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Connexin43 in Rat Oocytes: Developmental Modulation of Its Phosphorylation[1]

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ABSTRACT

It is well established that the 43-kDa connexin (Cx43) is predominantly expressed by ovarian somatic cells, whereas the identity of the connexins contributed by the oocyte to form gap junctions with its neighboring cells is not fully elucidated. Our study aimed to examine oocytes for the expression and regulation of Cx43 throughout oogenesis. Growing and fully grown rat oocytes that were meiotically incompetent and competent, respectively, were examined. Fully grown oocytes were analyzed either before or after reinitiation of meiosis as well as at the second meiotic metaphase. Immunofluorescent analysis of zona pellucida-free oocytes using conventional and confocal microscopy demonstrated a characteristic pattern of punctuated staining of Cx43 on the oolema. Immunogold electron microscopy localized Cx43 to the oocyte surface and the microvillar processes. Reverse transcriptase-polymerase chain reaction and Western blot analysis revealed similar amounts of Cx43 gene and protein in oocytes of different developmental stages. However, a relative increase in the phosphorylated forms of the protein was observed in fully grown oocytes that had completed their maturation. Our findings demonstrate that rat oocytes express a developmentally regulated Cx43. They further suggest that homotypic gap junctions that consist of Cx43 may be present between rat oocytes and their adjacent cumulus cells.

cumulus cells, gamete biology, gametogenesis, luteinizing hormone, meiosis, oocyte development

INTRODUCTION

The somatic cells of the ovarian follicle and the oocyte are interconnected by an extensively developed network of cell-to-cell communication, generated by gap junctions that allow the oocyte to send and receive regulatory signals [1–3]. Gap junctions are specialized regions in closely opposed membranes of neighboring cells that mediate the exchange of ions and small molecules [4, 5]. These transmembrane channels consist of protein subunits, referred to as connexins, that are members of a growing multigene family, and are distinguished by their molecular weight. Several types of connexins were detected in the ovary of different species [6, 7], with the rat ovary predominantly expressing the 43-kDa connexin, designated as connexin43 (Cx43) [8].

Information regarding the identity of the specific connexins contributed by the oocyte to form the gap junctions with their neighboring cumulus cells is somewhat controversial. Immunofluorescent staining demonstrated that cattle oocytes express both Cx32 and Cx43 proteins [9]. Later studies detected an additional connexin, Cx26, in oocytes of this animal species [10]. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis demonstrated that mouse oocytes express both the Cx32 and Cx43 genes [11]. However, using oocytes of the same species, this and a later study were unable to detect the presence of the Cx43 protein [12]. This last report demonstrated, however, that mouse oocytes express Cx37, which could not be detected in the surrounding cumulus cells.

The presence of Cx43 that is restricted to the granulosa cells and the exclusive expression of Cx37 by the mouse oocyte demonstrated in the aforementioned study seem to suggest that cell-to-cell communication between the female gamete and the somatic compartment of the ovarian follicle is established by heterotypic gap junctions. To further evaluate this intriguing possibility, we examined rat oocytes for the expression of Cx43. Using several complementary techniques for our analysis, we clearly detected the presence of Cx43, both gene and protein, in rat oocytes at different stages of their development. We also demonstrated, for the first time, posttranslational modification of the Cx43 protein in rat oocytes that have reached the second meiotic metaphase, completing their maturation.

MATERIALS AND METHODS

Animals

Sexually immature female Wistar rats, either 19 or 23 days old, were employed. The 23-day-old rats were injected with 10 IU of eCG (Gestyl; Organon, Oss, The Netherlands). When indicated, eCG injection was followed by 5 IU of hCG (Pregnyl; Organon, Oss, The Netherlands) 52 h later.

Collection of Oocytes

Ovarian oocytes are all arrested at prophase of the first meiotic division. However, because these oocytes vary in size and in their capacity to resume meiosis, they can be divided into 2 categories as follows: 1) growing oocytes that have not reached their final size and are incompetent to resume meiosis and 2) fully grown oocytes that are meiotically competent [13]. Resumption of meiosis in fully grown oocytes is physiologically stimulated by LH and can also be induced by exogenous administration of hCG. Upon exposure to these gonadotropins, oocytes complete the first meiotic division and progress to the second division, being arrested again at the second metaphase until fertilization. The exit from the first prophase-arrest and their progression to the second metaphase is also defined as oocyte maturation [1].

In order to study the pattern of Cx43 expression throughout oogenesis, rat oocytes at the 2 aforementioned developmental stages were employed. Growing oocytes were recovered from the ovarian follicles of 19-day-old rats. The fully grown oocytes were recovered from 25- and 26-day-old rats, and further divided into 3 subgroups: 1) immature oocytes that are arrested at the first prophase, 2) maturing oocytes that have reinitiated meiosis, and 3) mature oocytes that have reached the second metaphase. Immature and maturing oocytes were collected from ovarian follicles of untreated rats. Mature oocytes were recovered after ovulation from the oviductal ampullae.

Isolation of ovarian oocytes was performed as described previously

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[14]. Briefly, the ovaries were removed and placed into Leibovitz L-15 tissue culture medium (Life Technologies, Paisley, Scotland). The individual follicles were incised to release the cumulus-oocyte complexes. Removal of the cumulus cells was accomplished by gentle pipetting after 30 min of incubation in medium containing EDTA (40 mM) at 37°C. Postovulatory oocytes were recovered from the oviductal ampullae isolated into L-15 tissue culture medium containing hyaluronidase (1 mg/ml, Sigma Chemical Company, St. Louis MO). The cumulus-oocyte complexes were released into the medium and the cumulus cells were removed by gentle pipetting after 20 min of incubation at room temperature [13].

The zona pellucida (ZP) that encapsulates the cumulus-free oocyte contains a fairly large number of cumulus cell projections. For further removal of the ZP, the oocytes were immersed in acid Tyrode solution (pH = 3.5) for a few seconds, followed by several washings in a large volume of L-15 tissue culture medium. The resulting ZP-free oocytes were employed for immunofluorescent staining, Western blot analysis, and RT-PCR.

Immunofluorescent Staining

ZP-free oocytes were fixed in 3% paraformaldehyde for 15 min, permeabilized in 1% Triton X-100 for 2.5 min, and further incubated overnight at 4°C with specific monoclonal anti-Cx43 antibodies (Transduction Laboratories, Lexington, KY). Immunostaining was performed using the avidin-biotin protocol [15]. Briefly, oocytes were washed in 10 mM glycine and 10 mg/ml BSA in PBS (GB-PBS) and treated with biotinylated anti-mouse immunoglobulin G (IgG) for 45 min at room temperature, followed by incubation with fluorescein isothiocyanate (FITC)-avidin (Vector Laboratories, Burlingame, CA). The specimen was then mounted in GB-PBS and examined by either a confocal or a conventional microscope equipped with an epi-illuminator and a filter for FITC fluorescence.

Immunogold Electron Microscopy

Intact rat ovaries were fixed in 2% paraformaldehyde and 1% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 at room temperature for 2 h, and left overnight at 4°C. Samples were washed in the same buffer, osmicated in 1% OsO_4, dehydrated in an ascending series of ethanol followed by propylene oxide, and then embedded in either Epon-812 or LR Gold as described previously [16].

Ultrathin sections (70–90 nm) were incubated in blocking solution (0.5% BSA, 0.1% glycine, 1% Tween-20, 1% gelatin in PBS) for 1 h, followed by an overnight incubation at 4°C with specific anti-Cx43 antibodies (12.5 μg/ml) and a further incubation with second rabbit anti-mouse antibodies for 1 h. The sections were then incubated with gold-labeled (10 nm) third antibodies (IgG) for 1 h. Each incubation was followed by a 3-min washing with PBS (5 times). The tissue was stained by 2% uranyl acetate in 50% ethanol (4 min), followed by lead citrate (5 min). The sections were analyzed with a Philips EM-410 electron microscope.

Western Blot Analysis

Western blot analysis was performed as described previously [17] with some modifications. Groups of 1500 ZP-free oocytes were collected (100 oocytes per animal) into homogenization buffer (20 mM Tris [pH 7.5], 250 mM sucrose), supplemented with 10 mM dithiothreitol, 2 mM EDTA, 5 mM EGTA, 1 mg/ml pepstatin, 1 mg/ml leupeptin, and 1 mM PMSF (Sigma) dissolved in Laemmli sample buffer [18], boiled and loaded on 12.5% SDS-polyacrylamide gel. For better resolution of the different phosphorylated forms of Cx43 [17], the bisacrylamide in the monomer mixture was reduced from 0.8% to 0.12%. After electrophoresis, the proteins were transferred to a nitrocellulose membrane and probed with the above-mentioned anti-Cx43 antibodies. Detection of the protein-antibody complex was performed using horseradish peroxidase-linked goat anti-mouse antibodies followed by enhanced chemiluminescence detection reagents (ECL; Amersham Pharmacia Biotech, Little Chalfont Buckinghamshire, U.K.). Quantitation of the autoradiograms was performed by densitometric analysis (Computing Densitometer, PDI, 420 oe).

Analysis of Cx43 Gene Expression

The Cx43 gene was detected by RT-PCR. Total RNA of groups of 400 ZP-free oocytes each was extracted by the acid-guanidium-phenol-chloroform method [19] and reversed transcribed using random primers followed by PCR amplification. The RT reaction mixture contained 50 units of Moloney murine leukemia virus-RT, 200 μM dNTP, 6.5 mM $MgCl_2$, 20 units of RNAsin, 25 ng/ml oligo(dT), and 1.5× PCR buffer (Promega Corporation, Madison, WI). The reaction was performed at 37°C for 2 h. The reaction solution was then divided into 2 even aliquots that were further amplified by PCR using a labeled nucleotide ([α^{32}P]dCTP, Amersham) and primers for Cx43 and for S16 that served as our internal standard. The following pairs of primers were employed; 1) 5′-ATGGCTGCTCCTCACCAACG-3′ and 5′-GGTCGTTGGTCCACGATGGC-3′ for amplification of the 277-base pair (bp) Cx43 fragment correlating to the rat Cx43 cDNA 971–1248 (bp) sequence and 2) 5′-CGTTCACCTTGATGAGCCCATT-3′ and 5′-TCCAAGGGTCCGCTGCAGTC-3′ for S16 [20]. The PCR reaction was performed in test tubes that contained 250 ng of each primer, 200 μM dNTP, 2.5 mM $MgCl_2$, 2 μCi [α^{32}P]dCTP, 1× PCR buffer (Promega) and 2.5 units of *Taq* polymerase. A program of 30 and 28 cycles for Cx43 and S16, respectively, was employed as follows: incubation for 2 min at 94°C followed by the indicated number of cycles at 94°C for 30 sec; 60°C for 30 sec, and 72°C for 1 min, with a final extension for 5 min at 72°C. The radioactive products were electrophoresed on 5% nondenaturing polyacrylamide gel in 0.5× TBE buffer, and the gels were autoradiogrammed. Quantitation of the autoradiograms was performed by densitometric analysis (Computing Densitometer, PDI, 420 oe) and normalized according to the internal standard.

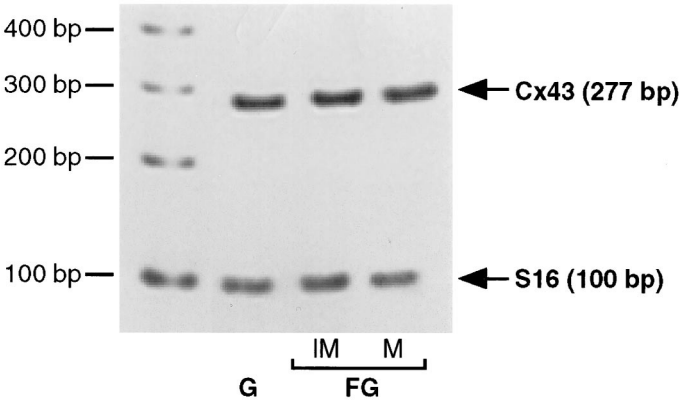

FIG. 1. RT-PCR analysis of Cx43 mRNA levels in rat oocytes at different developmental stages; growing (G) and fully grown (FG) oocytes. The latter category included immature (IM) and mature (M) oocytes. The results of 1 representative experiment out of 4 repetitions are presented.

RESULTS

Developmental Analysis of Cx43 Gene Expression

The Cx43 gene expression in the different groups of oocytes was analyzed by RT-PCR. Identification of the RT-PCR products was confirmed by sequence analysis, which revealed 100% identity to the relevant cDNA. Our results demonstrate that all the oocytes examined express the Cx43 gene (Fig. 1). A semiquantitative evaluation of Cx43 mRNAs, using the S16 mRNA as an internal standard, could not detect differences in the amount of the Cx43 gene expressed by oocytes representing the early and the various later developmental stages. Nevertheless, because growing oocytes are smaller in size than fully grown oocytes, the amount of the Cx43 transcript in each growing oocyte is apparently higher.

Localization of Cx43 Protein in Rat Oocytes

Immunofluorescent analysis of ZP-free rat oocytes, using specific anti-Cx43 antibodies, exhibited the characteristic punctuated pattern of Cx43 staining on cellular surfaces. Figure 2 represents a computerized summation of several serial sections of a growing rat oocyte analyzed by confocal microscopy. The obtained image clearly demonstrates the presence of Cx43 all over the oocyte surface. Immunogold staining of thin sections of ovarian follicles followed by electron microscopic examination confirmed that Cx43 is

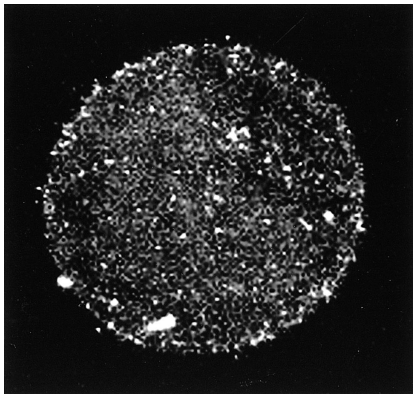

FIG. 2. Immunofluorescent staining of a ZP-free growing, incompetent rat oocyte using anti-Cx43 antibodies: a computerized summation of several serial sections analyzed by confocal microscopy. The results of 1 representative experiment out of 4 repetitions are presented.

indeed localized in the inner side of the oolema (Fig. 3a). Extensive immunogold staining localized on gap junctions between 2 somatic follicular cells that are known to express Cx43 served as a positive control (Fig. 3b). No staining was observed in sections incubated in the absence of the first antibody (Fig. 3c).

Developmental Analysis of Cx43 Protein Expression

Immunofluorescent staining was further employed to analyze the developmental pattern of expression of Cx43 in rat oocytes. Examination of the oocytes by conventional fluorescent microscopy revealed a clear fluorescent rim, demonstrating again the presence of Cx43 on the oolema. This pattern of fluorescent staining was observed in growing as well as fully grown oocytes with no difference between immature and mature oocytes (Fig. 4).

Having confirmed the presence of the Cx43 protein in oocytes of the different developmental stages and demonstrating their localization on the oocyte surface, we further used Western blot analysis to quantitate the amount of this protein expressed by oocytes of each of the aforementioned groups (Fig. 5). Similar to our previous observations in granulosa cells [17, 21], we herein demonstrate that the oocytes express a multiphosphorylated Cx43 protein. A previous treatment of our samples with alkaline phosphatase to remove putative phosphate groups resulted in an increased intensity of the lighter form of the Cx43 protein at the expense of the heavier forms that were entirely eliminated [17]. These results provided strong evidence that the proteins with the lower electrophoretic mobility, recognized by the specific Cx43 antibodies, indeed represent phosphorylated forms of Cx43. Furthermore, depletion of the Cx43 antibodies by their preincubation with a synthetic Cx43 peptide performed in the present study, completely eliminated the signal, indicating that all the proteins detected in this experiment represent Cx43 isoforms (Fig. 5).

Similar to our findings at the gene level, no substantial differences in the total amount of Cx43 protein between the various groups of oocytes could be shown (Fig. 5A). However, a difference in its phosphorylation state was clearly observed between immature and mature oocytes. The nonphosphorylated Cx43 in ovulated oocytes that have reached the second meiotic metaphase disappeared, and a concomitant increase in the abundance of the phosphorylated Cx43 isoforms could be observed. These changes could not be detected in maturing oocytes exposed to hCG for a shorter period of time (Fig. 5B).

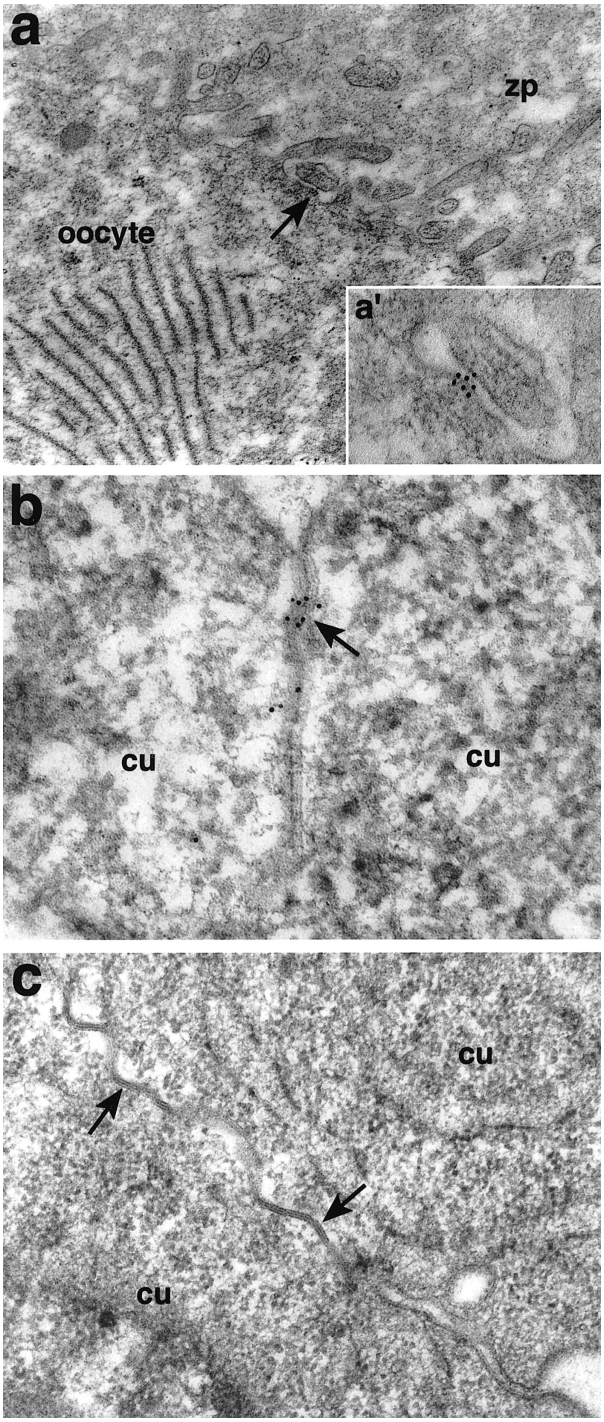

FIG. 3. Electron immunogold staining of thin sections of rat ovaries using anti-Cx43 antibodies. **a**) A contact area between the oocyte and the ZP (×7600); **a'**) an oocyte microvillar process folded over, exhibiting staining for Cx43 (×58 000); **b**) gap junctions (indicated by arrow) between 2 cumulus (cu) cells stained for Cx43 (×58 000); **c**) gap junctions between 2 cumulus cells (indicated by arrows). No first antibody was used for this sample (×44 000). The results of 1 representative experiment out of 4 repetitions are presented.

DISCUSSION

Our study clearly demonstrates that rat oocytes express Cx43. The protein is localized on the oocyte plasma mem-

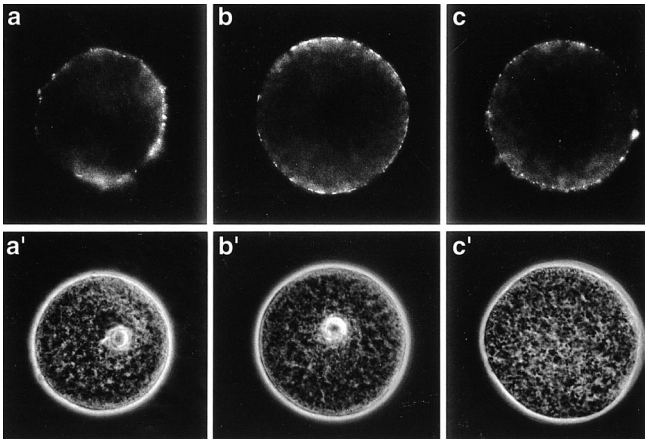

FIG. 4. Immunofluorescent staining of ZP-free oocytes at different developmental stages using anti-Cx43 antibodies. **a, b, c**) Growing and fully grown immature and mature oocytes, respectively, examined by fluorescent microscopy; **a′, b′, c′**) the same oocytes examined by light microscopy. The results of 1 representative experiment out of 4 repetitions are presented.

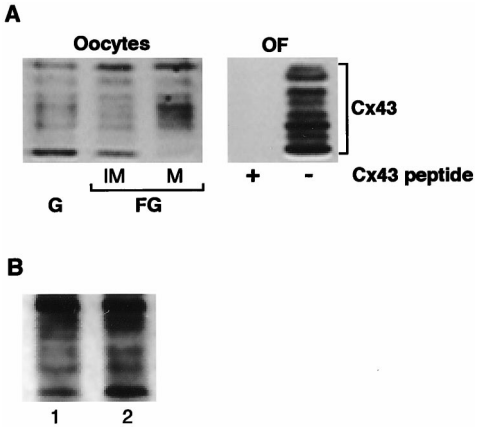

FIG. 5. **A**) Western blot analysis of Cx43 protein expression in rat oocytes at different developmental stages as follows: growing (G) and fully grown (FG) oocytes. The latter category included immature (IM) and mature (M) oocytes. Electrophoretically separated proteins of extracted membranes of intact ovarian follicles (OF) served as a positive control. Depletion of the anti-Cx43 antibodies by their preincubation with a Cx43 synthetic peptide completely eliminated the signal. **B**) Western blot analysis of Cx43 in fully grown oocytes isolated from eCG-treated rats before (lane 1) and 3 h after hCG administration (lane 2). The results of 1 representative experiment out of 4 repetitions are presented.

brane. No changes in the level of expression of the Cx43 gene nor its protein product occur upon acquisition of meiotic competence and throughout oocyte maturation. However, posttranslational modification manifested by hyperphosphorylation of Cx43 is observed in oocytes that underwent maturation, reaching the second metaphase of meiosis.

The ovarian follicle consists of 2 major cellular compartments; the somatic cells and the female gamete. Gap junction-mediated intercellular communication between these 2 compartments are responsible for orchestrating their development. Earlier studies demonstrated a dependency of oocyte growth on transmission of nutrients from the follicle cells [22–24]. Later reports showed that the meiotic status of the oocyte is subjected to regulation by its communication with the somatic follicular cells [25, 26]. More recent studies suggested that the oocyte receives not only regulatory signals, but also provides signals that control folliculogenesis [27–37]. Some of these messages could possibly be transmitted via gap junctions.

Gap junctions are transmembrane channels that consist of protein subunits referred to as connexins. The connexins are members of a growing protein family distinguished by their molecular weight. Multiple connexins have been detected in ovarian follicles of different species, among which Cx26, Cx32, Cx37, Cx40, Cx43, Cx45, and Cx60 are included [8, 10–12, 17, 38–45]. Depletion of the Cx32 gene in mice resulted in healthy fertile mice, indicating that this connexin is not necessary for normal ovarian function [46]. On the other hand, in Cx37-deficient mice, both the ovarian follicles and the oocytes were arrested at an early stage of their development pointing toward a major role of this protein in establishing bidirectional communication in the ovarian follicle [12]. Because mice that lack Cx43 die soon after birth as a result of cardiac malformation [47], analysis of the role of Cx43 in folliculogenesis required the establishment of a more complicated experimental strategy. These experiments used ovaries removed from prenatal Cx43 knockout mice, allowing them to further develop either in vitro, in organ culture, or in vivo under the kidney capsule of wild-type mice. In both cases, postnatal folliculogenesis in Cx43-deficient ovaries did not proceed beyond the primary follicle stage, and oocyte growth was retarded [48, 49]. These studies indicated that Cx43 plays an indispensable role in germ cell development and ovarian folliculogenesis.

Identification, localization, and regulation of the different ovarian connexins mentioned above have been extensively investigated in the somatic compartment of the follicle [6–8, 10, 17, 21, 38, 39]. However, information regarding connexin expression by the oocyte is fairly limited and somewhat confusing. RT-PCR analysis was successfully used to demonstrate Cx32 and Cx43 gene expression in mouse oocytes [11], and immunostaining localized the Cx43 protein on the oolema of cattle oocytes [9]. On the other hand, immunofluorescent analysis of mice ovarian sections using anti-Cx43 antibodies demonstrated an extensive staining that was restricted to the somatic follicular cells [10–12]. On the basis of these observations, Simon et al. [12] concluded that oocytes do not express Cx43. The other 2 studies, however, did not exclude the possibility that the staining observed at the interface between the oocyte and the cumulus cells could possibly represent Cx43 localized on the oocyte surface.

To overcome this confusion, our study examined oocytes that have been separated from the ovarian follicle and further treated to remove the surrounding cumulus cells. Moreover, because mechanical removal of the cumulus may leave residues of their cellular projections embedded in the ZP, we further dissolved this glycoprotein capsule to obtain optimal experimental conditions for exclusive examination of pure oocyte preparations. Immunofluorescent staining of these ZP-free oocytes demonstrated the presence of Cx43 on their oolema. Electron microscopic examination of immunogold stained ovarian sections confirmed that Cx43 is definitively contributed by the oocyte itself rather than by possibly attached residues of the cumulus cell projections. Our electron microscopic examination corroborates previous observations by our laboratory, and those of others, that rat cumulus cells express Cx43 [8, 17, 38, 50]. Additional RT-PCR experiments performed by us demonstrated that similar to mice [12], rat oocytes express Cx37 mRNA (data not shown). These findings taken together suggest that in

the rat, gap junction channels that communicate between the oocyte and its adjacent cumulus cells may be homotypic. Nevertheless, they do not exclude the presence of heterotropic gap junctions consisting of Cx37 and Cx43 expressed by the oocyte and cumulus, respectively [12].

Modifications of the gap junction protein in the oocyte throughout its development have not been studied as yet. Western blot analysis of isolated oocytes did not demonstrate variations in the amounts of Cx43 protein in oocytes of the different developmental stages. However, this analysis provided novel information regarding posttranslational modifications of Cx43 in oocytes. Specifically, we show herein for the first time that the relative amount of the phosphorylated forms of Cx43 increases after resumption of meiosis.

Sequence analysis of Cx43 shows consensus-phosphorylation sites of $p34^{cdc2}$ kinase in its C-terminal region. This kinase represents the catalytic subunit of the $p34^{cdc2}$/cyclin complex initially described as maturation promoting factor [51]. The activity of this kinase is elevated upon reinitiation of meiosis and transiently declines between the first and second meiotic divisions. Oocytes at the second metaphase of meiosis are characterized by a high $p34^{cdc2}$ kinase activity that is sustained until fertilization [52]. Taken together, this information may suggest that hyperphosphorylation of Cx43 in mature oocytes could possibly be mediated by $p34^{cdc2}$. A recent report demonstrating $p34^{cdc2}$-mediated phosphorylation of Cx43 during mitosis strongly supports this idea [53]. Along this line, changes in the phosphorylation state of Cx43, in FT2 10 cells that contain a temperature-sensitive mutation in the $p34^{cdc2}$ kinase, have also been reported [54]. Phosphorylation of Cx43 mediated by other kinases such as protein kinase C [54–56], Cyclic AMP-dependent protein kinase A [17, 56, 57], mitogen-activated protein kinase [56, 57], and tyrosine kinases in transformed cells [58], has previously been reported.

Phosphorylation of Cx43 has been implicated as a regulatory mechanism for the gating of gap junction channels [17, 21, 59–67]. Specifically, these studies showed that hyperphosphorylation of the Cx43 protein is associated with reduction in metabolic coupling. However, the hyperphosphorylation of Cx43 in oocytes that underwent maturation observed by us does not coincide with LH/hCG-induced reduction of metabolic coupling in the cumulus-oocyte complex [14, 25, 68]. This early interruption of the cell-to-cell communication that stops the transfer of the inhibitory cAMP to the oocyte, leading to resumption of meiosis, apparently represents the response of the somatic cells of the follicle to the preovulatory LH surge [1].

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