Human granulosa cells in culture exhibit functional cyclic AMP-regulated gap junctions

C.Furger1,5, L.Cronier2, C.Poirot3 and M.Pouchelet4

1INSERM U361 and Clinique Universitaire Baudelocque, Paris, 2Laboratoire de Physiologie Cellulaire, CNRS URA 1869, Poitiers, 3Laboratoire de Biologie de la Reproduction, Hôpital Cochin, Paris and 4INSERM Service Cinématographie, Le Vésinet, France

Numerous gap junctions exist between granulosa cells, between cumulus cells and between cumulus cells and the oocyte. They may play a role in the regulation of both follicular development and oocyte status. We used primary cultures of human granulosa cells to study the molecular nature and functionality of these gap junctions. As shown by a cinemicrographic technique, during the first 3 days of culture, cells flattened and extended in several directions by means of cytoplasmic extensions. An ultrastructural study showed the presence of both intercellular and annular gap junctions after 48 h of culture. As revealed by immunodetection analyses, connexin 43 was present. An analysis using a functional procedure, the gap fluorescence recovery after photobleaching (FRAP) method, indicated that: (i) diffusional communication existed among granulosa cells; (ii) the communication was delayed by treatment with 1-heptanol, a well-documented inhibitor of gap junction permeability; and (iii) permeability was up-regulated by incubation with 8-Br-cAMP, an analogue of cyclic AMP. The detection of connexin 43 and functional gap junctions in networks of cytoplasmic extensions indicated junction formation among cells during culture. In conclusion, our results show that human granulosa cells in culture exhibited functional gap junctions. Connexin 43 was present and the permeability of the gap junctions was up-regulated by cyclic AMP, an important modulator of human granulosa cell function.

Key words: connexin 43/cyclic AMP/FRAP method/gap junction/human granulosa-lutein cell

Introduction

The follicle is the main functional unit of the mammalian ovary and provides the support system for oocyte development. Granulosa cells (GC) surrounding the oocyte contribute in several ways towards accomplishing this function. They provide nutritive requirements for the growing oocyte, produce steroid hormones and control both nuclear and cytoplasmic maturation of the oocyte selected for ovulation. During follicular maturation, the function and differentiation of GC are regulated by the coordinated actions of the pituitary gonadotropins through their ability to stimulate the production of second messengers such as cyclic AMP (cAMP; Marsh, 1975). GC can communicate through the local production of intracellular factors such as cytokines (Adashi, 1992) or growth factors (Adashi et al., 1991) which act as paracrine and/or autocrine modulators. Furthermore, an intimate anatomical relationship links the GC. Numerous gap junctions have been observed among GC (Albertini and Anderson, 1974) and between the oocyte and the GC of the cumulus (Anderson and Albertini, 1976). Gap junction channels connect the cytoplasm of contacting cells, while the junctional aqueous pore (~14 Å in diameter) enables the transfer of small molecules (mol. wt <1.5 kDa) (for a review see Kumar and Gilula, 1996). Functional involvement of gap junctional communication during follicular development has been reported (for a review see Larsen et al., 1991). Gap junctions enable the passage of nucleotides, amino acids and sugars from GC to the oocyte for its growth and development (Eppig, 1979; Heller et al., 1981; Brower and Schultz, 1982). Furthermore, an intracellular factor, possibly cAMP, could in this manner control the status of oocyte meiosis (Dekel and Beers, 1978; Dekel et al., 1981). Therefore, the disappearance of gap junctions observed near the time of pre-ovulatory cellular dissociation might be responsible for meiotic resumption (Larsen et al., 1981, 1987; Racowsky et al., 1989).

In the human, mechanisms controlling gap junctional communication during follicular development are unknown. In the rat, some data suggest that the synthesis and turnover of gap junctions are promoted by gonadotrophin hormones (Burghardt and Anderson, 1981; Burghardt and Matheson, 1982). Follow-
Figure 1. Typical computer-generated fluorescence images of cultured human granulosa cells. Cells were loaded for 10 min with the fluorescent dye CFDA, before laser scanning and the photobleaching procedure. Fluorescence images represent selected areas corresponding to human granulosa cells: (a) before photobleaching; (b) just after photobleaching of cells 1 and 2; and (c) 12 min after photobleaching. (d) Same field seen by phase-contrast microscopy.

Wiesen and Midgley, 1993) and cattle (Sutovsky et al., 1993) ovaries. The expression of cx43 mRNA and protein seems to depend on the stage of follicular maturation and on intrafollicular cell position (Wiesen and Midgley, 1993, 1994).

The emergence of in-vitro fertilization (IVF) programmes has enabled the assessment of gap junctional communication to be made in human GC. Our investigations were designed to examine the presence of gap junctions and gap junctional communication in human GC. The use of human GC in primary culture requires a detailed knowledge of their in-vitro development process. Therefore, an analysis of their evolution in culture was performed by time-lapse cinematography. The presence of gap junctions was investigated by ultrastructural techniques and cx43 immunolocalization. The presence of a functional gap junction communication, and its possible modulation by the 8-Br-cAMP (a membrane-permeant analogue of cAMP), were investigated by fluorescence recovery after photobleaching (gap FRAP).

Materials and methods

Collection and culture of human GC

GC were aspirated along with follicular fluid and oocytes from patients participating in an IVF programme. Patients were stimulated by combined therapy with [D-Trp<sup>6</sup>]-gonadotrophin-releasing hormone (Decapeptyl; Ipsen-Biotech, Paris, France) and human menopausal gonadotrophin (HMG; Humegon; Organon, Serifontaine, France). Ovulation was triggered with HCG (5000 IU; Organon). The cumulus-oocyte complex was used in the IVF programme. Follicular fluids from several follicles of several patients were collected. Follicular fluid was removed after sedimentation of the cells by centrifugation (800 g, 10 min, 20°C) and replaced by phosphate-buffered saline (PBS; Sigma, St-Quentin-Fallavier, France). Red blood cells were removed by centrifugation on a preparation of Ficoll and sodium diatrizoate (Histopaque-1077; Sigma; 300 g, 35 min, 20°C). After mechanical dispersion, cells were counted in a haemocytometer and cultured (50 000 cells/cm<sup>2</sup>) in 35 mm plastic culture dishes (Poly Labo, Strasbourg, France) or 4-well glass slides (LAB-TEK; Poly Labo). Cell viability was assessed by Trypan Blue exclusion in a protein-free medium and was ~80%. Cells were then cultured in Ménézo B<sub>2</sub> medium (BioMérieux, INRA, France) supplemented with 10% fetal calf serum and maintained for 48 h at 37°C in a humidified environment with 5% CO<sub>2</sub> and 95% air. After 24 h and before replacing the medium, three washings with PBS at 37°C enabled the removal of dead granulosa and red blood cells. The morphology of the cells was similar after 48 h of culture in plastic or glass substrates. For comparable results, all the experiments were performed with the same cell density (50 000 cells/cm<sup>2</sup>). The cell density varied throughout the culture dish, but the same immunostaining and fluorescence recovery curve types were observed between cells grown from clusters and between well-separated cells.
Functional gap junctions between human GC

Figure 2. Behaviour of human granulosa cells in culture. Micrographs represent the selected images of a 16 mm film after (a) 5, (b) 20, (c) 40 and (d) 60 h of culture. Note the increase in the intercellular contact number with time, and the long cytoplasmic extensions emitted by cells during their spreading. Bars = 50 μm.

Time-lapse cinemicrography
Cell culture was performed using a ‘Rose chamber’, an airtight culture chamber comprising two circular glass coverslips separated by a silicon joint, the latter being pierced by two needles that allow the medium to be changed during culture (Rose, 1954). Cell behaviour was recorded 5 min after the introduction of collected cells in the Rose chamber. A record of cell behaviour over 72 h was taken with a Zeiss inverted microscope (ICM 405) equipped with a 16X Ph N.A. 0.40 Neofluar objective and a 16 mm Arriflex standard cinecamera driven by a hand-made time-lapse unit. The microscope was placed in a box maintained at 37°C with the use of an integrated temperature controller device (Multitop, Chauvin Arnoux, France). Frames were taken every minute. Between two successive frames, the light was turned off to avoid cellular damage. Agfa Copex Rapid AHU film was used. Cell behaviour was analysed by an analysis projector.

Transmission electron microscopy
After 2 days of culture, cells grown in 35 mm culture dishes were fixed in the dishes by 3% glutaraldehyde in Sörensen buffer, pH 7.3, for 1 h at room temperature. The cells were then scraped and centrifuged. The pellet was post-fixed with 4% OSO4 in the same buffer for 1 h at 4°C, dehydrated in a graded series of ethanol and propylene oxide, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined using a Zeiss electron microscope EM 10C/CR.

Immunofluorescence
After 2 days of culture, cells grown in four-well glass slides were washed three times with PBS and fixed for 10 min with absolute methanol at −20°C. After rinsing with PBS, cells were incubated overnight at 4°C in a light-tight humidified box with either cx43-specific rabbit antibody or preimmune serum (El Aoumari et al., 1990) [both diluted 1:20 in PBS-0.1% bovine serum albumin (BSA)]. Cells were washed three times with PBS for 10 min each, followed by incubation with a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (Nordic; Tébu, Le Perret en Yvelines, France) diluted 1:10 in PBS-0.1% BSA for 1 h at 20°C. Slides were washed three times with PBS for 10 min each and coverslips were mounted with a mixture of glycerol and mowiol. Photographs were taken with a microscope (DMRB; Leica, Rueil-Malmaison, France) equipped with an FITC filter (IC; Leica) or an interference contrast system.

Fluorescence recovery after photobleaching (gap FRAP) method
The cell–cell diffusion of a fluorescent dye was measured by the gap FRAP method, developed by Wade et al. (1986), using an interactive laser cytometer (ACAS 570; Meridian Instruments, Okemos, MI, USA), which allows for convenient digital video imaging and analysis. After 2 days of culture in 35 mm culture dishes, the medium was replaced by Tyrode solution containing (in mM) 144 NaCl, 5.4 KCl, 2.5 CaCl2, 1 MgCl2, 0.3 NaH2PO4, 5.5 glucose and 5 HEPES buffer at pH 7.4. Cells were loaded for 10 min at room temperature with 6-carboxyfluorescein (CF) diacetate (7 μg/ml in 0.25% dimethylsulphoxide), a membrane-permeant molecule which is hydrolysed by cytoplasmic esterases to CF, a hydrophilic derivative that remains trapped in the cells. The fluorescence intensity (excited by weak laser pulses) was recorded before and after photobleaching (Figure 1) and stored for analysis. If cells were interconnected by permeable gap junctions, fluorescence recovery following a slow exponential time course was observed in bleached cells because of the diffusion of CF from adjacent unbleached cells. The procedure for the local perfusion of 1-heptanol has been described previously (Cronier et al., 1994). Briefly, a needle was moved close to the selected cells under microscopic control and 1-heptanol (3 mM) was injected continuously over a 2 min period. At the end of the injection, 1-heptanol was not removed but its local concentration rapidly decreased because of dilution in the medium. For the cAMP study, cells showing a typical fluorescence recovery curve were chosen. GC were cultured in the presence of 8-Br-cAMP (5×10−4 M) at 37°C in...
constant $k$ was determined from recovery curves using the following equation:

$$\frac{(F_i - F)}{(F_t - F_0)} = e^{-kt},$$

where $F_i$, $F_0$, and $F_t$ are fluorescence intensities in tested cells before photobleaching, just after photobleaching and at time $t$ respectively. The determination of $k$ was performed during the 4 min following the photobleaching (Figure 6, inset). The mean value of rate constant $k$ of cells that recovered ($n = 24$) was $11.80 \pm 1.33 \times 10^{-2} \text{ min}^{-1}$. When isolated cells were photobleached, no fluorescence recovery was observed (Figure 7, O).

Aliphatic alcohols with chain length C6-C9 rapidly and reversibly interrupt the cell–cell communication through gap junctions (Johnston et al., 1980; Deleze and Hervé, 1983). To ascertain junctional communication type, an experiment was performed with 1-heptanol. A 2 min local perifusion of 1-heptanol delayed the recovery of fluorescence for 2 min (Figure 8), confirming the gap junctional nature of the dye diffusion.

To test the short time effect of cAMP on gap junctional communication between human GC, we measured the rate constant $k$ of four selected areas before and after incubation with 8-Br-cAMP ($5 \times 10^{-4} \text{ M}$) for 1 h. For all tested areas, 8-Br-cAMP increased the rate constant $k$ (Table I), suggesting a modulating role for cAMP in gap junctional communication between GC.

### Discussion

Our results demonstrate for the first time that human GC in culture exhibit contact-dependent communication by means of gap junctions. Cinematographic studies revealed that cytoplasmic extensions appear a few hours after the beginning of the culture, and that the cell–cell contact surface increases significantly with time. Cx43 and gap junctional communication are observed between GC connected by only one thin cytoplasmic extension. This argues for gap junction formation during cell spreading.

The ultrastructural analysis confirms the presence of the typical gap junctions reported previously in the same model (Amsterdam et al., 1989). Furthermore, it revealed that the two GC maturation states observed in fresh undissociated human GC, using cytoplasmic organelle development as a criterion of cell differentiation (Rotmensh et al., 1986), are also present in our in-vitro system. Therefore, we can hypothesize that both peri-ovulatory and less mature GC co-existed in our experiments. These different maturation stages are likely to reflect the diversity of the cell origin inherent in the IVF procedure. GC originated from several follicles of different sizes and probably different follicular compartments.

Our data demonstrate for the first time the expression of cx43 in human GC. In rat ovarian follicles, cx43 was immunolocalized within the GC layer (Beyer et al., 1989). Recently, immunolocalization and in-situ hybridization were used to specify cx43 distribution within the developing follicle of the rat ovary (Wiesen and Midgley, 1993). The protein and its mRNA were localized in the GC mural part of the mature

### Table I. Increase in the relative rate constant by treatment with 8-Br-cAMP

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>$k_{\alpha}$ ($10^{-2} \text{ min}^{-1}$)</th>
<th>$k_{\beta}$ ($10^{-2} \text{ min}^{-1}$)</th>
<th>Percentage of increase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13.63</td>
<td>16.29</td>
<td>19.52</td>
</tr>
<tr>
<td>2</td>
<td>8.98</td>
<td>15.92</td>
<td>77.28</td>
</tr>
<tr>
<td>3</td>
<td>15.48</td>
<td>18.82</td>
<td>21.57</td>
</tr>
<tr>
<td>4</td>
<td>11.61</td>
<td>18.22</td>
<td>56.93</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>12.42 ± 2.78</td>
<td>17.31 ± 1.42</td>
<td></td>
</tr>
</tbody>
</table>

$\alpha$ = relative rate constant measured under control conditions; $\beta$ = relative rate constant measured on the same cells after 8-Br-cAMP incubation for 1 h.
These results and ours indicate that cAMP, which has been protein kinase A-dependent phosphorylation of the channel-forming protein cx43. This protein has been found to be present in different phosphorylation states (Kadle et al., 1991; Musil and Goodenough, 1991) regulated by the cAMP pathway (Burt and Spray, 1988; Granot and Dekel, 1994). However, gonadotrophin-releasing hormone and 12-D-tetradecanoyl phorbol-13-acetate (TPA) have also been shown to regulate the phosphorylation state of cx43, and protein kinase C is also likely to be involved in that process (Granot and Dekel, 1994).

In summary, we show that human GC in culture exhibit functional gap junctions, the permeability of which is up-regulated by a cAMP analogue. It is likely that cAMP, which mediates the intracellular effect of LH and FSH, allows the gonadotrophins to control gap junctional status in human GC. Furthermore, the resumption of meiosis appears to be temporally correlated with the loss of gap junctions occurring in granulosa but not cumulus cells during the peri-ovulatory period (Moor et al., 1981; Eppig, 1982). Gap junctions between GC at a distant position from the oocyte may occupy a critical location in the follicle by providing a bridge between the entire oocyte–cumulus complex and the remaining membrana granulosa (Racowsky et al., 1989). Loss or closure of junctional channels could isolate the oocyte and some companion somatic cells from the regulatory influence of the underlying membrana granulosa. Further studies of gating mechanisms and connexin expression regulation in human GC in culture will contribute to a better understanding of pre-ovulatory intra-ovarian modifications.

Acknowledgements

The authors are very grateful to Dr Daniel Gros (CNRS UMR C9943, Luminy, Faculté des Sciences, Marseille, France) for providing human cx43-specific antibody, and to Dr André Malassine for constructive discussions. We also thank Gérard Delrue and his group (INSERM SC 6) for iconographic assistance, Nelly Gouhier for the cinematographic records and Mylène Navarro for providing the DMRB Leica fluorescence microscope. This work was supported by grants given by INSERM.

References

Brower, P.T. and Schultz, R.M. (1982) Intercellular communication between follicle, but were lacking in the corpus luteum (Wiesen and Midgley, 1993) and atretic follicles (Wiesen and Midgley, 1993, 1994). A dramatic loss of cx43 was also observed at the time of ovulation (Wiesen and Midgley, 1993). In our study, the use of a cx43-specific antibody revealed intercellular and macular stainings. Such localization supports the model of ultrastructural gap junction topology and is consistent with our ultrastructural localization. Both procedures indicate that gap junction components are present not only among cellular bodies, but also within networks of cytoplasmic extensions and between cytoplasmic extensions and cellular bodies. Moreover, it is likely that the intracellular macular fluorescent staining observed within some cells reflects the presence of the very common intracytoplasmic annular gap junctions observed by electron microscopy. The same pattern of cx43 intracytoplasmic staining was reported recently in both baboon and human luteal cells in culture using a different cx43 antibody (Khan-Dawood et al., 1996). However, one cannot exclude the possibility that cytoplasmic staining is caused by the presence of free hemi-connexons or free cx43 proteins localized within specific cytoplasmic compartments like the Golgi network.

The gap FRAP method, which was used successfully to investigate intercellular communication in rat primary (Stein et al., 1991) and spontaneous immortalized (Stein et al., 1993) GC, in bovine cultured luteal cells (Redmer et al., 1991) and in other endocrine cells (Cronier et al., 1994), has enabled us to demonstrate the presence of a cell–cell communication through gap junctions in human GC in culture. The rapid, reversible uncoupling observed after heptanol addition in the control medium confirmed that the initial dye diffusion, which follows an exponential time course, occurs via gap junctions. This finding was substantiated by the ultrastructural observation of gap junctions between GC membranes and by immunolocalization of cx43. Although dye coupling between contacting cells is not a direct measure of the flow of biologically active molecules from one cell to another, it unequivocally demonstrates the intercellular transfer of hydrophilic molecules (Wade et al., 1986). It is very likely that biologically important molecules of low molecular weight are also exchanged between cells that exhibit dye coupling. Our results show that the rate of transfer of molecules across human GC junctions is significantly and rapidly increased by the presence of 8-Br-cAMP (5×10⁻⁴ M). Previously this nucleotide has been associated with the modulation of gap junction communication in different cell types (Flagg-Newton et al., 1989; Sáez et al., 1986; Giaume et al., 1991). In swine cultured GC, communication was reduced sharply after the intracellular microinjection of protein kinase A inhibitor, but resumed with the injection of protein kinase A catalytic subunit or exposure to follicle stimulating hormone (FSH; Godwin et al., 1993). These results and ours indicate that cAMP, which has been implicated recently in the action of gonadotrophin hormones in human GC (Furger et al., 1996), could regulate its own diffusion rate through gap junctions within the GC layer. Moreover, one can hypothesize that the cAMP-dependent increase in junctional permeability is the consequence of direct protein kinase A-dependent phosphorylation of the channel-


