Antral follicle development influences plasma membrane organization but not cortical granule distribution in mouse oocytes

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In the present study, we evaluated the contributions of antral follicle development and antral granulosa cell-released factor(s) to the acquisition of a mature mouse oocyte plasma membrane organization and cortical granule distribution. This has been performed by comparing in vitro matured oocytes derived from early antral follicles (here referred to as denuded oocytes) or from pre-ovulatory follicles, and cultured either as cumulus-intact or cumulus-free oocytes, with in-vivo ovulated eggs. By using scanning and transmission electron microscopy, the denuded oocyte surface appears to be characterized by the presence of long microvilli, while that of pre-ovulatory oocytes and of ovulated eggs by shorter microvilli. However, denuded oocytes can acquire a pre-ovulatory-like plasma membrane configuration when matured in vitro in the presence of early antral granulosa or cumulus cells, but not of NIH-3T3 fibroblasts. On the contrary, fluorescence and confocal microscopy analyses after labelling with fluorescent Lens culinaris agglutinin show that all the oocyte classes analysed are characterized by similar cortical granule distribution and density. Thus, complete antral follicle development plays an important role in the process of oocyte surface differentiation, probably through the action of antral granulosa cell-released factor(s), but it does not affect oocyte capacity to normally distribute cortical granules.

Keywords: cortical granules/mouse oocyte/surface morphology

Introduction

In mammals, the recognition between spermatozoon and egg plasma membranes results in a binding event that is soon followed by membrane fusion (see Yanagimachi, 1994 for a review). Recently, some of the biochemical mechanisms regulating gamete interaction have been elucidated, with particular attention to the role played by a 94-kDa polypeptide (Kellog et al., 1992) or by integrins (Bronson and Fusi, 1990a,b; Blobel et al., 1992; Fusi et al., 1992; Tarone et al., 1993; Evans et al., 1995) as critical egg membrane components involved in this process. The region of the egg that participates in fusion is represented by the area containing microvilli and cortical granules (Johnson et al., 1975; Nicosia et al., 1977; Talansky et al., 1991; Green, 1993), suggesting that a specific organization of this area is relevant for the initial steps of sperm–egg interaction (Green, 1993; Myles, 1993; Foltz, 1995). Indeed, it has been demonstrated that the mammalian oocyte develops its fusion competence during the growth phase, in coincidence with the appearance of microvilli on the oocyte’s surface (Zuccotti et al., 1991).

Recently, it has been demonstrated that, in the mouse, the plasma membrane’s ability to fuse with a spermatozoon improves significantly during antral follicle development and reaches its full competence at the pre-ovulatory stage (Cecconi et al., 1996a). In a first attempt at explaining the differences in antral oocyte fusion kinetics, we have investigated whether oocytes obtained from follicles at different stages of antral development have different organization of their oolemma and/or of their cortical granule (CG) distribution. To this end, we have analysed the ultrastructural surface morphologies and the final CG localization in oocytes derived from early antral follicles (denuded oocytes, DO) or from pre-ovulatory follicles and matured either with (cumulus-intact, CI) or without (cumulus-free, CF) cumulus cells. A similar analysis has been performed also on DO matured in the presence of soluble factor(s) released by early antral granulosa or cumulus cells, as well as by NIH-3T3 fibroblasts.

Our results indicate that the oolemma of in-vitro matured oocytes derived from early antral follicles is characterized by a different ultrastructural organization from that of pre-ovulatory oocytes and ovulated eggs. However, a dramatic modification of the DO surface resulting in the acquisition of a pre-ovulatory oocyte-like plasma membrane configuration can be induced under the action of soluble factor(s) specifically produced by antral granulosa cells. From our results, the capacity of the oocyte to normally distribute CG is acquired independently of the stage of antral follicle development and the maintenance of functional communications between the oocyte and the surrounding cumulus cells during in-vitro maturation.

Materials and methods

Animals

Random-bred Swiss CD1 mice (Charles River, Italy) were used in these experiments.

Chemicals

All the reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Pregnant mare serum gonadotrophin (PMSG) was

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obtained from Intervet Laboratories (Boxmeer, The Netherlands), and human chorionic gonadotrophin (HCG) was obtained from Serono (Rome, Italy).

Collection of ovulated eggs and ovarian oocytes
Immature (22–23 days old) mice were primed with 5 IU PMSG, and 44–48 h later with 5 IU HCG. Mice were killed 15–16 h after HCG, and ovulated eggs were collected from the oviducts and denuded of cumulus cells by a brief treatment with 0.1% hyaluronidase in medium M16 (Whittingham, 1971) buffered with HEPES.

By puncturing early antral follicles (follicle diameter ranging from 200 to 300 µm; stage 7, Pedersen and Peters, 1968; stage 7a, Curci et al., 1991) of immature unprimed mice, oocytes denuded of adherent granulosa cells were obtained and are here referred as denuded oocytes (DO). Only DO with a cellular diameter ≥75 µm were selected for further experiments.

Cumulus-intact (CI) oocytes were obtained by puncturing the largest antral follicles (follicle diameter larger than 400 µm; stage 8, Pedersen and Peters, 1968) of PMSG-primed mice. Some of these CI oocytes were stripped of their cumulus cells by repeated micropipetting and used in the following experiments as cumulus-free (CF) oocytes. Follicle and oocyte diameters were measured by an ocular micrometer inserted on an inverted phase microscope.

In-vitro maturation of ovarian oocytes
Denuded oocytes, cumulus-intact and cumulus-free oocytes were cultured in 100 µl Waymouth MB 752/1 supplemented with 0.23 mM pyruvate, 50 mg/l streptomycin sulphate, 75 mg/l penicillin G potassium salt (Way-pyr; Van de Sandt et al., 1990), in 96-well tissue culture plates (microwells; Nunc, Roskilde, Denmark) at 37°C, 5% CO₂ in air for 16–18 h.

In another series of experiments, DO were matured in the presence of early antral granulosa or cumulus cells and, as a control, of NIH-3T3 fibroblasts. Early antral granulosa cells, recovered after puncturing the same early antral follicles utilized to obtain DO, and cumulus cells, stripped away from about 300 CI oocytes, were plated at 3×10⁶/ml; final concentration in microwells in 100 µl Way-pyr. To prepare feeder layers of NIH-3T3 fibroblasts, these somatic cells were cultured in microwells containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 7% fetal calf serum (FCS). When cells were at subconfluence, the culture medium was replaced by 100 µl Way-pyr. All the different somatic cell types were covered with a Cell Culture Insert Cyclopore Membrane (0.45 µm pore size, #3095 Falcon Plastics, Becton Dickinson Labware, Milano, Italy) adapted to microwell diameter, and germinal vesicle DO were placed on the top and matured as described above.

At the end of the maturation period, only MII-arrrested oocytes, as evidenced by polar body emission, were selected for further analyses. When required by experimental protocols, oocytes were deprived of the zona pellucida by a brief treatment with acidic Tyrode’s solution pH 2.5 (ZF, zona-free; Nicolson et al., 1975).

Processing for scanning (SEM) and transmission (TEM) electron microscopy
For SEM analysis, ZF oocytes were fixed for 90 min in 1% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2, post-fixed with 1% osmium tetroxide (OsO₄) in phosphate buffer and, after dehydration, critical-point dried. Microvilli density was determined by using the Cambridge-Quantimet 970 Program.

For TEM analysis, oocytes with (ZI, zona-intact) or without (ZF) zona pellucida were fixed for 2 h or 1 h, respectively, in 2.5% glutaraldehyde in cacodylate buffer and post-fixed with 1% OsO₄ in the same buffer. Dehydration and embedding followed standard procedures. In all the different oocyte classes, only the cortex was analysed, with the exclusion of the cortical granule-free domain. Microvilli lengths were determined by a KS 300 Kontron Program and expressed as the mean ± SD on n >40 microvilli measured for each oocyte class.

Lens culinaris agglutinin (LCA) labelling
For confocal analysis, ZF oocytes were fixed for 30 min at room temperature in 3% paraformaldehyde in PBS, washed twice in blocking solution (BS: 110 mM NaCl, 100 mM glycine, 1 mg/ml polyvinyl alcohol (PVA); pH 7.4) and permeabilized with 0.1% Triton X-100 in BS for 5 min. Oocytes were then rinsed in BS, and incubated in 100 µg/ml LCA–fluorescein isothiocyanate (LCA–FITC) for 30 min. After washing, oocytes were placed in 70% glycerol containing 1 mg/ml paraphenylendiamine and maintained between a coverslip and a glass slide supported by four columns of a mixture of vaseline and paraffin (9:1). Slides were viewed with a confocal microscope (Nikon, 2000, Molecular Dynamics, Sunnyvale, CA, USA) equipped with a 60X Plan Apo lens. For each oocyte class the same serial sections (2 µm thick) were recorded and downloaded to a digital image recorder.

For fluorescence analysis, oocyte semi-thin sections (1 µm thick) were prepared and treated with 30 µg/ml LCA–FITC as described by Talevi et al. (1997). Samples were then observed under the fluorescence microscope.

To quantify CG, oocytes were stained with LCA coupled to biotin and then with Texas red streptavidin following the procedure of Ducibella et al. (1988). For each oocyte class, CG density (expressed as number of CG/m² of egg cortex) was calculated as the mean of the counts of three equal areas of CG-occupied cortex according to Ducibella et al. (1988).

Statistical analysis
For the evaluation of differences between the groups, Student’s t-test was utilized, and P < 0.05 was considered significantly different.

Results
Ultrastructure of mouse oolemma
SEM analysis performed on MII-arrested ZF oocytes (25–30 oocytes/group) showed that the surface organization of in-vitro matured oocytes derived from early antral follicles (DO) was different from that of pre-ovulatory oocytes matured either with (CI) or without (CF) cumulus cells and that of ovulated eggs. In fact, while DO were characterized by the presence of long and deep microvilli (Figure 1A), both CI (Figure 1B) and CF oocytes (not shown) as well as ovulated eggs (Figure 1C) presented a surface densely covered with shorter microvilli. However, when DO were matured in the presence of early antral granulosa (Figure 1D) or cumulus cells (not shown), their surface morphology was very similar to that of pre-ovulatory oocytes. The microvilli density recorded for DO was 338 ± 44 (SD)/100 µm²; for DO matured in the presence of antral granulosa cells was 350 ± 27/100 µm²; and for pre-ovulatory oocytes and ovulated eggs were 345 ± 32/100 µm² and 350 ± 27/100 µm² respectively (not significant).

Given these results, a more detailed analysis of surface morphology was carried out on these different oocyte classes (20–25 oocytes/experimental group). TEM observations confirmed that the ultrastructural organization of DO plasma...
Figure 1. Representative scanning electron micrographs of the surfaces of MII-arrested zona-free oocytes: A: denuded oocytes (DO); B: cumulus-intact oocyte; C: ovulated eggs; D: DO matured in the presence of $3 \times 10^6$/ml early antral granulosa cells. Bar represents 1 µm.

membrane was completely different from that of pre-ovulatory and ovulated oocytes. In particular, the DO membrane was characterized by the presence of long microvilli (702 ± 135 µm, Figure 2A), while ovulated eggs (Figure 2B) as well CF (not shown) and CI pre-ovulatory oocytes (Figure 2C) presented a surface covered with shorter microvilli (MII: 324 ± 83 µm; CI oocytes: 296 ± 87 µm; CF oocytes: 316 ± 58 µm; DO vs MII: $P < 0.01$; MII vs CI or CF oocytes: not significant, $P > 0.01$). When matured in the presence of early antral granulosa cells (Figure 2D), the DO plasma membrane strongly resembled that of pre-ovulatory oocytes (microvilli length: 295 ± 54 µm; not significant, $P > 0.01$). Similar results were obtained by maturing DO in the presence of cumulus cells (not shown).

To exclude the possibility of a non-specific effect of co-culture procedures on oocyte surface morphology, DO were matured in vitro in the presence of NIH-3T3 cells. Under this specific experimental condition, DO microvillar organization was unmodified, since it was entirely comparable to that of DO matured without antral granulosa cells (not shown). Finally, the possibility that morphological modification of the oocyte surface could be due to zona pellucida removal by Tyrode’s solution was completely excluded by the finding that ZI and ZF oocytes presented similar oolemma configurations. A representative picture of a ZI pre-ovulatory oocyte is shown in Figure 3.

Cortical granule distribution in MII-arrested oocytes

From TEM observations, the cortex of all the different classes of oocytes seemed to be characterized by a similar distribution of dark granules, presumably mature CG (0.2–0.6 µm in diameter), localized below the microvillous region of the
Mouse oocyte surface and cortical granule distribution

Figure 3. Transmission electron micrograph of the surface of zona-intact cumulus-intact oocyte. Bar represents 1 µm.

Figure 4. Confocal images of MII-arrested zona-free oocytes labelled with Lens culinaris agglutinin–fluorescein isothiocyanate. A: denuded oocytes (DO); B: DO matured in the presence of 3×10⁶/ml cumulus cells; C: cumulus-intact oocytes; D: cumulus-free oocytes; E: ovulated eggs. Cortical granules form, in all the oocyte classes analysed, a monolayer in the cortex next to the plasma membrane with the exception of the region overlying the meiotic spindle. Bar represents 25 µm.

oolemma (Figure 2A–D). These results were confirmed by confocal analysis (20 oocytes/group), demonstrating that the same distribution pattern of LCA–FITC-labelled CG characterized DO matured in the absence (Figure 4A) or in the presence of early antral granulosa (not shown) or cumulus cells (Figure 4B) as well as pre-ovulatory oocytes (CI: Figure 4C; CF: Figure 4D). Fluorescent spots were distributed as a monolayer next to the plasma membrane with the exception of the region overlying the meiotic spindle, showing a pattern comparable to that of ovulated eggs (Figure 4E). Also fluorescence microscopic observations performed after LCA–FITC labelling of CG directly on ultra-thin sections of oocytes (five oocytes/group) confirmed the confocal microscopy observations. In Figure 5, a representative picture of the cortex of an in-vitro matured DO is shown.

Figure 5. Representative fluorescence micrograph of a denuded oocyte labelled with Lens culinaris agglutinin–fluorescein isothiocyanate (1 µm thick sections). Bar represents 10 µm.

CG density calculation was performed as reported by Ducibella et al. (1988) and the results were not significantly different between DO and the other oocyte classes examined (20 oocytes/group; DO: 30 ± 5 CG/100 µm²; CI oocytes: 32 ± 6 CG/100 µm²; CF oocytes: 31 ± 7 CG/100 µm²; ovulated eggs: 37 ± 5 CG/100 µm²).

Discussion

Results presented in this study demonstrate that in the mouse: (i) the surface of MII-arrested oocytes derived from early antral follicles (denuded oocytes) is significantly different from that of cumulus-intact and cumulus-free oocytes derived from pre-ovulatory follicles or from ovulated eggs; (ii) under the influence of soluble factor(s) released during the in-vitro maturation period by antral granulosa cells, the surface morphology of oocytes derived from early antral follicles resembles that of pre-ovulatory oocytes; and (iii) the stage of antral follicle development does not interfere with oocyte capacity to normally distribute CG in the cortex, since their final distribution, evidenced by the presence of a CG-free domain, and density are similar between ovulated eggs and all the classes of in-vitro matured antral oocytes analysed here.

Our observations showed that only at the end of a complete antral follicle development does the oocyte acquire the capacity to structurally modify its surface morphology into a mature configuration. This capacity is dependent upon the action of soluble factor(s) released by antral granulosa cells. In fact, under the influence of factor(s) specifically produced by antral granulosa cells, and not by fibroblasts, the oocytes derived from follicles at the beginning of antral development undergo surface changes, resulting in a microvillar morphology typical of pre-ovulatory oocytes and of ovulated eggs. In a previous report, we demonstrated that the kinetics of sperm–oocyte fusion showed by denuded oocytes were significantly slower than those of pre-ovulatory oocytes (Cecconi et al., 1996a).

By considering that a specific microvillar organization is required for an efficient sperm–oocyte interaction (Johnson et al., 1975; Philipps and Shalgi, 1980; Longo and Chen, 1981), our observations suggest that a specific microvillar organization is required for an efficient sperm–oocyte interaction (Johnson et al., 1975; Philipps and Shalgi, 1980; Longo and Chen, 1981).
1985; Myles, 1993; Green, 1993), the altered appearance of the DO microvillar apparatus may account for the delay of sperm–oocyte fusion exhibited by these oocytes, probably by interfering with the normal juxtapositioning of gametes.

The possibility that early antral oocyte plasma membranes may be characterized by a different composition with respect to those of pre-ovulatory oocytes cannot be excluded, even if in this paper we did not test this possibility. Indeed, it has been reported that ovulated mouse-egg plasma membranes show the existence of a mosaicism between the microvillar and amicrovillar regions, clearly evidenced by the presence of different Con-A binding sites (Johnson et al., 1975), different membrane protein diffusibility (Wolf and Ziomek, 1983), and an asymmetric pattern of integrin distribution (Tarone et al., 1993; Evans et al., 1995). However, the hypothesis that the low-fusion kinetics showed by demuded oocytes could be due to a different oolemma morphology is supported by the finding that the acquisition of a pre-ovulatory-like surface organization by denuded oocytes matured in the presence of antral granulosa cells corresponds to a significant improvement in their oolemma fusibility (Cecconi et al., 1996a). This result further highlights the role played by paracrine factor(s) in the acquisition/ regulation of specific activities of the follicle. Indeed, while the oocyte releases factor(s) essential for granulosa cell proliferation (Vanderhyden et al., 1992), steroidogenesis (Vanderhyden et al., 1993), cumulus expansion (Buccione et al., 1990; Salustri et al., 1990) and plasmogen activator inhibition (Canipari et al., 1995), granulosa cell-released factor(s) are specifically involved in the regulation of oocyte growth (Packer et al., 1994; Cecconi et al., 1996b) as well as in the acquisition of a mature oolemma (present results) and full oocyte fertilizability (Vanderhyden and Armstrong, 1989; Cecconi et al., 1996a).

With regard to CG distribution, previous reports (Nicosia et al., 1977; Ducibella et al., 1988) demonstrated that, in ovulated mouse eggs, CG are characteristically localized in the cortex with the exception of the region overlying the metaphase spindle (cortical granule-free domain). Our results on ovulated eggs are entirely consistent with those reported by Ducibella et al. (1988, 1990) and, in addition, demonstrate that this normal CG distribution characterizes all of the classes of antral oocytes analysed here. Therefore, this mouse oocyte ability can be achieved independently of the stage of antral follicle development and the maintenance of functional interactions between oocytes and surrounding cumulus cells during meiotic maturation. However, since cytoplasmic maturation of denuded oocytes, evaluated as the capacity to transform a sperm nucleus into a male pronucleus, is significantly lower with respect to that of pre-ovulatory oocytes (Cecconi et al., 1996a), a peripheral localization of CG cannot be considered indicative of a normal ooplasmic maturation.

In conclusion, our results demonstrated that in mouse oocytes the acquisition of a fusion-competent plasma membrane requires complete antral follicle development, and that this property is specifically regulated by antral granulosa cell-released factor(s). The fact that oocytes derived from incompletely developed antral follicles can also acquire a mature oolemma configuration, when matured in the presence of these factor(s), indicates that co-culture systems can contribute to the realization of a more suitable micro-environment for the in-vitro maturation of mammalian oocytes to be utilized for in-vitro fertilization.

Acknowledgements

The authors thank Dr S.Ullisse for critical reading of the manuscript, Prof. Floriana Rosati for her comments concerning TEM, Mrs L.Corona and Dr M.Di Cola for SEM observations, and Dr M. Giammatteo for confocal analysis observations. The work was supported by MURST 60% (to S.C. and R.C.), MURST 40% (to R.C.) and CNR (to R.C. and R.T.).

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Received on March 23, 1998; accepted on June 30, 1998