Zygote **10** (February), pp 59–64. © 2002 Cambridge University Press Printed in the United Kingdom

Oocyte Ca^{2+} spike acquisition during *in vitro* development of early preantral follicles: influence of age and hormonal supplementation

Odile G. Martins[1,2], Arlette Pesty[2], António Gouveia-Oliveira[3], António J. Cidadão[1], Carlos E. Plancha[1] and Brigitte Lefèvre[2]
Faculdade de Medicina de Lisboa, Lisbon, Portugal and INSERM Unité 355, Clamart, France

Date submitted: 1.6.01. Date accepted: 26.9.01

Summary

Calcium signalling is involved in important events in oocytes, such as meiotic competence acquisition. We have previously demonstrated the positive influence of animal age and gonadotropin stimulation *in vivo* regarding the ability of oocytes recovered from preantral follicles to exhibit calcium spikes. In the present work we determined whether preantral follicle development *in vitro* also allows oocytes to acquire calcium signalling activity. We also aimed to verify the influence of animal age, FSH + LH and/or insulin on oocyte calcium spike acquisition during preantral follicle culture. Early preantral follicles were isolated from 12-day-old and 1- to 3-month-old F_1 hybrid mice and cultured individually for either 2 or 6 days. At the end of the culture period the oocytes were processed for calcium imaging by confocal microscopy. We show that oocytes recovered from cultured preantral follicles exhibit variable calcium spike activity rates, depending on animal age, culture duration and hormonal supplementation. Oocytes recovered from adult animals continue to exhibit calcium spikes, and those recovered from juveniles acquire that activity after culture. Insulin and gonadotropins in combination account for an early and maintained inhibitory effect on calcium signalling acquisition by oocytes. Insulin alone also leads to an early inhibitory effect, which, however, disappears with longer culture periods. Contrary to the complex *in vivo* situation, the acquisition of calcium signalling by oocytes in a controlled *in vitro* environment does not seem to be dependent on gonadotropins alone.

Keywords: Calcium signalling, Follicle culture, FSH+LH, Gonadotropins, Growing oocytes, Insulin

Introduction

It has been shown that the acquisition of meiotic competence during folliculogenesis is related to the establishment of a fully functional IP_3-dependent calcium signalling pathway in the mouse oocyte (Carroll *et al*., 1994; Lefèvre *et al*., 1997). A detailed evaluation of Ca^{2+} spike activity in growing oocytes is necessary for understanding the mechanisms controlling acquisition of a functional calcium signalling pathway.

All correspondence to: Carlos E. Plancha, Instituto de Histologia e Embriologia, Faculdade de Medicina de Lisboa, Av. Prof. Egas Moniz, 1649–028 Lisbon, Portugal. Tel: +351 217 934 340. Fax: +351 217 951 780. e-mail: mcravo.cplancha@ mail.telepac.pt
[1]Instituto de Histologia e Embriologia, Faculdade de Medicina de Lisboa, Lisbon, Portugal.
[2]INSERM Unité 355, Maturation Gamétique et Fécondation and IFR 13, Clamart, France.
[3]Laboratório de Biomatemática, Faculdade de Medicina de Lisboa, Lisbon, Portugal.

In a previous work we showed that both age and exogenous gonadotropin stimulation *in vivo* increased the ability of oocytes recovered from preantral follicles to exhibit spontaneous Ca^{2+} spikes (Gomes *et al*., 1999*a*). In the present work we wanted to verify whether oocytes recovered from preantral follicles were able to acquire calcium activity during follicle culture. We also aimed to discover whether the reported age differences between oocytes were retained in our follicle culture system. Finally, the influence of gonadotropins and/or insulin was also analysed.

Materials and methods

Animals

Experiments were done with F_1 (C57B16 × CBA) juvenile (12-day-old) and adult (1- to 3-month-old) female mice, with a minimum of 4 animals per

https://doi.org/10.1017/S0967199402002083 Published online by Cambridge University Press

experimental group. Animals were killed by cervical dislocation.

Isolation and culture of preantral follicles

Ovaries were removed and mechanical dissection of preantral follicles was performed with fine 25 gauge sterile needles at 37 °C in Eagle MEM medium with Hank's salt and HEPES (25 mM) without L-glutamine (Gibco BRL), supplemented with penicillin 100 IU/ml (Gibco BRL), streptomycin 100 µg/ml (Gibco BRL) and 10% fetal bovine serum (FBS; Gibco BRL, Life Technologies, USA). All these procedures were performed under a Leica MZ12 stereomicroscope. Preantral ovarian follicles 100–130 µm in diameter with the theca layer, two or three granulosa cell layers, and no signs of isolation damage, atresia or oocyte abnormalities, were selected. Follicle horizontal diameters were measured perpendicularly at the beginning of culture with a precalibrated ocular micrometer, taking the basal lamina as reference. Follicle horizontal diameters are expressed as the mean of the two perpendicular measurements.

The culture system used was based on the work of Cortvrindt *et al*. (1996). Follicles were cultured individually in 60-well plates (Nunc, Intermed, Denmark) in 20 µl of Alpha MEM medium, covered with mineral oil (Sigma). The medium was supplemented with 5 µg/ml transferrin (Sigma), 5 µg/ml sodium selenite (Sigma), 0.2 mM sodium pyruvate (Sigma), 2 mM L-glutamine (Gibco BRL), penicillin 100 IU/ml, streptomycin 100 IU/ml and 10% FBS in 5% CO_2 air at 37 °C for 2 and 6 days. During culture, the medium was partially refreshed every 2 days. For each age group, four culture conditions were tested: control (no insulin, no gonadotropins), insulin only (5 µg/ml, Sigma), gonadotropins only (hrFSH, 100 mIU/ml, Gonal-F75, Serono; hrLH, 10 mIU/ml, LHADI, Serono) and insulin plus gonadotropins.

Isolation and fluorescent calcium indicator loading of the oocytes

At the end of the culture period, the oocytes were mechanically released from their follicles with fine 25 gauge sterile needles. The remaining granulosa cells were carefully removed by repeated aspirations through a fine glass pipette. Oocytes were then processed for calcium imaging within 40 min. All procedures were performed in M2 medium (Sigma, St Quentin Fallavier, France), in plastic Petri dishes, under a Leica MA12 stereomicroscope, at 37 °C. The cell-permeant fluorescent calcium indicator used was fluo-3/aceteoxymethyl-ester (fluo-3/AM; Molecular Probes, Eugene, OR), which was stored at –20 °C diluted (1 mM) in a 20% Pluronic F-127 solution in dimethyl sulfoxide (DMSO; Molecular Probes). Oocytes were placed in a drop of the fluo-3/AM dissolved to a final concentration of 5 mM in M2 medium, under mineral oil, and incubated in the dark for 15 min at 37 °C. They were then rinsed and placed in a fresh drop of M2 medium, under mineral oil, in a cell-culture chamber (Perfusion Open Closed chamber, Helmut Saur, Reutlingen, Germany) for subsequent confocal microscopic observation. All oocytes analysed were at the germinal vesicle (GV) stage. Oocyte diameters (excluding zona pellucida) were measured using the length profile utility of the Bio-Rad LaserSharp software, and expressed as the mean of two perpendicular measurements.

Confocal microscopy of living oocytes

All measurements of fluorescence emission were performed using a Bio-Rad MCR600 confocal laser scanning imaging system interfaced with a Nikon Diaphot inverted microscope as described previously (Gomes *et al*., 1999*a*). To perform time-lapse calcium imaging studies, dye-loaded oocytes were viewed through a ×20 objective (Fluor 20/0.75) at a single optical plane through the germinal vesicle. Calcium kinetics images of the oocytes were monitored continuously for 30–40 min with a 2–3 s interval between consecutive acquisitions. Full-screen sequential images were recorded for analysis, and a time-course curve of calcium indicator fluorescence emission was constructed using the Time Course Software for ratiometric Measurement (TCSM, Bio-Rad). Fluorescence intensity was measured in rectangles of similar areas (about 200 pixels) positioned in the oocyte cytoplasm. On these kinetic curves, data are expressed in arbitrary units uncalibrated in terms of calcium concentration.

Data collection and statistical procedure

We considered calcium activity to have occurred when an oocyte exhibited two or more Ca^{2+} spikes. The observation of only one spike during the evaluation period was interpreted as equivalent to the absence of Ca^{2+} spikes. The proportion of oocytes generating two or more Ca^{2+} spikes was calculated for each group and results were compared by Fisher's test using Intercooled Stata 6.0 software. Differences were considered significant at $p < 0.05$.

Results and discussion

Preantral follicles at the same developmental stage (two or three granulosa cell layers, 100–130 µm in diameter) were mechanically isolated from juvenile (12-day-old; n = 38) and adult (1- to 3-month-old;

https://doi.org/10.1017/S0967199402002083 Published online by Cambridge University Press

n = 62) animals. Follicles had a mean diameter of 114.6 ± 8.4 µm (n = 1592). Oocytes released from these preantral follicles were all at the GV stage with similar mean diameters (Table 1). Diameters were measured excluding zona pellucida. Whereas during the first 2 days of follicle culture the oocytes did not grow, their mean diameters increased significantly during the following days in culture. Neither hormonal supplementation of the medium nor animal age had an effect on oocyte growth (Table 1; see also Eppig *et al*., 2000).

As already shown in our previous study (Gomes *et al*., 1999*a*), functional differences as assessed by calcium activity occur between oocytes freshly isolated from juveniles and oocytes freshly isolated from adults. While all the oocytes recovered from non-cultured preantral follicles in juvenile mice were silent, 15% of those recovered from preantral follicles in adults exhibited two or more Ca^{2+} spikes during the 30–40 min period of data recording (Fig. 1). Maintaining early preantral follicles for 2 days in culture without hormonal supplementation allowed more oocytes to acquire a calcium activity, but the difference between the two age groups remained. In fact, after 2 days of culture within isolated preantral follicles, 70.8% of oocytes from adults exhibited two or more Ca^{2+} spikes during the observation period while only 21.7% of oocytes from juveniles showed the same type of signalling (Fig. 1*A*). Maintaining early preantral

Table 1 Mean oocyte diameters (± SD) related to female age, hormonal supplementation of preantral follicle culture medium and culture duration

Culture medium containing:	Juveniles Day 0 (43) 56.6 ± 3.9		Adults Day 0 (99) 61.3 ± 4.3	
	Day 2	Day 6	Day 2	Day 6
GO –/ insulin –	(61) 57.1 ± 2.5	(56) 64.0 ± 2.8	(62) 58.0 ± 3.3	(80) 64.2 ± 3.6
GO – / insulin +	(54) 55.7 ± 2.9	(68) 63.2 ± 3.4	(75) 59.8 ± 4.5	(44) 64.1 ± 3.2
GO + / insulin –	(50) 58.1 ± 2.4	(64) 63.9 ± 3.2	(72) 58.4 ± 4.0	(86) 66.4 ± 3.4
GO + / insulin +	(90) 57.7 ± 3.3	(85) 62.3 ± 3.5	(52) 61.8 ± 3.7	(63) 65.8 ± 4.1
Combined treatments	(255) 57.2 ± 3.0	(273) 63.2 ± 3.4	(209) 58.8 ± 4.1	(273) 65.3 ± 3.8

GO, gonadotropins: human recombinant follicle stimulating hormone and luteinising hormone (hrFSH + hrLH).
Number of oocytes evaluated is given in parentheses.

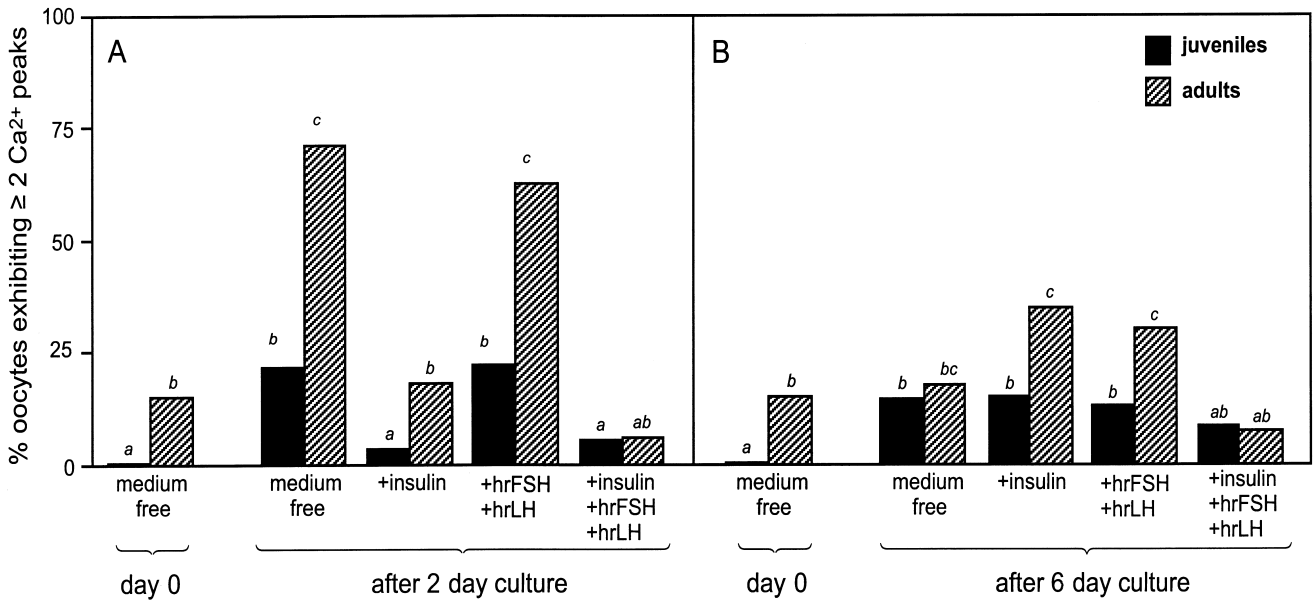

Figure 1 Percentage of oocytes exhibiting two or more Ca^{2+} spikes, in relation to female age, the hormonal supplementation of the preantral follicle culture medium and the time in culture. Culture conditions: no hormonal supplementation (medium free); + insulin alone, 5 µg/ml; + gonadotropins only (hrFSH, 100 mIU/ml; hrFSH, 10 mIU/ml); insulin + gonadotropins. Day 0, day of follicle isolation, without culture. For each panel (*A* or *B*), the groups are significantly different ($p < 0.05$) when there are no common letters over the bars.

https://doi.org/10.1017/S0967199402002083 Published online by Cambridge University Press

follicles for 6 days in culture without hormonal supplementation did not allow oocytes from juvenile mice to acquire more calcium activity than after a 2 day culture period, the percentage of oocytes exhibiting calcium signalling (14.3%, n = 56) being not significantly different from that observed after 2 days in culture (21.7%, n = 60) (Fig. 1). However, in the adult group, after a 6 day culture period the percentage of oocytes with calcium activity (17.4%, n = 69) was significantly smaller than that at day 2 (70.8%, n = 72) and similar to the percentage observed before culture (15.0%, n = 100) (Fig. 1). As a consequence, after 6 days of culture without hormonal supplementation the difference between the two age groups is lost.

In our preantral follicle culture system, insulin supplementation for 2 days strongly reduced calcium activity in oocytes from both age groups, to 3.7% in juveniles (n = 54) and 18.0% in adults (n = 89), leading to values similar to those observed before culture (Fig. 1*A*). After 6 days of culture we did not detect any significant effect of insulin supplementation in either juvenile or adult groups (Fig. 1*B*). The age difference observed prior to culture is always maintained in the presence of insulin. Regardless of animal age and culture duration, gonadotropin supplementation had no significant effect on the acquisition of calcium activity by the oocytes (Fig. 1). Consequently, the difference between juvenile and adult groups is also maintained in culture in the presence of gonadotropins. In our system the combined action of insulin and gonadotropins resulted in a marked inhibition of calcium activity in oocytes, yielding values similar to those observed before culture (Fig. 1). Interestingly, the difference between the two age groups is always lost in the combined presence of insulin and gonadotropins.

In this study, the Ca^{2+} spikes emitted by oocytes recovered from preantral follicles generally had irregular frequencies and irregular peak profiles (Fig. 2*A*, *B*). However, a subpopulation of oocytes from all groups began to exhibit regular spontaneous Ca^{2+} oscillations (considered as at least four Ca^{2+} spikes with a regular frequency during the 30–40 min of data collection) (Fig. 2*C*, *D*). In addition we noticed double Ca^{2+} spike profiles in some oocytes, as illustrated in Fig. 2*C*. These activities are interesting since they could represent an intermediate form of calcium signalling before full maturation of the phosphoinositide/calcium signalling pathway.

With this study we have confirmed and extended our previous findings demonstrating that preantral follicles with two or three granulosa cell layers freshly removed from juvenile and adult animals, although at the same developmental stage, contain oocytes with different Ca^{2+} spike activity (Gomes *et al*., 1999*a*). We have shown that oocytes recovered from cultured preantral follicles exhibit variable Ca^{2+} spike activity rates, depending on animal age, culture duration and hormonal supplementation.

The percentage of oocytes removed from our defined preantral follicle population that exhibit Ca^{2+} spikes increases with age and with *in vivo* pregnant male serum gonadotropin (PMSG) stimulation (Gomes *et al*., 1999*a*). The absence of response to gonadotropins that we observed in our culture system is consistent with the involvement of an intra-ovarian regulatory pathway, as previously suggested. Specifically, we favour an indirect mechanism for PMSG stimulation resulting from the release of local factors from PMSG-responsive follicles at more advanced stages. These factors would then act in a paracrine way to stimulate the population of preantral follicles under study (Gomes *et al*., 1999*a*). Our *in vitro* study uses a culture system where individual follicles are maintained in a controlled medium. Under these conditions, paracrine signalling between follicles is avoided and thus only intrafollicular effects of hormonal supplements can be tested. In other words, only in the case of direct gonadotropin action over our population of preantral follicles would we observe an effect. Further studies using group follicle culture approaches, namely co-culture of follicles at different developmental stages, should allow better understanding of interfollicular effects regarding the acquisition of calcium signalling by oocytes.

The acquisition of calcium signalling by oocytes *in vitro* is always impaired by the cumulative effect of insulin and gonadotropins. However, this inhibition of calcium signalling acquisition is seen with insulin alone, which agrees with the above-described lack of response of this preantral follicle population to gonadotropins in our culture system. The only difference found between supplementation with insulin alone and in combination with gonadotropins was the abolition of the age group differences in this latter culture condition. Taking into account that maintenance of age group differences may be relevant for proper follicular and oocyte development, this observation is consistent with the report of Eppig *et al*. (1998). That study demonstrated that addition of FSH to medium containing insulin had a deleterious effect on the percentage of mature oocytes competent to develop to the blastocyst stage (for review see Eppig *et al*., 2000). It was suggested that such a deleterious effect could result from the precocious differentiation of granulosa cells (Latham *et al*., 1999; Eppig *et al*., 2000). In future studies, it will be important to assess whether the long-term deleterious combined effect of insulin and gonadotropins on the 2-cell to blastocyst transition is related to the early inhibition of Ca^{2+} spike acquisition we report in this study.

Insulin is generally added to oocyte/COC/follicle cultures in order to promote a more efficient and

https://doi.org/10.1017/S0967199402002083 Published online by Cambridge University Press

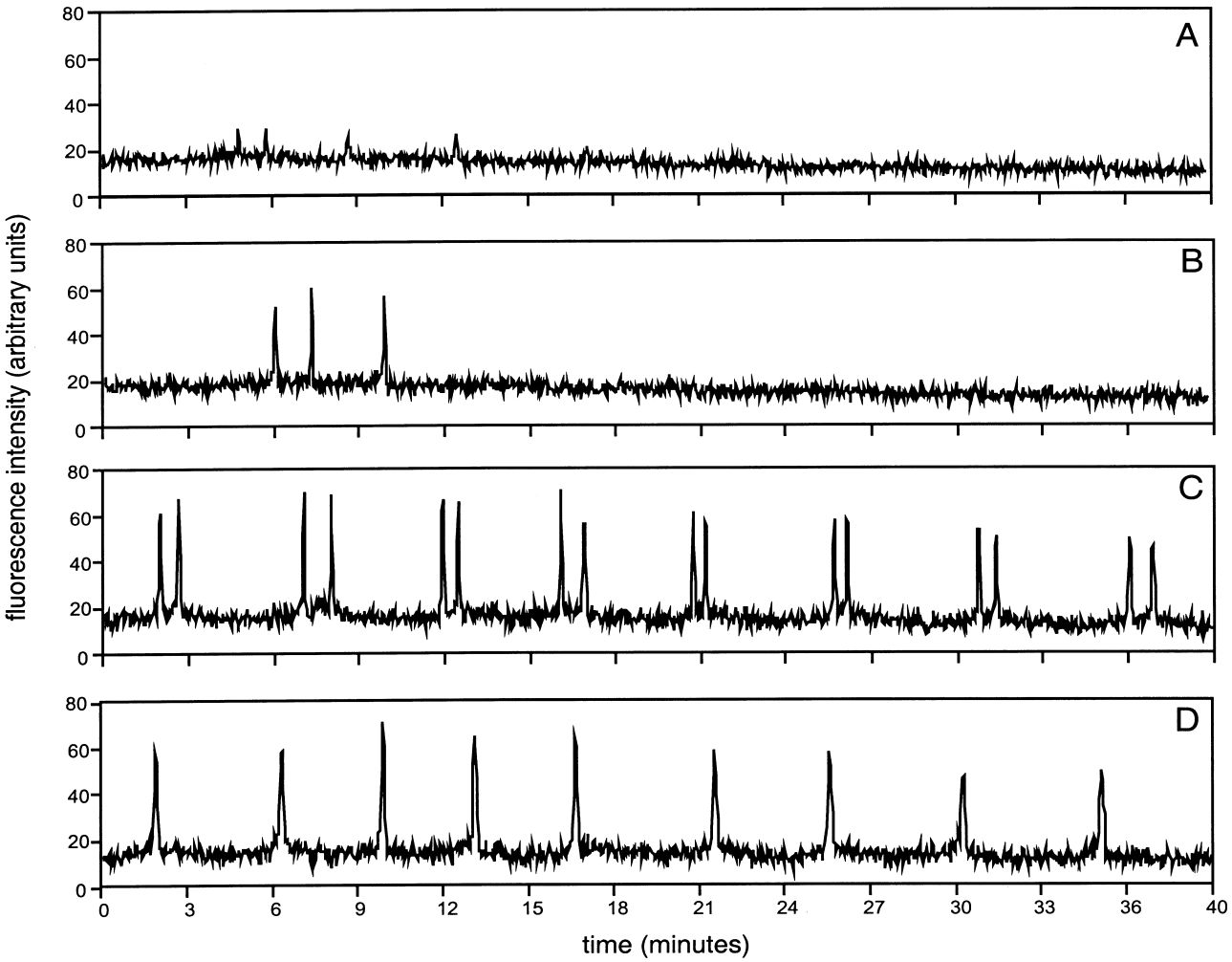

Figure 2 Different types of oocyte calcium activity. (*A*) No calcium activity (≤ 1 Ca^{2+} spike/30–40 min); (*B*) spontaneous calcium activity (≥ 2 Ca^{2+} spikes/30–40 min); (*C*) spontaneous regular calcium activity with double Ca^{2+} peaks (≥ 4 Ca^{2+} spikes/30–40 min regularly distributed); (*D*) spontaneous regular calcium activity with single Ca^{2+} peaks (≥ 4 Ca^{2+} spikes/30–40 min regularly distributed).

robust development of the female gamete (Eppig *et al.*, 1992, 1998). Our data point to an early inhibitory effect of insulin regarding *in vitro* acquisition of calcium signalling activity. It remains to be determined whether such early inhibition, maintaining oocyte calcium activity at a low level, could account for the beneficial effects of insulin supplementation. Indeed, the calcium activity observed after 2 days in culture could be too precocious and thus deleterious for the events that are occurring.

Oocyte calcium activity in germinal vesicle (GV) oocytes seems to develop from initially no activity in growing oocytes from very early preantral follicles, passes through a stage of rare Ca^{2+} spikes, and reaches clear calcium oscillations in fully grown oocytes recovered from antral follicles (Carroll *et al.*, 1994; Lefèvre *et al.*, 1997). Detection of calcium activity could be a valuable new way to functionally evaluate GV oocytes before meiotic competence acquisition. In the present study we were able to demonstrate what may be intermediate patterns of calcium activity, such as the regular double and single spikes. We are still far from understanding the molecular basis behind these patterns, although again we speculate that they may relate to the progressive functionality of the phosphoinositide/calcium signalling pathway (Gomes *et al.*, 1999*a*: Lefèvre *et al.*, 1997). The use of extended culture periods in three-dimensional matrices as a better approach to mimicking the complex ovarian microenvironment (Hartshorne, 1997; Gomes *et al.*, 1999*b*) could potentially allow the characterisation of such intermediate steps before acquisition by the oocyte of a fully functional phosphoinositide/calcium signalling pathway.

https://doi.org/10.1017/S0967199402002083 Published online by Cambridge University Press

Acknowledgements

The experimental part of this work was performed in Clamart, at INSERM unit 355, supervised by Jacques Testart. O.G.M. received a research scholarship from INSERM/ICCTI.

References

Carroll, J., Swann, K., Whittingham, D. & Whitaker, M. (1994). Spatiotemporal dynamics of intracellular [Ca^{2+}] oscillations during the growth and meiotic maturation of mouse oocytes. *Development* **120**, 3507–17.

Cortvrindt, R., Smitz, J. & Van Steirteghem, A.C. (1996). *In-vitro* maturation, fertilization and embryo development of immature oocytes from early preantral follicles from prepubertal mice in a simplified culture system. *Hum. Reprod.* **11**, 2656–66.

Eppig, J.J., Wigglesworth, K. & O'Brien, M.J. (1992). Comparison of embryonic developmental competence of mouse oocytes grown with and without serum. *Mol. Reprod. Dev.* **32**, 33–40.

Eppig, J.J., O'Brien, M.J., Pendola, F.L. & Watanabe, S. (1998). Factors affecting the developmental competence of mouse oocytes grown *in-vitro*: follicle-stimulating hormone and insulin. *Biol. Reprod.* **59**, 1445–53.

Eppig, J.J., Hosoe, M., O'Brien, M.J., Pendola, F.M., Requena, A. & Watanabe, S. (2000). Conditions that affect acquisition of developmental competence by mouse oocytes grown *in vitro*: FSH, insulin, glucose and ascorbic acid. *Mol. Cell Endocrinol.* **163**, 109–16.

Gomes, J.E., Pesty, A., Gouveia-Oliveira, A., Cidadão, A.J., Plancha, C.E. & Lefèvre, B. (1999*a*). Age and gonadotropins control Ca^{2+}-spike acquisition in mouse oocytes isolated from early preantral follicles. *Int. J. Dev. Biol.* **43**, 839–42.

Gomes, J.E., Correia, S.C., Gouveia-Oliveira, A., Cidadão, A.J. & Plancha, C.E. (1999*b*). Three-dimensional environments preserve extracellular matrix compartments of ovarian follicles and increase FSH-dependent growth. *Mol. Reprod. Dev.* **54**, 163–72.

Hartshorne, G.M. (1997). *In vitro* culture of ovarian follicles. *Rev. Reprod.* **2**, 94–104.

Latham, K.E., Bautista, F.D.M., Hirao, Y., O'Brien, M.J. & Eppig, J.J. (1999). Comparison of protein synthesis patterns in mouse cumulus cells and mural granulosa cells: effects of follicle-stimulating hormone and insulin on granulosa cell differentiation *in-vitro*. *Biol. Reprod.* **62**, 482–92.

Lefèvre, B., Nagyova, E., Pesty, A. & Testart, J. (1997). Acquisition of meiotic competence is related to the functionality of the phosphoinositide/calcium signalling pathway in the mouse oocyte. *Exp. Cell Res.* **236**, 193–200.