The ability to generate normal Ca\textsuperscript{2+} transients in response to spermatozoa develops during the final stages of oocyte growth and maturation

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Intracellular Ca\textsuperscript{2+} oscillations at fertilization are responsible for triggering egg activation. The aim of this study was to examine the effect of the age of the oocyte donor and in-vitro maturation on the generation of Ca\textsuperscript{2+} transients at fertilization. The results show that <10% of in-vivo and in-vitro matured oocytes from 19-day old mice develop to the blastocyst stage \textit{in vitro}. In contrast, 43% of in-vivo and 25% of in-vitro matured oocytes from 24-day old mice developed to the blastocyst stage. In parallel experiments, intracellular Ca\textsuperscript{2+} was monitored at fertilization. Oocytes from 19-day old mice generate significantly fewer transients than oocytes from 24-day old mice. In-vitro maturation significantly decreased the ability of oocytes from 19-day old mice but not 24-day old mice to generate Ca\textsuperscript{2+} transients in response to spermatozoa. Furthermore, we investigated the effect of oocyte maturation on Ca\textsuperscript{2+} signalling. Immature oocytes generated fewer Ca\textsuperscript{2+} oscillations and ceased oscillating earlier than mature oocytes. These studies suggest that the ability to generate Ca\textsuperscript{2+} transients in response to spermatozoa increases in the final stages of oocyte development and during oocyte maturation. This may contribute to the acquisition of developmental competence in the final stages of oogenesis.

Key words: calcium/fertilization/in vitro/mouse/oocyte

Introduction

The fertilization and development to term of oocytes matured \textit{in vitro} has been described for a number of mammalian species including humans (for review see Trounson et al., 1998). Nevertheless, development of in-vitro matured oocytes from all species, except possibly the mouse (Schroeder and Eppig, 1984), remains low compared to in-vivo matured oocytes (Barnes et al., 1996). Compromised development of in-vitro matured oocytes is likely to be related to the complex nuclear and cytoplasmic changes that occur during oocyte maturation. Oocyte maturation involves progression through the meiotic cell cycle from the first meiotic prophase to metaphase of the second meiotic division (MII). In addition to these cell cycle changes, a number of modifications in the cytoplasm are necessary in order that the oocyte can support fertilization and early development. These include the development of the capacity to decondense sperm chromatin, an increase in the levels of glutathione (Perreault et al., 1988), the capacity to release cortical granules (Ducibella et al., 1988) and an increase in the ability of the oocyte to release Ca\textsuperscript{2+} from intracellular stores (Carroll et al., 1996, for review). The decreased developmental capacity seen after in-vitro maturation may relate to deficiencies in either the cell cycle or the cytoplasmic events of maturation. It has been appreciated for many years that meiotic maturation occurs spontaneously on removal of the oocyte from an inhibitory follicular environment (Pincus and Enzman, 1935; Edwards, 1965). Furthermore, it is reasonable to expect that >80% of immature oocytes released from antral follicles progress to MII. Therefore progression through meiosis is not the major limiting factor in obtaining development after in-vitro maturation. Rather, the limitations appear to lie in components of the cytoplasm responsible for supporting the events of fertilization and early development.

The trigger that stimulates the transformation of the oocyte into a developing embryo is a sperm-induced increase in intracellular Ca\textsuperscript{2+} at the time of fertilization. In mammals, the increase in Ca\textsuperscript{2+} originates from intracellular stores and takes the form of a series of Ca\textsuperscript{2+} oscillations that continue for 3–4 h (Cuthbertson and Cobbold, 1985; Miyazaki et al., 1986; Kline and Kline, 1992; Jones et al., 1995). The repetitive nature of this signal is essential for complete oocyte activation. A single sperm-induced Ca\textsuperscript{2+} increase is insufficient to stimulate the completion of meiosis as indicated by the extrusion of the second polar body (Kline and Kline, 1992; Ozil and Swann, 1995). More recent studies indicate that multiple sperm-induced Ca\textsuperscript{2+} oscillations are necessary to ensure progression into the first embryonic cell cycle (Lawrence et al., 1998). The requirement of repetitive Ca\textsuperscript{2+} transients at fertilization is most likely due to the need to stimulate and maintain the degradation of cyclin B and hence ensure exit from meiosis and entry into interphase of the first mitotic division (Collas et al., 1995). Given the central role of sperm-induced Ca\textsuperscript{2+} transients in the initiation of early development, it is essential that the oocyte develops the capacity to respond to spermatozoa in such a manner.

The development of Ca\textsuperscript{2+} release mechanisms during oocyte maturation represents a major change in the physiology of the oocyte and ensures the appropriate response to the fertilizing spermatozoa. Ca\textsuperscript{2+} transients in response to spermatozoa, sperm factors, inositol trisphosphate and Ca\textsuperscript{2+} ionophores have all been shown to increase during oocyte maturation (for review see Carroll et al., 1996). A number of mechanisms have been proposed to explain these modifications, including
an increase in the levels and changes in the regulation of the inositol trisphosphate receptor (InsP3 R) (Fujiwara et al., 1993; Mehlman and Kline, 1994; Jones et al., 1995), changes in the structure of the endoplasmic reticulum (ER) (Mehlman et al., 1995), or changes in the size of the Ca\(^{2+}\) store itself (Carroll et al., 1994; Mehlman and Kline, 1994; Jones et al., 1995; Herbert et al., 1997).

Although it is well established that Ca\(^{2+}\) signalling systems are modified during oocyte maturation, there is considerable discrepancy between studies as to the extent of these changes (Mehlman and Kline, 1994; Jones et al., 1995). In addition, it is not known when during oogenesis the developing oocyte becomes competent to undergo these maturation-associated changes or whether they are affected by the conditions in which the oocyte is matured. Oocytes recovered from juvenile mice provide an excellent model system to investigate these questions, as they provide a relatively homogeneous cohort of oocytes of increasing developmental capacity (Eppig and Schroeder, 1989). Using this system, it has been established that the developmental competence of oocytes recovered from mice between the ages of 16–24 days increases with the age of the oocyte donor (Eppig and Schroeder, 1989). In order to determine if different patterns of Ca\(^{2+}\) signalling may account for these developmental differences, we have examined the ability of ovulated and in-vitro matured oocytes from mice of 19 and 24 days of age to generate Ca\(^{2+}\) transients in response to spermatozoa.

Materials and methods

Collection of oocytes

Immature and ovulated oocytes were collected from hormone-primed MF1 mice that were 17 or 22 days of age at the time of administering pregnant mare’s serum gonadotrophin (PMSG). For the collection of immature oocytes ovaries were collected 48 h after administration of 5 IU of PMSG when the mice were 19 or 24 days of age. The oocytes were released from the ovary into M2 (Fulton and Whittingham, 1978) culture medium at 37°C. Oocytes with a germinal vesicle and surrounded by cumulus cells were collected and washed in Minimum Essential Medium (MEM) containing 5% fetal calf serum (FCS). The oocytes were incubated for 16 h in 50 µl drops under paraffin oil at 37°C in a humidified atmosphere of 5% CO\(_2\) in air.

For the collection of ovulated oocytes mice were injected with 5 IU of human chorionic gonadotrophin (HCG) 48–52 h after PMSG. In order to standardize the nomenclature for determining the age of oocyte donors, the age of the donors of mature eggs as the age at which HCG was administered was defined. As such the mice are 19 or 24 days old when the oocytes were recovered. The oviducts were removed 14–16 h after HCG and the cumulus masses were released into M2 containing 0.3 mg hyaluronidase/ml. The cumulus-free oocytes were collected, washed three times in M2 and transferred to a drop of M2 under oil prior to IVF.

IVF and embryo culture

Insemination drops consisted of 100 µl drops of medium T6 containing 10 mg/ml of bovine serum albumin (BSA) (Fraction V, Sigma, Poole, Dorset, UK) under paraffin oil. Mice of proven fertility were killed by cervical dislocation and the epididymides were removed into 1 ml of T6 medium in a Petri dish. Sterile needles were used to puncture each epididymis and release the spermatozoa. The dish was placed in the incubator for 15 min allowing time for the spermatozoa to swim into a suspension. This suspension was diluted 1:10 into a pre-equilibrated insemination drop. The diluted sperm suspension was incubated for a further 2 h before adding the in-vitro matured or ovulated oocytes. The oocytes were incubated with spermatozoa for 4–5 h before the oocytes were collected and washed in CZB medium (Chatot et al., 1989). Development to the 2-cell stage and beyond was performed in 30 µl drops of CZB medium covered with paraffin oil and maintained in the incubator at 37°C in 5% CO\(_2\). In order to support development beyond the morula stage, it was necessary to transfer morulae into CZB containing 5.5 mmol/l glucose on day 3 of culture (Chatot et al., 1990). Throughout the culture period, development was assessed at 24 h intervals for a total of 72 h post-fertilization. At each assessment, the proportion of oocytes in the different stages of development was recorded.

Measurement of intracellular Ca\(^{2+}\)

To monitor changes in the level of intracellular Ca\(^{2+}\), oocytes were loaded with the Ca\(^{2+}\)-sensitive fluorescent dye, Fura-red (Molecular Probes, Eugene, OR, USA). For loading, oocytes were incubated in 2 µmol/l of Fura-red AM for 15 min at 37°C. To improve loading the medium also contained 0.05% pluronic F-127 (Molecular Probes) and to prevent extrusion and compartmentalization of the dye into organelles, 250 µmol/l of the anion pump inhibitor sulphinpyrazone was also included. After loading, the oocytes were removed from the Fura-red and maintained in M2 containing sulphinpyrazone. The zona pellucida was removed by a brief incubation in acidified Tyrode’s medium (pH 2.5, Sigma). The oocytes were washed in M2 and transferred to a heated chamber containing 500 µl of M2 without BSA on the stage of a Nikon Diaphot microscope. A further 500 µl of M2 with BSA and sulphinpyrazone was added to the stage and the medium was covered with oil.

Fura-red was excited sequentially at 440 and 490 nm and the fluorescence was collected using a 20×0.75 NA (numerical aperture) objective. The emitted light was passed through a long pass filter (>510 nm) and the data collected using a Newcastle Photometrics Multipoint system.

Statistics

Comparisons of the proportions of oocytes developing to different stages of development were compared using a χ\(^2\) test. For analysis of the Ca\(^{2+}\) transients the mean numbers of oscillations generated in each group were compared by Student’s t-test and the proportions that continued to oscillate for 1 or 2 h by χ\(^2\) test.

Results

Relationship between age of the oocyte donor, in-vitro maturation, and developmental capacity

In order to determine the effect of the age of the donor and in-vitro maturation on embryo development in our culture system, ovulated and in-vitro matured oocytes from 19- and 24-day old mice were fertilized and cultured in vitro for 72 h. Oocytes from all groups could be fertilized in vitro; however, in-vitro matured oocytes from 19-day old mice showed a significantly decreased rate of development to the 2-cell stage compared to the other groups (57% compared with 67–72%; P < 0.05; Figure 1).

Development in culture beyond the 2-cell stage was significantly affected by both the age of the oocyte donor and the method of maturation. Over 40% of ovulated oocytes from
Ca\textsuperscript{2+} transients in response to spermatozoa

Figure 1. The effect of the age of oocyte donor in-vitro maturation on subsequent development. Ovulated and in-vitro matured (IVM) oocytes from 19-day old and 24-day old mice were fertilized \textit{in vitro} and cultured for 72 h. The proportion of oocytes from each treatment reaching 2-cell stage (filled bars) or morula/blastocyst stage (unfilled bars) is shown. Columns representing the same developmental stage (2-cell or morula/blastocyst stage embryos) with different letters are significantly different ($P < 0.05$). Number of oocytes in each group: 24-day old ovulated, $n = 135$; 24-day old IVM, $n = 99$; 19-day old ovulated, $n = 166$; 19-day old IVM, $n = 100$.

24-day old mice developed to the morula/blastocyst stage compared with 27% after in-vitro maturation ($P < 0.05$). In comparison, $<10\%$ of ovulated and in-vitro matured oocytes from the 19-day old donors reached the morula/blastocyst stage (Figure 1), which was significantly less ($P < 0.05$) than that seen in oocytes from the older donors. This indicated that the age of the oocyte donor was the primary factor in limiting the development of these oocytes.

\textbf{Effect of oocyte donor and in-vitro maturation on the ability to generate Ca\textsuperscript{2+} transients in response to spermatozoa}

To investigate the possibility that oocytes with limited developmental capacity show an abnormal response to spermatozoa, we monitored intracellular Ca\textsuperscript{2+} in ovulated and in-vitro matured oocytes from 19- and 24-day old donors. Although oocytes from all groups were capable of generating Ca\textsuperscript{2+} oscillations in response to spermatozoa, it was clear that some conditions limited the ability to generate Ca\textsuperscript{2+} transients. Representative traces of fertilization-induced Ca\textsuperscript{2+} transients are shown for ovulated oocytes from young and old donors (Figure 2). In order to quantify different Ca\textsuperscript{2+} responses, the mean number of oscillations was measured in the first 2 h (Figure 3A) and the proportion of oocytes that continued oscillating after 1 or 2 h (Figure 3B). Oocytes from older donors generated significantly more oscillations in the first 2 h and continued to oscillate for longer than oocytes from younger donors (Figure 3A,B). In oocytes from 24-day old donors, in-vitro maturation had no effect on the ability to generate Ca\textsuperscript{2+} transients in response to spermatozoa. In contrast, in-vitro maturation significantly decreased the number of Ca\textsuperscript{2+} transients generated in response to spermatozoa in oocytes from younger donors (Figure 3A,B).

\textbf{Oocytes maturing in vitro show a reduced ability to respond to spermatozoa with Ca\textsuperscript{2+} transients}

The above studies indicated that during the final stages of the oocytes’ presence in the follicle the ability to generate Ca\textsuperscript{2+} transients increased. In light of this, and the previous discrepan-
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Figure 3. The effect of the age of the oocyte donor and in-vitro maturation on the ability to generate Ca\(^{2+}\)/H\(_{11001}\) transients in response to spermatozoa. Ovulated and in-vitro matured (IVM) oocytes from 19-day old and 24-day old mice were fertilized in vitro and Ca\(^{2+}\)/H\(_{11001}\) oscillations measured. The mean number of Ca\(^{2+}\)/H\(_{11001}\) oscillations in 2 h, for each oocyte population is shown in (A). In (B) the proportion of oocytes that continue to generate Ca\(^{2+}\) oscillations after 1 h (filled bar) and 2 h (open bar) is shown. Different letters above different columns indicate statistical significance (P < 0.05). Number of oocytes in each sample: 24-day old ovulated, n = 43, 24-day old IVM, n = 28; 19-day old ovulated, n = 30; 19-day old IVM, n = 32.

Figure 4. The ability to generate Ca\(^{2+}\) oscillations in response to spermatozoa increases during oocyte maturation. Representative traces from 24-day old mice of fertilization-induced Ca\(^{2+}\) transients in oocytes undergoing maturation in vitro (A) and in mature oocytes (B). Ca\(^{2+}\) was recorded as described in Materials and methods and Figure 2.

Discussion

The ability of oocytes to develop after fertilization requires that the oocyte has a mature cytoplasm and is arrested at the correct stage of meiosis, i.e. MII. In this study we show that one of the cytoplasmic components of oocyte maturation necessary for development is the ability to respond to spermatozoa with Ca\(^{2+}\) transients. Furthermore, the capacity to respond to spermatozoa is acquired in the final stages of oocyte growth in the follicle and during oocyte maturation.

Acquisition of developmental competence in the final stages of oocyte growth

Our studies confirm previous observations that suggest a gradual acquisition of developmental competence during oocyte growth. We found that oocytes from 19-day old donors cleaved to the 2-cell stage 10–15% less frequently than oocytes derived from 24-day old donors. However, the major difference was apparent in the relative abilities to develop to the blastocyst stage in vitro. Over 40% of ovulated oocytes from 24-day old females developed to the blastocyst stage compared with
Ca\(^{2+}\) transients in response to spermatozoa

A

![Graph showing mean number of oscillations in 2 h for immature and mature oocytes.](image)

B

![Graph showing percentage of oocytes undergoing calcium oscillations for 24-day old immature and mature oocytes.](image)

**Figure 5.** The number and duration of Ca\(^{2+}\) transients generated at fertilization increases during oocyte maturation. Ovulated oocytes and oocytes released from the follicles for 2–3 h were fertilized in vitro and Ca\(^{2+}\) oscillations measured. The mean number of oscillations in the 2 h period after fertilization is shown (A). In (B) the proportion of oocytes continuing to generate Ca\(^{2+}\) transients after 1 h (filled bars) or 2 h (hollow bars) is shown. Columns with different letters are significantly different (P < 0.05). Number of oocytes in each population: 24-day old immature, n = 19; 24-day old mature, n = 43.

<10% for oocytes from 19-day old females. The idea that growing oocytes acquire the ability to progress through the developmental process is well established. Much of this work has been carried out using the juvenile mouse as a model system, where, similar to the present study, the most dramatic effect is the increased ability to develop from 2-cells to blastocysts in culture (Eppig and Schroeder, 1989; Eppig et al., 1994). A major difference between our studies and those of Eppig and Schroeder (Eppig and Schroeder, 1989) is that the donors in our studies were primed with PMSG prior to recovery of the immature oocytes. The finding that the developmental differences are maintained, despite priming with PMSG, suggests that hormonal stimulation of the follicular environment with exogenous gonadotrophins is not capable of improving the developmental competence of oocytes from young mice. This result would indicate that follicles falling into the smaller antral stages contain oocytes that cannot be forced into a developmentally viable state by treatment with gonadotrophins. This is supported by our findings that ovulated and in-vitro matured oocytes from the young donors show a similar low rate of development. Thus it appears that the factors responsible for improving developmental competence accumulate between 19 and 24 days gestation, equivalent to the final few days of follicular development in the mouse. Furthermore, it is apparent that these factors are not induced by hormonal priming and are therefore likely to represent oocyte-specific modifications required for early embryonic development.

**Ability to generate Ca\(^{2+}\) transients in response to spermatozoa increases in the final stages of oocyte growth and during maturation**

A number of oocyte-specific factors are likely to be required for implementing the developmental programme. One necessary contribution to the successful transition from oocyte to embryo is the ability to generate a physiological response to the fertilizing spermatozoon. Here we provide evidence that the failure of oocytes to generate Ca\(^{2+}\) transients in response to spermatozoa may be one of the contributing factors to the poor rates of development in embryos derived from young oocytes. This is suggested by the association of the ability to generate Ca\(^{2+}\) transients in response to spermatozoa and the developmental competence of the oocyte. For example, oocytes from 24-day old donors generate more Ca\(^{2+}\) transients and have a greater developmental capacity than oocytes from 19-day old donors. Support for the idea that Ca\(^{2+}\) transients at activation can influence preimplantation development has been accumulating in recent years. The most dramatic example of this is the finding that the frequency and strength of electric field pulses at the time of oocyte activation dramatically influence the rate of preimplantation development (Ozil, 1990). Other studies have shown that the ratio of inner cell mass to trophectoderm cells can be changed by different patterns of Ca\(^{2+}\) oscillations in the first cell cycle (Bos-Mikich et al., 1997) and that the frequency of Ca\(^{2+}\) transients at activation influences the rate of implantation after embryo transfer (Swann and Ozil, 1994). Although the mechanism of this effect of Ca\(^{2+}\) transients remains unclear, it has been demonstrated that Ca\(^{2+}\) increases can lead to quantitative and qualitative differences in mRNA species in developing embryos (Rout et al., 1997). Further studies are required to determine a causal link between the ability to generate Ca\(^{2+}\) transients in growing oocytes and developmental competence.

Our studies clearly indicate that changes occur in growing oocytes that modify their capacity for releasing Ca\(^{2+}\). Previously it had been demonstrated that dramatic changes in Ca\(^{2+}\) release mechanisms occurred during oocyte maturation (Fujiwara et al., 1993; Carroll et al., 1994; Mehlman and Kline, 1994; Jones et al., 1995). Although all studies have described the same general phenomenon, a decrease in the capacity for Ca\(^{2+}\) signalling in immature oocytes, the effect of oocyte maturation on the ability to generate Ca\(^{2+}\) transients at fertilization was different in different laboratories. Mehlman and Kline (Mehlman and Kline, 1994) described smaller transients that persisted, while Jones et al. (Jones et al., 1995) found a rapid damping of the Ca\(^{2+}\) transients such that only one to three transients were generated. Our revisit to this question confirms that Ca\(^{2+}\) signalling is modified during...
In-vitro maturation can affect the ability to generate Ca$^{2+}$ transients in response to spermatozoa

Since the capacity to generate Ca$^{2+}$ transients in response to spermatozoa increases during oocyte maturation, systems for in-vitro maturation must support this developmental modification. Our study illustrates that in-vitro maturation further reduces the ability of oocytes from young donors to generate Ca$^{2+}$ transients in response to spermatozoa. This further decrease in the ability to release Ca$^{2+}$ may explain the decreased rate of 2-cell formation compared with ovulated controls. As described in the Introduction, there is a strong relationship between Ca$^{2+}$ oscillations and egg activation such that sufficient sperm-induced Ca$^{2+}$ transients are necessary to ensure complete oocyte activation. The requirement for multiple transients is most likely due to the observation that cyclin B is continuously synthesized in the MII oocyte (Kubiak et al., 1993) with the result that maturation promoting factor levels remain high unless Ca$^{2+}$-activated cyclin B degradation is maintained. In order to understand the limitations of in-vitro maturation, it will be necessary to elucidate the exact mechanisms responsible for the changes in Ca$^{2+}$ signalling that occur during oocyte maturation. This knowledge may provide the necessary basis for developing systems for in-vitro maturation that support optimal development.

The findings in this study have implications for the treatment of human infertility. Our results indicate that during the final stages of oocyte growth and during residence in the antral follicle oocytes accumulate factors, or undergo modifications, necessary for preimplantation development. One of these modifications is the ability to respond fully to spermatozoa at the time of fertilization. There are a number of observations illustrating that a significant proportion of apparently unfertilized human oocytes have in fact been penetrated by spermatozoa but have not formed pronuclei (Schmiadi and Kentenich, 1989; Calafel et al., 1991; Van Blerkom et al., 1994). Our results suggest that one possible reason for this developmental arrest is the lack of maternal modifications necessary to generate sufficient Ca$^{2+}$ oscillations in response to spermatozoa. It is also reasonable to conclude from our data that the use of in-vitro maturation systems in IVF programmes, where human oocytes also undergo maturation-associated changes in Ca$^{2+}$ signalling (Herbert et al., 1997), may limit the ability of oocytes to generate Ca$^{2+}$ transients in response to spermatozoa.

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