Calcium responses of human oocytes after intracytoplasmic injection of leukocytes, spermatocytes and round spermatids

Mario Sousa¹, Carmen Mendoza², Alberto Barros³ and Jan Tesarik²,⁴,⁵

¹Laboratory of Cell Biology, Institute of Biomedical Sciences, University of Oporto, Lg. Prof. Abel Salazar 2, 4000 Porto, Portugal, ²Department of Biochemistry and Molecular Biology, University of Granada Faculty of Sciences, Campus Universitario Fuentenueva, 18071 Granada, Spain, ³Laboratory of Medical Genetics, Faculty of Medicine, University of Oporto, Alameda do Prof. Hernani Monteiro, 4200 Porto, Portugal, and ⁴Laboratoire d’Eylau, 55 rue Saint Didier, 75116 Paris, France

To whom correspondence should be addressed at the Laboratoire d’Eylau, 55 rue Saint-Didier, 75116 Paris, France

Oocyte activation in mammals involves the action of a soluble sperm factor (SSF) that enables oocytes to develop a characteristic series of Ca²⁺ spikes (Ca²⁺ oscillations). SSF is also likely to be responsible for the Ca²⁺ oscillations driving oocyte activation after intracytoplasmic sperm injection (ICSI). With an appropriate injection technique, Ca²⁺ oscillations do not develop spontaneously after ICSI but can be triggered by subsequent treatment of sperm-injected oocytes with Ca²⁺ ionophore. Here we show that Ca²⁺ oscillations, quite similar to those developing after ICSI, can be triggered by the ionophore treatment in human oocytes previously injected with human round spermatids. In contrast, oocytes injected with earlier spermatogenic cells (primary and secondary spermatocytes) and with non-germ cells (polymorphonuclear leukocytes) did not develop Ca²⁺ oscillations after the ionophore challenge although the subsequent injection of SSF did induce typical Ca²⁺ oscillations in these oocytes. Disintegration of the plasma membrane of the injected cells was detected in all cases by transmission electron microscopy. Thus, the absence of the typical oscillatory Ca²⁺ response in spermatocyte-injected oocytes was due to the actual deficiency of SSF in the spermatocytes rather than to a defective responsiveness of the injected oocytes or to the failure of SSF release into the oocyte cytoplasm. The ability of human round spermatids to induce a response to calcium in oocytes that is similar to that induced by mature spermatozoa may be important for normal embryonic development after spermatid conception.

Key words: calcium/leukocyte/oocyte activation/spermatid/spermatocyte

Introduction

There are at least two theories to explain how the fertilizing spermatozoon triggers the Ca²⁺ release in oocytes that mediates oocyte activation. One theory implies one or more receptors on the oocyte surface; these receptors are postulated to be coupled to a G-protein or to a protein tyrosine kinase (Ciapa and Epel, 1991; Foltz et al., 1993; Moore et al., 1993; Shilling et al., 1994). However, spermatozoa also contain a soluble sperm factor (SSF) whose injection into oocytes causes oocyte activation (Dale et al., 1985).

In mammals, SSF is believed to facilitate the development of a characteristic series of Ca²⁺ oscillations in oocytes at fertilization by releasing Ca²⁺ from intracellular stores (reviewed by Swann, 1996). A sperm protein displaying this activity when injected into oocytes has been identified (Parrington et al., 1996). SSF is likely to be responsible for the development of Ca²⁺ oscillations in human oocytes after intracytoplasmic sperm injection (ICSI) (Tesarik, 1994; Tesarik et al., 1994). A direct injection of SSF into human oocytes can also induce Ca²⁺ oscillations (Homa and Swann, 1994) and oocyte activation (Dozortsev et al., 1995).

Recent reports on pregnancies and births after transfer of embryos obtained by injecting round and elongated spermatids into human oocytes (Fishel et al., 1995, 1996; Tesarik et al., 1995, 1996; Tesarik and Mendoza, 1996) and by injecting round spermatids and secondary spermatocytes into mouse oocytes (Ogura et al., 1994; Kimura and Yanagimachi, 1995a,b) raise the question of the stage of spermatogenesis at which the SSF activity first appears. In fact, SSF may be incompletely developed or absent in mouse round spermatids as spermatid-injected mouse oocytes require an extra activation stimulus to start normal embryonic development (Kimura and Yanagimachi, 1995a). This question is particularly relevant in light of the current debate concerning the mechanism and biological significance of sperm-induced Ca²⁺ oscillations in mammalian oocytes (Berridge, 1996; Swann and Lawrence, 1996; Tesarik and Sousa, 1996).

It has been shown previously that the action of SSF in human oocytes can be demonstrated by Ca²⁺ ionophore challenge which triggers Ca²⁺ oscillations in sperm-injected, but not sham-injected, human oocytes (Tesarik and Testart, 1994). In the present study, human oocytes donated for research purposes were injected with human spermatogenic cells at different developmental stages (primary spermatocytes, secondary spermatocytes, round spermatids) as well as with
non-spermatogenic cells (polymorphonuclear leukocytes). The Ca\(^{2+}\) responses elicited by the ionophore challenge in oocytes injected with each of these cell types were analysed by confocal laser scanning fluorescence microscopy and compared with those observed in oocytes injected with mature spermatozoa. The morphological status of the injected cells present in the oocyte cytoplasm was evaluated by transmission electron microscopy.

**Experimental design**

When spermatozoa are incorporated into oocytes by ICSI, the subsequent Ca\(^{2+}\) oscillations are presumably due to the action of SSF. In these special circumstances, however, SSF needs a booster to fully develop its activity. This booster can be provided by artificially increasing the oocyte Ca\(^{2+}\) load with the use of a Ca\(^{2+}\) ionophore (Tesariak and Testart, 1994). Alternatively, the intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) can be increased by performing a vigorous ooplasmic aspiration at the time of ICSI (Tesariak and Sousa, 1995a). In the latter case, Ca\(^{2+}\) oscillations develop after a lag period following the initial, aspiration-induced [Ca\(^{2+}\)]\(_i\) increase; however, this initial [Ca\(^{2+}\)]\(_i\) increase is not followed by any secondary [Ca\(^{2+}\)]\(_i\) changes when oocytes are sham-injected with medium alone (Tesariak et al., 1994), confirming the essential role of the release of SSF in the mechanism of the Ca\(^{2+}\) oscillations. On the other hand, when the action of SSF is not boosted by ionophore- or aspiration-induced [Ca\(^{2+}\)]\(_i\) increase, the development of Ca\(^{2+}\) oscillations in sperm-injected oocytes is substantially delayed or absent (Tesariak and Testart, 1994; Tesariak et al., 1994) although the oocyte-activation process can be reactivated by subsequent treatment of the oocytes with Ca\(^{2+}\) ionophore (Tesariak and Sousa, 1995). In this study, we injected different types of cells into oocytes avoiding oocyte cytoplasmic aspiration, thus preventing any SSF released from the sperm from inducing immediate Ca\(^{2+}\) oscillations. At 1 h after the injection, the oocytes were treated with Ca\(^{2+}\) ionophore to boost the Ca\(^{2+}\) oscillations. This experimental design was chosen because of the higher reproducibility of the ionophore-induced increase in Ca\(^{2+}\) oscillations when compared with those provoked by vigorous ooplasmic aspiration. The 1 h delay between injection and ionophore treatment was also needed to enable the oocytes to recover from the injection procedure and to be loaded with the Ca\(^{2+}\) reporter dye. Accordingly, a highly repeatable pattern of Ca\(^{2+}\) response was obtained when control oocytes were injected with mature spermatozoa using this technique, serving as a reference standard for the evaluation of oocyte responses to the other types of cells.

**Materials and methods**

**Cell source, microinjection technique and Ca\(^{2+}\)-monitoring experiments**

All gametes used in this study were donated by patients attending the in-vitro fertilization (IVF) programme of the Laboratory of Medical Genetics of University of Porto Faculty of Medicine. Human oocytes that failed to become fertilized in a clinical IVF protocol, and a limited number of fresh oocytes that were donated by consenting patients for research purposes were used. Round spermatids were isolated from ejaculates and identified as described previously (Tesariak and Mendoza, 1996). Primary and secondary spermatoocytes were isolated from the spermatid-enriched fraction of ejaculates and identified using previously described criteria (Kimura and Yangamichami, 1995b). Briefly, the main criterion for the distinction of individual stages of spermatogenic cells was a cell diameter which was \(\sim 15 \mu m\) for primary spermatoocytes, \(\sim 10 \mu m\) for secondary spermatoocytes and \(\sim 8 \mu m\) for round spermatids. In addition, the presence of developing acrosomal vesicles served to distinguish spermatogenic cells from non-sperm cells. In living cells observed using Hoffman modulation contrast optics, these vesicles appear as bright spots adjacent to the nucleus (Tesariak and Mendoza, 1996). From late pachytene onwards, human primary spermatoocytes contain two distinct acrosomal vesicles, which is also a finding typical for secondary spermatoocytes (Escalier et al., 1991; Bermudez et al., 1994), and this additional criterion was also applied in this study. Polymorphonuclear leukocytes were identified on the basis of their typical segmented nucleus. Only cells in which sufficient cytological details could be recognized to identify the cell type were injected. The microinjection technique for all types of cells was that described for round spermatid injection (Tesariak and Mendoza, 1996) except for omission of the vigorous cytoplasmic aspiration (see experimental design). SSF was prepared from ejaculated human spermatozoa as described (Homa and Swann, 1994; Swann, 1994). It was injected into oocytes in a volume of 5 pl using the same microinjection needles as were used for sperm injection (Tesariak and Sousa, 1995). After injection, oocytes were loaded with Fluo-3 and examined by confocal laser scanning microscopy as described previously (Tesariak and Sousa, 1995). Where indicated, oocytes were treated with 10 \(\mu M\) ionophore A23187 (Tesariak and Testart, 1994).

**Electron microscopy**

Oocytes were microinjected with different types of cells, loaded with Fluo-3 and recorded as described above. At the end of the recording period (3–4 h after microinjection), oocytes were fixed with Karnovsky fixative (2.3% glutaraldehyde, 0.6% paraformaldehyde, 0.08 M sodium cacodylate buffer, pH 7.3) for 1 h at room temperature, washed in 0.15 M sodium cacodylate (pH 7.3) containing 0.8% K\(_2\)Fe(CN)\(_6\) for 1 h at 4°C, washed in buffer, serially dehydrated in ethanol (75, 90, 95, 100, 100%, 30 min each at room temperature), equilibrated with propylene oxide (2\(\times\)10 min), and embedded in Epon 812. They were then serially sectioned from pole to pole in a Reichert Ultracut S ultramicrotome with a Diatome diamond knife (Sousa and Tesariak, 1994). Ultrathin sections were collected on celluloid-coated copper grids, contrasted with saturated alcoholic uranyl acetate for 60 min followed by Reynolds’ lead citrate solution for 5 min, and examined in a JEOL 100 CX II transmission electron microscope.

**Results**

When oocytes were injected with primary (data not shown) or secondary (Figure 1a) spermatoocytes, two types of response were observed after the subsequent ionophore treatment. In all, 30% (six out of 20) of oocytes did not develop any secondary Ca\(^{2+}\) changes during the recording period (85 min after ionophore addition) (Figure 1a), whereas 70% (14/20) of oocytes displayed a short series of irregular Ca\(^{2+}\) increases each of which was much smaller than that produced by the ionophore (Figure 1b). These secondary Ca\(^{2+}\) fluctuations are quite different from the typical Ca\(^{2+}\) oscillatory response of
Figure 1. Ca\textsuperscript{2+} reactivity of human oocytes injected with human spermatogenic cells at different developmental stages as revealed by analysis of Ca\textsuperscript{2+} changes following Ca\textsuperscript{2+} ionophore challenge of oocytes (ionophore addition and removal are marked with arrows) performed 1 h after injection. (a) Ca\textsuperscript{2+} response of an oocyte injected with a secondary spermatocyte showing an initial ionophore-induced Ca\textsuperscript{2+} increase which is not followed by any delayed Ca\textsuperscript{2+} response during the subsequent 85 min. (b) Ca\textsuperscript{2+} reactivity of an oocyte injected with a secondary spermatocyte showing a short series of small irregular Ca\textsuperscript{2+} fluctuations following the ionophore-induced Ca\textsuperscript{2+} increase. (c) Ca\textsuperscript{2+} reactivity of an oocyte injected with a round spermatid showing a series of Ca\textsuperscript{2+} oscillations following the ionophore-induced Ca\textsuperscript{2+} increase. (d) Ca\textsuperscript{2+} reactivity of an oocyte injected with a mature spermatozoan showing a series of Ca\textsuperscript{2+} oscillations following the ionophore-induced Ca\textsuperscript{2+} increase.

human oocytes to spermatozoa, which is characterized by clearly distinguished high-amplitude Ca\textsuperscript{2+} spikes (Taylor et al., 1993; Tesarik et al., 1994; Tesarik and Sousa, 1994; Tesarik and Testart, 1994), and most probably represents a noise artefact. In contrast, 76% (13/17) of oocytes injected with round spermatids developed high-amplitude Ca\textsuperscript{2+} oscillations (Figure 1c) similar to those observed, under identical conditions, in 80% (eight out of 10) of oocytes injected with mature spermatozoa (Figure 1d). The comparison of the percentages of oocytes developing different types of Ca\textsuperscript{2+} response after injection of different cell types is summarized in Table I in which both forms of the non-oscillatory Ca\textsuperscript{2+} record (Figure 1a,b) are represented as non-response.

To ensure that the low irregular Ca\textsuperscript{2+} increases of spermatoocyte-injected oocytes were actually a non-response and were not due to the action of a germ-line-specific factor, the same experiments were performed with oocytes injected with polymorphonuclear leukocytes. Interestingly, low irregular Ca\textsuperscript{2+} increases were also detected in these cases (Figure 2a). These records were quite similar to those seen in oocytes injected with spermatocytes (Figure 2b). In both cases, the capacity of oocytes to produce typical Ca\textsuperscript{2+} oscillations could be confirmed by subsequent injection of SSF.

Electron microscopic analysis of oocytes injected with polymorphonuclear leukocytes, secondary spermatocytes and round spermatids confirmed the identity of the injected cell type in all cases (Figure 3). At the comparable time after injection (3–4 h), different types of cells showed different degrees of degradation within the ooplasm. Polymorphonuclear leukocytes still had the main cytoplasmic structures relatively preserved although the plasma membrane displayed local deficiencies (Figure 3a). Of the injected secondary spermatocytes, only the nucleus with a locally disrupted nuclear envelope was identified (Figure 3b). Largely denuded chromatin with only locally persisting remnants of the nuclear envelope and fragments of axonemal structures was all that could be detected of the injected round spermatids (Figure 3c).

Discussion
The results of this study show that the injection of round spermatids into human oocytes sensitizes the oocytes to the development of Ca\textsuperscript{2+} oscillations in a way similar to the injection of mature spermatozoa. Because the development of Ca\textsuperscript{2+} oscillations in sperm-injected human oocytes is postulated to be due to the action of SSF released from the injected...
Table I. Overview of different types of Ca$^{2+}$ response observed after injection of different types of cells into oocytes and subsequent challenge with Ca$^{2+}$ ionophore

<table>
<thead>
<tr>
<th>Type of cell injected into oocytes</th>
<th>No. of oocytes injected</th>
<th>No. (%) of oocytes showing different types of reaction*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocyte</td>
<td>10</td>
<td>10 (100) 0 (0)</td>
</tr>
<tr>
<td>Primary spermatocyte</td>
<td>4</td>
<td>4 (100) 0 (0)</td>
</tr>
<tr>
<td>Secondary spermatocyte</td>
<td>16</td>
<td>16 (100) 0 (0)</td>
</tr>
<tr>
<td>Round spermatid</td>
<td>17</td>
<td>4 (24) 13 (76)</td>
</tr>
<tr>
<td>Spermatozoon</td>
<td>10</td>
<td>2 (20) 8 (80)</td>
</tr>
</tbody>
</table>

*Only changes occurring during a 3 h time period following the exposure to Ca$^{2+}$ ionophore were taken into account.

Figure 2. Evidence of a normal responsiveness to SSF of oocytes failing to produce Ca$^{2+}$ oscillations after the injection of human polymorphonuclear leukocytes and secondary spermatocytes. (a) Lack of the typical Ca$^{2+}$ oscillatory response in an oocyte injected with a polymorphonuclear leukocyte and subsequently challenged with Ca$^{2+}$ ionophore (addition and removal marked with arrows) followed by typical Ca$^{2+}$ oscillations after the later injection of SSF (arrow). (b) Lack of the typical Ca$^{2+}$ oscillatory response in an oocyte injected with a secondary spermatocyte and subsequently challenged with Ca$^{2+}$ ionophore (addition and removal marked with arrows) followed by typical Ca$^{2+}$ oscillations after the later injection of SSF (arrow).

Figure 3. Representative electron micrographs of oocytes injected with different types of cells. (a) Oocyte injected with a polymorphonuclear leukocyte. This picture shows the lobulated nucleus (N) and the cytoplasm of a polymorphonuclear leukocyte surrounded by the oocyte cytoplasm. The plasma membrane of the injected leukocyte (large arrows) is still apparent. Small arrows indicate an area in which the leukocyte plasma membrane is broken allowing a direct contact between cytoplasmic components of both cells. (b) Oocyte injected with a secondary spermatocyte (the corresponding Ca$^{2+}$ record is shown in Figure 1b). This picture shows the spermatocyte nucleus (N) surrounded by an incomplete nuclear envelope (arrows) and the adjacent oocyte cytoplasm. No structural components of the spermatocyte cytoplasm are visible. (c) Oocyte injected with a round spermatid (the corresponding Ca$^{2+}$ record is shown in Figure 1c). This picture shows the spermatid chromatin (C) with occasionally attached remnants of the nuclear envelope (large arrows) and with intermingled fragments of the axonemal structures (small arrows). The adjacent oocyte cytoplasm does not contain any identifiable structural components of the spermatid cytoplasm. Scale bar = 2 μm.
obtained after ionophore addition. In comparison with the experiments with mouse spermatid injection reported by Kimura and Yanagimachi (1995a) who were able to obtain activation of spermatid-injected oocytes only after application of an extra electrical stimulus, the present data suggest the possibility that mouse spermatids may contain sufficient SSF only to sensitize Ca\(^{2+}\) oscillations but not to induce them. The Ca\(^{2+}\) oscillations in the sensitized oocytes would thus be triggered by the electroactivation stimulus. If this hypothesis is correct, electroactivation of spermatid-injected mouse oocytes might lead to the development of Ca\(^{2+}\) oscillations although electroactivation of non-sensitized oocytes does not. This situation may be analogous to the action of Ca\(^{2+}\) ionophore upon human oocytes; in fact the ionophore treatment triggers Ca\(^{2+}\) oscillations in oocytes that have been previously injected with spermatozoa but never in control oocytes (Tesarik and Testart, 1994). Unlike the mouse, the SSF content in human round spermatids may not be limiting because identical Ca\(^{2+}\) responses were observed in this study in sperm-injected and spermatid-injected oocytes.

In contrast to sperm-injected and spermatid-injected oocytes, Ca\(^{2+}\) oscillations did not develop in oocytes injected with primary or secondary spermatocytes, and the Ca\(^{2+}\) records from these oocytes were similar to those observed in oocytes injected with non-germ cells. Because the same oocytes developed a typical oscillatory response after subsequent injection of SSF, and disintegration of the plasma membrane of injected cells was confirmed by electron microscopy, this lack of Ca\(^{2+}\)-oscillatory response was due to a deficiency of SSF activity in the injected cells and not to the lack of the oocyte's ability to respond to SSF or to the failure of SSF to escape from the injected cells and reach the oocyte cytoplasm.

Together, these data show that SSF activity develops between the secondary spermatocyte and round spermatid stages of human spermatogenesis. This explains why the boosting procedure (oooplasmic aspiration producing a Ca\(^{2+}\) influx) used for oocyte activation after sperm injection (Tesarik and Sousa, 1995) is also sufficient to activate human oocytes injected with round or elongated spermatids (Tesarik et al., 1995; Tesarik and Mendoza, 1996). On the other hand, SSF activity is deficient in human secondary spermatocytes. Looking towards the future of assisted conception therapy for patients whose spermatogenesis cannot progress beyond the secondary spermatocyte stage, this raises the question of whether SSF deficiency may influence embryo quality after fertilization with secondary spermatocytes. Further study is needed to understand the relationship between the action of SSF, the form of the fertilization-associated Ca\(^{2+}\) signals and the control of early embryonic development.

References


Received on July 27, 1996; accepted on October 10, 1996