Developmental changes in calcium dynamics, protein kinase C distribution and endoplasmic reticulum organization in human preimplantation embryos

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Developmental changes in the Ca²⁺ dynamics of human zygotes and preimplantation embryos were related to changes in the distribution of endoplasmic reticulum (ER) and protein kinase C (PKC). The fertilization-induced Ca²⁺ oscillations were typically observed over >5 h, were ryanodine-sensitive and showed a periphery-to-centre propagation of Ca²⁺ waves. At the same time, ER and PKC were accumulated in the cell periphery. After the appearance of pronuclei, ryanodine-sensitive Ca²⁺ oscillations of lower amplitude and frequency were observed until the pronuclear breakdown. However, Ca²⁺ waves then began in the perinuclear region, in the area of ER and PKC accumulation and spread towards the cell periphery. During the second to fourth cell cycle, small sinusoidal Ca²⁺ fluctuations were observed; sparse higher-amplitude Ca²⁺ spikes, superimposed on these basal fluctuations, appeared shortly before cell division. The sinusoidal Ca²⁺ fluctuations were asynchronous in individual blastomeres and disappeared progressively in arrested embryos. The direction of Ca²⁺ wave propagation and the distribution of ER and PKC were similar to the situation observed in pronuclear zygotes. In contrast to the zygotes, ryanodine did not arrest the Ca²⁺ oscillations but augmented their amplitude and frequency. These data suggest that human pre-embryos use different mechanisms of Ca²⁺ signalling in the early post-fertilization period, during the pronuclear development and during cleavage.

Key words: calcium dynamics/endoplasmic reticulum/human preimplantation embryos/protein kinase C/ryanodine

Introduction

Ca²⁺ signals play an essential role both at the time of conception, when they transduce a sperm-generated epigenetic message required for launching embryonic development (reviewed in Swann and Ozil, 1994), and during subsequent life when they modulate various cellular processes related to cell division and cell cycle control (e.g. Poenie et al., 1985; Whitaker and Patel, 1990; Lu and Means, 1993; Ciapa et al., 1994; Hepler, 1994). The nature of the Ca²⁺ signals employed at fertilization and during further development may be different; however, studies reporting long-term Ca²⁺ recordings of embryos, going on beyond the first cell cycle, are sparse and limited to non-mammalian species (Shantz, 1985; Yoshimoto et al., 1985; Speksnijder et al., 1989; Fluck et al., 1991; Grandin and Charbonneau, 1991; Kubota et al., 1993; Keating et al., 1994; Stricker, 1995). The only available information concerning the human species refers to the early sperm-induced series of Ca²⁺ oscillations occurring during the first hours following fertilization (Taylor et al., 1993; Tesarik et al., 1994; Tesarik and Sousa, 1994; Tesarik et al., 1995; Sousa et al., 1996a,b), whereas information about the Ca²⁺ dynamics during later phases of the first cell cycle and during the subsequent cell cycles of the early human development is lacking.

In spite of the current debate about the mechanism of Ca²⁺ oscillations in oocytes and other cell types and about the significance of this particular Ca²⁺ signal in early mammalian development (Berridge, 1996; Swann and Lawrence, 1996; Tesarik and Sousa, 1996), there is a general agreement as to the central role played by the endoplasmic reticulum (ER), which serves as the principal intracellular store of releasable Ca²⁺ signals (Streb et al., 1984; Clapham, 1995). ER is also believed to be the main source of Ca²⁺ released to the cytosol during the sperm-induced Ca²⁺ oscillations of mammalian oocytes (Homa et al., 1993; Whitaker and Swann, 1993; Swann and Ozil, 1994). The mechanism of the sperm-induced Ca²⁺ oscillations in oocytes is believed to involve a specific action of protein kinase C (PKC) (Swann et al., 1989; Miyazaki et al., 1990; Bird et al., 1993; Gallicano et al., 1993, 1995; Petersen and Berridge, 1994). Developmental changes in the Ca²⁺ dynamics may thus be accompanied by changes in the ER organization and PKC intracellular distribution.

In this study we examined the Ca²⁺ dynamics of human preimplantation embryos from the 1-cell zygote to the fourth cell cycle of embryonic development. These findings were related to the pattern of the intracellular distribution of ER and PKC at the corresponding developmental stages.
Menopausal gonadotrophin (HMG) and human chorionic gonadotrophin (HCG) after pituitary desensitization with a gonadotrophin-releasing hormone (GnRH) agonist; these oocytes were used in therapeutic IVF or intracytoplasmic sperm injection (ICSI) attempts. Details of the ovarian stimulation and of the IVF and ICSI techniques used have been described previously (Sousa and Tesarik, 1994; Tesarik et al., 1994; Tesarik and Sousa, 1995).

This study involves supernumerary oocytes from cases in which embryo freezing was not available. These oocytes were fertilized with husbands' spermatozoa and used for the study of events occurring during the early post-fertilization period and at the pronuclear stage. They were discarded after the breakdown of the pronuclear membrane.

Surplus embryos from IVF and ICSI treatment cycles which could not be frozen because of unavailability of the embryo freezing facility were also allocated to this study. Only normally fertilized (two pronucleated) zygotes and morphologically good embryos, classified as grade 4 (equal sized symmetrical blastomeres) or grade 3 (uneven blastomeres with <10% fragmentation) according to Steer et al. (1992), whose grade did not change during the time-course of experimentation, were included. The embryos were discarded immediately after the termination of each experiment. All of the gametes and embryos allocated to this study were used with the informed consent from the donating patients.

**Evaluation of the intracellular free Ca$$^{2+}$$ concentration**

Relative changes in the intracellular free Ca$$^{2+}$$ concentration ([Ca$$^{2+}$$]) were analysed using Fluo-3, a fluorescent probe for visualizing Ca$$^{2+}$$ in living cells (Minta et al., 1989) which was loaded into the embryos in the form of the cell-permeant acetoxymethyl ester Fluo-3-AM (Molecular Probes, Eugene, OR, USA). The advantages and disadvantages of the use of this non-ratiometric probe for the study of Ca$$^{2+}$$ dynamics in human oocytes have been discussed previously (Tesarik et al., 1995).

Oocytes, pronuclear zygotes and cleaving embryos were incubated with 9 µM Fluo-3-AM in SPM (prepared from a stock solution of 890 µM Fluo-3-AM in dimethylsulphoxide (DMSO)) for 30 min at 37°C under a gas phase of 5% CO$$\text{2}$$ in air (the final concentration of DMSO was 1%). They were then washed, placed in SPM and allowed to adhere to culture dishes coated with poly-L-lysine (Sigma, St Louis, MO, USA). The specimens were examined with a Biorad MRC 600 (Richmond, CA, USA) confocal laser scanning microscopy unit. The fluorescence emitted from the equatorial plane of the zygotes or of individual blastomeres of the cleaving embryos was monitored at intervals of 2 or 5 s depending on the experiment. Images were analysed using the Biorad Time Course Ratiometric Software Module. Specimens were scanned simultaneously in two optical channels. One channel served for the acquisition of the Fluo-3 fluorescence signal and the other was used for the visualization of a phase-contrast image of each specimen; the latter was required for concomitant evaluation of cytological changes, such as pronuclear breakdown or cell division, occurring in the specimens in the course of each experiment. At the end of each experiment, oocytes were treated with 10 µM ionophore A23187 (Sigma) which produced an increase in [Ca$$^{2+}$$], that was used for calibration of the whole record. Relative fluorescence intensity was thus expressed as percentage of this ionophore-induced [Ca$$^{2+}$$] increase.

Some Fluo-3-loaded oocytes were inseminated *in vitro* in the course of the Ca$$^{2+}$$ monitoring procedure. For *in vitro* insemination, oocytes were freed from the zona pellucida with 1% pronase (Sigma) in Tyrodé's salt solution (Sigma, cell culture grade) as described by Tesarik and Kopecky (1989a,b). After incubation for *in vitro* capacitation, spermatozoa were treated with ionophore A23187 to induce the acrosome reaction and thus shorten the time between sperm addition and sperm-oocyte fusion. Ionophore A23187 was added at
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Figure 2. Spatial characteristics of Ca^{2+} waves, protein kinase C (PKC) distribution and endoplasmic reticulum (ER) organization in the late pre-pronuclear period (5 h after the beginning of the fertilization-induced Ca^{2+} response). (A) Sequential confocal images of a Fluo-3-loaded oocyte showing a late fertilization-induced [Ca^{2+}]_{i} increase (representative of data obtained with 12 oocytes). Fluo-3 fluorescence emitted from the oocyte equatorial region was recorded at intervals of 5 s. The numbers indicate the time (s) after the recording of the first image of the series presented. (B) Confocal imaging of PKC (visualized with the use of Fim-1) in an oocyte undergoing late fertilization-induced Ca^{2+} oscillations (representative of data obtained with three oocytes). The same data were also obtained with three other oocytes fixed during the first h of the fertilization-induced Ca^{2+} oscillations. (C) Confocal imaging of ER visualized with the use of DiOC_{5}(3) in an oocyte undergoing late fertilization-induced Ca^{2+} oscillations (representative of data obtained with three oocytes). The same data were also obtained with three other oocytes fixed during the first hour of the fertilization-induced Ca^{2+} oscillations. In all parts of this composite figure, fluorescence intensity is expressed in pseudocolour according to the scale bar where the lowest values are coded black.

Pharmacological experiments

Ryanodine (Sigma) was used at a final concentration of 4 mM. This concentration of ryanodine has previously been shown to be most effective in producing a Ca^{2+} discharge from the ryanodine-sensitive Ca^{2+} stores of human oocytes without apparent toxic effects (Sousa et al., 1996a). The stock solution of 160 mM ryanodine was prepared in 40% DMSO in water and then diluted in SPM to double the final concentration desired. Ryanodine addition to cells was performed by mixing equal volumes of this solution with the cell-containing medium, resulting in final concentrations of 4 mM ryanodine and 1% DMSO. These concentrations were previously shown to give reproducible specific Ca^{2+} responses in human oocytes without apparent toxicity (Sousa et al., 1996a,b), and similar concentrations were also optimal for mouse oocytes (Swann, 1992).

Visualization of PKC and ER

For PKC staining, cells were fixed for 10 min in phosphate-buffered saline (PBS; Sigma, cell culture grade; pH 6.8) containing 3.75% depolymerized paraformaldehyde (Biorad). After fixation, cells were permeabilized with 100% methanol (−20°C, 10 min) and washed twice in PBS. Cells were then incubated in PBS containing 200 nM Fim-1 (Teflabs, Austin, TX, USA) prepared from a 0.297 mM stock solution in DMSO. Fim-1 is the fluorescein-conjugated K+ salt of an ATP-competitive catalytic site inhibitor of PKC-β, and is used here as a PKC reporter dye (Chen and Poenie, 1993).

For ER staining, cells were fixed with 0.25% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 3 min, stained for 30 s in the same buffer containing 2.5 μg/ml of the fluorescein-conjugated dye DiOC_{5}(3) (Molecular Probes) prepared from a stock solution of 0.5 mg/ml DiOC_{5}(3) in ethanol, and washed in buffer before observation.

Cells stained for PKC and ER were scanned by confocal microscopy from pole to pole with a 5 μm distance between sequential optical sections.

Results

Pre-pronuclear period

The sperm-induced Ca^{2+} oscillations were monitored in freshly fertilized human oocytes for ~6 h from their onset. To avoid a prolonged uninterrupted stay of oocytes outside the CO_{2} incubator and to limit the possibility of oocyte damage by laser light, periods of measurement by confocal microscopy were alternated with periods during which oocytes were washed in SPM and returned to the incubator. Of 12 fertilized oocytes that survived this long measurement procedure, all still exhibited Ca^{2+} oscillations at its end, i.e. 5−6 h after sperm−oocyte fusion (Figure 1A). In six of these oocytes, the sensitivity of the late fertilization-induced Ca^{2+} oscillations to ryanodine was tested; the addition of ryanodine inhibited the Ca^{2+} oscillations in all six cases (Figure 1B).

Spatial image analysis (12 oocytes) showed that each of the late fertilization-induced [Ca^{2+}]_{i} rises was introduced by a
focal release of Ca\textsuperscript{2+} at a variable site in the cortex of the oocyte followed by a global Ca\textsuperscript{2+} discharge, after which [Ca\textsuperscript{2+}]\textsubscript{i} returned relatively rapidly to basal values in the oocyte cortex and subcortex, whereas it remained elevated for a longer time in the oocyte central region (Figure 2A).

The six fertilized oocytes that still showed ongoing Ca\textsuperscript{2+} oscillations at the end of the [Ca\textsuperscript{2+}]\textsubscript{i} monitoring period (5–6 h after sperm–oocyte fusion) and that were not treated with ryanodine (see above) were subsequently processed either for PKC (three oocytes) or ER (three oocytes) visualization. In both cases, three other oocytes fixed in the course of the first hour of the fertilization-induced Ca\textsuperscript{2+} oscillations were also included. Irrespective of the time after fertilization, both PKC (Figure 2B) and ER (Figure 2C) were accumulated in the cell periphery.

**Pronuclear period**

Pronuclear zygotes (11 cases) also showed periodical [Ca\textsuperscript{2+}]\textsubscript{i} increases (Figure 3A); however, these occurred with a lower frequency as compared to the pre-pronuclear stage. In three cases, the sensitivity of these Ca\textsuperscript{2+} signals to ryanodine was tested, and a clear inhibitory effect (Figure 3B) was apparent in all of them. In two cases, it was possible to monitor [Ca\textsuperscript{2+}]\textsubscript{i} shortly before, during, and after the process of pronuclear envelope breakdown (Figure 3C). In both cases, the Ca\textsuperscript{2+} oscillations did not continue beyond the pronuclear stage.

The analysis of the spatial propagation of Ca\textsuperscript{2+} waves in pronuclear zygotes (11 cases) showed that the initial [Ca\textsuperscript{2+}]\textsubscript{i} increase of each Ca\textsuperscript{2+} spike invariably began from the central region of the cell (where the pronuclei were also located) and then spread towards the cell periphery (Figure 4A). It was also the central region in which [Ca\textsuperscript{2+}]\textsubscript{i} remained elevated for the longest time during each Ca\textsuperscript{2+} transient. Six of the pronuclear zygotes in which [Ca\textsuperscript{2+}]\textsubscript{i} was monitored were subsequently processed for PKC (three zygotes) and ER (three zygotes) visualization. Both PKC (Figure 4B) and ER (Figure 4C) were accumulated in the zygote central region, around the pronuclei.

**Cleavage period**

Only a limited number of supernumerary cleaving embryos was available for this study: three embryos at the 2-cell stage and seven embryos at the 4-cell stage. The 2-cell embryos were allocated to long-term observations in an attempt to visualize changes in [Ca\textsuperscript{2+}]\textsubscript{i} occurring during cell division, which proved possible in one case only. In this case, the embryo was subsequently treated with ryanodine. The remaining 2-cell embryos, in which we did not succeed in monitoring [Ca\textsuperscript{2+}]\textsubscript{i} at the time of cell division, were used for PKC (one embryo) and ER (one embryo) visualization. Six of the originally 4-cell embryos were used in experiments to evaluate the effect of ryanodine on Ca\textsuperscript{2+} dynamics (two embryos) and PKC (two embryos) or ER (two embryos) distribution. In one of the originally 4-cell embryos it proved possible to monitor [Ca\textsuperscript{2+}]\textsubscript{i} during cell division occurring simultaneously in two blastomeres and leading to the formation of a 6-cell embryo; this embryo was subsequently used to study the effect of ryanodine on Ca\textsuperscript{2+} dynamics.

Similar to the preceding developmental stages, cleaving embryos exhibited repetitive [Ca\textsuperscript{2+}]\textsubscript{i} increases; however, their characteristics were completely different. Unlike the first cell cycle, during which [Ca\textsuperscript{2+}]\textsubscript{i} baseline values were stable between...
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Figure 4. Spatial characteristics of Ca\(^{2+}\) waves, protein kinase C (PKC) distribution and endoplasmic reticulum (ER) organization in the pronuclear period. (A) Sequential confocal images of a Fluo-3-loaded two-pronuclear zygote showing a [Ca\(^{2+}\)]\(_i\) increase (representative of data obtained with 11 zygotes). Fluo-3 fluorescence emitted from the equatorial region was recorded at intervals of 5 s. The numbers indicate the time (s) after the recording of the first image of the series presented. (B) Confocal imaging of PKC in a pronuclear zygote undergoing Ca\(^{2+}\) oscillations (representative of data obtained with three zygotes). (C) Confocal imaging of ER in a pronuclear zygote undergoing Ca\(^{2+}\) oscillations (representative of data obtained with three zygotes). The dyes used and the mode of image presentation are those described in Figure 2.

individual Ca\(^{2+}\) spikes, cleaving embryos exhibited continuous sinusoidal changes in [Ca\(^{2+}\)]\(_i\) upon which higher-amplitude Ca\(^{2+}\) spikes were sometimes superimposed (Figures 5 and 6). These superimposed Ca\(^{2+}\) spikes were seen only in blastomeres of one 2-cell and one 4-cell embryo shortly before cell division (Figures 5 and 6). After the accomplishment of cell division, only the background sinusoidal Ca\(^{2+}\) fluctuations were detected (Figure 7), which represented the only Ca\(^{2+}\) changes seen in the case of embryos that did not cleave during the monitoring period (Figure 8).

The [Ca\(^{2+}\)]\(_i\) changes observed in cleaving embryos were asynchronous in individual blastomeres of the same embryos; this asynchrony became even more apparent when individual blastomeres began to display the cell-division-related Ca\(^{2+}\) spikes (Figures 5 and 6). Embryos that underwent spontaneous arrest of development between the 4-cell and 6-cell stages showed a progressive disappearance of the sinusoidal Ca\(^{2+}\) fluctuations, resulting in a straight-line [Ca\(^{2+}\)]\(_i\) record (data not shown).

Unlike the first cell cycle, the addition of ryanodine did not inhibit the Ca\(^{2+}\) signals of cleaving embryos. Instead, ryanodine elicited a marked increase in the amplitude and a moderate increase in the frequency of the sinusoidal Ca\(^{2+}\) fluctuations (Figure 9). The eventual effect of ryanodine on the cell-division-related Ca\(^{2+}\) spikes was not addressed.

Similar to pronuclear zygotes, cleaving embryos showed a centre-to-periphery propagation of Ca\(^{2+}\) waves (Figure 10A–C) and a central accumulation of PKC (Figure 10D) and ER (Figure 10E).

Discussion

The results of this study have shown that the fertilization-induced series of Ca\(^{2+}\) oscillations seen in human zygotes is followed by further temporal and spatial changes in [Ca\(^{2+}\)]\(_i\) during the first cell cycle, as well as during the subsequent cell cycles occurring during preimplantation embryo development. Based on evaluations of the frequency and amplitude of these Ca\(^{2+}\) transients and their sensitivity to ryanodine, the spatial propagation of Ca\(^{2+}\) waves and the observed intracellular distribution of PKC and ER, three developmentally related patterns of Ca\(^{2+}\) dynamics were distinguished. The distinctive features of each of the three patterns are summarized in Table I.

Pre-pronuclear period

The period of zygote development between sperm–oocyte fusion and the appearance of pronuclei is characterized by the occurrence of sperm-induced Ca\(^{2+}\) oscillations. The characteristics of these Ca\(^{2+}\) oscillations, as observed in this study, were similar to those described previously in human fertilized oocytes by ourselves (Tesarik et al., 1994, 1995; Tesarik and Sousa, 1994; Sousa et al., 1996a,b) and others (Taylor et al., 1993) and to those induced in human oocytes by injection of a cytosolic sperm fraction (Homa and Swann, 1994). However, in comparison with our earlier studies in which the overall duration of the period of Ca\(^{2+}\) oscillations rarely exceeded 2 h, oocytes usually showed this kind of Ca\(^{2+}\) response for >5 h in this study. This difference may be due to the fact that the Ca\(^{2+}\) monitoring was intermittent in this study, with periods of measurement alternating with periods of rest in a CO\(_2\) incubator, whereas the monitoring was continuous in our earlier studies. The continuous Ca\(^{2+}\) monitoring may have led to a higher degree of laser-light damage of embryos as compared to the present experiments. Fertilization-induced Ca\(^{2+}\) oscillations lasting for 4–5 h were reported by Taylor et al. (1993) who monitored oocytes in a perfusion chamber, which may also have modulated the adverse effect of light exposure.
Figure 5. \( \text{Ca}^{2+} \) signals of a 2-cell embryo shortly before cell division. (A) Record of the whole embryo. (B) Record of blastomere 1 (arrow and double arrow indicate low-intensity \( \text{Ca}^{2+} \) spikes corresponding to confocal images shown in Figure 10A and B respectively). (C) Record of blastomere 2 (arrow and double arrow indicate a high-intensity \( \text{Ca}^{2+} \) spike and an intermediate-intensity \( \text{Ca}^{2+} \) spike corresponding to confocal images shown in Figure 10A and B respectively). Fluo-3 fluorescence was recorded at intervals of 2 s.

Both early (Sousa et al., 1996a) and late (this study) sperm-induced \( \text{Ca}^{2+} \) oscillations can be inhibited by ryanodine and coincide with an accumulation of PKC and ER in the zygote periphery. Earlier confocal studies, performed with mouse (Mehlmann et al., 1995) and hamster oocytes (Shiraishi et al., 1995), also demonstrated a peripheral accumulation of ER. An accumulation of PKC in the cortical region of freshly fertilized oocytes has also been described in the sea urchin (Olds et al., 1995) and hamster (Gallicano et al., 1995) and may reflect an association of the activated enzyme with the plasma membrane or with cortical granules (Olds et al., 1995). However, the peripheral accumulation of ER and PKC in human oocytes is not a direct sequel of oocyte activation because a similar distribution of ER and PKC has also been observed in mature unfertilized human oocytes (M.Sousa and J.Tesarik, unpublished results). The \( \text{Ca}^{2+} \) stores present in the cortical and subcortical regions of human oocytes are essentially insensitive to ryanodine and presumably sensitive to inositol 1,4,5-trisphosphate (InsP\(_3\)) (Sousa et al., 1996a). At the beginning of each \( \text{Ca}^{2+} \) spike during the steady-state phase of the sperm-induced \( \text{Ca}^{2+} \) oscillations, these peripheral \( \text{Ca}^{2+} \) stores are responsible for the initial \( \text{Ca}^{2+} \) discharge which acts as a detonator for a global \( \text{Ca}^{2+} \)-induced \( \text{Ca}^{2+} \) release (CICR) involving the ryanodine-sensitive stores which are abundant in the rest of the oocyte cytoplasm (Tesarik et al., 1995; Sousa et al., 1996a). Accordingly, \( \text{Ca}^{2+} \) waves observed during each \( \text{Ca}^{2+} \) spike during this period show a periphery-to-centre direction of propagation.

**Pronuclear period**

A unique type of \( \text{Ca}^{2+} \) signal has been detected in pronucleated zygotes. Like the earlier fertilization-induced \( \text{Ca}^{2+} \) signal, this signal also consisted of repetitive \( \text{Ca}^{2+} \) spikes which were, however, of lower frequency and amplitude than those observed in the pre-pronuclear period. Furthermore, contrary to the fertilization-induced \( \text{Ca}^{2+} \) oscillations, the \( \text{Ca}^{2+} \) waves developing in the pronuclear period showed a centre-to-periphery direction of spatial propagation. This reversal of the direction of \( \text{Ca}^{2+} \) wave propagation coincided with a redistribution of PKC and ER, both of which became accumulated in the central cytoplasm, around the pronuclei. Notwithstanding, as in the pre-pronuclear period, the \( \text{Ca}^{2+} \) oscillations occurring in the pronuclear period were sensitive to ryanodine (Table 1). This study did not involve a continuous \( \text{Ca}^{2+} \) monitoring from fertilization through pronuclear development. Thus, it remains unknown what happens with \([\text{Ca}^{2+}]_i\) between the dissolution of the fertilization-induced series of \( \text{Ca}^{2+} \) oscillations and the appearance of the \( \text{Ca}^{2+} \) oscillations typical of the pronuclear stage. It cannot be excluded that, under physiological conditions, the series of fertilization-induced \( \text{Ca}^{2+} \) oscillations is not interrupted but transforms gradually into the pronuclear-type of \( \text{Ca}^{2+} \) oscillation. The arrest of \( \text{Ca}^{2+} \) oscillations in monitored oocytes may be artificial and due to the unphysiological environmental conditions including exposure to laser light (see above) and the action of the \( \text{Ca}^{2+} \) chelating dyes used for intracellular \( \text{Ca}^{2+} \) visualization.

Information about the \( \text{Ca}^{2+} \) dynamics of pronuclear zygotes of other mammalian species is scarce. Fissore and Robl (1993) demonstrated transient \([\text{Ca}^{2+}]_i\) rises in rabbit pronuclear zygotes; these \( \text{Ca}^{2+} \) transients decreased shortly before nuclear envelope breakdown. In the mouse, \( \text{Ca}^{2+} \) transients were also observed at the time of pronuclear breakdown and, in addition,
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Figure 6. Ca$^{2+}$ signals of a 4-cell embryo shortly before cell division. (A) Record of the whole embryo. (B) Record of blastomere 1 (arrow indicates a low-intensity Ca$^{2+}$ spike corresponding to confocal images shown in Figure 10C). (C) Record of blastomere 2. (D) Record of blastomere 3. (E) Record of blastomere 4 (arrow indicates an intermediate-intensity Ca$^{2+}$ spike corresponding to confocal images shown in Figure 10C). Fluo-3 fluorescence was recorded at intervals of 2 s.

at the time of the zygote's entry into the first embryonic mitosis (Tombes et al., 1992; Kono et al., 1996). Studies carried out in several non-mammalian species, in which fertilization induces a single [Ca$^{2+}$]$_i$ rise rather than a series of Ca$^{2+}$ oscillations, also revealed additional [Ca$^{2+}$]$_i$ rises at the pronuclear stage (Poenie et al., 1985; Steinhardt, 1990; Gillot and Whitaker, 1994; Ciapa et al., 1994; Stricker, 1995). Similar to the present observations, the spatial propagation of these Ca$^{2+}$ signals differed from the fertilization-induced Ca$^{2+}$ response in sea urchin (Gillot and Whitaker, 1994) and starfish zygotes (Stricker, 1995). This change in the spatial propagation of Ca$^{2+}$ waves may be related to the migration of the Ca$^{2+}$ stores with the highest sensitivity to CICR, which serve as the source of a local detonating Ca$^{2+}$ discharge triggering the global CICR in human oocytes (Tesarik et al., 1995), from the oocyte periphery to the perinuclear region of pronuclear
Table I. Developmental changes in the characteristics of the Ca\(^{2+}\) signalling system of human preimplantation embryos

<table>
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<tr>
<th>Period</th>
<th>Features of [Ca(^{2+})], rises</th>
<th>PKC accumulation</th>
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<td>Temporal pattern</td>
<td>Spatial propagation</td>
<td>Ryanodine effect</td>
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<td>Spiking</td>
<td>Periphery-to-centre</td>
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<td>Sinusoidal plus spiking</td>
<td>Centre-to-periphery</td>
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PKC = protein kinase C; ER = endoplasmic reticulum.

Figure 7. Ca\(^{2+}\) signals recorded in a representative blastomere of a 4-cell embryo that has developed from the 2-cell embryo whose records are shown in Figure 5. Fluo-3 fluorescence was recorded at intervals of 2 s. The other blastomeres showed a similar record.

Figure 8. Ca\(^{2+}\) signals recorded in a representative blastomere of a 4-cell embryo in which no cell division occurred either during, or close to, the recording period (representative of data obtained with two 2-cell embryos and six 4-cell embryos). Fluo-3 fluorescence was recorded at intervals of 2 s. The other blastomeres showed a similar record.

Figure 9. Effect of the addition of 4 mM ryanodine (Ry) on Ca\(^{2+}\) signals recorded in the embryo whose previous Ca\(^{2+}\) records are shown in Figure 7 (representative of data obtained with one 4-cell embryo, two 4-cell embryos and one 6-cell embryo). (A) Record of the whole embryo. (B) Record of a representative blastomere. Fluo-3 fluorescence was recorded at intervals of 2 s.

zygotes. The observed reorganization of ER and redistribution of PKC coinciding with this reversal of the spatial dynamics of Ca\(^{2+}\) signals may reflect this underlying mechanism. Earlier electron microscopic observations on human pronuclear zygotes also showed the presence of abundant ER structures in the perinuclear region (Soupart and Strong, 1974; Sundström et al., 1981; Sathanathan and Trounson, 1985), contrasting with the accumulation of this organelle in the periphery of unfertilized human oocytes (Zamboni et al., 1972; Sundström et al., 1985; Sundström and Nilsson, 1988).

It is possible that at least part of the Fim-1 fluorescence signal detected in the perinuclear region of human pronuclear zygotes in this study corresponded to protein kinase M rather than PKC. This is corroborated by recent observations on hamster ionophore-activated oocytes probed with Rim-1 (Gallicano et al., 1995), a PKC reporter dye analogous to Fim-1 used in the present study. These authors have demonstrated...
Figure 10. Spatial characteristics of Ca$^{2+}$ waves, protein kinase C (PKC) distribution and endoplasmic reticulum (ER) organization in the cleavage period. (A) Sequential confocal images of a 2-cell embryo showing a small [Ca$^{2+}$]$_{i}$ increase in blastomere 1 (b1) (marked with an arrow in the corresponding record shown in Figure 5B) and of a large [Ca$^{2+}$]$_{i}$ increase in blastomere 2 (b2) (marked with an arrow in the corresponding record shown in Fig. 5C). (B) Sequential confocal images of a 2-cell embryo showing a small [Ca$^{2+}$]$_{i}$ increase in blastomere 1 (b1) (marked with a double arrow in the corresponding record shown in Figure 5B) and an intermediate-intensity [Ca$^{2+}$]$_{i}$ increase in blastomere 2 (b2) (marked with a double arrow in the corresponding record shown in Figure 5C). (C) Sequential confocal images of a 4-cell embryo showing a small [Ca$^{2+}$]$_{i}$ increase in blastomere 1 (b1) (marked with an arrow in the corresponding record shown in Figure 6B) and an intermediate-intensity [Ca$^{2+}$]$_{i}$ increase in blastomere 4 (b4) (marked with an arrow in the corresponding record shown in Figure 6E). Fluo-3 fluorescence emitted from the equatorial plane of the embryos shown in panels A–C was recorded at intervals of 2 s. The numbers displayed in these panels indicate the time (s) after the recording of the first image of each series presented. (D) Confocal imaging of PKC in a 4-cell embryo (representative of data obtained with one 2-cell and two 4-cell embryos). (E) Confocal imaging of ER in a 4-cell embryo (representative of data obtained with one 2-cell embryo and two 4-cell embryos). The dyes used and the mode of image presentation are those described in Figure 2.
a shift in the Rim-1 fluorescence intensity maximum from the oocyte periphery, where the dye labelled PKC, towards the central region of the oocyte, where the dye mainly labelled protein kinase M. This latter represents the isolated catalytic subunit of PKC devoid of the membrane-binding domain, probably the result of proteolytic down-regulation of PKC (Gallicano et al., 1995).

**Cleavage period**

Two types of Ca\(^{2+}\) signals were detected in this study: a background sinusoidal rhythm, observed at any phase of the cell cycle, and superimposed clusters of higher Ca\(^{2+}\) spikes occurring shortly before cell division. Although the temporal pattern of this Ca\(^{2+}\) signal was quite different when compared with the first cell cycle after fertilization, the spatial propagation of Ca\(^{2+}\) waves was similar to the pronuclear period. ER and PKC also showed a perinuclear accumulation in blastomeres of cleaving embryos, similar to pronuclear zygotes. Surprisingly, ryanodine exhibited a quite opposite effect on these Ca\(^{2+}\) signals when compared with the previous stages. The early fertilization-induced series of Ca\(^{2+}\) oscillations has been suggested to rely on cyclic exchanges of Ca\(^{2+}\) ions between the InsP\(_3\)-sensitive and the ryanodine-sensitive Ca\(^{2+}\) stores (Sousa et al., 1996a; Tesarik and Sousa, 1996), and a similar mechanism may underlie the Ca\(^{2+}\) oscillations of pronuclear zygotes which show a similar sensitivity to ryanodine. The absence of ryanodine inhibition of the [Ca\(^{2+}\)]\(_i\) rises occurring in cleaving human embryos may be related to a change in the Ca\(^{2+}\) oscillation mechanism involving a modification of the role of the ryanodine-sensitive Ca\(^{2+}\) stores. Alternatively, the responsiveness of the ryanodine receptors may have changed, either as a consequence of the synthesis of new classes of the receptor or owing to conformational changes of the structure of the existing receptors. Further research is needed to elucidate these questions. Experiments carried out in the sea urchin also have suggested that the mechanism of the cell-cycle-related Ca\(^{2+}\) transients in embryonic cells is different from that of the fertilization-induced Ca\(^{2+}\) signals of freshly fertilized oocytes (Ciapa et al., 1994).

Similar to our observations, a unique type of Ca\(^{2+}\) signal typically preceded mitosis in cleaving starfish embryos (Stricker, 1995). In spontaneously cleavage-arrested embryos observed in this study, fluctuations of [Ca\(^{2+}\)]\(_i\), disappeared progressively. This finding suggests that the changes in [Ca\(^{2+}\)]\(_i\), reported in this study represent developmentally regulated signals required for cleavage of human embryos. The dependence of cleavage on InsP\(_3\)-driven Ca\(^{2+}\) signals has been demonstrated experimentally in the sea urchin (Ciapa et al., 1994) and starfish (Stricker, 1995). These Ca\(^{2+}\) signals are believed to be governed by a ‘cytoplasmic clock’ that is independent of cytokinesis because they still continue in embryos whose cleavage has been arrested artificially by colchicine or protein synthesis inhibition (Kubota et al., 1993; Ciapa et al., 1994; Keating et al., 1994; Stricker, 1995). Hence, these Ca\(^{2+}\) signals appear to be important for, but independent of, cell division in early embryos.

**Calcium signals and early embryonic development**

Previous studies have focused on the fertilization-induced Ca\(^{2+}\) signals in human zygotes (Taylor et al., 1993; Tesarik and Sousa, 1994; Tesarik and Testart, 1994; Tesarik et al., 1994, 1995; Sousa et al., 1996a,b) and on the possible consequences of artificial modifications of these signals in special conditions associated with different techniques of micromanipulation-assisted fertilization (Tesarik and Sousa, 1994; Tesarik, 1995, 1996). This study is the first to show that developmentally regulated changes in [Ca\(^{2+}\)]\(_i\), presumably playing a signalling role, continue at later stages of the first cell cycle as well as during cleavage of human embryos. These data should stimulate further study of the relationship between these Ca\(^{2+}\) signals, on the one hand, and embryo quality on the other, including participation in the early materno-embryonic crosstalk necessary for embryo implantation (Edwards, 1995). The action of many of the factors known to be implicated in this crosstalk, such as platelet-activating factor or components of the interleukin-1 system, involve Ca\(^{2+}\) as a second messenger. The evaluation of the eventual role of embryonic Ca\(^{2+}\) signals in the synthesis of, or response to, implantation-preparing molecules is an intriguing challenge for future research.

**References**


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