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Prolonged, repetitive calcium transients in rat oocytes fertilized in vitro and in vivo

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Zona-free rat oocytes inseminated with capacitated sperm, under conditions that allow polyspermic fertilization, exhibited a rapid, transient elevation of cellular calcium (from 147 ± 10 to 607 ± 55 nM, $n = 19$, measured by Fura 2 fluorescence ratio imaging) immediately after sperm attachment. This peak was followed by a series of dramatic calcium transients of high amplitude (maximal 847 ± 32 nM) and frequency (range 2.1 ± 0.07 – 3.9 ± 0.07 min), which continued for several hours. A similar pattern was seen also in zona-free oocytes fertilized with low sperm density (i.e. producing mainly monospermic attachment) and in zona-enclosed oocytes fertilized in vitro. Moreover, single or repetitive calcium transients were observed in rat oocytes fertilized in vivo. These findings indicate that in normal fertilization in vivo, sperm–oocyte interaction initiates a prolonged train of cyclical calcium changes in the oocyte. This activity may be necessary for the early events in the fertilization process.

Calcium transient; Fertilization, in vivo; Fertilization, in vitro; Oocyte; Rat

1. INTRODUCTION

There is a consensus regarding the importance of early changes in oocyte calcium following fertilization (e.g. [1,2]). The combined picture, collated from experiments performed using various techniques in different species, suggests an initial rise in cellular calcium concentration, followed by a series of repetitive transients. The full quantitative description of this phenomenon, however, has not been previously reported in mammalian oocytes under conditions that most closely mimic the in vivo process.

The purpose of this work was to establish an experimental model system that will display the full kinetics of calcium changes initiated by sperm attachment in mammalian oocytes. In our hands, the quantitation of oocyte free calcium ([Ca]) could be readily performed using Fura 2-AM as a fluorescent probe. We demonstrate that the sperm–oocyte interaction in rat triggers a train of calcium transients that continues for an extended period of time and is highly reproducible in a large number of oocytes. Moreover, we show that oocytes fertilized in vivo exhibit single or repetitive calcium transients, suggesting that the transients observed in in vitro fertilization faithfully mimic the in vivo process.

2. MATERIALS AND METHODS

Isolation of zona-enclosed and zona-free oocytes from immature superovulated female rats has been previously described [3]. Capacitated sperm for in vitro fertilization was prepared as described elsewhere [4]. Isolation of oocytes from superovulated immature females, following mating, has been also previously described [5]. All manipulations were performed at 25–37°C. Zona-free or zona-enclosed oocytes were loaded with Fura 2-AM (Molecular Probes, 3 μM) for 30 min at 37°C in Toyoda medium supplemented with 15 mM HEPES [6]. Oocytes were washed free of the dye and allowed to attach to poly-L-lysine (Sigma) coated coverslips. Insemination with capacitated sperm was performed either directly on the coverslip during the actual measurements of cellular calcium (zona-free oocytes) or prior to the measurements (zona-enclosed oocytes). The coverslip was placed in a thermostated chamber (Applied Imaging), adjusted to 35–38°C with continuous monitoring of medium temperature.

Calcium concentration was measured by monitoring fluorescence ratio at 340/380 nm using the Applied Imaging Magiscan system in the Magical mode. The sampling was performed every 0.16–15 s per ratio, according to the design of the experiment. Oocytes were examined with X10–X40 objectives on an inverted microscope (Nikon TMD), allowing a high number of oocytes to be analyzed simultaneously. The calibration was performed in 100 mM KCl, 20 mM NaCl solution, buffered to pH 7.4 with 25 mM HEPES, using $Ca^{2+} = 0$ (2 mM EGTA) and saturating Ca^{2+} (5 mM) as reported by Grynkiewicz et al. [7], assuming Ca^{2+}-Fura 2 dissociation constant of 224 nM. Backgrounds of medium in oocyte-free area were routinely subtracted from all frames at the respective excitation wavelengths. All results were presented as mean ± S.E.M.

3. RESULTS

The free calcium concentration in unfertilized oocytes was variable (range 70–270 nM, mean 138 ± 8 nM, $n = 31$). The addition of capacitated sperm to zona-free oocytes resulted in a microscopically visible sperm attachment. Immediately following sperm attachment, we observed a rapid, transient increase in oocyte [Ca]. In a representative experiment, the initial calcium transient

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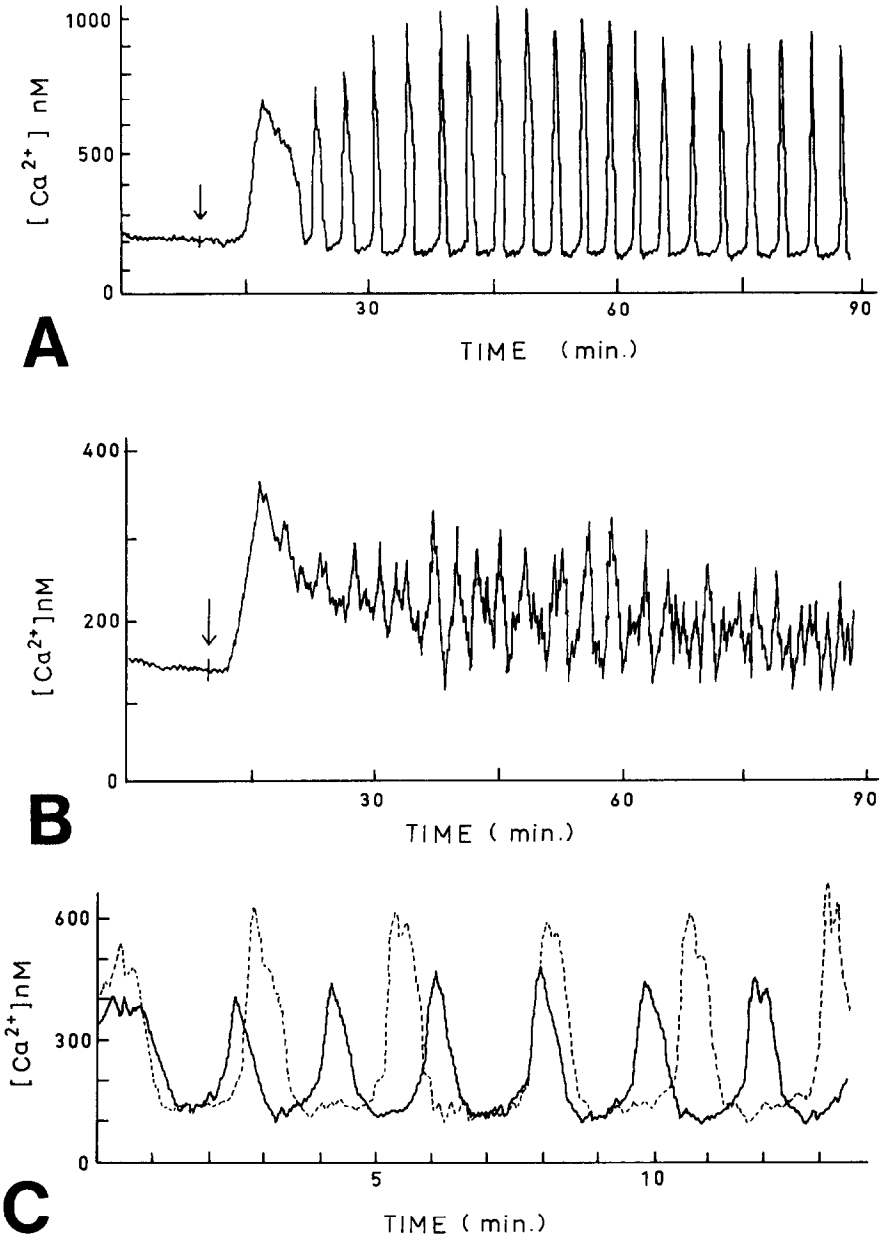

Fig. 1. Concentration of calcium in oocytes after fertilization in vitro. (A) A representative pattern of changes in cellular [Ca] in zone-free oocyte inseminated with high sperm density at the time indicated by the arrow. (B) An integrated graph of [Ca] changes of 19 zona-free oocytes inseminated with high concentration of sperm at a point denoted by an arrow. Note the almost simultaneous appearance of the first peak of [Ca] elevation. (C) A representative tracing of [Ca] repetitive transients in two zona-enclosed oocytes, 4.5 h after insemination.

displayed variability in its amplitude and duration (607 ± 55 nM and 6.3 ± 0.2 min, respectively, n = 19). This elevation was followed by a series of narrower calcium transients of high amplitude (847 ± 32 nM, maximal amplitude). A representative pattern is shown in Fig. 1A. In individual oocytes, the transients were cyclical with regular peak-to-peak intervals (range 2.1 ± 0.07 to 3.9 ± 0.07 min). The repetitive [Ca] transients continued for a variable period of time and in some oocytes were followed for 5 h (see also Fig. 1C).

In zona-free oocytes inseminated with a high concentration of sperm, an almost simultaneous appearance of the first [Ca] transient was observed in most of the oocytes examined (time to the beginning of the first peak 5.2 ± 0.7 min, Fig. 1B). Under these conditions, polyspermy occurs and could be verified microscopically. To ensure that the pattern of [Ca] dynamics described above was not a result of simultaneous multiple sperm–oocyte interactions, we conducted similar experiments using very low sperm concentrations. In these experiments, the onset of the first transient was not simultaneous for all the oocytes tested and many

oocytes did not respond within the time of the experiment. Nevertheless, oocytes, in which sperm attachment (1–2 per oocyte, $n = 10$) was visually confirmed, displayed an identical phenomenon.

Fertilization in vivo follows the interaction between sperm and zona-enclosed oocytes. Our experiments performed on zona-free oocytes may thus not faithfully reflect the physiological process. Hence, we inseminated zona-enclosed oocytes, which were subsequently loaded with Fura 2 and analyzed for [Ca] changes. This protocol produces almost exclusively monospermic fertilization. Oocytes treated in this manner exhibited also repetitive [Ca] transients over an extended period of time ($n = 15$, see Fig. 1C for representative experiment). We could not monitor the initial [Ca] transient in zona-enclosed oocytes, since their attachment was not firm enough to avoid rapid movements caused by sperm attachment.

To rigorously verify that [Ca] transients are indeed characteristic of normal fertilization, we have mated immature superovulated females and isolated these in vivo fertilized-oocytes after five hours. The in vivo fertilized oocytes were loaded with Fura 2 after their isolation. [Ca] measurements and visual inspection for fertilization were performed 7–8 h after mating, i.e. 3–4 h after sperm penetration [8]. A series of repetitive calcium transients or a single wide transient were observed in a high proportion of fertilized oocytes ($n = 11$, see Fig. 2A,B for representative responses). Hence, oocytes fertilized in vivo exhibit patterns of [Ca] changes similar to those fertilized in vitro, either poly- or monospermically.

4. DISCUSSION

The importance of fertilization-associated changes in oocyte [Ca] have been proposed already by Jaffe [1]. Although repetitive [Ca] spikes have been reported in sea urchin [9] and in ascidia [10], the only comparable findings in mammals are the recent reports in hamster [11], mouse [12,13] and pig [14] oocytes. Although Fissore et al. [15] have reported several [Ca] transients in bovine oocytes, these were variable, irregular and very late. Recently, Miyazaki et al. have described the kinetics of repetitive [Ca] transients in hamster oocytes fertilized in vitro under conditions that promote polyspermy [16].

The present report provides several novel observations in this area. Firstly, although the basal [Ca] values exhibit a large range among individual oocytes in the same experiment (90–270 nM), this variability does not affect the dramatic changes associated with fertilization.

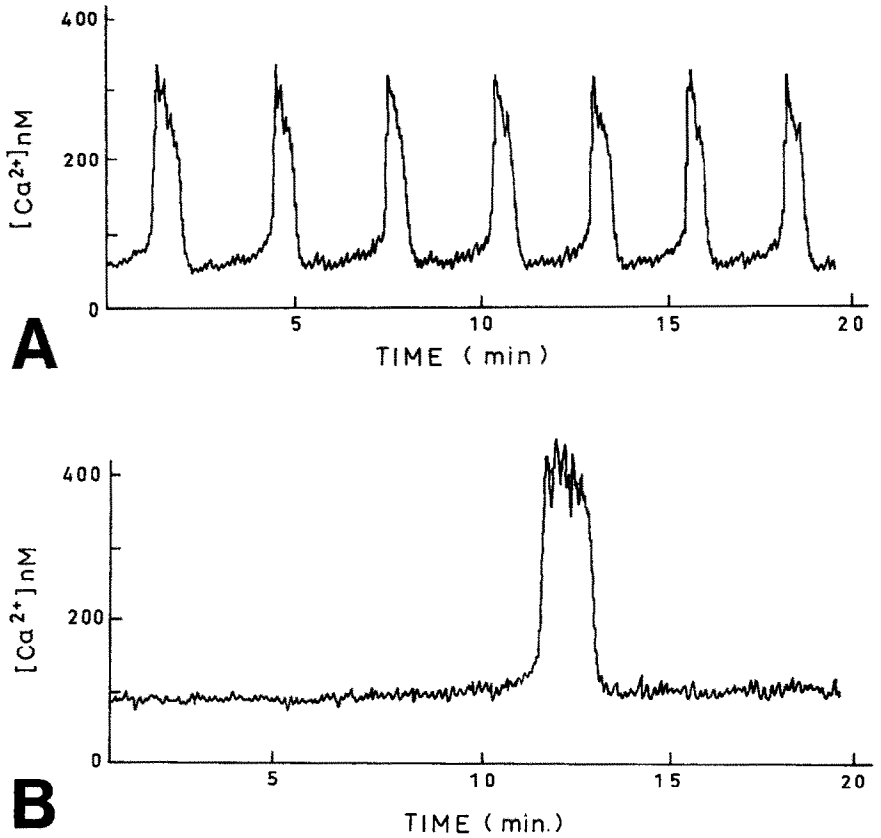

Fig. 2. [Ca] transients in in vivo fertilized oocytes. Superovulated immature females were mated and oocytes were isolated and loaded with Fura 2. [Ca] was assayed in individual oocytes at 7 h after mating. (A) A representative tracing of repetitive [Ca] transients. (B) A representative tracing of a single, broad [Ca] tracing observed within the time window of the experiment (20 min).

Secondly, the repetitive [Ca] transients persist for a long time following sperm attachments. Thirdly, and more importantly, while all of the published data (with the exception of Fissore et al. [15]) were performed on zona- free oocytes, we demonstrate that monospermic fertilization of either zona-free or zona-enclosed oocytes produces the same pattern of [Ca] transients. This observation was confirmed in a large number of both zona-free and zona-enclosed oocytes. Hence, it is most likely that this phenomenon reflects the physiological events occurring during fertilization in vivo in mammals. Indeed, repetitive [Ca] transients were seen in oocytes isolated from females following in vivo fertilization. In a few oocytes, a single, longer transient was observed within the examined period (10–25 min). It should be further investigated whether these single transients represent repetitive events of much lower frequency, a different mode of [Ca] activity that is characteristic of in vivo fertilized oocytes or a result of the time of oocyte isolation after sperm penetration. Previous reports suggest that fertilization is accompanied by a prolonged train of calcium transients. The most complete description was reported by Miyazaki et al. [17] in hamster oocytes, using the luminescent probe, aequorin and by Kline and Kline in mouse oocytes [13] using fluo-3. Both methods preclude the assignment of absolute calcium concentrations. Following the report of Miyazaki et al. [16] in hamster, this is the second report that quantitatively describes the entire sequence of [Ca] changes. Further, to the best of our knowledge, it is the first report of cyclical [Ca] transients in mammalian oocytes fertilized in vivo. Our results demonstrate the validity of studies performed on in vitro inseminated oocytes, as they closely mimic the in vivo conditions. The highly reproducible pattern of fertilization-induced [Ca] changes in rat oocytes should allow a future study of its spatio-temporal relationship to the early events of fertilization in this model system.

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