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Spatiotemporal pattern of calmodulin and $[Ca^{2+}]_i$ is related to resumption of meiosis in mouse oocytes

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Abstract

During meiotic maturation, mammalian oocytes undergo a series of morphological and physiological changes that prepare them for fertilization. Calcium-initiated signaling is thought to trigger these processes. In this study, we examine the spatio–temporal pattern of calcium and calmodulin (CaM), its downstream receptor, in order to investigate their association with meiotic maturation. Intracellular free calcium and activated CaM levels were measured using the fluorescent probes Calcium Green™-1 and TA-CaM, respectively. The distribution patterns were examined using confocal microscopy. Both calcium and activated CaM showed a dynamic spatiotemporal distribution during meiotic maturation. After release from IBMX buffer, calcium was found to periodically translocate from the perinuclear region to the germinal vesicle (GV) in 90 s intervals. After 90 min, calcium stopped oscillating and became concentrated within the GV. After a further 60 min, the GV broke down and calcium dispersed into the ooplasm, but calcium levels were slightly lower here than in the original nuclear region. Activated CaM also showed a dynamic patterning process similar to calcium. Taking the data from calcium chelation and CaM inhibition together, our results suggest that the dynamic distribution patterns of calcium and activated CaM are crucial for oocyte maturation.

Keywords: Oocyte; Meiosis; Calcium; Calmodulin; Spatiotemporal distribution

1. Introduction

In mammalian ovaries, fully grown oocytes are arrested at the dictyate stage of meiosis. They are distinguished by having a large nucleus with several nucleoli, and are known as germinal vesicles (GV). Oocytes resume meiosis only in response to a pre-ovulatory surge of gonadotrophic hormones in vivo, which is marked by corruption of the nuclear envelope and disappearance of the nucleoli, a process known as germinal vesicle breakdown (GVBD). It is believed that initiation of GVBD is controlled by a number of different cellular signals. Previous work in our laboratory and by other groups have shown that calcium oscillations or waves play an important role in the resumption of meiotic maturation of mammalian oocytes, including human, mouse, bovine and porcine oocytes (Balakier et al., 2002; Carroll et al., 1994; He et al., 1997; Homa et al., 1993; Li et al., 1994a,b; Sedmikova et al., 2003; Tosti et al., 2002).

Calcium oscillations occur before GVBD, and the latter can be prevented when the oscillations are inhibited (Li et al., 1994a,b). Other reports suggest that changes in intracellular Ca^{2+}, although they may occur, are not essential for GVBD (Carroll and Swann, 1992). A study about the gap-junction communication in mouse cumulus–oocyte complexes found that FSH and EGF, both of which stimulate maturation in vitro, had no effect on Ca^{2+} in such complexes (Webb et al., 2002). Thus, the question of whether Ca^{2+} oscillations are needed for oocyte maturation remains controversial. This may be due to differences among species and between mechanisms governing spontaneous maturation in vitro and gonadotrophin-induced maturation in vivo.

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In in vitro studies, chemicals that block the degradation of cAMP, such as 3-isobutyl-1-methyl-2,6(1H,3H)-purinedione (IBMX), are used to maintain higher levels of cAMP in oocytes, to inhibit their spontaneous maturation. Varying follicle fluid dose performs this function in vivo. The release of oocytes from the inhibition medium imitates the action of the preovulatory gonadotrophin surge, which may be resposible for the controversy over calcium requirement.

Previous studies have shown that the transduction of calcium signaling in the oocyte is different from that in most cultured mammalian cells. Only a uniform rise in calcium was seen in small somatic cells during signal activation (Tombes and Borisy, 1989), but a sharp $[Ca^{2+}]_i$ gradient between the sources and sinks is required for calcium function in big oocytes. Fertilization events can trigger dramatic changes in this gradient, from the sperm entry point to the opposite area of the oocyte. Thus, embryonic cells may use a different strategy to somatic cells in the transduction of calcium signaling (Ducibella et al., 2002).

CaM is a ubiquitous intracellular mediator of calcium signaling. The binding of calcium to CaM enables it to activate various target enzymes, such as calcium/CaM dependent protein kinases, and therefore regulate many physiological processes (Lu et al., 1993; Niki et al., 1996; Rasmussen and Means, 1989; Vogel, 1994; Wang et al., 1985). Calcium/calmodulin-dependent protein kinase II (CaMKII) is tightly associated with the meiotic spindle after egg activation. There is also a transient, tight association of calmodulin with the meiotic spindle that colocalizes with CaM kinase II (Johnson et al., 1998). It has been found that CaMKII also participates in the maturation of mouse and porcine oocytes (Fan et al., 2003; Su and Eppig, 2002).

We have found that CaM is dynamically redistributed during the cell cycle in HeLa cells (Li et al., 1999a,b). Each stage of the cell cycle has its special distribution pattern: CaM is mainly found in the cytosol during G1, then translocated into the nucleus from S to G2 phase, and highly concentrated in the nucleus before G2/M transition. Through the spatio–temporal distribution of CaM, calcium signalling regulates physiological processes such as G1/S transition, DNA replication, chromatin condensation, nuclear envelope breakdown, spindle structure maintenance, cortex flow and cytokinesis (Baitinger et al., 1990; Li et al., 1999a,b; Means, 1994; Santella, 1998). So we proposed a hypothesis of CaM functioning in cultured mammalian cells: CaM functioning depends on its spatiotemporal distribution. CaM is activated only when freely diffused calcium shows up in areas of CaM distribution (Li et al., 1999a,b). But the distribution of CaM in oocytes during GVBD has not been well studied, and whether the localized aggregation of CaM is involved in the regulation of cell function in the oocyte still remains unclear.

In this study, we addressed this question by examining the dynamic distribution of calcium and CaM during GVBD in live oocytes. We found that both calcium levels and activated CaM showed a dynamic spatiotemporal distribution during GVBD. In addition, both chelating calcium and inhibiting CaM activation blocked the occurrence of GVBD. This suggests that the oscillation of calcium and the dynamic activation of CaM are crucial for meiotic maturation.

2. Materials and methods

2.1. Oocyte collection and culture

Oocytes were collected from superovulated 21 day ICR mice. The mice were injected intraperitoneally with 5 IU pregnant mare serum gonadotrophin (PMSG, Tianjin hormone factory, China). The ovaries were excised 48 h after PMSG treatment and punctured with a fine needle under a stereomicroscope at ambient temperature, to release oocytes from follicles. Oocytes with surrounding cumulus cells were blown in and out of a fine glass tube to release denuded oocytes. The collected oocytes were cultured in DMEM supplemented with 10% NCS. All the collecting procedures were carried out in M2 medium with 50 μM IBMX (Sun et al., 1999), to inhibit spontaneous meiotic resumption.

2.2. Microinjection

The calcium probe, Calcium Green™-1, was purchased from Molecular Probes (Eugene, OR, USA). Calcium Green™-1 emits green fluorescence when excited at the wavelength 488 nm. The activated CaM probe, TA-CaM, was a gift from Prof. D. C. Chang (Hong Kong University of Science and Technology). TA-CaM can be used to measure the activation of CaM in living cells because it increases in fluorescence intensity 9-fold when binding to calcium and its target protein (Torok and Trentham, 1994). TA-CaM emits blue fluorescence when exited by ultraviolet light.

Calcium Green™-1 (200 μmol/l) (Carroll et al., 1994) and TA-CaM (1 mmol/l) (Li et al., 1999a) were dissolved in injection buffer (Hepes 10 mM, $MgCl_2$ 3 mM, sodium glutamate 30 mM, sucrose 80 mM, potassium phosphate 40 mM, pH 7.2) and injected into the cytosol of oocytes using an Eppendorf microinjection system mounted on a Nikon microscope. Rhodamine-Dextron (10 kDa) (Molecular Probes) was co-injected with the probes as an indicator of microinjection. Injected oocytes were then washed four times in DMEM without IBMX, and then observed with a confocal microscope (Bio-Rad MRC-1024 ES) mounted with a Zeiss Axiovert 100.

2.3. Confocal observation

To observe the oscillation of $[Ca^{2+}]_i$, we chose a focal plane that crossed the GV. Images of the sections were recorded every 2 s and the fluorescence intensity within the selected areas (inside GV and perinuclear region) were analyzed with Lasersharp software (Bio-Rad). To observe the spatial distribution of calcium and CaM, the oocytes were optically scanned from top to bottom with 1 μm Z-step. All the sections were 3-dimensionally reconstructed with Lasersharp software. For observation of live oocytes, microinjection and confocal observations were conducted in a chamber mounted on the microscope, to maintain a constant humidity and temperature.

2.4. Antagonism of calcium and CaM

To investigate the functions of calcium and CaM during meiotic maturation, EGTA (Sigma, St Louis, MO, USA) was either administered directly to the culture medium to chelate extracellular calcium, or microinjected into the ooplasm to block intracellular calcium (Tosti et al., 2002). The volume of EGTA injected into oocytes was carefully controlled and the dead oocytes were excluded. Only the oocytes that were alive after injection were calculated in the results (n=40). A calmodulin antagonist, W7 (Calbiochem, La Jolla, CA, USA), was added directly to the medium to block calmodulin activity (Fan et al., 2003; Su and Eppig, 2002). After treatment, the oocytes were cultured in medium without IBMX.

2.5. Statistical analysis

The significance of the difference between oocytes undergoing GVBD after treatment and the corresponding controls was analyzed using a pooled t-test. Each treatment was repeated three times. A probability value of $P<0.05$ was taken as statistically significant.

3. Results

3.1. Calcium periodically translocated into GV during meiosis resumption

When oocytes were released from IBMX inhibition medium, denuded oocytes showed large germinal vesicles with several nucleoli inside (Fig. 1B, left panel). Confocal observation found that calcium was mainly aggregated in the perinuclear region, but was lower in GVs and cytosol, as indicated by the dim fluorescence (Fig. 1B, middle panel). We chose a small area in the perinuclear region and inside a GV to measure calcium level changes after release from IBMX buffer. Calcium showed synchronized oscillations in different compartments of the oocyte (Fig. 1A). Calcium in the perinuclear region and GV oscillated synchronously over intervals of 90 s and lasted for more than 1 h (n=8) (because the time period was too long, we have just shown one part of the oscillation, lasting about 6 min; the oscillation pattern remained unchanged until GVBD occurred). The increases in fluorescence intensity in the perinuclear region and GV were also similar, although calcium levels were different.

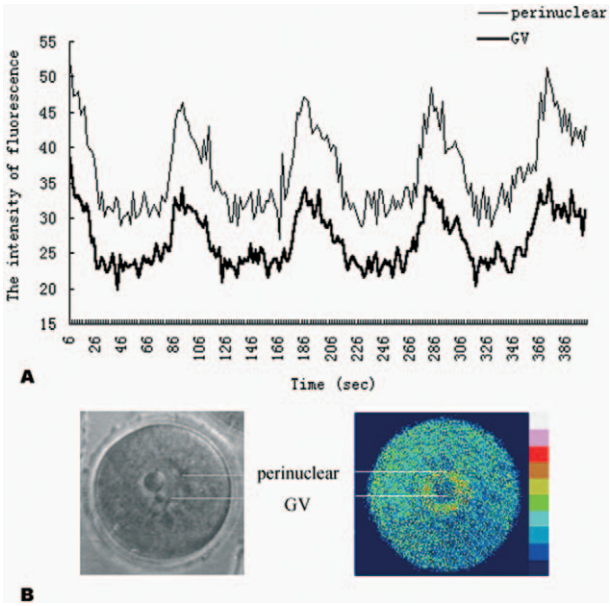

Fig. 1. Calcium oscillations in the optical section across GV of oocytes after release from follicles. The images were recorded every 2 s and the fluorescence intensity in the selected areas (inside GV and perinuclear region) were analyzed by Lasersharp software (Bio-Rad). Calcium levels inside the GV changed concurrently with those of the perinuclear region. The calcium oscillations continued for more than 1.5 h; this figure shows the oscillations of a period before GVBD. A total of 8 oocytes were analyzed and all of them displayed the same pattern of calcium oscillation. A: calcium oscillations before GVBD; B: an oocyte with GV and calcium localization when released from follicle.

3.2. Calcium aggregated at the GV after oscillation ceased

About 90 min after release from IBMX, calcium oscillation stopped and the majority of the calcium was found dispersed in the cytosol, with some remaining around the GV. But as Fig. 2A shows, calcium also aggregated in one hemisphere of the oocyte. When the oocyte was ready for GVBD (about 2 h after release from IBMX buffer), almost all the calcium aggregated in the GV (Fig. 2B) and remained there until GVBD (around 2.5 h after release from IBMX buffer). After GVBD, calcium in the GV dispersed into the whole ooplasm and became evenly distributed (Fig. 2C). Intracellular calcium level was higher after GVBD than at the GV stage.

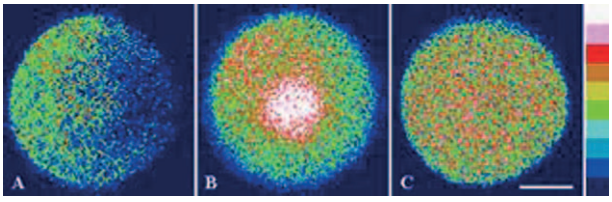

Fig. 2. The distribution of $[Ca^{2+}]_i$ during GVBD in mouse oocytes cultured without IBMX. Calcium was first aggregated around GV before GVBD (A) and highly concentrated into GV during GVBD (B), then dispersed and evenly distributed into ooplasm (C) (scale bar: 20 μm) (magnification: 200×, stained with Calcium Green™-1). In this experiment, 7 out of 8 oocytes displayed the same distribution of calcium. The color bar represents the fluorescence intensity from lowest (dark blue) to highest (white).

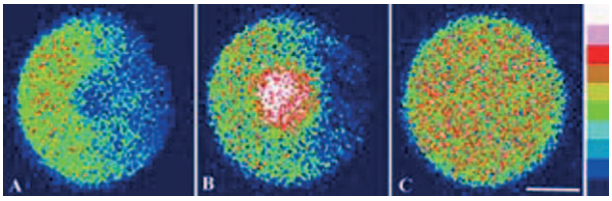

Fig. 3. The distribution of TA-CaM during GVBD in mouse oocytes. The pattern of change for CaM is similar to that of $[Ca^{2+}]_i$. CaM was first aggregated in one hemisphere of the oocyte and surrounded the GV before GVBD (A). CaM highly concentrated into GV during GVBD (B), then dispersed and evenly distributed into ooplasm (C) (scale bar: 20 μm) (magnification: 200×). Eight out of 11 oocytes displayed the same distribution of CaM during GVBD. The color bar represents the fluorescence intensity from lowest (dark blue) to highest (white).

3.3. CaM distribution patterns during GVBD

Using TA-CaM as the probe for activated CaM, we found that CaM had a similar distribution pattern to calcium during GVBD. It also aggregated in one hemisphere of the oocyte before GVBD (Fig. 3A), then translocated into GV (Fig. 3B), and finally became evenly distributed after GVBD (Fig. 3C). These results suggest that both calcium and CaM aggregate in the GV, and this colocalization could facilitate the activation of CaM during GVBD.

3.4. Chelating intracellular $[Ca^{2+}]_i$ may inhibit meiosis resumption

To investigate the function of calcium during GVBD, we chelated extracellular and intracellular calcium to observe their effect on GVBD in mouse oocytes. We did this by administering EGTA directly to the culture medium or microinjecting it into GV. Chelating extracellular calcium had no effect on GVBD; almost all the oocytes underwent GVBD after 3 h in culture (Fig. 4). But when intracellular calcium was chelated by EGTA microinjection, GVBD was blocked in a dose-dependent manner (Table 1).

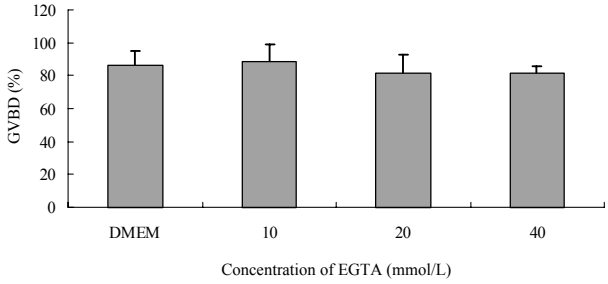

Fig. 4. Meiosis was resumed when the oocytes were cultured with EGTA. Chelating extracellular Ca^{2+} did not affect the resumption of meiosis, whilst intracellular Ca^{2+} was enough to trigger GVBD. Every experiment was repeated three times and more than 40 oocytes were cultured in each concentration of EGTA.

Table 1
The effect of microinjection of EGTA on GVBD of mouse oocytes. The volume of solution injected into oocytes did not exceed 1% of oocyte volume. Dead oocytes due to damage by microinjection were excluded

	Concentration (mM)	Number of injected oocytes	Number of GVBD oocytes
Control	Injection buffer	18	16 (16/18)
EGTA	1	22	16 (16/22)
EGTA	10	31	17 (17/31)[a]
EGTA	20	36	2 (2/36)[b]

[a] $P<0.05$ vs control group.
[b] $P<0.01$ vs control group.

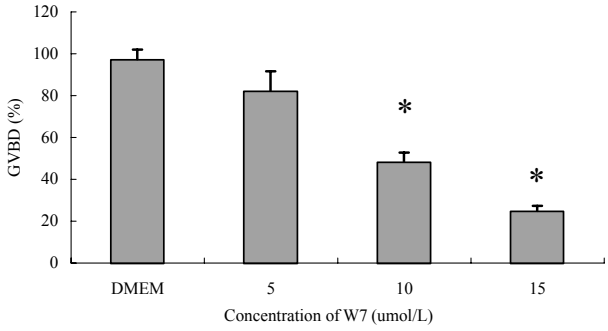

Fig. 5. The influence of W7 on GVBD. When W7 was added to the medium to inhibit the activation of CaM, GVBD was blocked. After treatment with 15 μM W7, 75% of oocytes remained in the GV stage. Every experiment was repeated three times and more than 40 oocytes were cultured in each concentration of W7.

3.5. Inhibiting CaM blocks GVBD in mouse oocytes

As a CaM inhibitor, W7 blocks the binding of $[Ca^{2+}]_i$ to CaM. When the cell membrane-permeable W7 was added to the medium to inhibit CaM activation, the oocytes were arrested at the GV stage. After treatment with 15 μM W7, 75% of oocytes remained in the GV stage ($P<0.01$, Fig. 5). These results indicate that activation of CaM is crucial for GVBD. When released from W7, most of the oocytes continued through meiosis.

4. Discussion

It has been reported that calcium release from intracellular stores leads to spontaneous calcium oscillation in oocytes, which may trigger the resumption of meiosis maturation. In this study, we also found a localized calcium change before GVBD in mouse oocytes. Calcium oscillated inside the GV from the perinuclear region. Moreover, we found most free calcium aggregated into the GV just before GVBD. Calcium levels throughout the whole oocyte became evenly distributed after GVBD. These localized changes in the GV region reflect extensive morphological changes in GV, such as chromatin condensation and nuclear membrane dissociation. We have previously found that most mitochondria translocate to one hemisphere of the oocyte; the one from which the first polar body is extruded (Li and Fan, 1997).

We have also found that calcium aggregates in one hemisphere of the oocyte before GVBD, but we do not know whether it is the same one as the first polar body is extruded from. CaM is the most abundant and well-known Ca^{2+} sensor in eukaryotic cells. It remains inactive until it binds to calcium. It modulates many basic cellular processes, such as cell growth, differentiation, proliferation, cell survival and cell motility (Agell et al., 2002; Bähler and Rhoads, 2002; Chin and Means, 2000). There have been many studies of the function of the Ca^{2+}/CaM compound during mitosis (Bootman and Berridge, 1995; Lu et al., 1993; Rasmussen and Means, 1989).

Activation of CaM is also believed to mediate the calcium signal that regulates meiosis resumption in oocytes. We have found that the spatial–temporal distribution of CaM is critical to its function during the cell cycle in cultured mammalian cells. CaM aggregates into the nucleus before G2/M transition in living HeLa cells when monitored using the green fluorescent protein (GFP) labeled fusion protein method (Li et al., 1999a,b). Because the resumption of meiosis maturation in mouse oocytes is similar to G2/M transition in mammalian somatic cells, we examined whether calcium signaling is also required for the spatial–temporal distribution of calcium and CaM in oocytes. Our results showed that activated CaM, in addition to calcium, aggregates inside the GV before GVBD.

We also confirmed that chelating calcium and inhibiting CaM activity both blocked resumption of meiosis maturation, indicating that the Ca^{2+}/CaM compound inside GV regulates the nuclear events of meiosis resumption. Our observation supports the hypothesis that CaM activation depends on its spatial–temporal distribution and its interaction with freely diffused calcium in situ. Previous studies have suggested that Ca^{2+}/CaM regulates the progress of mitosis by its downstream molecular CaMKII. Baitinger et al. (1990) found that CaMKII was necessary for nuclear membrane breakdown at early metaphase in mitosis. CaMKII could also be the target molecule of Ca^{2+}/CaM during meiosis regulation. Abbott et al. (2001) found that the protein levels of a 60 kDa CaMKII isoform increased 150% during oocyte maturation.

Calcium/calmodulin may also regulate other events during meiosis maturation, in addition to GVBD. Calcium oscillation has been found to be responsible for cortical granule exocytosis (Abbott and Fissore, 1999). Koninck and Schulman (1998) reported that CaMKII could act as a frequency decoder of calcium oscillation in vitro. More recent data indicate that CaMKII participates in oocyte activation and fertilization in mammals. It has been found that CaMKII, and hence the calcium signaling pathway, is potentially involved in regulating the meiotic maturation of mouse oocytes. This kinase participates in the gonadotrophin-induced resumption of meiosis, as well as promoting the metaphase I to anaphase I transition (Su and Eppig, 2002; Tatone et al., 2002).

Fan et al. (2003) found that CaMKII is a multifunctional regulator of the meiotic cell cycle and spindle assembly in porcine oocytes, and that it may exert its effect via regulation of MPF and MAPK/p90rsk activity during the meiotic maturation and activation of pig oocytes. A calcium response mechanism develops during meiosis maturation in mouse oocytes, which is also important in fertilization and subsequent events. How CaM interacts with its downstream signaling molecules will be an interesting research topic for the future.

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