Spindle function in *Xenopus* oocytes involves possible nanodomain calcium signaling

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Abstract

Intracellular calcium transients are a universal phenomenon at fertilization and are required for egg activation, but the exact role of Ca\(^{2+}\) in second polar body emission remains unknown. On the other hand, similar calcium transients have not been demonstrated during oocyte maturation and, yet, manipulating intracellular calcium levels interferes with first polar body emission, in mice and frogs. To determine the precise role of calcium signaling in polar body formation, we employed live cell imaging coupled with temporally precise intracellular calcium buffering. We found that BAPTA-based calcium chelators caused immediate depolymerization of spindle microtubules in meiosis I and meiosis II. Surprisingly, EGTA, at similar or higher intracellular concentrations, had no effect on spindle function or polar body emission. Using two calcium probes containing permutated GFP and the calcium sensor calmodulin (Lck-GCaMP3 and GCaMP3), we demonstrated enrichment of the probes at the spindle but failed to detect calcium increase during oocyte maturation, at the spindle or elsewhere. Finally, endogenous calmodulin was found to co-localize with spindle microtubules throughout all stages of meiosis. Our results, most importantly the different sensitivity of the spindle to BAPTA and EGTA, suggest that meiotic spindle function in frog oocytes requires highly localized, or nanodomain, calcium signaling.
Introduction

Intracellular calcium release, triggered by fertilization, is a dominant biochemical event responsible for egg activation, leading to completion of meiosis and subsequent embryonic development (Miyazaki, 2007; Whitaker, 2006). In mammalian eggs which arrest at metaphase II, fertilization-induced calcium signaling is manifested in a series of calcium waves across the whole egg, lasting several hours and overlapping with sister chromatid segregation (anaphase II) and cytokinesis to emit the second polar body (Ozil, 1998). In frog eggs which also arrest at metaphase II, only a single calcium wave is evident, which originates at the sperm entry site and travels rapidly through the cortical region to reach the antipode within 10 minutes (Busa and Nuccitelli, 1985; Fontanilla and Nuccitelli, 1998). A key role of calcium signaling at fertilization appears to be the activation of calmodulin-dependent protein kinase II, CMKII, (Johnson et al., 1998) leading to the activation of anaphase promoting complex (APC/C) and destruction of cyclin B and securin, thus initiating anaphase II and emission of the second polar body (Lorca et al., 1993; Madgwick et al., 2005). The repetitive calcium transients in mammalian eggs, in addition to the role in anaphase II initiation, are thought to be required for metabolic egg activation in preparation for embryonic development (Whitaker, 2006). However, a more recent mouse study has suggested that “calcium insulation”, in which both calcium efflux and calcium influx are blocked but intracellular calcium transients are unperturbed, blocks second polar body emission but allows anaphase II and many other egg activation events to proceed normally (Miao et al., 2012). It was suggested that these eggs fail to rotate the metaphase II spindle and hence fail cytokinesis (Miao et al., 2012).
Oocyte maturation, on the other hand, is triggered by species-specific intra-ovarian hormonal signals. Although calcium waves have been detected in mouse oocytes undergoing spontaneous maturation in vitro, prior to germinal vesicle breakdown (GVBD) (Carroll and Swann, 1992), they do not appear to be required for GVBD in mice (Tombes et al., 1992). On the other hand, multiple studies have reported the lack of global calcium transients during first polar body emission in mice (Hyslop et al., 2004; Marangos and Carroll, 2004; Tombes et al., 1992), and yet depletion of external calcium, and to a lesser degree chelating intracellular calcium with BAPTA, inhibits first polar body emission (Tombes et al., 1992). Moreover, in frog oocytes, removal of external calcium, depletion of intracellular calcium stores by thapsigargin, or injection of BAPTA, inhibited first polar body emission (Sun and Machaca, 2004).

This paradox, the clear inhibition of first polar body emission by disrupting calcium signaling and yet no global calcium transients are evident, could be explained if polar body emission requires localized calcium transients. In this study, we have combined live cell imaging coupled with timely application of calcium chelators to examine the immediate effect to intracellular calcium buffering on polar body emission. We have also employed two calcium probes, Lck-GCaMP3 and GCaMP3, membrane and non-membrane versions of the same permutated GFP-calmodulin-M13 core (Shigetomi et al., 2010a; Tian et al., 2009), to determine possible local calcium transients during polar body emission.
Results

Lck-GCaMP3 and GCaMP3 failed to detect calcium transients during oocyte maturation

To detect possible calcium transients during polar body emission, we employed Lck-GCaMP3, a membrane-bound calcium probe with EC₅₀ of 153 nM for calcium (Shigetomi et al., 2010a). The core of this probe (GCaMP) is a permutated GFP containing the Ca²⁺ sensor calmodulin at one end and M13 peptide of myosin light chain kinase at the other. Calcium binding triggers calmodulin-M13 binding, resulting in a circularized and fluorescent GFP (Nakai et al., 2001).

As a control, we generated Lck-Cherry, a red fluorescence version of GFP (von Dassow G. et al., 2009). The Lck sequence contains sites of protein myristylation and palmitoylation (Yurchak et al., 1996) anchoring the fusion proteins to cellular membranes. We first determined the response of these probes in Xenopus eggs to the well-characterized calcium wave triggered by fertilization. Oocytes were injected with mRNAs coding for Lck-GCaMP3 and Lck-Cherry, and incubated with progesterone to complete meiosis I (Fig. 1A). Mature (metaphase II) eggs were imaged to determine the basal fluorescence signals of the two probes, focusing on the animal pole including the first polar body (Fig. 1B, 00:00, arrow). The eggs were then pricked (mimicking fertilization (Leblanc et al., 2011)) followed immediately by time lapse confocal imaging. Robust Lck-GCaMP3 (eGFP) signal was observed at the animal pole cortex 2 minutes after pricking (Fig. 1B, 00:02, upper panel, and the graph), consistent with the travelling speed of the calcium wave (Miller et al., 1993) initiated at the site of pricking (1/4 of pole-to-pole distance). The signal subsided 2 minutes later (Fig. 1B, 00:04, upper panel, and the graph) indicating the passing of the calcium wave peak. In contrast, cortical Lck-Cherry signal
remained unchanged throughout (Fig. 1B, middle panels, and the graph). These results clearly indicated that Lck-GCaMP3 functioned as a calcium sensor in frog oocytes, responding robustly to the calcium increase at egg activation.

We next carried out live cell imaging of oocytes during oocyte maturation, focusing on first polar body emission. Oocytes were injected with mRNAs for RFP-H2B and Lck-GCaMP3, incubated with progesterone and monitored for GVBD. Lck-GCaMP3 exhibited a slight plasma membrane signal in GV oocytes (not shown), and this remained unchanged up to the time of metaphase to anaphase transition (Fig. 2A, 00:00). Shortly after anaphase initiation (Fig. 2A, 00:04), the cortical signal appeared to increase around the position of the contractile ring (Fig. 2A, 00:06) (Zhang et al., 2008), continuing to increase as the membrane constricted to sever the polar body chromosomes (Fig. 2A, 00:08 and 00:10). This signal persisted for hours while the egg was arrested at metaphase II (not shown).

Oocytes injected with Lck-Cherry, together with eGFP-H2B, were subjected to the same time lapse imaging experiments. These experiments clearly indicated the Lck-Cherry exhibited very similar increase in cortical signals during polar body emission (Fig. 2B, 00:00-00:10). The similarity between the two probes was more evident in XZ plane view (Fig. 2C), which indicated membrane thickening around the base of the polar body, in contrast to the increase of brightness of Lck-GCaMP3 signal after pricking (Fig. 1B). Co-localization of the two probes was further confirmed in oocytes injected with the two fluorescence probes imaged during first polar body emission (Fig. 2D). One obvious difference was the presence of Lck-GCaMP3 signal at the
spindle pole (arrow in Fig. 2A and Fig. 2D) and the corresponding lack of Lck-Cherry signal (Fig. 2B and Fig. 2D).

Similar Lck-GCaMP3 signal was observed during second polar body emission (Fig. 2E). In this case both the initial calcium wave (Fig. 2E, 00:03-00:06) and later accumulation at the base the polar body (00:20-00:22) were observed. Anaphase II was noted at 00:12 in this oocyte. Interestingly, the (metaphase II) spindle pole signal was initially very weak (Fig. 2E, 00:00, arrow), but became brighter (00:02) slightly ahead of the neighboring cortical region (00:03). The spindle pole signal remained stronger as the calcium wave subsided (00:06-00:09). These results suggest that the spindle pole signal exhibited by Lck-GCaMP3 in meiosis I (Figs. 2A and 2D) and in metaphase II prior to egg activation (Fig. 2E, 00:00) likely represent enrichment of the fluorescence probe due to a combination of Lck sequence (membrane-targeting) and the calcium sensor calmodulin (spindle-targeting, see later).

To further determine possible calcium transients in the spindle area, we carried out similar imaging experiments with GCaMP3, the same permutated GFP core but without the membrane-targeting Lck sequence (Tian et al., 2009). During oocyte maturation, GCaMP3 was slightly enriched at the spindle (Fig. 3A, 00:00-00:08) and remained so through anaphase I (Fig. 3A, 00:11). Similar enrichment was also observed at metaphase II (Fig. 3B, 00:00). However, following pricking (egg activation), GCaMP3 signal increased dramatically in the cortical area and especially at the spindle (Fig. 3B, 00:02), and subsided quickly (Fig. 3B, 00:03) in a time course similar to that seen with Lck-GCaMP3 (Fig. 1B). These data suggest that the signals seen at the spindle (GCaMP3) or spindle pole (Lck-GCaMP3) before egg activation likely represent
enrichment of the probe. At egg activation, these probes sense the calcium wave (Busa and Nuccitelli, 1985), producing robust fluorescence. Therefore, prior to egg activation, the overall calcium concentrations at the spindle are not significantly higher than that in the surrounding area.

Taken together, these data (Figs. 1-3) indicated that unlike egg activation which features prominent global calcium transients, Xenopus oocyte maturation does not involve global calcium transients, or overall calcium elevation at the spindle. Furthermore, these data suggest that the apparent accumulation of Lck-GCaMP3 and Lck-Cherry around the site of polar body emission was likely due to new membrane addition.

**BAPTA, but not EGTA, caused rapid depolymerization of spindle microtubules**

Previous studies (Sun et al., 2008; Sun and Machaca, 2004) have demonstrated that interfering with intracellular calcium signaling does not inhibit GVBD, but the oocytes do not form polar body and exhibit abnormal spindles. To determine the immediate effect of disrupting calcium signaling, we carried out live cell imaging coupled with timed injection of DB-BAPTA (Kd=1.6 µM), a calcium buffer previously shown to be effective in inhibiting cleavage in Xenopus embryos (Miller et al., 1993). Oocytes injected with fluorescent probes for microtubules and chromosomes were treated with progesterone and monitored by time lapse confocal imaging. At prometaphase when the meiosis I spindle is anchored to the oocyte cortex with one pole (Fig. 4A-DBB alone, 00:00), DB-BAPTA was injected. Within a few minutes of DB-BAPTA injection, corresponding to the time for the chelator to diffuse from the injection site to the
animal pole where the spindle resides (Miller *et al.*, 1993), the bulk of spindle microtubules started to disappear, accompanied by the clustering of chromosomes to the cortex (Fig. 4A-DBB alone, 00:04-00:08). Chromosomes remained at the cortex for hours with no sign of polar body emission or oocyte degeneration (not shown), indicating metaphase arrest (14/14 in five experiments). In contrast, oocytes injected with water were not perturbed and, at approximately 2 hours after GVBD, initiated anaphase (not shown) and progressed to emit the first polar body and arrested at metaphase II (10/10 in four experiments), as we have shown previously (Shao *et al.*, 2013; Zhang *et al.*, 2008).

To test the calcium specificity of DB-BAPTA, we carried out a series of similar buffer injection experiments except that DB-BAPTA solution had been mixed with CaCl$_2$ in a ratio of 20:1, 5:1, and 1:1, giving free Ca$^{2+}$ concentrations in the buffer of 0.08 µM, 0.4µM and 1.6 µM respectively (Kline, 1988). Injection of DB-BAPTA buffer with 20:1 ratio caused rapid spindle collapse, indistinguishable from DB-BAPTA alone (not shown). DB-BAPTA buffer with 5:1 ratio caused similar but more gradual spindle collapse (Fig. 4A, middle row, representative of 19 oocytes in four experiments). DB-BAPTA buffer with 1:1 ratio did not collapse the spindle in the same time frame (Fig. 4A, bottom row, representative of 11 oocytes in two experiments). Instead, it caused cortical contraction, similar to egg activation (see Materials and Methods). These data clearly indicate that the immediate spindle collapse caused by DB-BAPTA is due to its calcium buffering action.

To further demonstrate the effect of calcium buffering on spindles, we employed a UV-labile caged calcium chelator, diazo-2. Diazo-2 is a caged BAPTA with a low affinity (2.2 µM) but
becomes a high affinity calcium chelator (BAPTA; about 0.1 µM) after photoactivation (Mulligan and Ashley, 1989). Trial experiments indicated that while high concentrations of the caged diazo-2 caused microtubule depolymerization similarly to DB-BAPTA, intracellular concentrations lower than ~1 mM had no effect on spindle stability or polar body emission (Fig. 4B, top, representative of 24 oocytes in three experiments). Therefore we injected 30 nL of 10 mM diazo-2 (or 0.9 mM final intracellular concentration) at prometaphase. The oocytes were incubated for 5-10 minutes to allow diazo-2 to equilibrate throughout the oocyte before confocal imaging (Fig 4B, bottom, 00:00). Immediately after, the oocyte was subjected to UV excitation through the same microscope lens for three minutes (00:00 to 00:03). Simultaneous confocal imaging indicated rapid depolymerization of spindle microtubules and chromosome clustering (00:02 to 00:06), similarly to oocytes injected with DB-BAPTA alone (Fig. 4A, top). UV excitation of oocytes not injected with diazo-2 did not affect spindle morphology nor polar body emission (not shown), as we have reported previously (Shao et al., 2013).

The above results, rapid depolymerization of spindle microtubules in the presence of BAPTA-based calcium buffers and yet no evidence of overall increase of calcium at the spindle compared to the surrounding cytoplasm, suggest the involvement of highly localized, or nanodomain, calcium signaling (Neher, 1998; Wang and Augustine, 2014). In a calcium nanodomain, the calcium channel and the calcium sensor are within tens of nanometers, often in the same molecular complex. High concentrations of calcium released at the mouth of the channel binds rapidly (microsecond scale) to the calcium sensor, producing a biological response, before falling rapidly. The nanodomain calcium is so transient both spatially and temporally that it is not amenable to conventional microscopy using mobile calcium indicators (Tay et al., 2012).
Nanodomain calcium signaling is effectively buffered by the fast calcium buffer BAPTA and its derivatives but not to EGTA (Adler et al., 1991), which is a slow calcium buffer at physiological pH (Tsien, 1980). To test this possibility, we carried out similar injection experiments with EGTA. Indeed, EGTA injection (30 nl of 25 mM per oocyte) at prometaphase caused no noticeable change in spindle structure, nor did it inhibit first polar body emission (Fig. 4C, representative of 38 oocytes in five experiments). Injecting 60 nL of 25 mM EGTA (4.5 mM intracellular concentration), or mixing EGTA with various concentrations of calcium (5:1 and 1:1) before buffer injection, produced identical results (not shown).

To ascertain that both EGTA and DB-BAPTA function properly to buffer calcium in Xenopus oocytes, we determined the effect of DB-BAPTA and EGTA on egg activation, which requires global calcium elevation (Fig. 1B). Oocytes were injected with chromosome and microtubule probes, and treated overnight with progesterone. Mature (metaphase II) eggs were injected with water (Fig. 5A, 00:00), immediately followed by time lapse imaging. The injection served to activate the egg (pricking), as evident by cortical contraction, anaphase II (00:12-00:14) and emission of the second polar body (00:31). Injection of DB-BAPTA to metaphase II eggs (Fig. 5B, 00:00) did not cause cortical contraction, indicating that DB-BAPTA inhibited egg activation. However, metaphase II spindle collapsed rapidly accompanied by chromosome clustering (Fig. 5B, 00:02-00:15), similarly to prometaphase oocytes injected with DB-BAPTA (Fig. 4A, top). EGTA similarly inhibited egg activation (no cortical contraction) but, in contrast to DB-BAPTA, did not cause spindle collapse at all (Fig. 5C, 00:00 to 00:32). As summarized in Table 1, with the exception of DB-BAPTA-1:1, all buffers inhibited egg activation. DB-BAPTA buffered with equal molar ratio of Ca^{2+} has a free Ca^{2+} concentration of 1.6 μM, similar to the
amplitude of calcium wave (1.2 µM) at fertilization previously determined by others (Busa and Nuccitelli, 1985). Similarly buffered EGTA has 123 nM free Ca²⁺ thus capable of inhibiting egg activation (Table 1). These results confirmed that while both DB-BAPTA and EGTA effectively buffered the global calcium transients at egg activation, only DB-BAPTA (and diazo-2 after uncaging) was able to buffer the calcium transients required for spindle function.

**Differential effects of DB-BAPTA on Cdc42 and RhoA GTPases**

To further demonstrate the effect of calcium buffering at later steps of polar body emission, we injected DB-BAPTA at the beginning of anaphase. Specifically, we wished to determine the effect of calcium buffering on Cdc42-mediated membrane protrusion and the simultaneous RhoA contractile ring function (Zhang et al., 2008). In control oocytes, a small amount of eGFP-wGBD was seen at the spindle (Fig. 6A-Ctrl, 00:00), as previously reported (Zhang et al., 2008). Shortly after anaphase (00:04), a bright eGFP-wGBD “cap” was seen overlaying the protruding polar body chromosomes (00:10, arrow; note the outline of oocyte surface in faint green) and enveloping the polar body (00:16, arrow). Injection of DB-BAPTA at the beginning of anaphase (Fig. 6A-DB-BAPTA, 00:04) eliminated eGFP-wGBD signal and prevented membrane protrusion, trapping all chromosomes inside the eggs (00:08-00:22; 8/8 in two experiments). While control oocytes emitted the first polar body (PB) and arrested with a metaphase II spindle similarly anchored to the cortex (Fig. 6A-Ctrl, 01:23), DB-BAPTA-injected oocytes failed to emit a polar body, with condensed chromosomes close to the cortex (Fig. 6A-DB-BAPTA, 01:50). Therefore, DB-BAPTA inhibited Cdc42 activation and prevented membrane protrusion.
Interestingly, and in contrast to the complete inhibition of Cdc42 activation, DB-BAPTA did not inhibit RhoA activation, nor inhibit the formation of a contractile ring (Fig. 6B). Instead, while the contractile ring constricted “below”, and hence extricating, the polar body chromosomes (Fig. 6B-Ctrl, 00:11-00:16) in control oocytes, the contractile ring in DB-BAPTA-injected oocytes constricted, futilely, “above” all chromosomes (Fig. 6B-DB-BAPTA, 00:08-00:12). The effect of DB-BAPTA injection on RhoA contractile ring is reminiscent of the phenotype caused by a dominant-negative mutant (Cdc42N17) (Zhang et al., 2008).

**Calmodulin is intimately associated with meiosis spindles**

The above results, particularly the different sensitivity of spindle integrity to BAPTA and EGTA, strongly suggest that polar body emission in Xenopus oocytes requires nanodomain calcium signaling. Nanodomain calcium signaling is not sensitive to EGTA because of the close proximity, within tenths of nm, and the calcium source and calcium sensor, and that the binding of calcium to the sensor is much more rapid than binding of calcium to EGTA (Augustine et al., 2003; Neher, 1998). One possible calcium sensor is the calcium-binding messenger protein calmodulin. Xenopus oocytes contain extremely high concentration, 44-59 µM, of calmodulin (Cartaud et al., 1980). Furthermore, calmodulin has been shown in association with the spindle in meiosis I pig oocytes (Fan et al., 2003), meiosis II mouse oocytes (Johnson et al., 1998) and in somatic cells (Li et al., 1999).
Oocytes were fixed at the various stages during polar body emission, and co-stained with antibodies against calmodulin and β-tubulin, and counter-stained with DAPI. Calmodulin was enriched at the spindle, starting at early (E) prometaphase when short microtubules emanating from the single patch-shape microtubule organizing center (MOTC) capture cortical chromosomes (Gard, 1992; Shao et al., 2012), and throughout prometaphase, metaphase I, anaphase I and metaphase II, with patterns very similar to those of anti-β-tubulin staining (Fig. 7; co-localization coefficients, at any stage, of greater than 0.8). Interestingly, the microtubule remnant present within the first polar body was not associated with calmodulin (Fig. 7, arrow, right most panels), suggesting calmodulin’s role in functional (spindle) microtubules. We could not consistently inhibit polar body emission using W7 (Su and Eppig, 2002), either added to the medium or by injection (up to 50 µM final intracellular concentration), or calmodulin-binding domain peptide 290-309 of CMKII (Ichida et al., 2000) (10-100 nl of 0.5 mM stock solution, per oocyte), or using morpholino oligos antisense to Xenopus calmodulin (not shown). However, the same inhibitors also did not block egg activation (not shown). Thus, further work with more efficient inhibitor/strategy will be required to establish the functional role of calmodulin in polar body emission.
**Discussion**

In this study we combined live cell imaging with temporally precise application of calcium chelators: timed injection of DB-BAPTA or EGTA, and UV-induced activation of caged calcium chelator diazo-2 (Mulligan and Ashley, 1989). Our approach has the distinct advantage of revealing the direct effect of intracellular calcium buffering. Our data clearly indicate that BAPTA-based calcium chelators (DB-BAPTA and uncaged diazo-2), but not EGTA, caused rapid collapse of meiosis spindles. The effect of DB-BAPTA was clearly due to calcium buffering as indicated by the inverse relationship between concentrations of free calcium in the buffer and the speed of spindle collapse (Fig. 4A). DB-BAPTA alone caused immediate collapse of the spindle and clustering of chromosomes to the cortex, subject to diffusion of the buffer from the injection site to the spindle location (Miller *et al.*, 1993). DB-BAPTA/Ca$^{2+}$ mixture with a ratio of 5 (free Ca$^{2+}$=400 nM) caused much more gradual spindle collapse and chromosomes clustering. The buffer mixture with DB-BAPTA/Ca$^{2+}$ ratio of 1 (free Ca$^{2+}$=1.6 µM) did not collapse the spindle in the same time frame. Instead it caused cortical contraction, mimicking the fertilization calcium wave of 1.2 µM amplitude (Busa and Nuccitelli, 1985). With time, the spindle in these oocytes eventually became smaller (not shown), presumably because excess free Ca$^{2+}$ eventually dissipates through a combination of sequestration, by endogenous organelles (i.e. ER) and calcium buffers, and efflux, allowing DB-BAPTA to buffer the spindle-based calcium transients.
The spindle collapse caused by DB-BAPTA injection or UV activation of diazo-2 exhibited a speed identical to nocodazole treatment (Shao et al., 2013), suggesting similar mechanism: inhibition of microtubule polymerization. Consistent with this interpretation, injection of DB-BAPTA at the junction of metaphase-to-anaphase transition inhibited cortical Cdc42 activation and membrane protrusion (Fig. 6A), both of which are dependent on spindle microtubules (Leblanc et al., 2011) and spindle positioning, with one pole anchored to the cortex (Zhang et al., 2008). In contrast, the complete collapse of microtubule spindle, caused by DB-BAPTA, did not inhibit RhoA activation or formation of the contractile ring (Fig. 6B), suggesting that chromosomes and/or the closely associated MTOC in these acentrosomal oocytes (Gard, 1992; Schuh and Ellenberg, 2007; Shao et al., 2012), were sufficient to induce the contractile ring.

Interestingly, in sea urchin embryos, the two asters (MTOCs), placed far enough from each other, can activate RhoA and support complete cytokinesis, without microtubule spindle or chromosomes (von Dassow G. et al., 2009).

However the most interesting discovery is the different sensitivity of spindle integrity to BAPTA and EGTA: rapid and complete microtubule depolymerization in the presence of DB-BAPTA but none at all in the presence of EGTA, at equal or higher concentrations. This is despite EGTA having an affinity for calcium (123 nM at physiological pH) (Qin et al., 1999) that is at least an order of magnitude greater than that (K_d=1.6µM) of DB-BAPTA, and despite the ability of EGTA to inhibit egg activation in all buffer compositions (Table 1). This different sensitivity is reminiscent of that found in another calcium-mediated process: neurotransmitter release in the presynaptic nerve terminals of Squid Giant Synapse (Adler et al., 1991). A large body of literature has yielded detailed mechanistic understanding of this system and the different
responses to BAPTA and EGTA (Neher, 1998; Wang and Augustine, 2014). The close proximity, and indeed the direct molecular contact, of the calcium source (voltage-gated calcium channels) and calcium sensor (the vesicular protein synamptotagmin) form a calcium signaling unit, called nanodomain (the calcium channel and the sensor are within 20 nm of each other), such that the high local calcium concentration directly, with a time scale of microseconds, drives synamptotagmin-mediated vesicle fusion (exocytosis) and neurotransmitter release. Such calcium signaling is completely insensitive to EGTA which is a slow Ca\(^{2+}\) chelator, because at physiological pH EGTA is protonated and needs to be completely de-pronated before Ca\(^{2+}\)-binding can occur (Qin et al., 1999). In fact, it was its pH dependence and slow calcium-binding kinetics that prompted the design and synthesis of BAPTA and the various BAPTA derivatives, calcium chelators (Tsien, 1980). Whereas BAPTA, and its derivatives, has the same calcium binding motifs as EGTA (and hence the same Ca\(^{2+}\) selectivity over other divalent cations), BAPTAs are fast calcium chelators, with on-rate being diffusion-limited, and therefore are effective in buffering nanodomain calcium transients (Wang and Augustine, 2014). Our data strongly suggest that the spindle formation, more specifically microtubule polymerization, requires spindle-based nanodomain calcium signaling.

The requirement of calcium transients for spindle microtubule polymerization appears to contradict the longstanding view that microtubule polymerization does not require calcium and, in fact, is inhibited by calcium (Olmsted and Borisy, 1975; Weisenberg, 1972). Interestingly, the conclusion of calcium-noninvolvement was based on the insensitivity of microtubule polymerization to EGTA. Also, only very high and non-physiological concentrations of calcium, 0.5 mM or higher, inhibit microtubule polymerization (Olmsted and Borisy, 1975; Weisenberg,
Physiological calcium concentrations (e.g. 1.2 µM at fertilization (Busa and Nuccitelli, 1985)) clearly do not destabilize spindle microtubules or interfere with spindle function. As shown here, pricking (or water injection) caused immediate calcium elevation (Fig. 1B), but metaphase II spindle remained stable and completed anaphase and polar body emission (Fig. 5A). This is in contrast to BAPTA-type buffers (Figs. 4A, 4B and 5B) or nocodazole (Shao et al., 2013), which caused immediate collapse of microtubule spindles and clustering of chromosomes.

The calcium source in the proposed nanodomain calcium signaling is currently unknown, but it appears not to involve calcium influx. Incubating oocytes in OR2 media (Ma et al., 2006) without CaCl₂ resulted in normal spindle and first polar body (not shown). Interestingly, numerous studies have reported the association of membrane structures, particularly of nuclear envelop/ER (endoplasmic reticulum) origin, with mitotic apparatus (i.e. microtubules and kinetochores) in both plant and animals (Hepler and Wolniak, 1984) including animal oocytes (Rieder and Nowogrodzki, 1983). Depletion of ER calcium stores using thapsigargin caused spindle defects (Sun and Machaca, 2004), directly implicating IP3 receptor since it is the only ER calcium channel in Xenopus oocytes (Kume et al., 1993; Parys et al., 1992). Intriguingly, IP3 receptors in Xenopus oocytes exhibits sub-micrometer clusters (ca 50 nm diameter) containing several tens of channels, with a mean cluster-cluster spacing of ~3 µm (Dargan and Parker, 2003). Intra-cluster calcium-induced calcium transients are disrupted by BAPTA but minimally affected by EGTA (Dargan and Parker, 2003). However, physiological functions of these IP3 receptor clusters remain unknown since the calcium transients are measured under artificial IP3 introduction (Dargan and Parker, 2003).
On the other hand, calmodulin might be a calcium sensor in this proposed calcium signaling system. First, Xenopus oocytes contain extraordinarily large amount (44-59 µM) of calmodulin (Cartaud et al., 1980). Second, calmodulin is highly enriched at the spindle at all stages of meiosis (Fig. 7). Similarly, BAPTA buffers caused immediate microtubule depolymerization at all stages (Figs 4A, 4B, 5B). Given its complete insensitivity to EGTA, the calcium transients required for spindle function must be spindle-based (Wang and Augustine, 2014). Third, calmodulin is a common calcium sensor with two calcium binding domains, each capable of binding two Ca^{2+}. The C-terminal calcium binding lobe exhibits on-rate of 350-500 µs and that of the N-lobe is much slower (20 ms) (Malmendal et al., 1999; Park et al., 2008). Calmodulin is also known to bind IP3 receptor and regulates its calcium channel activity (Kasri et al., 2006). Intriguingly, calmodulin has also been implicated in regulating microtubule dynamics via microtubule-associated proteins (Lefevre et al., 2013).

The complete insensitivity of the spindles to high concentrations of EGTA implies that the functional relevant calcium transients are restricted to each nanodomain, dissipating rapidly and within the immediate vicinity of the calcium channels (i.e. IP3 receptors) without any functional inter-nanodomain interaction (calcium-induced calcium transients). As such, a global calcium elevation in the spindle area may not be expected, likely explaining the failure of the two calcium probes, GCaMP3 and Lck-GCaMP3, to detect significant calcium signals at the spindle prior to egg activation. Further imaging work, with far better spatial and temporal resolution, will be required to determine the presence of nanodomain calcium signals at the spindle.
Materials and methods

Oocyte collection

Sexually mature Xenopus laevis females were obtained from Nasco (Fort Atkinson, Wisconsin). The females were primed with 50 units of pregnant mare gonadotropin 3-10 days prior to sacrifice. Oocytes were manually defolliculated (Liu and Liu, 2006) in OCM media (prepared weekly by mixing 480 mL of Lebbovitz L-15 medium, 320 mL sterile water, 0.32 g of bovine serum albumin, and gentamycin to 0.5 mg/mL, pH 7.6-7.8) and kept at 18°C until use. When stored sparsely with frequent medium changes and cleaning out the dead, isolated oocytes can be used for 2-4 days.

Fluorescent probes and other reagents

The following plasmids have been described previously: pCS2-eGFP-H2B (histone 2B), pCS2-mRFP-H2B (Miller and Bement, 2009), pRN3-β5-tubulin-GFP (Verlhac et al., 2000), pCS2-3GFP-EMTB (von Dassow G. et al., 2009), eGFP-wGBD (for active Cdc42) and eGFP-rGBD (for active RhoA) (Benink and Bement, 2005; Zhang et al., 2008). Lck-GCaMP3 and GCaMP3 (Shigetomi et al., 2010a; Tian et al., 2009) was purchased from Addgene and subcloned between EcoRI and Bgl II sites of pCS2+ vector (Turner and Weintraub, 1994). The DNA sequence encoding the N-terminal 26 amino acids of Lck was amplified by PCR using Lck-GCaMP3 (Shigetomi et al., 2010b) as a template and inserted into pCS2+-C’mCherry vector (von Dassow
G. et al., 2009), generating Lck-Cherry. All cDNA constructs were linearized before in vitro transcription using Ambion SP6 mMMESSAGE kit.

Polyclonal anti-calmodulin antibodies (SAB4503194) and monoclonal antibodies against β-tubulin (T5201) were from Sigma.

**Oocyte injection, oocyte maturation and prick activation**

All fluorescent probes (mostly mRNAs) were injected at the germinal vesicle stage (GV or intact nucleus) oocytes, at least several hours before progesterone treatment to allow protein translation. Oocytes were incubated in OCM plus 1μM progesterone to trigger oocyte maturation. Oocytes were monitored every 10 minutes, starting about 2-3 hours after the addition of progesterone, for the appearance of a “maturation spot” (Fig. 1A), indicative of nuclear envelop breakdown (or GVBD). GVBD oocytes were transferred to fresh OCM without progesterone. DB-BAPTA is dissolved in water, and mixed with CaCl₂ to the indicated molar ratio, according to Kline (Kline, 1988). EGTA was dissolved in water and adjusted to pH 7 using KOH, and mixed with CaCl₂ to the indicated molar ratio. The buffer was injected half way between the equator and the animal pole (typically 30 nL of 25 mM DB-BAPTA or EGTA per oocyte, or 2.25 mM final intracellular concentration, based on the estimated water accessible cytoplasm of 333 nL (Snow and Nuccitelli, 1993)). To activate mature eggs (arrested at metaphase II), the egg was pricked with a glass needle, or injected with water, half way between the animal pole and the equator. Egg activation (Table 1) was assessed by contraction of the
pigmented animal hemisphere (cortical contraction) (Sive et al., 2000), and confirmed by assessing the presence of second polar body (typically 30 minutes or longer after egg activation).

**Time-lapse confocal laser scanning microscopy**

Previously, it has been demonstrated that anaphase initiation commences approximately 120 min following GVBD (Ma et al., 2006; Zhang et al., 2008). Accordingly oocytes were placed in the imaging chamber prior to anaphase initiation and imaged for various length of time, as indicated in figures and/or legends. Imaging is acquired with a 60× oil objective on a Zeiss Axiovert with a BioRad 1024 laser scanning confocal imaging system. Time-lapse imaging is taken at various time intervals (1-3 minutes) with pixel size of 0.6×0.6×2 µm, x, y and z respectively. Images were 3D-rendered (reconstructed) using Volocity software, with no image deconvolution or any other manipulations. Shown are 3D images in “top” view (same direction of the microscope objective) or the perpendicular “side” view, or XY/XZ/YZ plane/slice images (see Figs. 1B and 2C). All images in a time series are acquired with the same settings during time lapse imaging of the same oocyte, and processed with identical 3D rendering parameters. Time (hh:mm) 00:00 refers to the start of time lapse of each oocyte.

Due to the cortical contraction, especially during the first few minutes following pricking of metaphase II eggs, time lapse imaging of second polar body emission was technically more challenging. Typically, multiple image segments were generated because of the need to reposition the oocytes. Some image distortion may also occur because each of our confocal stacks took up to 20 seconds to complete.
“Uncaging” of diazo-2

Diaz-2 (D-3034, Invitrogen) is a caged, weak calcium chelator ($K_d = 2.2\mu M$), but once
photoactivated by UV (~360nm), it is released as a strong scavenger of calcium ($K_d = 73nM$). To
“uncage” diazo-2, the oocytes were exposed to UV excitation (Chroma’s 11000V3, 350/50 nm;
100W mercury bulb) through a 60× oil objective. The oocyte was simultaneously subjected to
confocal imaging through the same objective.

Statistics

All data were analyzed by student t-test. P values of less than 0.05 are considered statistically
significant.
Acknowledgment

We thank the anonymous MBC reviewers for constructive comments, and for suggesting the EGTA experiments. We thank Dr. William Bement (University of Wisconsin) for fluorescent probes, and Drs. Bement and Wayne Chen (University of Calgary) for discussion. This work was supported by a research grant from Canadian Institutes of Health Research (MOP 89973) to XJL. Ms. Sofia Zhang participated in this project as a summer student intern in 2016.
Reference List


Figure 1. Lck-GCaMP3 detected global calcium transients at egg activation
A. Time line of experimental approach: All fluorescent probes (mostly mRNAs) were injected at GV stage. Frog oocytes (1.4 mm diameter) exhibit dark-pigmented animal hemisphere and light vegetal hemisphere. GVBD is manifested with depigmentation at the center of the animal hemisphere (“maturation spot”) where all meiosis steps occur (and imaged). Pg: progesterone; ProM: prometaphase; MI: metaphase I; Ana I: anaphase I; MII: metaphase II; PB: polar body; red lines: spindle microtubules; blue dots: chromosomes.

B. An MII oocyte injected with mRNAs for Lck-GCaMP3 and Lck-mCherry was imaged before (00:00) and at the indicated time after prick activation. The relative position shift, as marked by the 1st polar body (arrows) was due to the cortical contraction characteristic of egg activation. The graph shows relative fluorescence of the cortical region surrounding the first polar body (white dash line box), with values at 00:00 set as 1 (n=3 oocytes, two experiments). **: P<0.001.
Li_Fig. 2
Figure 2. Polar body emission involves significant membrane addition

A. Time series of an oocyte injected with mRNAs for Lck-GCaMP3 and RFP-H2B during 1\textsuperscript{st} polar body emission. Arrow depicts the cortical spindle pole.

B. Time series of an oocyte injected with mRNAs for Lck-mCherry and GFP-H2B during 1\textsuperscript{st} polar body emission.

C. The graph shows summary of relative fluorescence at the cortical region around the spindle (white dash line box) at 00:00 and 00:10 of the same oocyte as in C, with values at 00:00 set as 1. n=3 oocytes, two experiments. NS=not significant.

D. Time series of an oocyte co-expressing Lck-GCaMP3 and Lck-Cherry imaged during similar time points as in A and B. Arrow depicts cortical spindle pole.

E. Time series (slightly tilted side view, with the coordinates shown) of an oocyte injected with Lck-GCaMP3 and RFP-H2B imaged at metaphase II (00:00) and the indicated time after pricking. Arrow depicts position of the cortical pole of metaphase II spindle. Anaphase was noted at 00:12. 1\textsuperscript{st} and 2\textsuperscript{nd}: first and second polar body respectively.
Figure 3. GCaMP3 failed to detect spindle-based calcium signals

A. Time series of an oocyte injected with GCaMP3 and RFP-H2B imaged during first (1\textsuperscript{st}) polar body emission. Arrow depicts metaphase I spindle (00:00=115 min after GVBD). Representative of three oocytes.

B. Time series of an oocyte injected with GCaMP3 and RFP-H2B imaged at metaphase II (00:00) and the indicated time after pricking. Arrow depicts metaphase II spindle. 1\textsuperscript{st} and 2\textsuperscript{nd}: first and second polar body. Representative of 6 oocytes.

Figure 4. BAPTA Ca\textsuperscript{2+} chelators, but not EGTA, causes spindle microtubule depolymerization in meiosis I
A. DB-BAPTA, MT/RFP-H2B

- Side view
- DBB alone
  - 00:00
  - 00:02
  - 00:04
  - 00:06
  - 00:08

- Top view
  - 00:00
  - 00:04
  - 00:06
  - 00:10
  - 00:22

- DBB/Ca^{2+} = 5
  - 00:00
  - 00:04
  - 00:06
  - 00:10
  - 00:22

- DBB/Ca^{2+} = 1
  - 00:00
  - 00:04
  - 00:10
  - 00:15
  - 00:23

B. Diazo-2, MT/RFP-H2B

- No UV
  - 00:00
  - 00:05
  - 00:09
  - 00:13

- Side view
  - 00:00
  - 00:02
  - 00:04
  - 00:06

- Top view
  - UV (00:00-00:03)

C. EGTA, MT/RFP-H2B

- 00:00
- 00:08
- 00:18
- 00:20
- 00:28
A. **Top:** Time series of an oocyte injected with 30nl of 25mM of DB-BAPTA at 90 minutes after GVBD. The oocyte was subjected to time lapse imaging immediately following DB-BAPTA injection (00:00). The schematics on the right depict side view of oocytes at 0 and 8 min after DB-BAPTA injection, respectively.

**Middle:** Time series (side view) of an oocyte injected (60 min after GVBD; 00:00) with 30nl of 25mM DB-BAPTA mixed with 5mM CaCl₂, depicting more gradual spindle collapse and chromosome clustering.

**Bottom:** Time series (side view) of an oocyte injected with 30nL of 25mM DB-BAPTA mixed with 25mM CaCl₂ 60 minutes after GVBD, depicting no spindle collapse within similar time frame. This series is composed of four image segments of the same oocyte, necessitated due to the cortical contraction.

B. **Top:** Time series (side view) of an oocyte injected with 30nl of 10mM diazo-2 at 90 minutes after GVBD (00:00) followed by time lapse imaging.

**Bottom:** Time series of an oocyte injected with 30nl of 10mM diazo-2 at 90 minutes after GVBD. Five minutes after diazo-2 injection, the oocyte was imaged (00:00) followed immediately by UV illumination for 3 minutes (00:00 to 00:03) and simultaneous time lapse imaging.
C. Time series (side view) of an oocyte injected with 30nl of 25mM of EGTA at 90 minutes after GVBD. The oocyte was subjected to time lapse imaging immediately following EGTA injection (00:00), depicting normal anaphase (00:20) and first polar body emission (00:28).
Figure 5. Both DB-BAPTA and EGTA buffers inhibited egg activation, but only DB-BAPTA caused collapse of metaphase II spindle

A. Time series (side view) of a mature egg injected with 30nL of water followed immediately by time lapse imaging (00:00), depicting anaphase (00:12) and emission of the second polar body (00:31). This series is composed of three image segments of the same oocyte, necessitated due to the significant cortical contraction requiring re-position of the oocyte.

B. Time series of a mature egg injected with 30nL of 25mM DB-BAPTA (00:00) followed by immediate time lapse imaging, depicting the lack of egg activation (no cortical contraction) but rapid collapse of metaphase II spindle.

C. Time series (side view) of a mature egg injected with 30nL of 25mM EGTA (00:00) followed by immediate time lapse imaging, depicting no cortical contraction or changes of metaphase II spindle.
Li Fig. 6
Figure 6. DB-BAPTA inhibits Cdc42 activation but not RhoA activation

A. Top: Time series (side view) of a control oocyte showing the active Cdc42 (green) cap overlaying the polar body chromosomes (00:10, arrow) and active Cdc42 at the polar body cortex (00:16, arrow). Bottom: Time series of an oocyte injected with 20nL of 30mM DB-BAPTA immediately after anaphase initiation (upward arrow), depicting the lack of Cdc42 activation or polar body emission.

B. Top: Time series (side view) of a control oocyte showing the active RhoA (green) contractile ring (00:11) and constriction “below” the polar body chromosomes (00:13 and 00:16, arrow). Bottom: Time series of an oocyte injected with 20nL of DB-BAPTA immediately after anaphase initiation (upward arrow) showing active RhoA contractile ring (00:08) and constricting “above” both sets of chromosomes (00:12, arrow).
Figure 7. Co-localization of calmodulin with spindle microtubules during polar body emission

Typical confocal images of oocytes fixed at the indicated stages, stained with anti-calmodulin and anti-tubulin, and counter-stained with DAPI. The projection orientation is indicated for each oocyte, where xy plane is parallel to the oocyte surface and z is perpendicular, pointing toward the center of the oocyte. The approximate time (minutes from GVBD) of each stage is indicated at the bottom. E: early (prometaphase); red: tubulin; green: calmodulin; blue: DNA.
Table 1. Buffer injection and egg activation

<table>
<thead>
<tr>
<th>Buffer:Ca^{2+} ratio</th>
<th>Free Ca^{2+} in buffer</th>
<th>Egg activation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DB-BAPTA (25mM)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>alone</td>
<td>No (28/28)</td>
<td></td>
</tr>
<tr>
<td>5:1</td>
<td>400nM</td>
<td>No (12/12)</td>
</tr>
<tr>
<td>1:1</td>
<td>1.6µM</td>
<td>Yes (9/12)</td>
</tr>
<tr>
<td><strong>EGTA (25mM)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>alone</td>
<td>No (32/32)</td>
<td></td>
</tr>
<tr>
<td>5:1</td>
<td>25nM</td>
<td>No (8/8)</td>
</tr>
<tr>
<td>1:1</td>
<td>123nM</td>
<td>No (6/6)</td>
</tr>
<tr>
<td><strong>Water</strong></td>
<td></td>
<td>Yes (10/11)</td>
</tr>
</tbody>
</table>

Each egg was injected with 30 nL of water or calcium buffers with the indicated buffer:Ca^{2+} ratio and corresponding free Ca^{2+} in the buffer. Numbers in parentheses indicate the number of injected eggs showing the indicated phenotype, yes or no egg activation, over the total number of injected eggs.