Meiosis, Egg Activation, and Nuclear Envelope Breakdown Are Differentially Reliant on Ca^{2+}, Whereas Germinal Vesicle Breakdown Is Ca^{2+} Independent in the Mouse Oocyte

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Abstract. During early development, intracellular Ca^{2+} mobilization is not only essential for fertilization, but has also been implicated during other meiotic and mitotic events, such as germinal vesicle breakdown (GVBD) and nuclear envelope breakdown (NEBD). In this study, the roles of intracellular and extracellular Ca^{2+} were examined during meiotic maturation and reinitiation at parthenogenetic activation and during first mitosis in a single species using the same methodologies. Cumulus-free metaphase II mouse oocytes immediately resumed anaphase upon the induction of a large, transient Ca^{2+} elevation. This resumption of meiosis and associated events, such as cortical granule discharge, were not sensitive to extracellular Ca^{2+} removal, but were blocked by intracellular Ca^{2+} chelators. In contrast, meiosis I was dependent on external Ca^{2+}; in its absence, the formation and function of the first meiotic spindle was delayed, the first polar body did not form and an interphase-like state was induced. GVBD was not dependent on external Ca^{2+} and showed no associated Ca^{2+} changes. NEBD at first mitosis in fertilized eggs, on the other hand, was frequently, but not always associated with a brief Ca^{2+} transient and was dependent on Ca^{2+} mobilization. We conclude that GVBD is Ca^{2+} independent, but that the dependence of NEBD on Ca^{2+} suggests regulation by more than one pathway. As cells develop from Ca^{2+}-independent germinal vesicle oocytes to internal Ca^{2+}-dependent pronuclear eggs, internal Ca^{2+} pools increase by approximately fourfold.

THE release of Ca^{2+} from intracellular stores is a universal stimulus of fertilization or parthenogenetic activation (Steinhardt and Epel, 1974; Steinhardt et al., 1974, 1977; Ridgway et al., 1977; Yoshimoto et al., 1986; reviewed by Jaffe, 1985) as well as the resumption of meiosis II (Masui et al., 1977; Cuthbertson et al., 1981; Kaufman, 1983). Upon fertilization, Ca^{2+} spreads across the egg as a wave in diverse deuterostome species (Gilkey et al., 1978; Jaffe, 1983; Busa and Nuccitelli, 1985; Miyazaki et al., 1986; Hafner et al., 1988; Hamaguchi and Hamaguchi, 1990; Speksnijder et al., 1990) and is frequently followed by Ca^{2+} oscillations (Cuthbertson and Cobbold, 1985; Miyazaki, 1988; Speksnijder et al., 1989). Inositol triphosphate-dependent (Swann and Whitaker, 1986) and independent (Rakow and Shen, 1990) pathways have been implicated to mediate the sperm-triggered Ca^{2+} wave. Calmodulin and protein kinase C(PKC)[1] appear to be the relevant species-specific targets of such Ca^{2+} changes to mediate cortical granule exocytosis (Steinhardt and Alderton, 1982; Bement and Capco, 1989; Heinecke and Shapiro, 1990; Heinecke et al., 1990; Shapiro, 1991). In species where meiosis is reinitiated at fertilization by the degradation of cytostatic factor (CSF) (Masui and Shibuya, 1987) or its active component, c-*mos* (Sagata et al., 1989), calpain II, the Ca^{2+}-dependent protease (Watanabe et al., 1989), is the likely Ca^{2+}-responsive effector. Our understanding of the thresholds, kinetics, and sources of Ca^{2+} at activation of metaphase II mammalian oocytes, however, comes from only a few studies (Whittingham and Siracusa, 1978; Cuthbertson et al., 1981; Colonna et al., 1989).

Little is known about the role of Ca^{2+} during first meiosis and first polar body formation, in any species. It is known that Ca^{2+} flux across the cell surface (Leibfried and First, 1979; Paleos and Powers, 1981; Jagiello et al., 1982) and PKC activity (Bornslaeger et al., 1986) are important during first polar body formation in mammals, however, dependencies on Ca^{2+} and potential Ca^{2+} targets remain undetermined.

Changes in the level of intracellular free Ca^{2+} ion have also been strongly implicated to trigger the initiation of M phase (Whitaker and Patel, 1990). For example, nuclear envelope breakdown (NEBD) has been shown to be dependent upon Ca^{2+} mobilization and frequently accompanied

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1. *Abbreviations used in this paper*: AM, acetoxymethyl; CG, cortical granules; dbcAMP, N^6,2'-O-dibutyryl adenosine 3':5'-cyclic monophosphate; DIC, differential interference contrast; GV, germinal vesicle stage; GVBD, germinal vesicle breakdown; hCG, human chorionic gonadotropin; MPF, M phase promoting factor; MT, microtubule; NEBD, nuclear envelope breakdown; PKC, protein kinase C; PN, pronuclear formation.

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by transient rises in Ca^{2+} (Schuetz, 1975; Poenie et al., 1985; Steinhardt and Alderton, 1988; Silver, 1989; Kao et al., 1990). Furthermore, a potential target for Ca^{2+} at NEBD, the multifunctional Ca^{2+}/calmodulin kinase, has been identified in sea urchin embryos (Baitinger et al., 1990). The subsequent events of mitosis, including anaphase onset and cytokinesis, also appear to be dependent upon Ca^{2+} mobilization (Hepler and Callaham, 1987; Ratan et al., 1988; Hepler, 1989; Tombes and Borisy, 1989; Kao et al., 1990) and several potential Ca^{2+}-binding targets, such as calmodulin and calpain, associate with the spindle (reviewed by Whitaker and Patel, 1990). The onset of M phase, which is marked by the disassembly of the envelope of the meiotic nucleus (germinal vesicle breakdown [GVBD]) or the mitotic nucleus (NEBD), has been strongly associated with M phase promoting factor (MPF) (Nurse, 1990). MPF consists of at least two conserved components, one of which, $p34^{cdc2}$ kinase, is a ubiquitous eukaryotic serine/threonine protein kinase (Nurse, 1990). NIMA, another serine/threonine kinase in *Aspergillus nidulans*, represents a pathway separate from cdc2 required to initiate mitosis (Osmani et al., 1991). No clear link has been reported among members of the $p34^{cdc2}$ or NIMA pathways and Ca^{2+} at M phase (Luca and Ruderman, 1989; Picard et al., 1990).

Initial reports that GVBD in invertebrates and amphibians was either induced by Ca^{2+} (Moreau et al., 1978), accompanied by a Ca^{2+} transient (Wasserman and Masui, 1975), or dependent on internal Ca^{2+} mobilization (Masui et al., 1977) have been succeeded by analyses suggesting that GVBD can be accompanied by Ca^{2+} transients, but is not Ca^{2+} dependent (Picard and Doree, 1983; Witchel and Steinhardt, 1990). In mammalian oocytes, reports of the role of Ca^{2+} as a mediator or trigger of GVBD (Powers and Paleos, 1982; Jagiello et al., 1982; Tsafriri and Bar-ami, 1978) conflict with reports of its Ca^{2+} independence (Paleos and Powers, 1981). In fact, Ca^{2+} has been implicated in GVBD suppression through protein kinase C (Bornslaeger et al., 1986).

Our results indicate that parthenogenetic activation and NEBD in mouse eggs occur independently of external Ca^{2+} and immediately upon the release of Ca^{2+} from internal stores. In contrast, GVBD occurs completely independently of Ca^{2+}. Other aspects of oocyte maturation are dependent on the apparent influx of Ca^{2+}. Our results are presented with respect to our observed gradual filling of internal Ca^{2+} stores, which we interpret provides a mechanism to reduce the chances of precocious activation before nuclear maturation in the oocyte.

Materials and Methods

Collection of Oocytes and Developmental Assays

Reagents were obtained from Sigma Chemical Co. (St Louis, MO), unless otherwise indicated.

ICR mice (Sprague-Dawley, Indianapolis, IN) were used for this investigation. Cells were cultured in calcium-containing or calcium-free ($CaCl_2$ replaced with 1 mM EGTA) M-2 culture medium (Fulton and Whittingham, 1978) at 37°C. Developmental stages examined in this study were assayed by differential interference contrast (DIC) microscopy of living oocytes, as shown in Fig. 1 with an approximate time line.

Germinal vesicle stage (GV) oocytes were obtained by dissecting ovaries and physically removing cumulus cells in 0.1-mM dibutyryl cAMP (dbcAMP); GVBD occurred ~2-3 h after dbcAMP removal (Wassarman et al., 1976). A portion of GV oocytes was found to be sensitive to the absence of external calcium in M-2 culture medium, as reported previously (Powers and Paleos, 1982; De Felici and Siracusa, 1982). These degenerated oocytes, which could easily be identified with phase or DIC microscopy, were physically removed from the population before subsequent experimental analysis. They typically accounted for ~50% of the population, but the variability was significant. Nonetheless, the surviving half of oocytes showed no visible abnormalities and underwent GVBD with normal kinetics.

Oocytes in first meiosis were obtained by allowing GV oocytes to mature after dibutyryl cAMP (dbcAMP) withdrawal. Completion of meiosis I was assayed by first polar body formation, unless otherwise indicated, and occurred 6 h after GVBD. Subsequent maturation of the oocyte to metaphase II occurred within 5 h of meiosis I completion, where the oocyte arrested until fertilization/activation.

Naturally arrested oocytes in metaphase II were collected from mice superovulated by 7.5 IU pregnant mare serum gonadotropin, followed 48 h later with 5.0 IU human chorionic gonadotropin (hCG). The completion of meiosis II (assayed by second polar body and female pronucleus formation

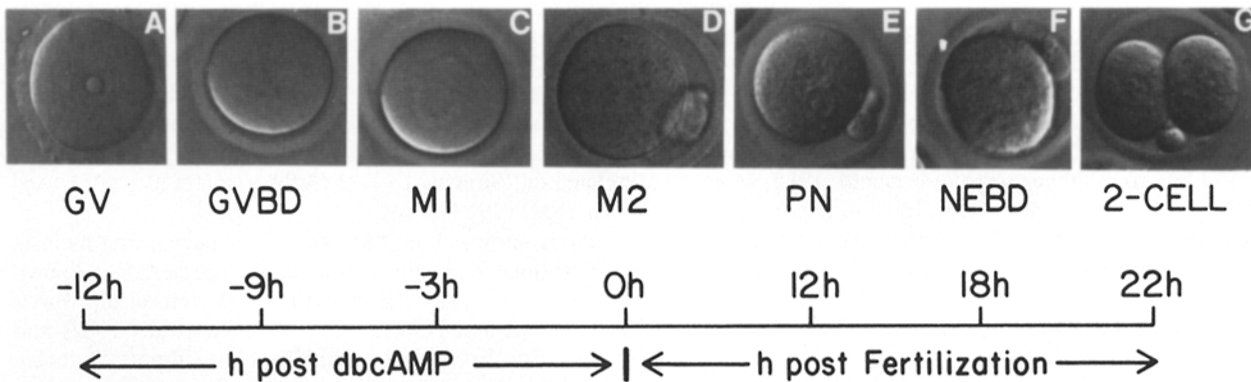


Figure 1. Time line of developmental stages of mouse/oocytes eggs. DIC microscopy of living oocytes. Stages include: (*A*) germinal vesicle (*GV*); (*B*) germinal vesicle breakdown (*GVBD*); (*C*) first meiotic spindle (*MI*); (*D*) first polar body (*M2*) and second meiotic spindle (*invisible*); (*E*) fertilized egg with male and female pronucleus and second polar body (*PN*); (*F*) pronuclear envelope breakdown (*NEBD*); (*G*) first cleavage (*2-cell*). Since maturing oocytes naturally arrest at M2, we set that time to 0. Events before 0 are those after dbcAMP removal, but are assigned negative values from M2, while events afterwards have positive values, starting with h after estimated fertilization. Times represent when 50% of the population reached the indicated stage. For parthenogenetic activation studies, haploid female pronuclear formation occurred 5 h after artificial stimulation; all other times are similarly adjusted by 7 h less.

and eventual cell division) was achieved with equal success using either 7% ethanol for 7 min or 10 μM ionomycin for 15 min (Kaufman, 1983). Ionomycin, a nonfluorescent Ca^{2+}-specific ionophore (Molecular Probes, Inc., Eugene, OR), was dissolved in DMSO at 5 mM and stored at −20°C. A single aliquot was always thawed and diluted just before addition to cells. Using BCECF, a pH sensitive fluorescent compound, we ascertained that ionomycin specifically elevated Ca^{2+}, not H^+ (data not shown).

To examine first mitosis, fertilized oocytes were flushed from oviducal ampullae beginning 24 h after hCG (Schatten et al., 1985). NEBD began 28-30 h after hCG and cytokinesis was completed 2-3 h later. Statistically significant differences between values were determined at the 99% confidence level by two-tailed *t* tests.

Oocytes studied by immunofluorescence microscopy were permeabilized and fixed in a microtubule-stabilizing buffer (Schatten et al., 1985). Chromosomes were visualized with the DNA-specific compound: 4,6 diamidino-2-phenylindole (DAPI), at 2.5 μg/ml, cortical granules were observed with fluorescently labeled lens culinaris agglutinin (Sigma Chemical Co., Ducibella et al., 1988) and microtubules were labeled by indirect immunofluorescence with a rabbit anti-tubulin antibody, as described (Schatten et al., 1985).

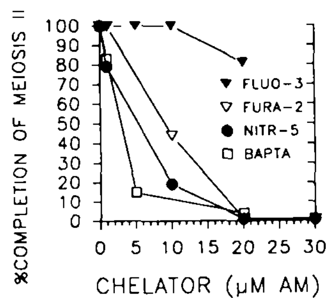


Figure 2. Parthenogenetic activation of metaphase II oocytes is blocked by Ca^{2+}-binding compounds in proportion to their Ca^{2+} affinity. The percentage parthenogenetic activation or resumption of second meiosis (second polar body and pronuclei formation) in Ca^{2+}-containing M2 culture medium of between 20 and 50 oocytes at each data point was compiled from three separate trials. Activation was with either 7% ethanol or 10 μM ionomycin and each compound was loaded at the specified μM concentration of its AM ester for 1 h at 37°C. Reported Ca^{2+} affinities are: *BAPTA*, 110 nM; *nitr-5*, 150 nM; *fura-2*, 225 nM, and *fluo-3*, 400 nM.

Introduction of Molecules and Fluorescence Imaging

We used the fluorescent Ca^{2+} probes fura-2 and fluo-3 and the nonfluorescent calcium chelators EGTA, BAPTA (Molecular Probes, Inc.) and nitr-5 (Calbiochem Corp., La Jolla, CA) in both Ca^{2+}-containing and Ca^{2+}-free media. These probes were either injected as free acids or loaded into cells as their acetoxymethyl ester (AM) derivatives. Microinjection was as described (Simerly et al., 1990). Typically, AM dyes were dissolved in 2% Pluronic F-127 in 100% DMSO, then diluted 1000-fold into loading medium to final concentrations of 1 μM indicator dye and 10 μM BAPTA. Final DMSO concentrations never exceeded 0.2%. Loading was typically for 1 h at 37°C followed by at least two washes of medium ~1-2 h before the developmental event of interest. The dye/chelator concentration eventually selected was experimentally optimized (see Fig. 2). We found that all stages of oocytes loaded AM dyes equally well, that dyes were completely internalized since added extracellular $MnCl_2$ could not rapidly quench fluorescence, but that some compartmentalization of dye occurred by the end of the experiment (<20%), as indicated by the average percentage of fluorescence persistence within a single oocyte or egg after 0.01% digitonin treatment (Tsien and Tsien, 1990). Compartmentalization of tetracarboxylate organic anion Ca^{2+} dyes into intracellular vesicles is common (Malgaroli et al., 1987); we observed it to be equivalent at all developmental stages. We typically worked with these compounds under indirect room lights.

The temperature was carefully maintained at 37°C with a thermocoupled hot air blower in a plexiglass enclosure which surrounded the entire stage and objective. Oocytes were imaged through coverslips mounted in dishes and overlaid with medium and mineral oil. Calcium-EGTA solutions, buffered to different free Ca^{2+} concentrations as described (Tombes and Borisy, 1989), were microinjected into oocytes when indicated.

Fluorescent video imaging of living cells was performed using an intensified silicon intensified target video camera (ISIT) camera (model 66; Dage-MTI, Inc., Michigan City, IN) coupled to an inverted microscope (Nikon Diaphot, Nikon, Inc., Melville, NY) equipped with a UV-transmitting, 20× "Fluor," 0.75 numerical aperture, dry, phase-contrast objective. Images were acquired and quantitatively processed with "Image-1" image analysis software (Universal Imaging Corp., West Chester, PA) which controlled dual excitation/neutral density filter wheels (Eastern Scientific, Charlotte, NC). Illumination was at 340 and 380 nm for fura-2. Illumination was typically attenuated 1,000-fold from a 75 W xenon power source for a total of 0.25 s (8 frames) per ratio half. Each half of the ratio pair was separated by 0.25 s. Images were acquired at various frequencies not exceeding one every 10 s.

Images are presented as their calibrated internal Ca^{2+} level, calculated as described (Tombes and Borisy, 1989) using a Ca^{2+} dissociation constant of 225 nM for fura-2. Integrated and averaged Ca^{2+} levels were calculated for each oocyte within an image frame as described (Florman et al., 1989) and plotted as averages versus time with standard deviations. Our calibrations routinely yielded 340:380 nm fura-2 fluorescent ratio values of 0.2 and 5.0 for the unsaturated and saturated forms of fura-2, respectively.

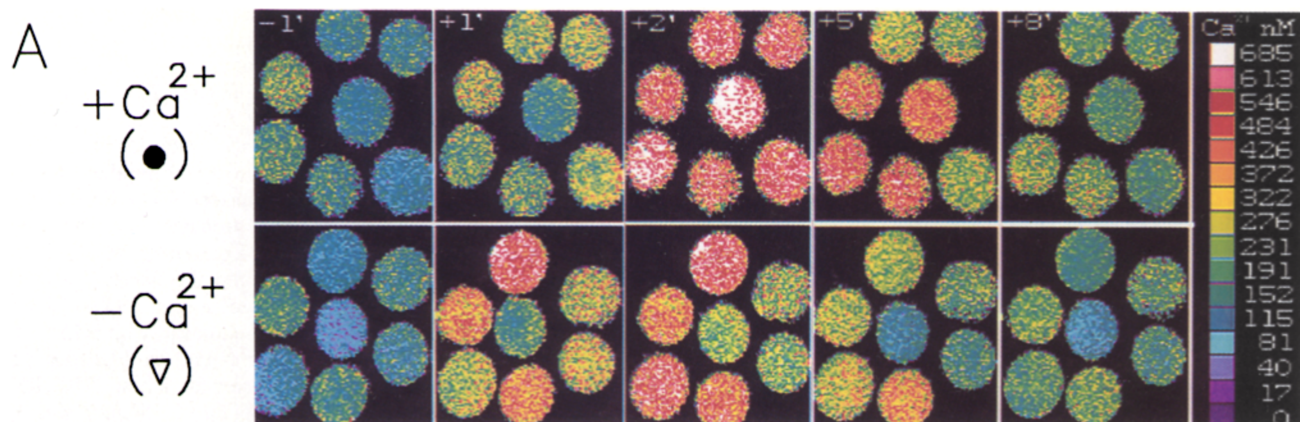
For NEBD, we pooled eggs from a single mother to improve synchrony and loaded them for 1 h, starting 2 h before we expected the first NEBD to occur. Imaging then continued through NEBD for several more hours. For GVBD, we loaded and then imaged oocytes before and after release from dbcAMP.

Results

An examination of the role of Ca^{2+} in mouse oocytes at the time of meiosis II reinitiation provided a reference point both experimentally and temporally for this entire study. The kinetics of all developmental stages assayed in this study are shown in Fig. 1 relative to metaphase II (M2). Second meiosis completion was induced by both parthenogenetic activation and natural fertilization. Relative to natural fertilization, the kinetics of events subsequent to M2 were approximate and set by the time when mice were mated. Relative to the stimulus of parthenogenetic activation, the kinetics of events after activation were more accurate and occurred 7 h sooner. For example, pronuclear formation (PN) occurred at +5 h, NEBD occurred at +11 h, and first cytokinesis (2-cell) at +15 h. Events before M2 are indicated in negative hours, relative to the time that maturing oocytes reach M2. To obtain meiosis I (M1) oocytes, we isolated GV oocytes free from cumulus cells and allowed GVBD and subsequent development to M2 arrest to occur spontaneously. Our results, like those of other investigators (Schroeder and Eppig, 1984; Schroeder et al., 1988), indicate that these developmental events are not dependent on attached cumulus cells.

Parthenogenetic Activation Is Blocked by Ca^{2+} Chelators

It was important to select concentrations and loading times of (*a*) fluorescent dyes which gave a sufficient signal without inhibiting the cellular event of interest and (*b*) nonfluorescent chelators which were just sufficient to achieve maximal inhibition. We fixed loading times at 1 h and titrated the AM esters of the Ca^{2+} indicators, fura-2 and fluo-3, and the Ca^{2+} chelators, BAPTA and nitr-5, on parthenogenetic activation of cumulus cell-free oocytes in calcium-containing medium (Fig. 2). Dye import was uniform, at least for fluo-3 and fura-2, based on their similar extinction coefficients and fluorescence intensity levels after loading. All of these Ca^{2+}-binding compounds blocked activation, half-maximally at 3 (BAPTA), 5 (unphotolyzed nitr-5), 10 (fura-2), and above (fluo-3) 20 μM. This result was consistent with their relative Ca^{2+} affinities: 110 (BAPTA), 150 (nitr-5, unphotolyzed), 225 (fura-2), and 400 (fluo-3) nM, as described (Grynciewicz et al., 1985; Kao et al., 1989). These results suggested that the inhibitory effect of each of these Ca^{2+}-specific compounds was due to the chelation of Ca^{2+} and not a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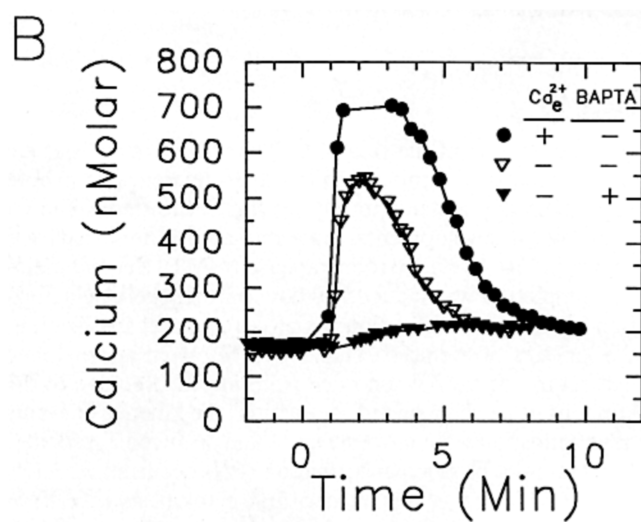
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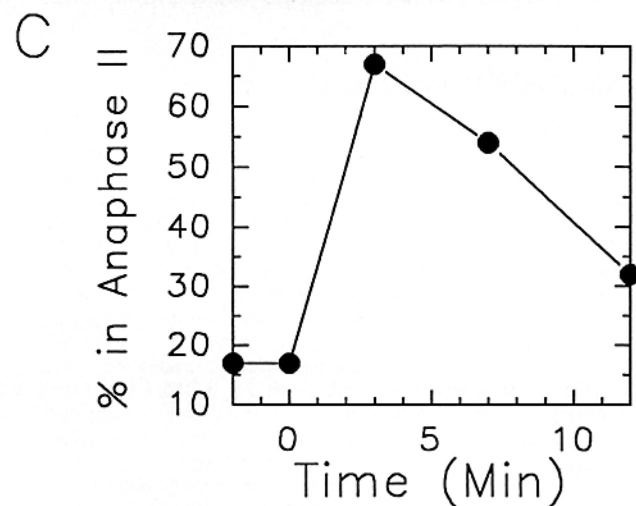
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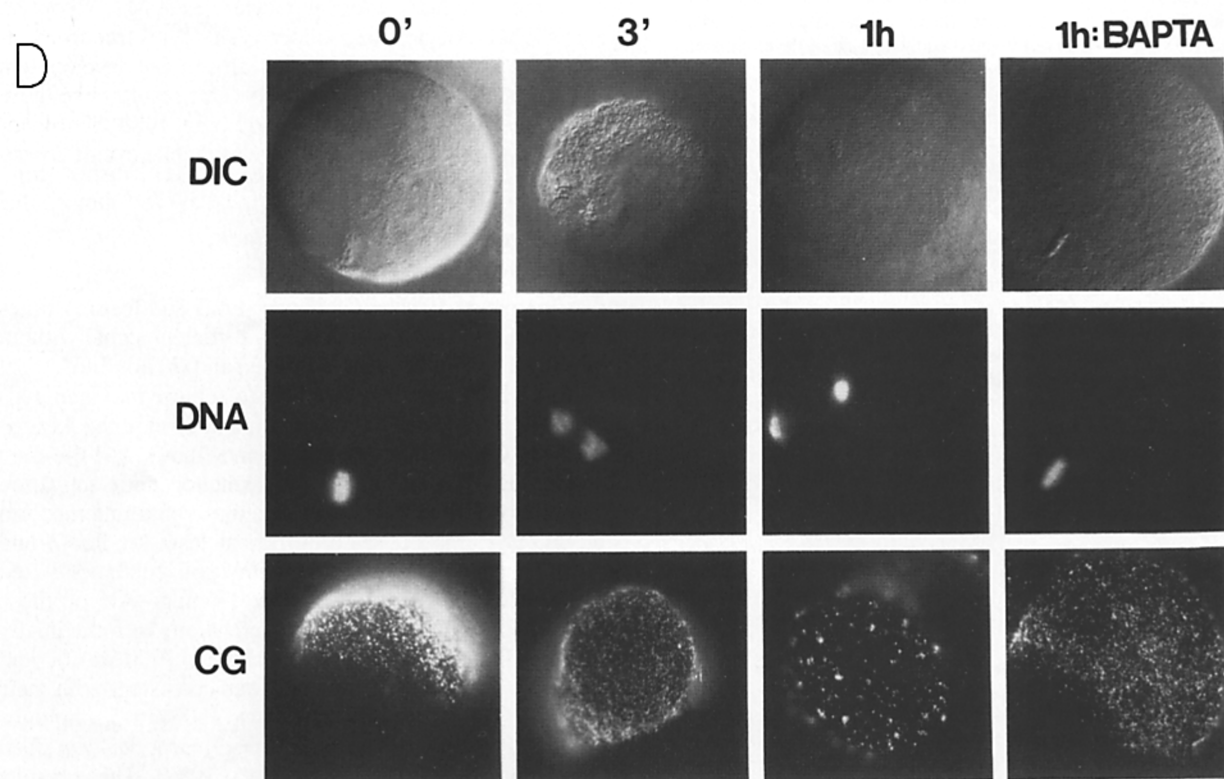
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result of a nonspecific side reaction associated with dye import and AM ester hydrolysis. These results also established working concentrations of 1 μM for fura-2/AM and 10 μM for BAPTA/AM. These concentrations were used in all other events examined in mouse oocytes/eggs in this study.

Parthenogenetic Activation Mobilizes Ca^{2+} from Internal Stores

To determine whether the Ca^{2+} dependency at activation was associated with an elevation of Ca^{2+}, metaphase II oocytes were fura-2 AM loaded and ratiometrically imaged before and after activation with 10 μM ionomycin. A selection of five of the resultant calibrated images of oocytes activated in Ca^{2+}-containing and Ca^{2+}-lacking medium are displayed as nM (10^{-9} M) Ca^{2+} versus time (Fig. 3 *A*). In both the presence and absence of external Ca^{2+}, ionomycin elicited an elevation of internal Ca^{2+}, which peaked within a few minutes and then returned to preactivation levels. Within populations, each oocyte had slightly different Ca^{2+} transient kinetics, but between populations, the average time course was similar. Activation occurred in both populations of imaged oocytes at 80%, the standard activation rate without indicator or imaging, indicating that imaging itself was noninhibitory and that the activation stimulus was sufficient regardless of external Ca^{2+}. The transient behavior of ionomycin in the presence of external Ca^{2+} was initially surprising, although it has been documented in single cells (Foskett and Melvin, 1989) and in populations (Albert and Tashjian, 1985) and may be because of both the instability of ionomycin and cellular mechanisms which compensate for increased Ca^{2+}. We observed a similar Ca^{2+} amplitude when oocytes were activated with 7% ethanol in Ca^{2+}-containing medium (data not shown), although Ca^{2+} remained elevated until ethanol was removed.

The population-averaged, ionomycin-induced Ca^{2+} transient in the absence of external Ca^{2+} subsided more rapidly and reached a lower amplitude than in the presence of external Ca^{2+} (Fig. 3 *B*). The integrated Ca^{2+} transient for oocytes activated in the absence of Ca^{2+} was 46% of that which occurred in the presence of external Ca^{2+}. Activation rates were similar in this experiment (80 vs 77%), indicating that less than half of the control level of Ca^{2+} change was necessary to activate oocytes. When oocytes were first loaded with 10 μM BAPTA/AM, which was the minimum concentration of Ca^{2+} chelator required to block activation (Fig. 2), and then imaged in the absence of external Ca^{2+}, the ionomycin-induced intracellular Ca^{2+} rise was dampened to baseline levels, as shown in Fig. 3 *B*. Oocytes microinjected with EGTA and fura-2, then activated with ethanol, also showed a negligible elevation of Ca^{2+} and a complete block of activation (data not shown).

To determine the temporal coupling between the Ca^{2+} elevation and the onset of anaphase II, we fixed oocytes at various time points after activation in Ca^{2+}-containing medium and stained their chromosomes with DAPI (Fig. 3 *D*; DNA). Chromosomal patterns and/or the presence of nuclear envelopes observed in DIC allowed scoring oocytes as in either metaphase, anaphase, or interphase. The percentage of cells in anaphase is shown on the same time scale in Fig. 3 *C* as the ionomycin-induced Ca^{2+} transient in Fig. 3 *B*. Both anaphase II and Ca^{2+} peak simultaneously within 3 min of addition of activating agent. The chromatin patterns of oocytes preactivation, 3 min and 1 h after activation are shown in Fig. 3 *D*. Cells which were blocked from activation by BAPTA/AM persist in the metaphase configuration even 1 h after activation (Fig. 3 *D*).

The release of cortical granules (CG), another Ca^{2+}-mediated activation event, was also examined during the same time course (Fig. 3 *D*). Cortical granule-free zones (Fig. 3 *D*, *O′* and *CG*) were observed near meiotic spindles and did not clump or decrease in density until at least 1 h after activation (Fig. 3 *D*, 1 h and *CG*), as reported (Ducibella et al., 1988). However, like chromatid separation, the preactivation configuration of cortical granules was maintained in BAPTA/AM loaded oocytes (Fig. 3 *D*, *1 h:BAPTA*, and *CG*).

The percentage of oocytes that activated (as judged by the formation of both a pronucleus and second polar body) under all conditions of Ca^{2+} removal or chelation was averaged from all activations with either ionophore or ethanol (Fig. 4 *A*). Control oocytes were activated in Ca^{2+} and showed the

Figure 3. Ca^{2+} ion elevation at activation is linked to anaphase II onset. (*A*) One time point before (−1′) and four time points after (*+1′*, *+2′*, *+5′*, and *+8′*) activation with 10 μM ionomycin of two populations of oocytes, in Ca^{2+}-containing and Ca^{2+}-lacking medium are shown with times indicated in min and Ca^{2+} in nM, according to the scale bar. (*B*) The mean calibrated Ca^{2+} ion concentration from the two populations of oocytes in *A*, plus a third population, are plotted at the indicated times before and after activation by 10 μM ionomycin at T = 0 min (ionomycin was removed at T = 10 min). The third population was loaded with both fura-2/AM (1 μM) and BAPTA/AM (10 μM) and then activated in Ca^{2+}-free M2 culture medium. The legend shows the presence or absence of BAPTA loading and the presence or absence of Ca^{2+} in the activation medium. Activation occurred in 8/10 oocytes loaded with fura-2 alone and imaged in Ca^{2+}-containing medium, 10/13 oocytes loaded with fura-2 alone and imaged in Ca^{2+}-free medium, and 0/11 oocytes loaded with fura-2 and BAPTA and imaged in Ca^{2+}-free medium. Standard error bars were omitted for clarity, since the variability could be as high as 30% of the signal as is apparent in images in *A*. (*C*) On the same time scale as in *B*, the percentage of oocytes in anaphase II, as determined by fixation and staining with DAPI, at various time points after activation with ionophore in Ca^{2+}-containing medium, is plotted. For each point, 10–20 oocytes were determined to be either in metaphase II, anaphase II, telophase II or interphase as shown below in *D*. (*D*) Control oocytes before activation (*0′*) and 3 min (*3′*) and 1 h (*1 h*) after activation with ethanol in Ca^{2+}-containing medium as well as a BAPTA-loaded oocyte 1 h after activation in Ca^{2+}-free medium (*1 h: BAPTA*) were fixed, photographed with DIC optics, and with fluorescent DAPI staining to image chromatin (*DNA*) and fluorescein-lens culinaris agglutinin for cortical granules (*CG*). Notice the cortical granule-free zone at 0′ on the same side of the oocyte as the metaphase spindle. The 3′ time point shows the immediate resumption of meiosis, without any noticeable change in cortical granule density or distribution photographed at a point on the opposite side from the spindle, where normally penetration is more likely and where there is not a cortical granule-free zone. Only after 1 h does the obvious change in cortical granules occur as the second polar body is being formed. Notice the similarity between DNA and cortical granule patterns between control (*0 min*) and the BAPTA loaded oocyte even 1 h after ethanol treatment.

standard 80% activation rate (■). External Ca^{2+} removal alone (□) did not significantly prevent any aspect of activation. Internal Ca^{2+} chelation alone (10 μM BAPTA/AM preloading) significantly inhibited activation, even in Ca^{2+}-containing medium (▨). If both internal Ca^{2+} was chelated and external Ca^{2+} removed, activation was completely blocked. Two-tailed t tests confirmed that internal Ca^{2+} chelation, but not external Ca^{2+} removal alone, significantly blocked activation.

Meiosis I is Dependent upon External Ca^{2+}

In contrast to meiosis II, we found that external Ca^{2+} removal completely blocked formation of the first polar body, while internal Ca^{2+} chelation alone was only slightly inhibitory (Fig. 4 *B*), as confirmed by two-tailed t tests. To examine the specific locus of this external Ca^{2+} dependency, we fixed oocytes and stained their chromosomes and microtubules (MTs). Although first polar bodies had indeed not formed after incubations of GV oocytes in the absence of external Ca^{2+}, oocytes were not prevented from proceeding through meiosis I (Fig. 5). Oocytes incubated in Ca^{2+}-containing medium for 11 or 22 h after dbcAMP removal reach M2 (Fig. 5 *A*). In contrast, oocytes cultured without dbcAMP for 4 h and then switched to medium lacking external Ca^{2+} for 7 h remained in meiosis I (Fig. 5 *B*). After 18 h without Ca^{2+}, oocytes had decondensed their chromatin within two small clumps (Fig. 5 *C*). These clumps did not resemble pronuclei (Fig. 1). Although MTs persisted, the spindle did not. Because chromosomes had decondensed and the spindle had disassembled, we termed these oocytes "interphase-like." These results suggested that external Ca^{2+} is important for first polar body formation, normal meiosis I kinetics, and for preserving the chromosome and spindle configuration which is normally maintained between M1 and M2.

The inhibition of first polar body formation was reversible since oocytes, which still contained the first meiotic spindle after 7 h of incubation without Ca^{2+} (Fig. 5 *B*), could achieve M2 if returned to Ca^{2+}-containing medium for another 11 h (Fig. 5 *D*). None of these oocytes had the interphase-like morphology found in oocytes that were kept in Ca^{2+}-free medium for the additional 11 h. Once achieved, however, the interphase-like morphology could not be reversed by returning Ca^{2+} to the medium.

Despite the apparent dependence of first polar body formation on external Ca^{2+}, we observed no Ca^{2+} changes during the 4–5-h natural time course of meiosis I in over 20 oocytes loaded with 1 μM fura-2 AM and imaged every 15 s in Ca^{2+}-containing medium (data not shown). Meiotic kinetics were normal during imaging and imaged oocytes developed normally to arrest at metaphase II.

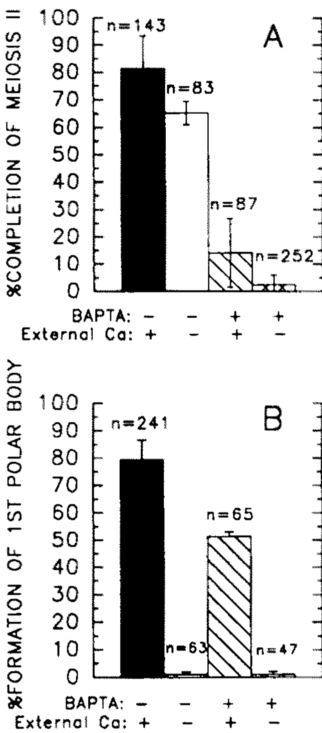

Figure 4. (*A*) Completion of meiosis II is dependent on intracellular Ca^{2+}. The percentage of oocytes induced to complete meiosis II (as assayed by second polar body and pronuclei formation) were averaged from three–five trials in which oocytes were treated by either ionomycin or ethanol as described in Materials and Methods. n indicates the number of oocytes assayed under each condition and standard deviation error bars are shown. Where indicated, BAPTA/AM was loaded at 10 μM and Ca^{2+}-free medium lacks calcium and contains 1 mM EGTA. The first bar shows control conditions: no BAPTA loading and normal (calcium-containing) activation medium used. The second bar represents no BAPTA loading, but activation in Ca^{2+}-free medium. The third bar shows BAPTA loaded oocytes in the presence of calcium and the fourth bar is BAPTA loaded oocytes in the absence of external calcium. (*B*) Extracellular Ca^{2+} removal blocks first polar body formation. Oocytes loaded with or without 10 μM BAPTA/AM and then allowed to progress through meiosis I (assayed by first polar body formation) in the presence or absence of external calcium are displayed as in *A*. When used, BAPTA was loaded at least 3 h before first polar body formation (at least 2–4 h before the onset of meiosis I) and incubations were carried out overnight (at least 16 h) before assaying. Each data point represents an average of three trials and the indicated number of oocytes (n).

GVBD Is Not Dependent on Ca^{2+}

Since our results suggested that oocytes during first meiosis were more sensitive to Ca^{2+} deprivation than oocytes during second meiosis, we wanted to test this sensitivity at earlier meiotic stages, such as GVBD. Our interest in GVBD was also based on the reported role of Ca^{2+} during mouse and echinoderm oocyte GVBD (see introduction). We examined oocytes undergoing GVBD for their dependence on Ca^{2+} and their potential association with transient elevations of Ca^{2+}. To assay for any natural transients at this developmental stage, we loaded oocytes with fura-2 AM and imaged them both before and after the removal of dbcAMP. Of 20 oocytes monitored over the 4-h time course after dbcAMP removal leading beyond GVBD, we observed no change in intracellular Ca^{2+} (Fig. 6). The baseline Ca^{2+} in these oocytes was around 100 nM, similar to that in eggs (Fig. 3), but did not change.

With significantly large numbers of oocytes, we characterized the dependence of GVBD on internal and external Ca^{2+} as we had for other meiotic events. Our results indicated that GVBD was not blocked by any of the Ca^{2+} chelation conditions known to be effective in blocking first or second polar body formation (Fig. 7 *A*).

To directly test whether Ca^{2+} could stimulate GVBD, we treated GV oocytes arrested with dbcAMP with 10 μM ionomycin in Ca^{2+}-containing medium. Although these oocytes exhibited a transient rise in Ca^{2+} (data not shown), they did not undergo GVBD. These conditions did not inhibit GVBD, since 90% of these ionophore-stimulated oocytes underwent GVBD when dbcAMP was subsequently removed.

NEBD Is Dependent on Internal Ca^{2+} Stores and Is Frequently Associated with a Ca^{2+} Transient

Existing evidence strongly suggests that Ca^{2+} transients exist

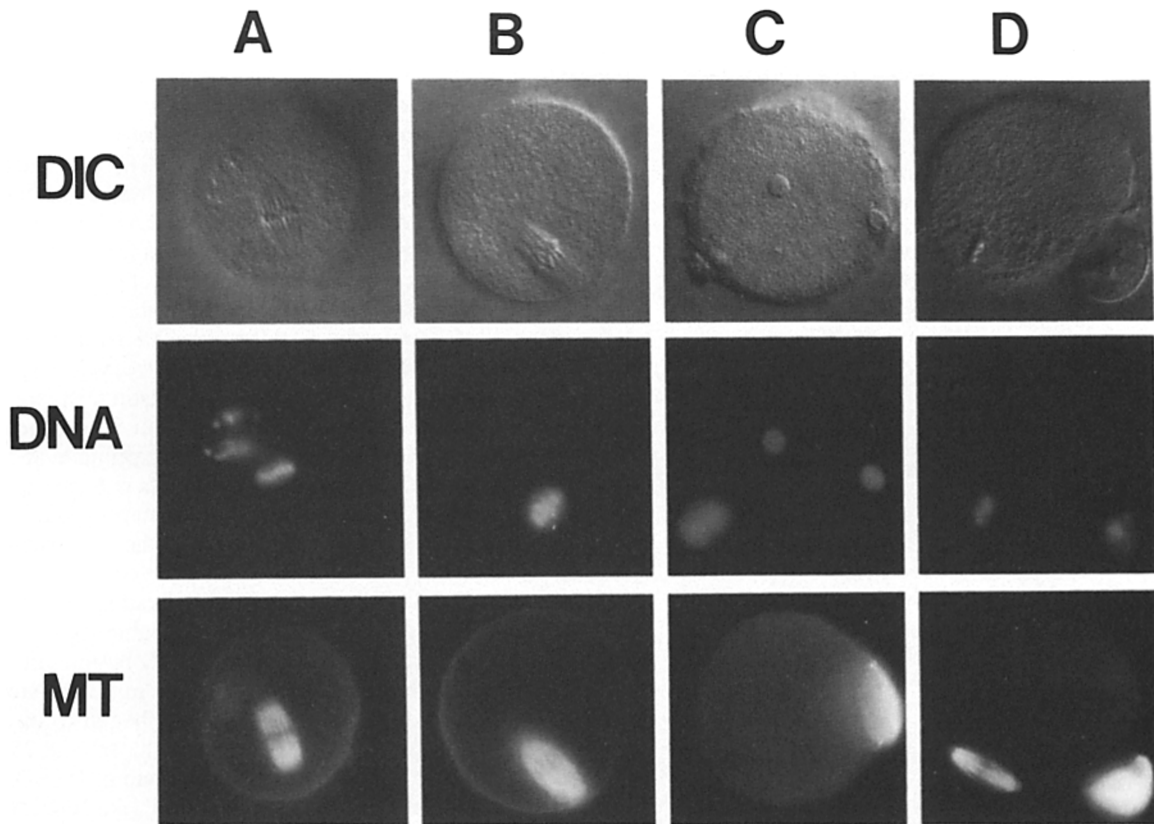

Figure 5. External calcium deprivation has pleiotropic effects on microtubule and chromatin configurations in meiosis I oocytes. Oocytes were incubated starting 4 h after GVBD (just before meiosis I spindle formation) for the indicated times in the indicated mediums and were fixed as described in methods and stained with DAPI (*DNA*) and an anti-tubulin antibody (*MT*). (*A*) Control oocytes showing normal metaphase II spindle microtubule and DNA patterns after 7 h of incubation in Ca^{2+}-containing medium. An 18-h incubation showed the same pattern. The first polar body is partially out of focus and additional masses of DNA are residual cumulus cells. (*B*) Oocytes incubated in the absence of Ca^{2+} for 7 h show a normal, but delayed first meiotic spindle; no first polar body had formed. (*C*) Oocytes incubated in the absence of Ca^{2+} for 18 h show two immature nuclei and the absence of a well-defined second meiotic spindle. The peripheral DNA mass is residual cumulus cells. The immunoreactive tubulin staining structure located at the site of the aborted first polar body is a spindle remnant (compare to tubulin staining structure in completed first polar body in Fig. 7 *D*). (*D*) Oocytes incubated in the absence of Ca^{2+} for 7 h as in *B*, were then returned to Ca^{2+}-containing medium for the last 11 h. Note the completion of the first polar body and arrest of the oocyte at metaphase of second meiosis.

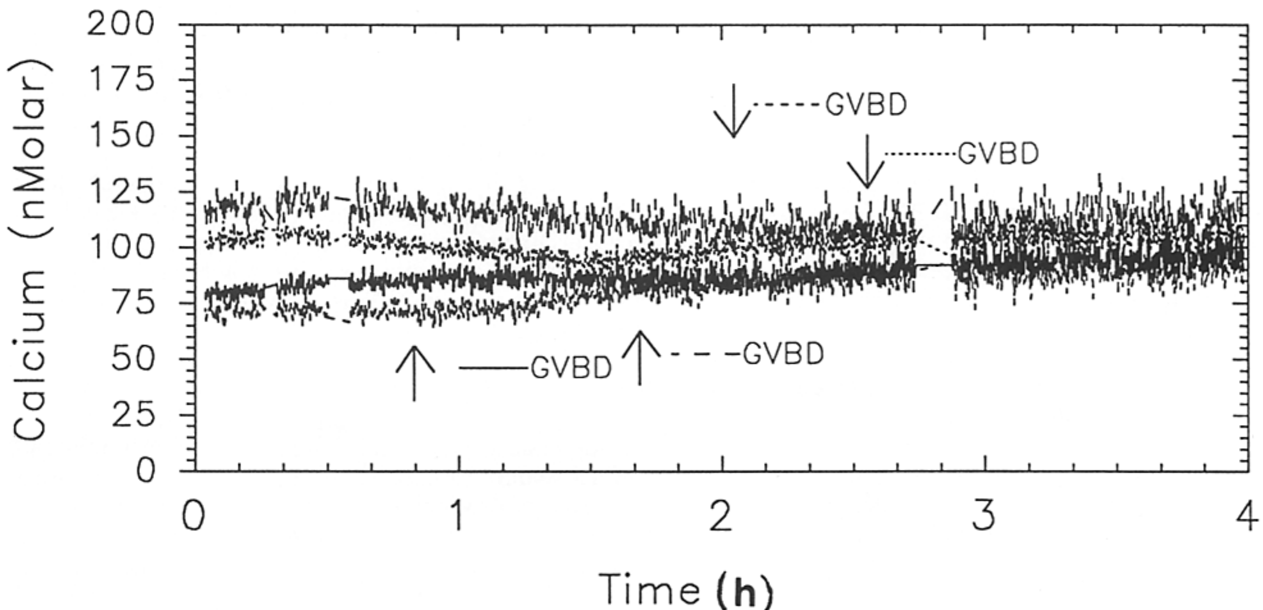

Figure 6. GVBD is not associated with transient elevations of Ca^{2+}. Four representative oocytes monitored around the time of GVBD showed no changes in Ca^{2+}. Although oocytes were monitored from the time of dbcAMP removal, time zero is arbitrary here. GVBD occurred as indicated for each oocyte.

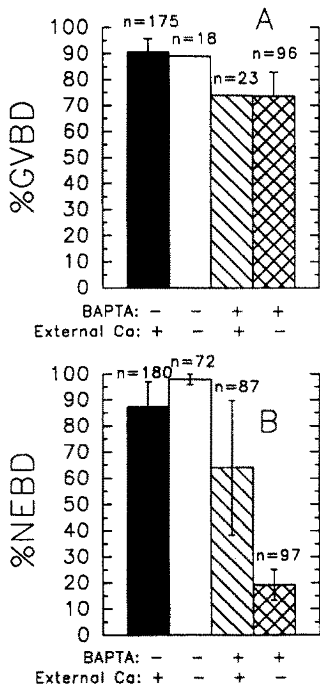

Figure 7. (*A*) GVBD is not dependent on external or internal Ca^{2+}. Oocytes at the germinal vesicle stage were removed from dbcAMP blockage in the presence or absence of external calcium without loading or after loading with 10 μM BAPTA/AM and scored for GVBD. The first bar shows control conditions: no BAPTA loading, oocytes developed in standard (calcium-containing) medium. Second bar shows GVBD is not dependent on external calcium. The third and fourth bars indicate that GVBD occurs in oocytes loaded with BAPTA/AM and then allowed to develop in the presence or absence of external calcium. Results were obtained from three trials and the number (*n*) of oocytes tested under each condition. (*B*) NEBD is dependent on internal, not external Ca^{2+} stores. Fertilized eggs were placed in the presence or absence of external calcium, sometimes after loading with 10 μM BAPTA/AM and scored for NEBD. The first bar shows control conditions: no BAPTA loading before eggs developed in standard (calcium-containing) medium. The second bar shows NEBD in the absence of calcium. The third and fourth bars indicate the percentage NEBD in eggs loaded with BAPTA/AM and then allowed to develop in the presence or absence of external calcium. Results were obtained from three trials and the total number (*n*) of eggs assayed under each condition.

and may trigger mitotic M phase (NEBD; see introduction). Unlike GVBD, but like activation, NEBD in pronucleate eggs was dependent on Ca^{2+} (Fig. 7 *B*). Simply removing extracellular Ca^{2+} neither blocked NEBD nor any other mitotic event, including anaphase and cytokinesis (□). Intracellular Ca^{2+} chelation alone with BAPTA/AM partially inhibited NEBD (▨). Significant inhibition, as judged by two-tailed *t* tests, was achieved only when eggs were loaded with BAPTA/AM and then developed in the absence of extracellular Ca^{2+} (▩).

To determine whether fertilized mouse eggs entering mitosis exhibited Ca^{2+} transients at NEBD, we loaded eggs with 1 μM fura-2 AM just before we expected the first eggs of a population to undergo NEBD after natural fertilization. Imaging conditions were identical to other stages and in many eggs we observed single Ca^{2+} transients, from a baseline of 100 ± 40 nM to 250 ± 90 nM, with half-maximal widths of ~1 min, just preceding NEBD. From three trials, eight of 19 eggs imaged rapidly enough to detect such transients exhibited them. Such rapid imaging was noninhibitory; no aspect of mitosis was delayed by imaging. Three such eggs exhibiting transients are shown in color (representing nM Ca^{2+}) in hours after imaging had begun (Fig. 8). The single, rapid Ca^{2+} transient occurred within 5 min before NEBD and appeared to be global in nature. By averaging and plotting Ca^{2+} in the three eggs shown in color versus time, the quantitative variability in amplitude is more evident. Notice that in each egg the kinetics of the transient are similar and there is a slight overshoot when Ca^{2+} returns to baseline. No Ca^{2+} transients were detected during mitotic spindle formation, chromatid separation, or cleavage.

To further examine the role of Ca^{2+} at NEBD, we tested whether an ionomycin-induced Ca^{2+} transient was sufficient to precociously induce NEBD in late G2 fertilized eggs. Because of the natural asynchrony in the timing of fertilization, we attempted to induce NEBD in populations of eggs where at least 10% had already undergone NEBD naturally and in which we expected at least an additional 30% to undergo NEBD in the next hour. Our population size was large enough that we would expect to statistically distinguish precocious from normal NEBD. Ionomycin induced a rapid, transient increase in Ca^{2+} which exceeded in amplitude and duration that observed naturally at NEBD (data not shown). However, in 37 eggs treated in two trials, we never induced NEBD, precociously, even when we repeated the ionomycin stimulus after 30 min. Nevertheless, all of the eggs eventually underwent NEBD at a normal pace, indicating that the ionomycin-induced Ca^{2+} transients were not inhibitory.

We also tried to precociously trigger NEBD by microinjecting Ca^{2+}–EGTA-buffered solutions. We injected late pronucleate eggs, in a population which had begun to undergo NEBD, with Ca^{2+}–EGTA solutions set to 1.3 and 25 μM Ca^{2+}, but did not see an increase in the rate of NEBD. These microinjections did not prevent NEBD, like BAPTA or EGTA alone, and all treated eggs eventually underwent NEBD within the expected time. We conclude that internal Ca^{2+} mobilization is necessary for NEBD, although we cannot conclude that Ca^{2+} transients are necessary or even sufficient to trigger NEBD, despite their compelling temporal association.

Maturation of Ca^{2+} Stores during Meiotic Maturation

We investigated whether the difference between the external Ca^{2+} dependence of first and second meiosis was related to different levels of internally stored Ca^{2+} during oocyte maturation. As in Fig. 3, we estimated the level of internal Ca^{2+} by integrating the Ca^{2+} peak induced by ionomycin in the absence of external Ca^{2+}, averaged from at least six oocytes. Fig. 9 shows these results for oocytes at the GV, MI, MII arrested and fertilized pronucleated egg stages, using the time scale of Fig. 1. There was a fourfold increase in internally stored Ca^{2+} from GV to PN, with the greatest increase occurring between MI and MII, in parallel to the shift in Ca^{2+}-source dependencies. This was because of an increase in both the amplitude and duration of the average transient at each stage.

Discussion

Ca^{2+}-dependent events shift from a reliance on external to internal sources as intracellular Ca^{2+} stores mature during mouse oocyte development. We find that: (*a*) during meiosis II, internal Ca^{2+} release in metaphase II–arrested oocytes is necessary and sufficient to immediately stimulate the onset of anaphase II and eventually activate the release of cortical granules; (*b*) during meiosis I, spindle formation is delayed,

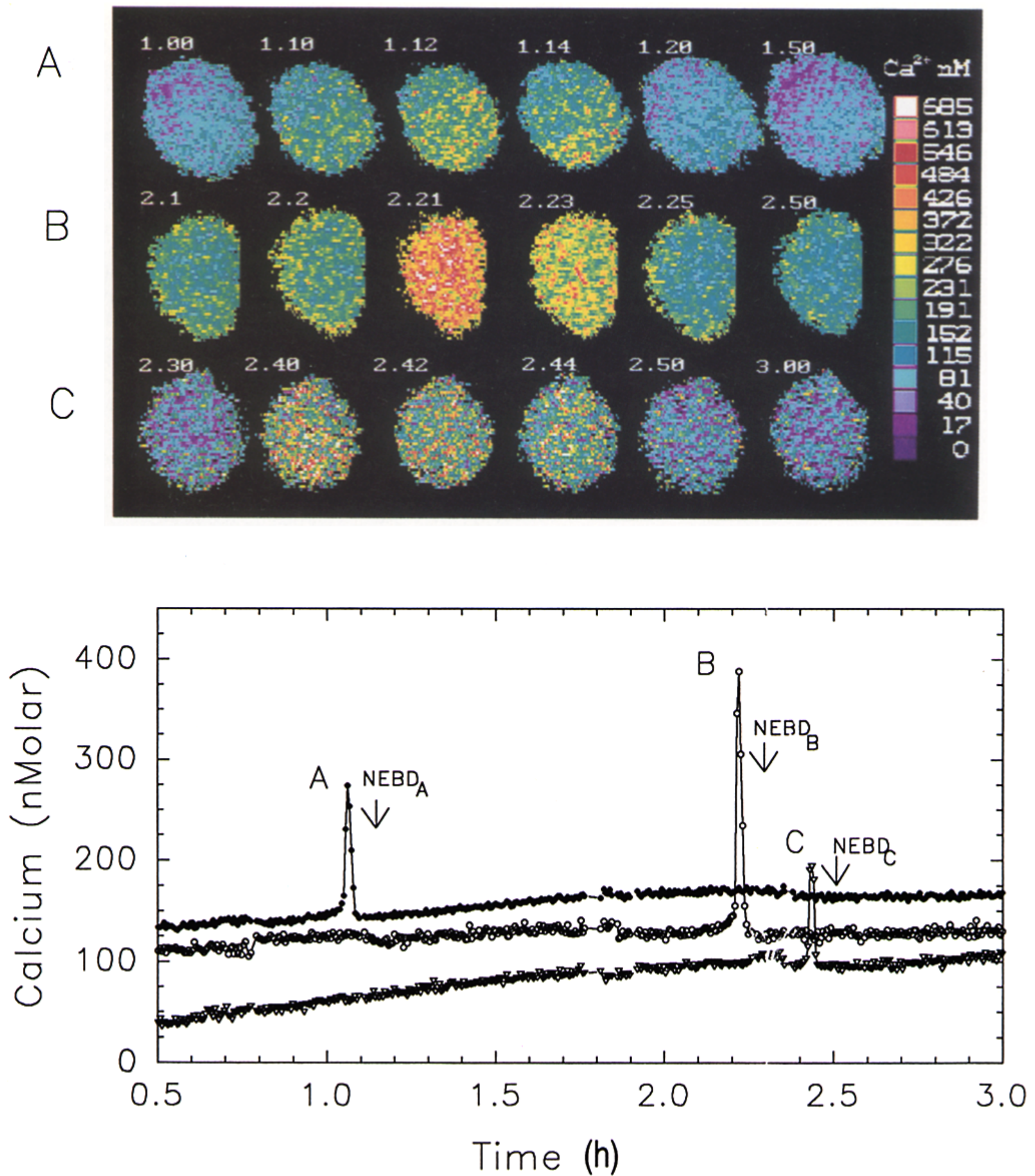

Figure 8. First mitotic NEBD is frequently coupled to a Ca^{2+} transient. Calibrated Ca^{2+} images from three eggs (*A–C*) undergoing NEBD (times are indicated in fractions of hrs after imaging had begun). Scale represents nM free Ca^{2+} ion. Ca^{2+} increases were global in nature. Egg B was at the edge of a field of view. The Ca^{2+} signal was averaged over the entirety of the three eggs and plotted here on the same time scale. In these eggs, NEBD occurred within 5 min of the Ca^{2+} transient, as marked. Cytokinesis occurred approximately 1 h after NEBD and both it and anaphase, whose timing was not precisely determined, were not associated with a detectable transient.

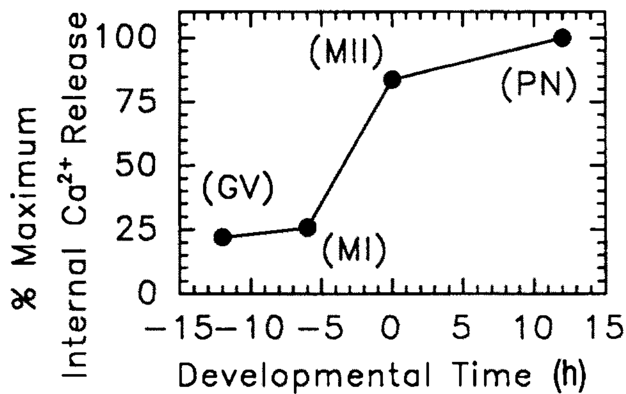

Figure 9. Kinetics of intracellular Ca^{2+} store maturation. Oocytes and eggs obtained at germinal vesicle (*GV*), meiosis I (*MI*), metaphase II (*MII*), and pronucleate (*PN*) stages were loaded with 1 μM fura-2 and stimulated by 10 μM ionomycin in medium lacking calcium. The Ca^{2+} transients in each oocyte were determined, averaged, plotted with time, and integrated. The integrated value is plotted here as the percentage of the maximum value (obtained at the PN stage) versus its developmental stage, with times indicated as in Fig. 1.

first polar body formation is prevented, and oocytes escape from M phase as a result of the removal of external Ca^{2+}; (*c*) NEBD is frequently associated with Ca^{2+} transients and is dependent on Ca^{2+} mobilization, but GVBD is not; and (*d*) between GVBD and metaphase II arrest, internal Ca^{2+} stores increase fourfold.

Delayed Internal Ca^{2+} Store Maturation May Reduce the Risk of Precocious Activation

The maturation of mammalian oocytes to metaphase II, where they become competent for fertilization, has been associated with several specific events. The ability of metaphase II oocytes to be parthenogenetically activated increases dramatically as soon as, but not before, oocytes reach metaphase II (Whittingham and Siracusa, 1978; Kubiak, 1989). The ability of cortical granules to undergo exocytosis during oocyte maturation is acquired around metaphase I and progressively increases with the time of maturation (Ducibella et al., 1988). Oocytes from juvenile mice, induced to mature in vitro, are incapable of proceeding past metaphase I, even though spindle chromatin and MTs appear normal (Wickramasinghe et al., 1991). The development of internal Ca^{2+} stores can be added to this list; it may be a necessary condition for the acquisition of activation and cortical granule firing potential. The full development of internal Ca^{2+} stores does not occur until oocytes reach metaphase II. The regulated build up of internal Ca^{2+} stores may provide some reproductive advantage by reducing the chances of precocious activation before nuclear maturation due to premature Ca^{2+} release. Such spontaneous activation occurs in aged metaphase II oocytes (Whittingham and Siracusa, 1978) possibly as a result of the increased risk of spontaneous Ca^{2+} release from mature stores.

Ca^{2+}-triggered Events at Parthenogenetic Activation Are Not Necessarily Immediate

In mammalian and amphibian oocytes arrested at metaphase II, the destruction of cytostatic factor (CSF) is thought to be triggered by Ca^{2+} to immediately induce the resumption of meiosis II (Masui and Shibuya, 1987). We demonstrate that anaphase II onset in the mouse oocyte is also strictly temporally coupled to a large Ca^{2+} transient and is dependent on Ca^{2+} mobilization. The normal amplitude and duration of the Ca^{2+} transient is significantly greater than the threshold required for activation for two reasons. First, internal Ca^{2+}, which can fully drive activation, supplies only half of the Ca^{2+} provided when external Ca^{2+} also contributes to the transient. Second, anaphase II initiates before the Ca^{2+} transient has begun to subside. This immediate activation of chromatid separation is a clear example of an event which is tightly coupled to the Ca^{2+} transient. In contrast, other Ca^{2+}-dependent events reported in this study, such as cortical granule exocytosis and polar body formation, show no temporal coupling to cytoplasmic increases in Ca^{2+}.

The lack of temporal coupling of mouse CG discharge to the Ca^{2+} rise, in this system, may be surprising considering the instantaneous Ca^{2+}-induced firing of CGs at fertilization in invertebrates like the sea urchin (Heinecke and Shapiro, 1990). However, the relatively slow (at least 15 min) kinetics of CG firing in mouse oocytes, as reported here and by others (Fukuda and Chang, 1978; Ducibella et al., 1988; Colonna et al., 1989), may reflect other functions. In mammals, where fertilization is internal and polyspermy is not a potentially great problem, partial CG exocytosis may occur during meiosis I to generate CG-free zones to regulate the site of sperm penetration (Ducibella et al., 1988), while complete CG exocytosis after activation may protect the embryo. Nonetheless, the changes in CGs accompanying activation of the mouse oocyte are blocked by chelators of internal Ca^{2+} and are not affected by external Ca^{2+} removal. This finding is consistent with the extracellular Ca^{2+}-independent modifications of zona pellucida proteins (Kurasawa et al., 1989) and the proposed role of PKC in mouse CG firing (Colonna et al., 1989).

First Meiosis has Pleiotropic Dependencies on Ca^{2+}

Our results confirm previous reports that first polar body formation is dependent on external Ca^{2+} in cumulus-free mammalian oocytes (Leibfried and First, 1979; Paleos and Powers, 1981; Jagiello et al., 1982). We extend those studies to show that, although first polar body formation is prevented by the absence of external Ca^{2+}, first meiotic spindle formation is not, although it is delayed. Ultimately, oocytes incubated in the absence of external Ca^{2+} exit meiosis, perhaps after chromosome pair separation, but still without polar body formation. Inhibitors of PKC also allow chromosome pair separation, but block first polar body formation, suggesting that part of this Ca^{2+} dependency may be mediated by PKC (Bornslaeger et al., 1986). First polar body formation is another Ca^{2+}-dependent event which shows no associated transient Ca^{2+} elevation. Such cell surface events may have Ca^{2+} flux cortically restricted, away from detection by cytoplasmic dyes. We expect that second polar body formation is also dependent on Ca^{2+}, but that it acquires Ca^{2+} from internal sources, which are mature at that point. Those Ca^{2+} stores may also be cortically restricted, thus precluding the detection of associated cytoplasmic Ca^{2+} elevations.

In addition, as now concluded for mitotic anaphase in many systems (Hepler, 1989; Tombes and Borisy, 1989),

first meiotic anaphase in the mouse oocyte as well as first mitotic anaphase in the mouse egg is not accompanied by a detectable Ca^{2+} transient. The lack of associated transients does not signal a Ca^{2+}-independent event. The delay in first meiosis observed in the absence of Ca^{2+} may reflect a dependence of chromosome movement on Ca^{2+} mobilization.

The most striking effect of culturing first meiotic oocytes in the absence of external Ca^{2+} was that oocytes did not simply arrest at some point during meiosis I or take longer than usual to reach the normal arrest point of metaphase II. Rather, oocytes exited M phase and achieved an interphase-like state. Normally, there is no interphase between meiosis I and II in the mouse oocyte, as defined by the reformation of nuclei; interphase occurs after meiosis II. In this case, the interphase-like configuration could have been reached before or after segregation of chromosome pairs, although it appeared to occur after segregation, since two "nuclei" were visible. This interphase-like state could also have been achieved after meiosis II, since oocytes naturally arrested at metaphase II have been reported to activate and form pronuclei by long-term incubation in Ca^{2+}-free medium (Whittingham and Siracusa, 1978). Interestingly, a similar interphase-like state is induced when either *Xenopus* (Jessus et al., 1991) or mouse (Rime et al., 1989) oocytes are treated with agents which inhibit protein phosphorylation. By assaying molecular markers of M phase, such as lamin and histone H1 phosphorylation, it should be possible to determine to what molecular degree Ca^{2+} removal causes oocytes to exit M phase.

Ca^{2+} Transient Heterogeneity at NEBD Implies that Transients Are Dispensable

The temporal association of Ca^{2+} transients, when they occur, with NEBD suggests a causal role of Ca^{2+} in nuclear envelope disassembly. The sensitivity of NEBD to chelator inhibition supports this reasoning. However, some eggs undergo NEBD without any apparent Ca^{2+} transient, indicating that even though Ca^{2+} mobilization is required, a detectable transient may not be.

The possibility that Ca^{2+} can be mobilized, i.e., transferred from a source to a target, without the appearance of a visible transient, has been discussed (Kao et al., 1990; Tsien and Tsien, 1990). Our results support the conclusion that the apparent absence of transients is not because of technical limitations in our ability to detect them. Although there was significant variation in the amplitude of transients, which nonetheless was at least 10-fold higher than our estimated threshold for detection, there was not variability in their kinetics. Had we sampled the wrong focal plane, we would still have expected to detect transients of the same duration, but smaller amplitude. Transients were global in nature, indicating little, if any spatial limitations on detection. We found two equally populated subgroups of eggs undergoing NEBD simultaneously, one which exhibited Ca^{2+} transients at NEBD and the other which did not. In addition, global elevations of Ca^{2+} alone induced at time points near natural NEBD are insufficient to trigger NEBD precociously.

How then can Ca^{2+} transients at NEBD be explained? The appearance of a Ca^{2+} transient requires that a source release Ca^{2+} into the cytoplasm and that it diffuse to and be bound by the indicator dye faster than a target binds it or a sink sequesters it. If sources and targets are juxtaposed, it would be possible for a Ca^{2+} transfer to occur from source to target without any significant increase in cytoplasmic Ca^{2+}. Useful indicators such as fura-2 have lower affinities for Ca^{2+} and are used at lower concentrations than useful chelators such as BAPTA. Consequently, it is possible for a chelator to effectively compete with the natural target for released Ca^{2+} under conditions where no detectable indicator signal would be produced.

Ca^{2+} Dependence at NEBD Implies Multiple Essential Regulatory Pathways

The observation of Ca^{2+} transients prompts the question of how transients and the mobilization of Ca^{2+} integrate with other regulatory pathways believed to trigger NEBD, such as MPF. A relevant Ca^{2+} target exists in the Ca^{2+} calmodulin-dependent protein kinase, whose activity can be triggered by a brief transient and which appears to be essential for NEBD (Baitinger et al., 1990). From extensive work, NEBD has also been shown to be induced by the phosphorylation of nuclear lamins, which is accomplished by $p34^{cdc2}$, the catalytic subunit of MPF, and other kinases (Nurse, 1990). No definitive relationships have been identified between Ca^{2+} and MPF ($p34^{cdc2}$).

Perhaps both Ca^{2+} transfer and MPF activation, but not Ca^{2+} transients, are necessary, but not independently sufficient to trigger NEBD. This model requires that the timing of these two necessary events relative to each other is not precisely determined, but that they are interregulated by feedforward and feedback mechanisms. If MPF activation occurs first, then when Ca^{2+} is released, NEBD is immediately triggered and feeds back to shut off Ca^{2+} release rapidly before it is manifest in the form of a transient cytoplasmic elevation, but after Ca^{2+} has bound to its target. On the other hand, if MPF is not fully active when Ca^{2+} channels are opened, Ca^{2+} activates its target and cytoplasmic Ca^{2+} levels rise and register as a transient until MPF becomes fully active to trigger NEBD and feedback to close channels. Whichever pathway is fully activated first, it should also stimulate the activation of the second pathway.

Such a link between Ca^{2+} and MPF may be represented by results obtained in starfish oocytes, where a peptide modeled on the conserved "PSTAIR" domain of $p34^{cdc2}$ stimulates internal Ca^{2+} release, without any associated effect on $p34^{cdc2}$ kinase activity (Picard et al., 1990). If full activation of MPF is related to a conformational change in $p34^{cdc2}$ to expose the PSTAIR domain, then this could represent a feedforward mechanism from MPF to Ca^{2+}.

Such a multipathway model also has support from *Aspergillus nidulans*, where Nima and cdc2, both encoding protein kinases, represent independent, yet essential pathways to initiate mitosis (Osmani et al., 1991). Feedforward mechanisms between these two essential kinases are implied by experiments where premature mitosis is induced by the hyperactivation or overexpression of just one of the kinases at a time (Russell and Nurse, 1987; Osmani et al., 1988).

GVBD Does Not Depend on Ca^{2+}

Unlike NEBD, GVBD showed neither transients nor a requirement for Ca^{2+} mobilization. This was surprising, since the fundamental properties and dynamics of nuclear envelopes are believed to be conserved between meiosis and mi-

tosis (Stick, 1987). However, the same conclusion, that GVBD is not dependent on Ca^{2+}, has been reached in the starfish oocyte (Witchel and Steinhardt, 1990). More strictly interpreted, although the results with mouse and starfish oocytes are not consistent with a role for Ca^{2+} transfer at the time of GVBD, they do not exclude the possibility of a Ca^{2+}-dependent event well upstream (>2 h in the mouse) of GVBD. The rapidly expanding analyses of cell cycle control indicate the possibility of dissimilarities in the protein kinase components of meiotic and mitotic MPF (Nurse, 1990). Additional studies of protein kinase and Ca^{2+} targets may help resolve differences in the Ca^{2+} dependencies between NEBD and GVBD. Clearly, the interaction between Ca^{2+} transfer and MPF activation at many M phase loci is an area demanding further examination.

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Note Added in Proof. D. Kline and J. T. Kline corroborate our findings by showing that BAPTA blocks cortical granule exocytosis and mouse egg activation, and that natural fertilization is associated with a large transient elevation of Ca^{2+} (Kline, D., and J. T. Kline. 1992. *Dev. Biol.* 149: 80–89.

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