A Cell Cycle–associated Change in Ca^{2+} Releasing Activity Leads to the Generation of Ca^{2+} Transients in Mouse Embryos During the First Mitotic Division

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Abstract. We have used Ca^{2+}-sensitive fluorescent dyes to monitor intracellular Ca^{2+} during mitosis in one-cell mouse embryos. We find that fertilized embryos generate Ca^{2+} transients at nuclear envelope breakdown (NEBD) and during mitosis. In addition, fertilized embryos arrested in metaphase using colcemid continue to generate Ca^{2+} transients. In contrast, parthenogenetic embryos produced by a 2-h exposure to strontium containing medium do not generate detectable Ca^{2+} transients at NEBD or in mitosis. However, when parthenogenetic embryos are cultured continuously in strontium containing medium Ca^{2+} transients are detected in mitosis but not in interphase. This suggests that mitotic Ca^{2+} transients are detected in the presence of an appropriate stimulus such as fertilization or strontium.

The Ca^{2+} transient detected in fertilized embryos is not necessary for inducing NEBD since parthenogenetic embryos undergo nuclear envelope breakdown (NEBD). Also the first sign that NEBD is imminent occurs several minutes before the Ca^{2+} transient. The Ca^{2+} transient at NEBD appears to be associated with the nucleus since nuclear transfer experiments show that the presence of a karyoplast from a fertilized embryo is essential. Finally, we show that the intracellular Ca^{2+} chelator Bapta inhibits NEBD in fertilized and parthenogenetic embryos in a dose-dependent manner.

These studies show that during mitosis there is an endogenous increase in Ca^{2+} releasing activity that leads to the generation of Ca^{2+} transients specifically during mitosis. The ability of Ca^{2+} buffers to inhibit NEBD regardless of the presence of global Ca^{2+} transients suggests that the underlying cell cycle-associated Ca^{2+} releasing activity may take the form of localized Ca^{2+} transients.

At the time of ovulation the mammalian oocyte is arrested in metaphase of the second meiotic division. Fertilization triggers a series of Ca^{2+} transients that are responsible for oocyte activation, including the completion of the second meiotic division and entry into the first mitotic cell cycle (Kline and Kline, 1992; Whitaker and Swann, 1993). The Ca^{2+} transients stimulated at fertilization continue until the formation of pronuclei in the one-cell mouse embryo (Jones et al., 1995*a*). Oocyte activation can also be stimulated parthenogenetically by agents that lead to a transient rise in intracellular Ca^{2+} (Whittingham, 1980). After the initiation of embryonic development the preimplantation embryo undergoes a series of endogenously regulated cell division cycles. The role of intracellular Ca^{2+} in the initiation of cell division at fertilization is well established, however, the endogenous pathways that control subsequent cell division are unknown.

In other systems increases in intracellular Ca^{2+} are associated with cell-cycle checkpoints, including entry into and exit from mitosis (Whitaker and Patel, 1990). Ca^{2+} transients occur at nuclear envelope breakdown (NEBD)[1], the first sign of entry into mitosis in sea urchin embryos (Poenie et al., 1985; Steinhardt and Alderton, 1988; Twigg et al., 1988; Ciapa et al., 1994), mouse embryos (Tombes et al., 1992), and in fibroblasts (Kao et al., 1990). A direct role for Ca^{2+} in the initiation of NEBD has been suggested since NEBD is delayed in the presence of intracellular Ca^{2+} buffers, and advanced by raising intracellular Ca^{2+} (Steinhardt and Alderton, 1988; Twigg et al., 1988; Kao et al., 1990). However, the role of Ca^{2+} transients in the stimulation of mitosis is controversial (Hepler, 1989). Some studies have failed to detect mitotic Ca^{2+} transients consistently (Whitaker and Patel, 1990; Tombes et al., 1992), and in fibroblasts the generation of mitotic Ca^{2+} transients requires the presence of serum (Tombes and Borisy, 1989; Kao et al., 1990). Thus a role for Ca^{2+} as a cell cycle signal at fertilization is well established while in other cases of cell division the evidence is strong but not yet conclusive.

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1. *Abbreviation used in this paper*: NEBD, nuclear envelope breakdown.

In a recent study we found that fusing karyoplasts (plasma membrane-bound nuclei) from one- and two-cell mouse embryos with metaphase II arrested oocytes stimulates Ca^{2+} transients and oocyte activation (Kono et al., 1995). This activity was specific to karyoplasts from fertilized embryos and was not a property of karyoplasts from parthenogenetic embryos or of cytoplasts from any source. Therefore, we concluded that fertilization provides karyoplasts of early embryos with a Ca^{2+} releasing activity. Ca^{2+} transients have been reported to occur at NEBD in ~ 50% of fertilized mouse embryos (Tombes et al., 1992) raising the possibility that the nuclear-associated Ca^{2+} releasing activity may lead to the generation of Ca^{2+} transients at NEBD in the first mitotic cell cycle.

In this study we have investigated the ability of fertilized and parthenogenetic mouse embryos to generate Ca^{2+} transients in mitosis, and the role of intracellular Ca^{2+} in stimulating NEBD. We found that fertilized but not parthenogenetic embryos show a large Ca^{2+} transient at NEBD and suggest that the Ca^{2+} transients reflect an underlying cell cycle-associated modification in the mechanisms of Ca^{2+} release. Further, chelation of intracellular Ca^{2+} inhibits NEBD suggesting a role for Ca^{2+} in cell cycle progression.

Materials and Methods

Collection and Preparation of Oocytes and Embryos

Female C57B/6JLac × CBA/CaLac (B6CBF1) mice were superovulated by intraperitoneal injections of pregnant mares' serum gonadotrophin and human chorionic gonadotrophin (hCG) 48 h apart. Cumulus masses were collected from the oviducts 15–16 h after hCG injection. For in vitro fertilization, the cumulus complexes were placed into a drop of T6 (100 μl) medium containing capacitated sperm (~ 1×10^6 /ml) as described previously (Kono et al., 1993). After 4–6 h incubation at 37°C in 5% CO_2 in air the cumulus-free oocytes were recovered and examined for the presence of pronuclei.

Parthenogenetic embryos were produced by exposure of oocytes to 7% ethanol in M2 Medium (Fulton and Whittingham, 1978) for 7 min at room temperature (Kono et al., 1995) or by a 90-min incubation in Ca^{2+}-free M16 medium supplemented with 10 mM Sr^{2+} (Kline and Kline, 1992; Bos-Mikich et al., 1995). In some experiments embryos were continuously incubated in strontium containing medium. To produce diploid parthenogenones, the oocytes were cultured for a further 5 h in M16 medium containing 5 μg/ml cytocholasin B. Fertilized and activated zygotes were cultured in M16 medium (Whittingham, 1971) until used for nuclear transfer or the recording of intracellular Ca^{2+}.

Nuclear Transfer

Pronuclei were exchanged between fertilized and parthenogenetic zygotes using standard micromanipulation techniques (Kono et al., 1991, 1993). Before transfer of nuclei the zonae pellucidae of embryos was slit with a glass needle. Nuclei were removed from donor embryos in a plasma membrane-bound vesicle (karyoplast) and transferred with inactivated Sendai virus (HVJ, 2700 hemagglutinating activity unit/ml) to the perivitelline space of recipient enucleated zygotes. Sendai virus caused the fusion of the transferred karyoplast with the recipient embryo within 20 min. Cytoplasm was transferred in a similar manner in membrane-bound vesicles (cytoplasts) that were then fused with recipient embryos using Sendai virus as described above.

Measurement of Intracellular Calcium

Cytosolic Ca^{2+} levels were recorded using the Ca^{2+}-sensitive dyes indo-1 (Sigma) and calcium green dextran, MW 10,000 (Molecular Probes, Cambridge, UK). For loading of indo-1, embryos were incubated for 25 min at 37°C with 50 μM of the acetoxymethyl ester (AM) form of indo-1in M2 medium containing 0.02% pluronic F127. Embryos loaded with these dyes develop to blastocysts in culture at rates similar to controls (unpublished). Ca^{2+}-green dextran was pressure injected into one-cell embryos 1–2 h before the predicted time of NEBD. For microinjection embryos were held using a wide-bore holding pipette and injection was through a broken-tipped injection pipette. The pipette contained 100 μM calcium green dextran and the injection was ~2–5% of the egg volume.

Fluorescence was recorded as described previously (Carroll and Swann, 1992), with some minor modifications. Single embryos loaded with fluorescent dye were placed in 1 ml of M16 medium under paraffin oil in a heated chamber. The chamber was heated to 35–37°C and was gassed with 5% CO_2 in air. Fluorescence was monitored with 9924B photomultiplier tubes connected to A1 current to voltage converters and analogue signals recorded on computer. Indo-1 records are presented as the ratio of the 405- and 490-nm emission wavelength. An estimation of intracellular Ca^{2+} was obtained using the method of Grynkiewicz et al. (1985). Fluorescence emission from Ca^{2+}-Green dextran was collected at 525 nm and is presented with an arbitrary fluorescence scale.

Chelation of Intracellular Calcium

Embryos were loaded with different concentrations (1–20 μM) of the calcium chelator Bapta, or 20 μM indo-1 (controls), by incubation in the AM ester in M16 for 30 min 11–12 h after exposure to sperm or strontium containing medium. At this time all embryos had visible pronuclei. After loading, the embryos were transferred to drops of M16 under paraffin oil.

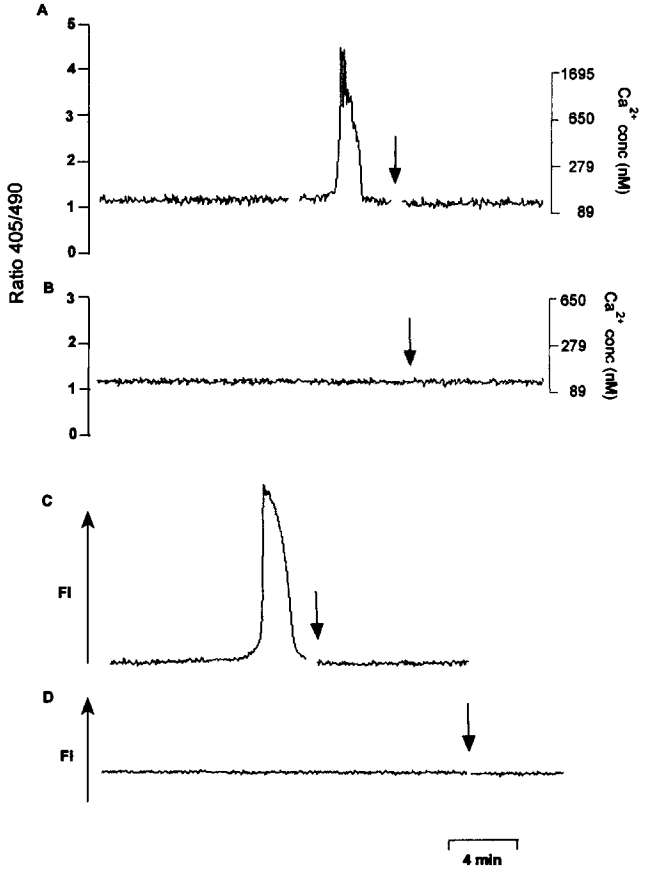

Figure 1. Ca^{2+} transients at NEBD occur in fertilized but not parthenogenetic one-cell mouse embryos. One cell mouse embryos were loaded with indo-1 (*A* and *B*) or Ca^{2+}-green dextran (*C* and *D*) as described. Fertilized embryos show a Ca^{2+} transient close to the time of NEBD (*A* and *C*) while parthenogenetic embryos show no change in Ca^{2+} levels (*B* and *D*). The arrow indicates the time that embryos were examined for NEBD. In all cases NEBD was complete at the arrow. The precise relationship between NEBD and the Ca^{2+} transient is shown in Fig. 3.

Table I. Characteristics of Ca^{2+} Transients at NEBD in Fertilized and Parthenogenetic One-Cell Mouse Embryos

Type of embryo	No. of embryos with Ca^{2+} transients at NEBD*	Duration	Mean peak Ca^{2+} concentration
		min	*nM*
Fertilized	11/11	1.9 ± 0.3	1512 ± 519
Parthenogenetic	0/6	–	–

*The number of fertilized embryos loaded with indo-1 in which NEBD Ca^{2+} transients were detected. Data from Ca^{2+}-green loaded embryos is not included as it is not calibrated for Ca^{2+} concentration.

Progression through mitosis was monitored on a dissecting microscope at 50× magnification. To avoid prolonged exposure to nonambient conditions cleavage, which is easily recognizable, rather than NEBD, was monitored at 30-min intervals. After control embryos had cleaved to the two-cell stage, Bapta-treated embryos that were arrested were examined in more detail under bright field (×200) and were stained for 10 min with the DNA-specific fluorochrome Hoechst 33258 and examined using a Leitz fluorescent microscope.

Results

Ca^{2+} Transients Occur at NEBD of Fertilized Embryos

To determine whether Ca^{2+} transients were associated with NEBD of the first mitosis in mouse embryos we monitored fluorescence of indo-1 through the period of NEBD. A single Ca^{2+} transient was recorded around the time of NEBD in all of the fertilized one-cell embryos examined (n = 11) (Fig. 1 *a*). Observation of the embryo immediately after the Ca^{2+} transient revealed that NEBD had occurred, as indicated by the arrow in Fig. 1, by the time the Ca^{2+} had returned to baseline levels. However, the precise temporal relationship between NEBD and the Ca^{2+} transient could not be determined in this experiment. This question was examined in a separate series of experiments (see below). The Ca^{2+} transient lasted 1.9 ± 0.3 min, reached an estimated average peak Ca^{2+} of 1512 ± 519 nM (Table I). While all fertilized embryos showed a Ca^{2+} transient at NEBD similar experiments with strontium or ethanol activated embryos failed to detect any increase in intracellular Ca^{2+} (n = 6) (Fig. 1 *b*, Table II). Despite the absence of a measurable Ca^{2+} transient, the parthenogenetically activated embryos progressed through mitosis and cleaved to the two-cell stage.

To examine the possibility that a smaller Ca^{2+} transient occurred in parthenogenetic embryos but was not detected using the Ca^{2+} dye indo-1, we microinjected Ca^{2+}-green dextran (MW 10,000). Dextran linked dyes do not become compartmentalized into cellular organelles and therefore are more sensitive to cytosolic changes in Ca^{2+} (Gillot and Whitaker, 1993; Carroll et al., 1994). Similar to embryos loaded with indo-1, Ca^{2+} transients were seen at NEBD in all fertilized embryos (n = 5) (Fig. 1 *c*), however, parthenogenetic embryos showed no evidence of Ca^{2+} transients at NEBD (n = 5) (Fig. 1 *d*).

The Ca^{2+} Releasing Activity in Fertilized Embryos Is Associated with the Pronuclei

We have previously found that karyoplasts but not cytoplasts from fertilized embryos transferred to metaphase II arrested oocytes cause Ca^{2+} transients that lead to oocyte activation (Kono et al., 1995). Ca^{2+} transients were detected only after the donor nucleus had undergone NEBD. This result predicts that at NEBD in the first cell cycle the increase in intracellular Ca^{2+} originates from the region of the nucleus. To investigate this possibility, karyoplasts and cytoplasts were exchanged between fertilized and parthenogenetic embryos and intracellular Ca^{2+} monitored at the time of NEBD.

Table II. Characteristics of Ca^{2+} Transients That Occur during Mitosis in Fertilized and Parthenogenetic One-Cell Mouse Embryos

Type of embryo	No. of embryos with mitotic Ca^{2+} transients	Interspike interval	Duration	Mean peak Ca^{2+} concentration
		min	*min*	*nM*
Fertilized*	4/4	36.7 ± 8.8	2.1 ± 0.3	852 ± 344
Parthenogenetic‡				
(i) 2 hours	0/4	–	–	–
(ii) continuous	7/7	21.5 ± 10.4	3.9 ± 1.1	408 ± 52

*Fertilized data includes Ca^{2+} transients detected in embryos arrested in metaphase with colcemid.
‡After activation for 2 h in strontium containing medium embryos were either removed and cultured further in M16 (*2 hours*) or were transferred to fresh strontium containing medium (*continuous*). Only the embryos exposed continuously to strontium generated Ca^{2+} transients during mitosis.

The generation of a Ca^{2+} transient at NEBD was consistently associated with the presence of a karyoplast from a fertilized embryo. Thus, a Ca^{2+} transient at NEBD was detected in parthenogenetic embryos that received a karyoplast from a fertilized embryo (n = 9; Fig. 2 *a*). However, the reciprocal transfer of a karyoplast from a parthenogenetic embryo to an enucleated fertilized embryo was not associated with an NEBD Ca^{2+} transient (n = 8; Fig. 2 *b*). The association of the Ca^{2+} releasing activity to karyoplasts from fertilized embryos was further examined by transferring cytoplasts from fertilized to parthenogenetically activated embryos. Cytoplasts from fertilized embryos did not lead to the generation of Ca^{2+} transients at NEBD (n = 5; Fig. 2 *c*) supporting the observation that these endogenously driven Ca^{2+} transients arise from the region of the nucleus.

The Ca^{2+} Transient Occurs after the Initiation of NEBD

The close temporal relationship between NEBD and the Ca^{2+} transient prompted a more detailed examination to determine the relationship between the two events. Fluorescence was monitored from indo-1 or Ca^{2+}-green dextran-loaded one-cell embryos while every few minutes the nucleus was observed using Nomarski optics. NEBD could be divided into several distinct morphological stages (see Fig. 3). Before NEBD the two pronuclei were closely apposed with a clear demarcation between cytoplasmic and nuclear compartments at the nuclear membrane. In addition, each pronucleus contained 1–3 distinct nucleoli. The first indication that NEBD was imminent was a shrinking in the size of the nucleoli and a blurring of the border between nucleus and cytoplasm. This process continued until the nucleoli and the distinction between the cytoplasm and nucleus were no longer visible. From the first indication of NEBD, i.e., the shrinking of the nucleoli, to completion

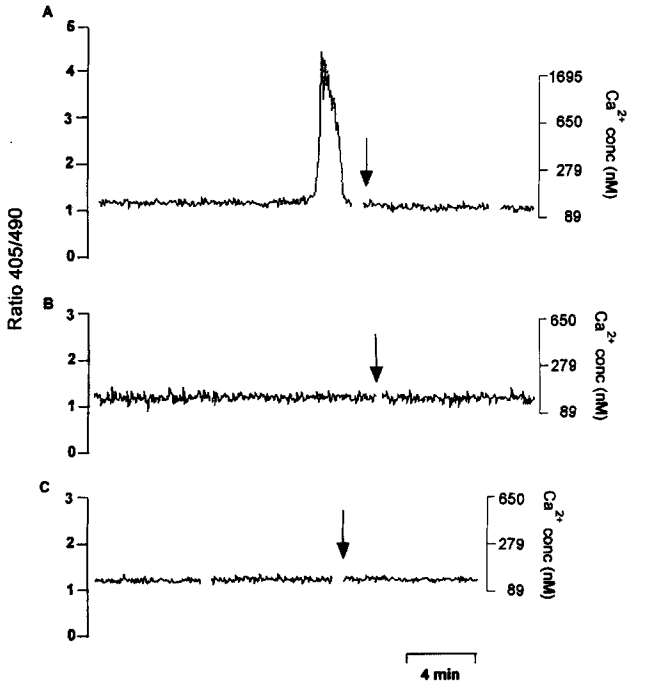

Figure 2. The Ca^{2+} releasing activity seen at NEBD is associated with the nucleus of fertilized embryos. Karyoplasts or cytoplasts were exchanged between fertilized and parthenogenetic one-cell mouse embryos. Parthenogenetic embryos containing a karyoplast from fertilized embryos generate a Ca^{2+} transient at the time of NEBD (*A*). Fertilized embryos containing a nucleus from parthenogenetic embryos show no changes in intracellular Ca^{2+} at NEBD (*B*). The transfer of a similar volume of cytoplasm from a fertilized embryo to a parthenogenetic embryo does not provide the ability to release intracellular Ca^{2+} at NEBD (*C*).

was ~5 min. As illustrated in Fig. 3 the clear nuclear-cytoplasmic demarcation was lost and the nucleoli difficult to visualize before the Ca^{2+} transient was detected. Similar observations were made in five other one-cell embryos. The increase in Ca^{2+} at NEBD occurred 3–5 min after it was possible to determine that NEBD had been initiated. By the time the Ca^{2+} transient had returned to basal levels NEBD was completed. Thus a Ca^{2+} transient was observed in fertilized one-cell embryos at the time of NEBD, but after the first morphological signs that NEBD had begun.

Ca^{2+} Transients Are Detected after NEBD and Are Specific to Mitosis

After NEBD, Ca^{2+} was monitored to determine whether any further transients occurred during mitosis. In four fertilized embryos in which Ca^{2+} was monitored from just after NEBD for up to 60 min, three embryos generated at least one Ca^{2+} transient while in one of the embryos, where the recording was continued, several Ca^{2+} transients were detected (Fig. 4 *a*). During the recording the embryo cleaved to the two-cell stage after which no further Ca^{2+} transients were detected. These mitotic Ca^{2+} transients were not detected in parthenogenetic embryos activated by a 2-h exposure to strontium ($n = 6$) (Fig. 4 *b*). Thus mitosis-specific Ca^{2+} transients are detected only in fertilized embryos.

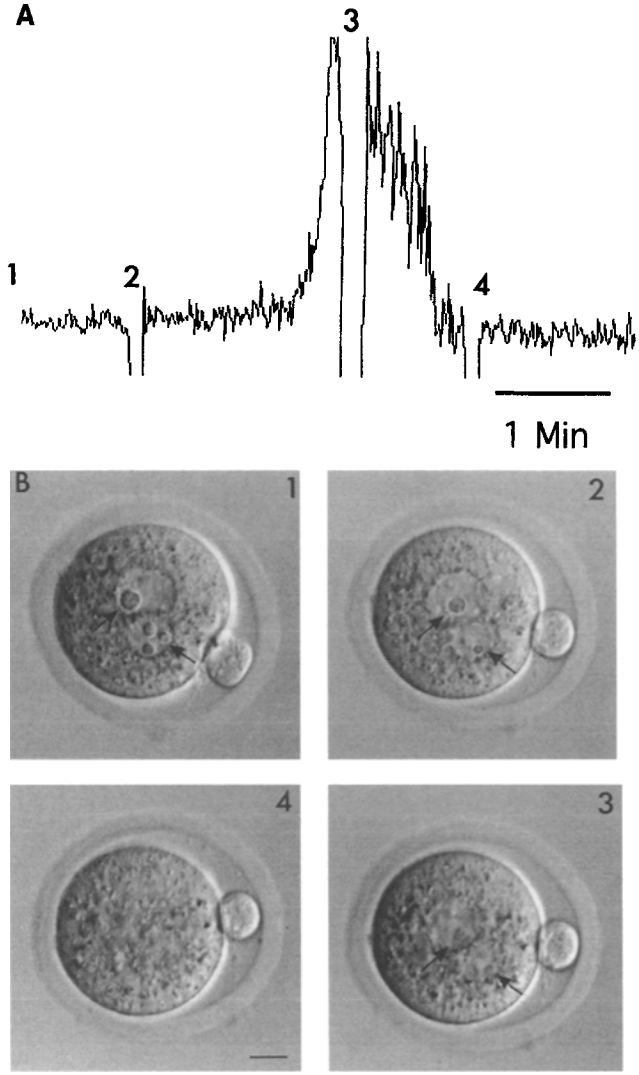

Figure 3. The first signs that NEBD is imminent occur before the Ca^{2+} transient. Photomicrographs of an embryo undergoing NEBD and the corresponding Ca^{2+} record of the NEBD Ca^{2+} transient. At the downward lines in the Ca^{2+} record the recording was stopped and a photograph taken. Before NEBD the two pronuclei and nucleoli are clearly visible. About 2 min before the rising phase of the Ca^{2+} transient the nucleoli have reduced in size and the border between nucleus and cytoplasm is less distinct. As the Ca^{2+} transient is generated the process of NEBD continues, the nuclear membrane and nucleoli becoming increasingly less distinct. Bar, 20 μm.

To examine the relationship between the generation of Ca^{2+} transients and mitosis in more detail Ca^{2+} was monitored in embryos arrested in mitosis using the microtubule inhibitor colcemid, and in control embryos allowed to progress through mitosis to the two-cell stage. Colcemid was added to fertilized embryos just before the estimated occurrence of NEBD and Ca^{2+} was monitored in the metaphase arrested one-cell embryos and in the control two-cell embryos 3–5 h after NEBD. In all of the metaphase-arrested embryos ($n = 6$) at least one Ca^{2+} transient was detected during a recording of at least 60 min ($n = 2$) while in longer recordings of 90–180 min 2 ($n = 2$), 3 ($n = 1$), or 4

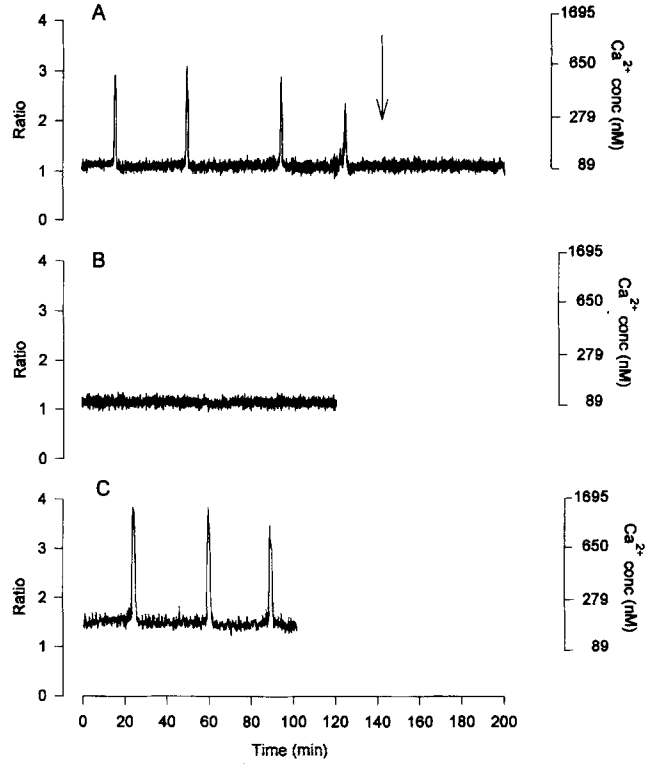

Figure 4. Ca^{2+} transients are specific to mitosis. Monitoring of intracellular Ca^{2+} in embryos after NEBD demonstrates Ca^{2+} transients occur during mitosis. The arrow indicates cleavage to the two-cell stage that occurred during the recording (*A*). Note that Ca^{2+} transients do not occur after cell division (*A*). Parthenogenetic embryos do not show any increase in intracellular Ca^{2+} after NEBD (*B*). Embryos arrested in metaphase with colcemid show Ca^{2+} transients (*C*), while control two-cell embryos do not show any changes in baseline Ca^{2+} (not shown).

(n = 1) Ca^{2+} transients were seen (Fig. 4 *c*). The transients occurred on average every 36.7 ± 8.8 min and lasted for 2.1 ± 0.3 min. The mean peak Ca^{2+} concentration was 852 ± 344 nM (Fig. 4 *c*, Table II). In contrast, no Ca^{2+} transients were detected in the control two-cell embryos (n = 4; not shown). These findings support the idea that the ability to generate Ca^{2+} transients is specific to mitosis, however, why parthenogenetic embryos are an exception is not clear.

Sensitization of Ca^{2+} Release in Parthenogenetic Embryos in Mitosis Leads to the Generation of Ca^{2+} Transients

The finding that Ca^{2+} transients are seen only during mitosis suggests that there is an endogenous change in the Ca^{2+} release systems during this stage of the cell-cycle. However, mitotic Ca^{2+} transients do not appear to be universal because they are not apparent in parthenogenetic embryos. A number of possible explanations exist for this difference between fertilized and parthenogenetic embryos. One is that the sperm provides additional factors that modify the Ca^{2+} releasing activity that, in combination with the proposed endogenous change in Ca^{2+} releasing activity, is sufficient to cause Ca^{2+} transients during mitosis. To test this idea parthenogenetic embryos were continuously incubated in strontium containing medium and Ca^{2+} was monitored at different times in the first cell cycle. Strontium causes Ca^{2+} oscillations in unfertilized oocytes (Kline and Kline, 1992; Bos-Mikich et al., 1995) that downregulate after 3–4 h (unpublished observations). Ca^{2+} was monitored in parthenogenetic embryos before (8–13 h after activation) and after (14–16 h after activation) NEBD for a period of at least 60 min. In the presence of strontium containing medium no Ca^{2+} transients were detected in any pronuclear stage embryos (n = 9; Fig. 5 *a*). In contrast, after NEBD, all of the parthenogenetic embryos generated repetitive Ca^{2+} transients (n = 5; Fig. 5 *b*). The Ca^{2+} transients were 3.9 ± 1.1 min in duration, occurring every 21.5 ± 10.4 min and reaching an estimated mean peak Ca^{2+} of 408 ± 42 nM. The strontium-induced Ca^{2+} transients, similar to those seen in fertilized embryos, stopped after cleavage to the two-cell stage. Thus the presence of strontium containing medium during mitosis reveals a mitosis-specific modification in Ca^{2+} release in par-

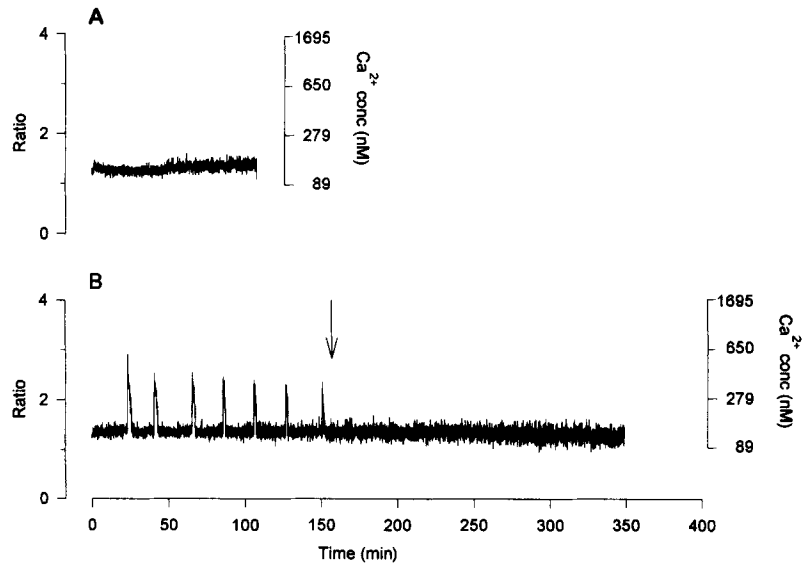

Figure 5. Parthenogenetic embryos generate mitotic Ca^{2+} transients in the presence of strontium. Oocytes were activated and cultured in the presence of strontium. During interphase, 8–13 h after activation, no Ca^{2+} transients were detected in any oocytes in which Ca^{2+} was monitored for at least 1 h. In the record shown Ca^{2+} was monitored for 100 min starting at 9 h after activation (*A*). After NEBD, 12–14 h after activation, Ca^{2+} transients were seen in all embryos examined (*B*). Similar to fertilized embryos (see Fig. 4 *a*) no Ca^{2+} transients were detected after cell division, indicated by the arrow (*B*).

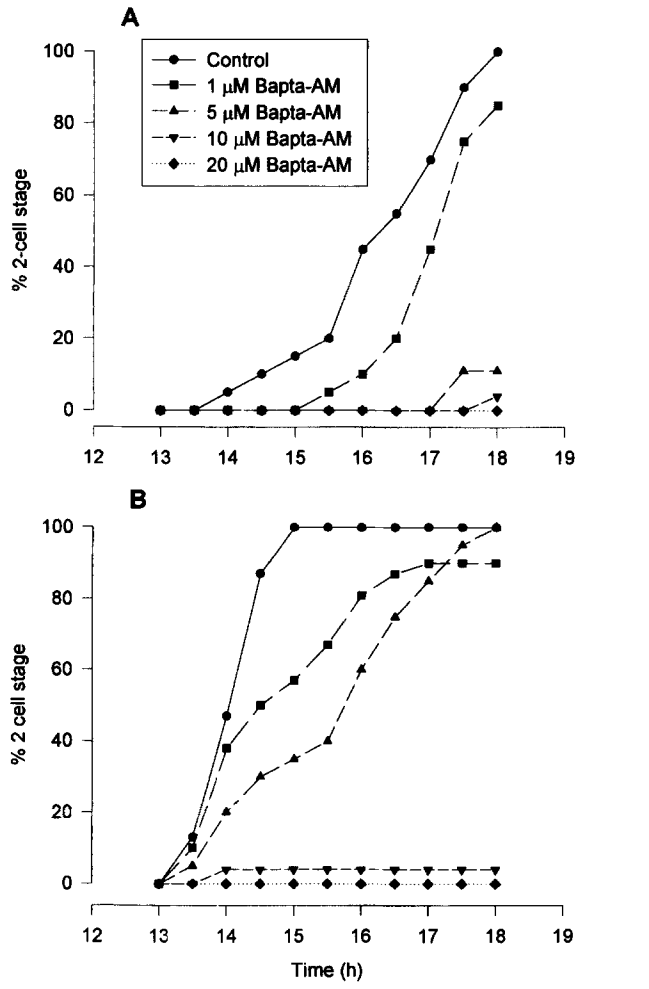

Figure 6. Chelation of intracellular Ca^{2+} inhibits NEBD in fertilized and parthenogenetic one-cell mouse embryos. One-cell embryos with visible pronuclei were loaded with Bapta AM, or indo-1 AM (controls), 11–12 h after activation or fertilization and scored for cleavage to the two-cell stage at 30-min intervals. The time scale indicates the number of hours after the addition of sperm or strontium to the oocytes. In fertilized (*A*) and parthenogenetic (*B*) one-cell embryos, cleavage to the two-cell stage is dependent upon the concentration of Bapta AM used for the loading period of 30 min. Examination of arrested embryos with Hoechst 33258 revealed that fertilized (*C*) and parthenogenetic (*D*) embryos incubated with 20 μM Bapta had intact pronuclei. The out of focus fluorescence in *C* is the second polar body. In parthenogenetic embryos extrusion of the second polar body was suppressed using cytocholasin D.

thenogenetic embryos, similar to that seen in fertilized embryos.

Chelation of Intracellular Ca^{2+} Inhibits NEBD in Both Fertilized and Parthenogenetic One-Cell Embryos

To determine a possible role for changes in intracellular Ca^{2+} in the progression through mitosis, fertilized and parthenogenetic embryos with visible pronuclei were incubated in various concentrations of Bapta AM (1–20 μM) to buffer intracellular Ca^{2+}. Control embryos were incubated in identical conditions with 20 μM indo-1 AM. Preliminary experiments revealed that the rate of progression through to the two-cell stage was not affected by loading with indo-1. The number of two-cell embryos was scored at 30-min intervals. Parthenogenetic embryos began to cleave ~1–2 h before fertilized embryos (Fig. 6, *a* and *b*), which is probably accounted for by the time taken for sperm to penetrate the zona pellucida and fuse with the oolemma (unpublished observations). The data shows that Bapta inhibits cell cleavage in a dose-dependent manner such that concentrations of 10 μM loaded for 30 min was sufficient to inhibit cell division (Fig. 6, *a* and *b*). Closer examination of the arrested embryos that were loaded with 20 μM Bapta AM revealed that the pronuclei were visible and the chromatin remained decondensed suggesting that NEBD had been inhibited (Fig. 6, *a* and *b*). This inhibition was not due to the AM loading technique since control embryos were loaded with indo-1 AM in identical conditions.

Discussion

Here we show that sperm provide a Ca^{2+} releasing activity that becomes associated with the pronuclei and leads to the generation of Ca^{2+} transients during mitosis of the first cell cycle. We provide evidence that the mitotic Ca^{2+} transients result from an endogenous cell cycle-associated change in Ca^{2+} homeostasis. The ability to undergo NEBD is independent of the generation of detectable Ca^{2+} transients, but NEBD is inhibited in all embryos if Ca^{2+} is buffered with Bapta. This infers that NEBD is a Ca^{2+}-dependent event but that the generation of global Ca^{2+} transients is not necessary.

Ca^{2+} Transients at NEBD Reflect an Underlying Cell Cycle-associated Change in the Mechanisms of Ca^{2+} Release

A number of findings in the present study suggest that Ca^{2+} release in one-cell mouse embryos is regulated in a cell cycle-dependent manner. First, endogenous Ca^{2+} transients were detected consistently in embryos undergoing mitosis and never during interphase. Second, embryos arrested in metaphase continue to show Ca^{2+} transients long after the non-arrested controls cleave to the two-cell stage. Third, parthenogenetic embryos, which do not normally generate mitotic Ca^{2+} transients do so if continuously incubated in strontium containing medium. These findings are consistent with a previous study where we have found that the ability of the sperm to generate repetitive Ca^{2+} transients in oocytes is specific to metaphase (Jones et al.,

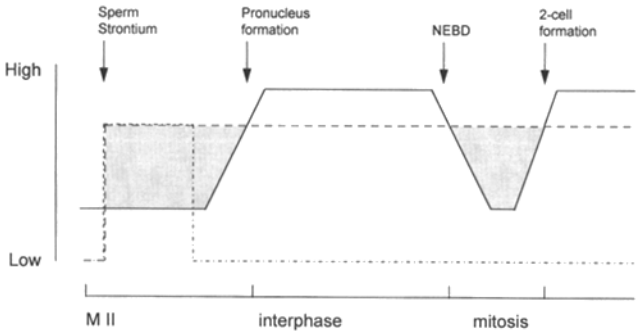

Figure 7. A model suggesting the origin of mitotic Ca^{2+} transients. Central to this model is a cell cycle-associated change in the threshold for Ca^{2+} release. This is represented by the solid line. The threshold is low during mitosis (and in metaphase of meiosis) and therefore supports the generation of Ca^{2+} transients, while in interphase the threshold increases and the generation of Ca^{2+} transients is less likely. Imposed on this cycle is an exogenous stimulus provided by sperm or strontium that sensitizes Ca^{2+} release sufficient to generate Ca^{2+} release in mitosis but not in interphase. The periods during which Ca^{2+} transients are generated are depicted by the shaded areas. As these areas indicate, fertilization or continual exposure to strontium containing medium initiates Ca^{2+} transients in metaphase II oocytes and in mitosis of the first cell cycle when the threshold for Ca^{2+} transients is low. In contrast, in interphase when the threshold increases no Ca^{2+} transients are seen. Normally, parthenogenetic activation is induced by a short 2-h incubation in strontium containing medium. In this case Ca^{2+} transients are seen in the metaphase II oocyte at the time of activation but are not detected during mitosis because the stimulus has been removed.

1995*a,b*). These findings mark mitosis as a period of increased Ca^{2+} releasing activity.

A model that fits with the present data is shown in Fig. 7. Central to this model is a cell cycle-associated change in the threshold for Ca^{2+} release, which is low in mitosis and high in interphase. The occurrence of Ca^{2+} transients is therefore dictated by fluctuations in the threshold for Ca^{2+} release and the physiological state of the Ca^{2+} release systems of the embryo. Fertilization is known to sensitize Ca^{2+} release mechanisms in oocytes (Igusa and Miyazaki, 1983) thus Ca^{2+} transients are seen in metaphase II oocytes and in embryos in the first mitosis, presumably stimulated by the presence of residual Ca^{2+} releasing sperm factors (Swann, 1990). Similarly, strontium containing medium provides a stimulus sufficient to support the generation of Ca^{2+} transients at mitosis but not in interphase where the threshold for the generation of Ca^{2+} transients is high.

The cell cycle-associated change in Ca^{2+} homeostasis shown in the model may be applicable to a number of other systems. Mitotic Ca^{2+} transients have also been observed in sea urchin embryos (Steinhardt and Alderton, 1988; Twigg et al., 1988), frog embryos (Grandin and Charbonneau, 1991; Kubota et al., 1993; Keating et al., 1994), and in fibroblasts (Tombes and Borisy, 1989; Kao et al., 1990). Also the generation of spontaneous Ca^{2+} transients in growing mouse oocytes is tightly correlated with the commitment to enter the first meiotic division (Carroll et al., 1994). Therefore a cell cycle-associated increase in Ca^{2+} releasing activity appears to be conserved from echinoderms to mammals.

However, while many studies have reported Ca^{2+} transients during mitosis, the results are often inconsistent (Hepler, 1989; Whitaker and Patel, 1990; Tombes and Borisy, 1989; Kao et al., 1990). By analogy with our findings in fertilized and parthenogenetic embryos it is possible to offer an explanation for these inconsistencies. First, the requirement of serum for the generation of mitotic Ca^{2+} transients (Tombes and Borisy, 1989; Kao et al., 1990) may be a result of serum causing an increase in basal inositol trisphosphate ($InsP_3$) levels such that Ca^{2+} release mechanisms are sensitized sufficiently to generate Ca^{2+} transients during mitosis, but not during interphase. Second, in sea urchin embryos the mitotic Ca^{2+} transients are smaller and seen less consistently in parthenogenetic and monospermic embryos compared to polyspermic embryos (Whitaker and Patel, 1990). This suggests that a threshold level of sperm-derived factors is required to reveal the mitotic increase in the sensitivity of Ca^{2+} release, which is consistent with our finding of Ca^{2+} transients in fertilized but not parthenogenetic embryos.

The mechanism that leads to an increase in Ca^{2+} releasing activity may involve a direct modification in the sensitivity of Ca^{2+} channels, perhaps through changes in phosphorylation (Nakade et al., 1994). Alternatively, there may be a cell cycle-associated production of Ca^{2+} mobilizing second messengers such as $InsP_3$ as shown in sea urchin and *Xenopus* embryos (Stith et al., 1993; Ciapa et al., 1994). Thus while it is clear that there is an endogenous activity that leads to the sensitization of Ca^{2+} release mechanisms during mitosis, the underlying clock that controls the timing, and the mechanism it uses to generate Ca^{2+} transients is unclear. The temporal association between the increased Ca^{2+} releasing activity and the activation of maturation promoting factor at each mitosis (Murray and Kirschner, 1989) suggests a possible relationship between the two events. Further work is required to examine the possibility of any such relationship and to determine if one is driving the other.

Ca^{2+} Transients at NEBD Are Stimulated by Sperm-derived Ca^{2+} Releasing Activity That Is Associated with the Pronuclei

The finding that Ca^{2+} releasing activity is associated exclusively with karyoplasts from fertilized embryos suggests that NEBD Ca^{2+} transients are driven by the nuclear region and are not a result of a cytoplasmic modification or of cell cycle-associated changes in ion channel activity (Day et al., 1993). The Ca^{2+} releasing activity of karyoplasts from fertilized embryos has been demonstrated previously by their ability to activate MII-arrested oocytes (Kono et al., 1995). It is not entirely clear, however, whether the Ca^{2+} releasing activity is localized to the perinuclear region or whether it is localized within the nucleus and released at NEBD, as has been shown for a number of other proteins (Millar et al., 1991; Ookata et al., 1992; Hepler et al., 1994). The sperm-derived factor/s responsible for Ca^{2+} releasing activity of karyoplasts has many properties similar to that responsible for stimulating Ca^{2+} transients at fertilization. First, the amplitude and dura-

tion of the Ca^{2+} transients are similar (see Jones et al., 1995*a*). Second, while the frequency of Ca^{2+} transients at fertilization is greater than that at mitosis, it is similar to that occurring in metaphase-arrested oocytes, 10–14 h after fertilization (Jones et al., 1995*a*), i.e., one transient every 30–60 min. Third, karyoplasts from embryos activated by the injection of a purified Ca^{2+} releasing sperm extract (Swann, 1990) also activate unfertilized oocytes after nuclear transfer (Kono et al., 1995). Therefore, our data are consistent with the idea that the Ca^{2+} releasing activity provided at fertilization becomes associated with the pronuclei, and is then reactivated when the Ca^{2+} release mechanisms become modified at mitosis (see Fig. 7).

An Increase in Intracellular Ca^{2+} Is Necessary for NEBD

Our finding that Bapta inhibits NEBD in fertilized and parthenogenetic embryos suggests that an increase in intracellular Ca^{2+} is necessary for NEBD. This is consistent with previous studies in sea urchin embryos (Steinhardt and Alderton, 1988; Twigg et al., 1988) and fibroblasts (Kao et al., 1990). In a previous study on one-cell mouse embryos it has been shown that Bapta alone does not completely inhibit NEBD and is more effective in combination with Ca^{2+}-free medium (Tombes et al., 1992). The improved effectiveness of Bapta in the present study may be due to the use of a higher concentration, closer to the time of NEBD.

To explain why some cells progress through mitosis in the absence of a detectable increase in Ca^{2+}, while Ca^{2+} buffers inhibit mitosis, it has been proposed that the relevant Ca^{2+} movements (or gradients) are too local to be detected (Kao et al., 1990; Whitaker and Patel, 1990; Snow and Nuccitelli, 1993). It is possible that local Ca^{2+} movements at NEBD may go undetected since they are small (Ciapa et al., 1994) and thus may occupy only a fraction of the oocyte volume from which fluorescence is being monitored. Such an explanation is consistent with our findings that NEBD-associated global Ca^{2+} transients are only detected in the presence of an exogenous stimulus such as fertilization or strontium that may serve to amplify small localized Ca^{2+} transients. The recent findings of local Ca^{2+} changes (Cheng et al., 1993; Yao and Parker, 1994) and that the nuclear envelope releases Ca^{2+} (Gerasimenko et al., 1995) supports the possibility that local Ca^{2+} transients in the nuclear region may induce NEBD and progression through mitosis.

A Role for Ca^{2+} Transients in Embryonic Development?

Finally, does the generation of global Ca^{2+} transients in fertilized embryos have any role in the development of the early embryo? There is some evidence that Ca^{2+} can modify early development since imposing Ca^{2+} transients during early embryogenesis improves the development of embryos to the blastocyst stage (Vitullo and Ozil, 1992; Stachecki et al., 1994*a*,*b*). Our finding of endogenous Ca^{2+} transients in fertilized mouse embryos suggests that these mitotic Ca^{2+} transients may be a paternally derived mechanism for increasing early embryonic development.

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