Calcium and Endoplasmic Reticulum Dynamics during Oocyte Maturation and Fertilization in the Marine Worm Cerebratulus lacteus

Stephen A. Stricker, Roberto Silva, and Toni Smythe
Department of Biology, University of New Mexico, Albuquerque, New Mexico 87131

To monitor calcium and endoplasmic reticulum (ER) dynamics during oocyte maturation and fertilization, oocytes of the marine worm Cerebratulus lacteus were injected with the calcium-sensitive indicator calcium green dextran and/or the ER-specific probe “DiI.” Based on time-lapse confocal imaging of such specimens, prophase-arrested immature oocytes failed to develop normally after insemination and typically produced non-wave-like calcium transients that were lower in amplitude and less persistent than the wave-like oscillations observed during fertilizations of mature oocytes. Accordingly, the ER of DiI-loaded immature oocytes lacked an obvious substructure, whereas ER clusters, or “microdomains,” began to form in maturing specimens at about the time that these oocytes became competent to undergo normal fertilization-induced calcium dynamics and cleavage. The ER microdomains of mature oocytes typically reached widths of 1–8 μm and disappeared approximately 1 h after fertilization, which in turn coincided with the termination of the calcium oscillations. Collectively, these findings indicate: (i) changes in ER structure are temporally correlated with the onset and cessation of the calcium oscillations required for subsequent cleavage, and (ii) such ER reorganizations may play an important role in early development by enabling mature oocytes to generate a normal calcium response. © 1998 Academic Press

Key Words: Ca²⁺ oscillations; calcium waves; meiotic maturation; DiI; confocal microscopy; nemertean; mouse oocyte; ICSI.

INTRODUCTION

At fertilization, the concentration of intracellular calcium ions within the egg must undergo a transient rise for development to proceed normally (Nuccitelli, 1991; Whitaker and Swann, 1993; Schultz and Kopf, 1995). Before a proper fertilization response can be generated, however, eggs must complete a maturation process that allows their calcium stores to become more reactive to sperm and other calcium-releasing agents (Chiba and Hoshi, 1989; Chiba et al., 1990; Tombes et al., 1992; Fujiwara et al., 1993; Mehlmann and Kline, 1994; Jones et al., 1995; Shiraishi et al., 1995; He et al., 1997; Machaty et al., 1997). Although the exact mechanisms of this sensitization have not been fully elucidated, it seems likely that the endoplasmic reticulum (ER) of the egg is integrally involved, given that the ER is the major storage site for bound calcium that is released at fertilization (Eisen and Reynolds, 1985; Terasaki and Sardet, 1991). Thus, attempts have been made to correlate the enhanced calcium response displayed by mature eggs with fertilization-induced changes that may occur in the ER (Gardiner and Grey, 1983; Charbonneau and Grey, 1984; Shiraishi et al., 1995; Mehlmann et al., 1996; Kume et al., 1997).

To assess possible ultrastructural alterations, the ER has been examined by electron microscopy in eggs fixed at various stages of maturation (Campanella et al., 1984; Ducibella et al., 1988; Larabell and Chandler, 1988). More recently, microinjection of the carbocyanine probe “DiI” was developed as a relatively specific and simple way of tracking ER morphology within living cells (Terasaki and Jaffe, 1993). Based on DiI injections and in vivo analyses, the ER has been shown to undergo structural changes during oocyte maturation in starfish (Jaffe and Terasaki, 1994), rodents (Mehlmann et al., 1995; Shiraishi et al., 1995), and frogs (Kume et al., 1997). Fertilization-induced reorganizations of the ER have also been documented in DiI-loaded oocytes and eggs obtained from ascidians (Speksnijder et al., 1993), sea urchins (Terasaki and Jaffe, 1991; Jaffe and Terasaki, 1993), and starfish (Jaffe and Terasaki, 1994). Collectively, such studies demonstrate that the ER is a dynamic organelle whose structure changes...
in response to maturation and/or fertilization. However, additional analyses are needed to determine the precise timing of the ER reorganizations and how these structural alterations might relate to changes that occur in the spatiotemporal patterns of calcium transients during maturation and fertilization.

The marine nemertean worm Ceratobasulus lacteus produces oocytes that possess a large nucleus ("germinal vesicle," or GV) during prophase I of meiosis. Such GV-containing oocytes spontaneously begin germinal vesicle breakdown (GVBD) by ~45–60 min after being removed from the ovary, complete GVBD within about 30 min, and subsequently mature to a metaphase I (MI) arrest point by ~2 h postremoval from the ovary. After fertilization, mature oocytes generate a series of wave-like calcium transients, or “oscillations,” for about 45–90 min before completing meiosis and undergoing cleavage (Stricker, 1996). 

MATERIALS AND METHODS

Gametes from six female and four male Cl. lacteus adults (Marine Biology Laboratory, Woods Hole, MA) were prepared in "MBL" artificial seawater according to methods described previously (Stricker, 1996, 1997). In this study, “mature” refers to oocytes that completed GVBD and reached an MI arrest. “Immature” corresponds to: (i) “maturing” oocytes that were actively progressing toward MI and (ii) “prophase-arrested” specimens that for undetermined reasons remained blocked in prophase and retained an intact GV for at least 2 h after maturing oocytes had attained MI arrest.

Prior to confocal microscopy, washed oocytes were attached to protamine-coated specimen dishes and routinely given 0.5–1% injections of calcium green (CG) and rhodamine B (Rh) dextrans, 10,000 MW [Molecular Probes, Inc.] as described by Stricker (1997). For studies of ER structure, a saturated solution of Dil [DiIC18(6); 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes Inc.] was prepared in soybean oil (Wesson) and injected into 1-2% oocyte volume, based on subsequent measurements of the injected oil droplet. Simultaneous monitoring of calcium and ER dynamics within the same oocyte was carried out after making separate injections of Dil and CG.

Time-lapse calcium imaging was conducted at 12–16°C on a Bio-Rad MRC-600 confocal system that was equipped with a thermoelectric cooling stage and a Nikon Diaphot microscope using a 20×, 0.7 NA objective or a 60×, 1.3 NA objective (Stricker, 1996). For such studies, the CG and Rh signals were collected every 5 or 15 s within an ~1- to 5-μm-thick plane situated near the oocyte center (Stricker, 1996). The dual-channel images were then ratioed and either graphed in a normalized form as R-F/R0, where R0 is the initial CG/Rh ratio, and R is subsequent CG/Rh ratios, or converted into pseudocolor montages, in which blues and reds corresponded to relatively low and high [Ca2+]i, respectively (Stricker, 1995, 1996). Alternatively, some oocytes were injected with just CG dextran and subjected to single-channel ratioing to produce montages and F-F0 normalized graphs, where F0 is the initial CG fluorescence intensity, and F is subsequent CG fluorescence intensities (Stricker et al., 1994). Insensations were routinely conducted: (i) 3–8 h postremoval from the ovary for mature oocytes; (ii) 5–8 h postremoval for prophase-arrested specimens; or (iii) 30–45 min postremoval for actively maturing oocytes. Based on previous calibrations, the fertilization-induced calcium transients of normally developing mature specimens typically corresponded to several hundred nanomolar increases over baselines (Stricker, 1996).

To examine ER structure, the Dil signal was sometimes viewed in a fixed optical plane either by itself or along side the CG signal in specimens dually injected with ER and calcium probes. More commonly, however, the ER was monitored in Dil-loaded specimens to determine if there were structural changes in the ER that could account for different calcium-mobilization patterns in immature vs mature oocytes. Collectively, such analyses reveal: (i) prophase-arrested immature oocytes of Cl. lacteus fail to develop normally following insemination and typically display a fertilization-induced calcium response that lacks the amplitude, kinetics, and spatiotemporal patterns observed in oocytes that were fertilized after having undergone maturation; (ii) the ER exhibits major structural reorganizations during maturation and fertilization; and (iii) such reorganizations are correlated with the timing of normal fertilization-induced oscillations, supporting the results of previous studies on mammalian oocytes that there is an important link between calcium and ER dynamics during early development.

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accumulated from at least three separate experiments involving oocytes from two or more females. Three exceptions to this rule were: (i) measurements of polyspermic calcium dynamics that were based on only two experiments, (ii) analyses of GVBD in three cultures of Hoechst-labeled oocytes that were obtained from a single female, and (iii) a single experiment assessing cleavage rates in non-dye-loaded specimens inseminated before MI arrest, at 30–45 postremoval from the ovary.

RESULTS
Calcium Dynamics and Development in Immature Oocytes

Unlike the oocytes of some animals which can be reversibly arrested in prophase I after removal from the ovary (Stricker et al., 1994; Mehlman et al., 1995), well-developed oocytes of C. lacteus spontaneously completed GVBD by 1.5 h postremoval from the ovary, and no drug or hormone treatment has yet been reported that reversibly blocks isolated C. lacteus oocytes in prophase. However, in two of the six females examined in this study, ~10–30% of the oocytes that reached full-sized diameters of ~120 μm lacked any overt signs of degradation or abnormal morphology, but nevertheless continued to contain a GV when examined >2 h after other fully grown oocytes had reached MI. To make extended recordings of calcium dynamics in oocytes possessing an intact GV, such prophase-arrested specimens (N = 10) were examined by time-lapse confocal microscopy for 45–60 min prior to sperm addition and were found to lack calcium fluxes (Fig. 1A), suggesting that these immature oocytes do not spontaneously undergo calcium oscillations before fertilization.

Following insemination of 25 prophase-arrested specimens, however, 16 displayed an essentially synchronous "cortical flash" of elevated calcium around the entire periphery of the oocyte (Fig. 2A), as is typically generated by external calcium influx at the onset of fertilization in mature oocytes (Stricker, 1996). Subsequently 5 of the 16 positively responding oocytes failed to undergo additional calcium transients (Fig. 1A), whereas the other 11 oocytes generated one to a few more calcium fluxes that started 11.7 ± 6.0 min (N = 11) after sperm addition (Fig. 1B).

As in mature oocytes (Stricker, 1996), the fertilization-induced calcium transients of prophase-arrested specimens: (i) reached full peak height within ~1 min, (ii) lasted 1–5 min/transient, and (iii) preceded the next transient by 2–15 min. However, compared to the calcium transients of mature oocytes, the fluxes generated by prophase-arrested oocytes were significantly lower in amplitude and more ephemeral (Fig. 1B; Table 1). In addition, 10/12 mature oocytes situated next to prophase-arrested specimens generated multiple calcium waves from a discrete onset site beginning 10.1 ± 5.2 min after sperm addition (Fig. 2B), whereas the calcium fluxes of prophase-arrested immature specimens typically propagated as cortical flashes that lacked a point-source origin or a well-defined wavefront (Fig. 2A).

To assess the developmental potential of prophase-
FIG. 2. The spatiotemporal properties of fertilization-induced calcium transients differ in immature vs mature oocytes. Pseudocolored montages of ratioed confocal images depicting fertilization-induced calcium transients in a fixed optical plane every 5 s (A, B) or 15 s (C). Time progresses from left to right (A–C) and top to bottom (A, C). Blues represents low free calcium concentrations; yellows and reds correspond to progressively higher calcium levels. (A) Three consecutive calcium transients in a prophase-arrested immature oocyte fertilized 6 h after removal from ovary. First frame depicts a nonratioed, prefertilization image, showing site of germinal vesicle (double arrows) just below plane of focus. Note: fertilization triggers multiple cortical flashes rather than point-source calcium waves. (B) Two neighboring mature oocytes, each showing a normal fertilization-induced calcium wave when inseminated 4 h after removal from ovary. (C) Two consecutive calcium waves generated 60 and 66 min postfertilization in a maturing oocyte inseminated 30 min after removal from ovary. Scale bar, 50 μm.
Table 1
Fertilization-Induced Ca\textsuperscript{2+} Oscillations at Various States of Maturation

<table>
<thead>
<tr>
<th>Maturation state of oocyte at fertilization</th>
<th>Amplitude of Ca\textsuperscript{2+} transients( \times 10^{-3} )</th>
<th>Overall length of oscillatory sequence (min) ( \times 10^{-3} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prophase-arrested( f )</td>
<td>0.19 ± 0.08 (N = 55)</td>
<td>23.5 ± 17.9 (N = 11)</td>
</tr>
<tr>
<td>Maturing( g )</td>
<td>0.36 ± 0.09 (N = 144)</td>
<td>137.2 ± 92.8 (N = 17)</td>
</tr>
<tr>
<td>Early response( a )</td>
<td>0.30 ± 0.10 (N = 62)</td>
<td>—</td>
</tr>
<tr>
<td>Late response( a )</td>
<td>0.41 ± 0.09 (N = 82)</td>
<td>—</td>
</tr>
<tr>
<td>Maturing, polyspermic( h )</td>
<td>0.40 ± 0.08 (N = 80)</td>
<td>296.4 ± 65.1 (N = 7)</td>
</tr>
<tr>
<td>Mature( i )</td>
<td>0.44 ± 0.15 (N = 72)</td>
<td>70.2 ± 15.3 (N = 10)</td>
</tr>
</tbody>
</table>

\( a \) Average heights of fertilization-induced calcium transients as determined from R-Rh/Rh, where R is the initial baseline CG/Rh ratio before calcium transient; R is subsequent CG/Rh ratios, and a value of 0.44 corresponds to a 44% increase over baseline ratio; N is the number of calcium transients measured.

\( b \) Time from addition of sperm to end of calcium oscillations; N is the number of oocytes measured.

\( c \) Full-sized, immature specimens that remained arrested in prophase I and failed to undergo GVBD by 4 h after removal from ovary.

\( d \) Actively maturing oocytes that were fertilized 30-45 min postremoval from ovary.

\( e \) The early calcium response occurring <90 min postremoval from ovary in the 17 maturing oocytes that were fertilized 30-45 min after removal from ovary.

\( f \) The early and late responses occurred over a total of 137.2 ± 92.8 min for these 17 maturing oocytes.

\( g \) The late calcium response occurring >90 min postremoval from ovary in the same 17 maturing oocytes as listed in the row above.

\( h \) Maturing oocytes fertilized 55 min after removal from ovary with high concentrations of sperm to yield polyspermic inseminations as judged by correlative Hoechst labeling and the aberrant cytokineses displayed; amplitudes calculated from initial transients produced within 60 min after sperm addition.

\( i \) Mature oocytes fertilized after reaching MI arrest 2-8 h after removal from ovary.

* Significantly lower in amplitude than in the other specimens (P < 0.05).

** Significantly shorter in overall duration than in the other specimens (P < 0.05).

Arrested oocytes, 18 of the 25 specimens examined by confocal microscopy were monitored for prolonged periods after insemination. Of these 18 specimens, only 3 had cleaved at 4–6 h after sperm addition, whereas the others either had an intact GV (N = 10) or had undergone GVBD without cleavage (N = 5). Accordingly, in three cultures of Hoechst-labeled oocytes that were obtained from one of the females producing high levels of prophase-arrested specimens, none of the oocytes cleaved, 79.4 ± 3.0% underwent GVBD, and 20.6 ± 3.4% were in prophase I arrest before sperm was added to the specimen dishes. However, at 4–6 h postinsemination, there was 82.3 ± 4.7% cleavage, 14.2 ± 6.5% GVBD without cleavage, and 3.5 ± 1.8% continued prophase arrest among the three cultures, indicating that fertilization could trigger GVBD. Collectively, these data obtained from confocal studies and Hoechst-labeled cultures suggest that although prophase-arrested oocytes did not typically cleave, such cells were also not fully moribund given that they could initiate and complete GVBD after being inseminated.

For analyses of calcium dynamics in unfertilized maturing oocytes that had not reached MI arrest, 9 oocytes were rapidly processed after removal from the ovary and examined by confocal microscopy within 5 min postinjection with the calcium indicator. In 5 of 9 specimens, no discrete calcium transients were observed at the onset of imaging, whereas in 4 oocytes, one or a few calcium fluxes occurred within 30 min after removal from the ovaries (Fig. 3). Whether such transients represented normally occurring fluxes prior to GVBD or simply reflected a transiently activated state caused by the injection could not be ascertained. In any case, all 9 oocytes matured to MI without any calcium fluxes occurring from 0.5 to 2.3 h after removal from the ovary, and this lack of oscillations was not simply due to oocyte morbidity, since 8 of 9 specimens immediately generated calcium oscillations after reaching MI and undergoing fertilization at 2.3 h postremoval from the ovary (Fig. 3).

To determine if maturing oocytes can produce calcium oscillations when inseminated prior to MI, 24 dye-loaded oocytes were treated with sperm at 30–45 min postremoval from the ovary. In 17 of 24 cases, the maturing specimens generated a cortical flash and calcium oscillations. Such postflash oscillations typically began 10–25 min after insemination, reached peak heights within 1 min, lasted for 1–5 min per transient, and occurred every 2–10 min. Unlike the ephemeral response to prophase-arrested oocytes, the oscillations of all 17 maturing oocytes persisted at least 40 min, and were even more prolonged (P < 0.05) than those observed in mature oocytes (Table 1), although it remains to be determined if the lengthened response of maturing oocytes was a normal feature of fertilization or simply the result of some polyspermic specimens being included in the dataset.

When examined up to 90 min postremoval from the ovary (i.e., the time point when GVBD is generally completed),
the initial calcium transients produced by these fertilized maturing oocytes were significantly lower in amplitude than the fertilization-induced transients generated by mature specimens (Table 1). However, maturing oocytes inseminated 30–45 min postremoval from the ovary eventually produced high-amplitude transients that typically began ~105–140 min after removal from the ovary (range, 100–205 min; average, 122.2 ± 29.8 min; N = 17) (Figs. 4A and 4B). Thus, the normalized peak heights of transients generated >90 min after removal from the ovary by these fertilized maturing specimens averaged 0.41 ± 0.09 (N = 17), which in turn was similar to the 0.45 ± 0.15 value produced by fertilizations of mature oocytes (N = 10) (Table 1; Stricker, 1996, 1997). The spatial patterns of the later transients also resembled the point-source calcium waves in fertilized, mature oocytes (Fig. 2C). Moreover, 10 of 17 (59%) of the dye-loaded oscillating specimens that were fertilized before MI at 30–45 min postremoval from the ovary underwent a normal, although delayed, first cleavage (Fig. 5A), and 90 of 132 (68.2%) of non-dye-loaded oocytes in one specimen dish cleaved normally by ~5 h after being inseminated at 40 min postremoval from the ovary. Collectively, such data indicate that although GV-containing maturing oocytes initially generated abnormal fertilization-induced calcium transients, post-GVBD development was often associated with normal calcium dynamics and delayed cleavage.

The production of a weak calcium response by maturing oocytes at the onset of fertilization could have been due to: (i) unsuccessful sperm fusion/incorporation, (ii) inadequate stores of bound calcium, and/or (iii) an incompletely developed capacity to mobilize bound calcium. However, in three cultures of Hoechst-labeled maturing oocytes that were fertilized 30 min after removal from the ovary and fixed 45–60 min postfertilization, 70.0 ± 16.9% of the ~80 maturing specimens examined in each culture possessed an incorporated sperm (Fig. 5B), which suggests that the initial

![FIG. 3](image-url) Maturing oocytes do not produce sustained oscillations prior to fertilization. In a maturing oocyte that displayed a few calcium transients (double arrows) before GVBD, a prolonged set of calcium oscillations occurred only after the oocytes had matured and sperm was added to the specimen dish at 2.3 h postremoval from ovary. Changes in intracellular calcium are indicated by R-Ro/Ro, where Ro is the initial CG/Rh ratio and R is subsequent CG/Rh ratios.

![FIG. 4](image-url) Maturing oocytes initially generate low-amplitude calcium waves upon monospermic fertilization. (A, B) Fertilization-induced calcium waves in maturing oocytes that were inseminated before MI arrest, at 47 min postremoval from ovary, showing initially low-amplitude transients prior to an increase in the peak heights of the transients during later stages of imaging. Changes in intracellular calcium are indicated by R-Ro/Ro, where Ro is the initial CG/Rh ratio and R is subsequent CG/Rh ratios.
abnormal calcium dynamics were not simply due to an inhibition of sperm fusion and incorporation.

Similarly, the hypothesis that maturing oocytes contain insufficient stores of bound calcium was not supported by inseminations of maturing oocytes using high sperm concentrations (5–10$^3$ sperm/ml) that caused numerous sperm incorporations per oocyte, based on correlated Hoechst staining (unpublished observations) and the very abnormal cleavages that were observed (Fig. 5C). In such polyspermic fertilizations, all seven maturing specimens began to generate high-frequency oscillations whose amplitudes essentially matched those of mature oocytes (Table 1; Figs. 6A and 6B), suggesting that maturing oocytes had sufficient stores of bound calcium for immediately producing a prolonged, high-amplitude calcium response, provided that multiple sperm triggered the calcium release.

Changes in the Endoplasmic Reticulum during Oocyte Maturation

To monitor ER dynamics during meiotic maturation, DiI injections were conducted as quickly as possible after removal of oocytes from the ovary. In such specimens, the DiI took approximately 30 min to spread completely throughout the ooplasm, and thus the first full images of ER morphology were obtained 40–50 min after removal of the oocytes from the ovary.

In low-magnification optical planes taken near the equator of immature oocytes, no obvious substructuring of the DiI signal was typically observed other than an overall brighter fluorescence at the periphery compared to that displayed by the central ooplasm (Fig. 7A). Such heterogeneity was presumably due to: (i) an unequal distribution of ER and/or (ii) a decreased capacity to detect the centrally located signal in these somewhat opaque oocytes. In any case, since the center of the oocyte contributed relatively little signal that would confound projected renderings, ER structure was routinely examined in confocal z series that were compressed into single-plane images, to display a

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more expansive view of the ER than can be seen in a fixed optical section. As in low-magnification single-section views, the DiI staining in compressed z series was relatively homogeneous at 40 min after removal from the ovary in the 19 maturing specimens examined, except for one to several fluorescent masses of unknown significance that were visible in about half of the specimens (Fig. 7B). Similarly, 8 of 8 prophase-arrested oocytes that failed to undergo GVBD also lacked any pronounced substructuring in their DiI signal when viewed at low magnification at 6 h after injection (Fig. 7B, inset).

However, as maturation proceeded, the ER of unfertilized maturing oocytes began to form subspherical clumps of DiI fluorescence, or “ER microdomains” (Speksnijder et al., 1993) that were visible in low-magnification images (Figs. 8A, 8B, 9A, 9B, and 9F–9H). Such microdomains typically measured 1–8 μm wide along their longest horizontal dimension and were most obvious in the outermost 10–15 μm of ooplasm, based on: (i) low-power optical sections or serial z sections showing a peripheral localization (Figs. 8B and 9H); and (ii) higher-resolution sections that also suggested a comparatively lower concentration of microdomains in centrally located areas of the oocyte (Fig. 9A).

In confocal z series collected every 10 to 15 min through a total of 23 DiI-loaded maturing oocytes, widespread ER microdomains first became clearly visible 136.1 ± 24.7 min postremoval from the ovary in 5 specimens (range, 105-180 min) (Fig. 9B), whereas in another 4 oocytes such microdomains appeared at an undetermined time between the end of the time-lapse run at 3 h postremoval and the
collection of another z series at 4–5 h postremoval. Alternatively, 6 specimens reached MI arrest but failed to show any noticeable ER microdomains by 5 h postremoval, thereby yielding a total of 17 of 23 (73.9%) mature oocytes with ER microdomains. Similarly, in 31 specimen dishes containing Dil-loaded specimens, 231 of 301 (76.8%) of the injected oocytes exhibited ER microdomains when examined 3–8 h after removal from the ovary.

Such ER microdomains did not simply arise from non-Dil-specific staining or a rapid restructuring of the ER invariably caused by Dil injections, given that: (i) the CG dextran signal did not show microdomain-like structures (Fig. 9E); (ii) prophase-arrested oocytes injected with Dil continually lacked microdomains, and Dil-injected maturing specimens failed to show microdomains for >1 h; (iii) in four experiments, ER microdomains were observed in 10 of 15 mature oocytes that were either fixed within 2 min after Dil injection or injected 15 min after fixation (Fig. 9C).
which in turn indicated that microdomains were present prior to injection and not simply caused by vesicular trafficking or a DiI-induced reorganization of the ER; and (iv) after sectioning noninjected oocytes, subspherical inclusions that resembled ER microdomains were visible in mature specimens fixed 3-5 h after removal from the ovary but not in GV-containing oocytes fixed <20 min post-removal (Figs. 9D and 11).

In 30 DiI-loaded oocytes with a clearly defined animal-vegetal axis, morphometric analyses revealed that there was no statistical difference in the size or number of ER microdomains in the animal vs vegetal hemispheres (Table...
TABLE 2
Morphometry of ER Microdomains in Mature Oocytes

<table>
<thead>
<tr>
<th></th>
<th>Animal hemisphere</th>
<th>Vegetal hemisphere</th>
</tr>
</thead>
<tbody>
<tr>
<td>Width of ER microdomain (µm)(^a)</td>
<td>4.8 ± 0.94</td>
<td>4.3 ± 0.97</td>
</tr>
<tr>
<td>Total number of ER microdomains/oocyte hemisphere</td>
<td>32.8 ± 20.1</td>
<td>31.4 ± 12.6</td>
</tr>
<tr>
<td>Total area occupied by ER microdomains (µm(^2))</td>
<td>512.1 ± 258.0</td>
<td>408.9 ± 279.8</td>
</tr>
</tbody>
</table>

Note. None of the values is significantly different in animal vs vegetal halves at P = 0.05.
\(^a\) N.o. of microdomains measured, 1926; no. of oocytes examined, 30.
\(^b\) Measured along maximum horizontal dimension.

Endoplasmic Reticulum Dynamics after Fertilization

Following insemination of mature specimens that had been doubly injected with DiI and CG, the ER microdomains did not display any gross changes in morphology or position within the oocyte for at least the first several calcium waves that propagated through the oocyte (Fig. 10A). However, subsequent monitoring revealed that 80 of 93 (86%) of the DiI-loaded mature specimens that originally had ER microdomains prior to fertilization lacked such inclusions when examined 1.5–2 h after insemination (Figs. 10B and 10C). A similar reduction in the number of specimens containing microdomain-like inclusions was observed in sections of plastic-embedded material.

To determine when the microdomains disappeared more precisely, an additional 45 DiI-loaded specimens with ER microdomains were inseminated, and serial confocal z sections were collected every 10 min for 2 h following fertilization (Fig. 10D). Of these 45 oocytes, 37 (82%) underwent a fertilization-induced loss of microdomains. The microdomains began to disappear 40.1 ± 11.9 min postfertilization (N = 37) and were essentially gone at 64.1 ± 19.0 min postinsemination (N = 37), without showing any marked shifting within the oocyte during disaggregation. The loss of ER microdomains over the time frame of ~1 h seemed to be due to fertilization rather than the imaging procedure or a nonspecific aging process, since: (i) 8 of 8 unfertilized specimens examined by time-lapse microscopy for up to 6 h retained their microdomains (Fig. 8A); and (ii) essentially identical numbers of ER-containing specimens were observed in newly mature, unfertilized oocytes as in unfertilized oocytes that had been allowed to age for several hours (i.e., data presented in the previous section).

The disappearance of ER microdomains was also associated with normal development because all 21 of the inseminated oocytes that continued to contain ER microdomains during postinsemination imaging failed to undergo maturation or cleavage during 5 h of time-lapse runs. Conversely, more than half of the oocytes that lost their ER microdomains after fertilization: (i) completed maturation (Fig. 10C), (ii) cleaved without reestablishing their microdo-
FIG. 10. Fertilization causes loss of ER microdomains. (A) Single-plane, time-lapse series at 15-s intervals of a fertilization-induced calcium wave propagating through cortex of mature oocyte doubly injected with calcium green dextran (upper row) and Dil (lower row). (B) Compressed confocal z series through two mature oocytes before (left side) and 2 h after (right side) fertilization, showing a fertilization-induced loss of ER microdomains in each of the specimens. (C) Compressed confocal z series through mature oocyte before (left side) and 1.75 h after (right side) fertilization, showing a postfertilization loss of ER microdomains and polar body production (arrows) from the former Dil-free zone corresponding to the meiotic apparatus. (D) Time-lapse sequence of compressed confocal z series collected at 10-min intervals directly after sperm addition (first frame, upper row), showing: (i) loss of ER microdomains—50 min postfertilization (sixth frame, upper row), (ii) maturation, and (iii) cleavage. Scale bars: 10 μm (A); 50 μm (B-D).
mains at least during sporadic checks made up to the 4-cell stage (Fig. 10D), and (iii) eventually formed blastulae (Fig. 12).

After a dilute solution of sperm was added to one specimen dish, a few oocytes lost their microdomains only after several hours had elapsed. Since sperm–oocyte interactions were not monitored, it remains unknown if the delay in ER microdomain loss was due to a lengthy delay before sperm reached these oocytes, or because there was a prolonged lag between sperm–oocyte interaction and microdomain loss (Fig. 12). In either case, cleavages were also delayed in these oocytes compared to the timing of their neighbors that underwent microdomain loss on schedule, and the two specimens in the dish that did not undergo fertilization-induced microdomain loss failed to cleave (Fig. 12), suggesting that the loss of ER microdomains may be required before normal cleavage can proceed.

**DISCUSSION**

Inseminations of Immature Oocytes Trigger Abnormal Calcium Dynamics and/or Delayed Cleavage

Following insemination, the calcium response of prophase-arrested *C. lacteus* oocytes was lower in amplitude, more short-lived, and less wavelike than the calcium oscillations generated by fertilizations of mature oocytes. Prophase-arrested specimens also typically remained uncleaved after sperm addition. The absence of proper oscillations and cleavage in these specimens may have simply been due to a nonspecific moribund state. However, prophase-arrested oocytes showed no overt signs of degradation, and many initiated and completed GVBD after insemination, indicating that the oocytes could at least mature after sperm addition. Accordingly, the initial fertilization-induced calcium waves produced by GV-containing, maturing oocytes that characteristically went on to cleave were also lower in amplitude than those of mature specimens. Until a method is devised that blocks *C. lacteus* oocytes in prophase and then releases them from this arrest to undergo full development, the possibility cannot be precluded that the prophase-arrested oocytes examined in this study underwent abnormal Ca\(^{2+}\) dynamics owing to a reduced viability. However, the alternative view that the incomplete calcium response of prophase-arrested oocytes is a biologically relevant characteristic coincides with previous studies that have used direct measurements of calcium levels (Chiba et al., 1990; Fujiwara et al., 1993; Mehlmann and Kline, 1994; Stricker et al., 1994) or observations of cortical reactions and polyspermy (Barrios and Bedford, 1979; Duciella and Buetow, 1994; Wang et al., 1997) to show that immature oocytes generate abnormal calcium responses, even in species which can be reversibly arrested in prophase before undergoing normal development.

In any case, normally maturing *C. lacteus* oocytes that were inseminated at 30–45 min after removal from the ovary eventually produced fertilization-induced calcium waves that reached comparable amplitudes and propagated with spatiotemporal properties similar to those generated by mature oocytes. One possible explanation for such findings is that maturing oocytes possessed inadequate calcium stores that gained sufficient levels of mobilizable calcium only after completing maturation (Tombes et al., 1992). However, maturing oocytes of *C. lacteus* could indeed produce high-amplitude transients directly following insemination provided that the oocytes were subjected to polyspermic inseminations. Accordingly, after sensitiza-
tion with the sulphydryl reagent thimerosal, immature mouse oocytes produced an initial fertilization-induced calcium response with nearly the same amplitude as that generated by mature oocytes (Mehlmann and Kline, 1994). Collectively, such findings suggest that immature oocytes possess ample stores of bound calcium, but such stores are not sufficiently sensitized for monospermic fertilizations until maturation is completed (Chiba et al., 1990; Mehlmann and Kline, 1994).

In addition to generating a normal calcium response at later postinsemination time points, maturing oocytes of *C. lacteus* eventually cleaved, although such cleavages were delayed by about 2 h compared to the cleavages of zygotes that had been fertilized after reaching M1 arrest. Coupled with the findings that fertilization-induced calcium oscillations in *C. lacteus* depend on the release of internal calcium stores (Stricker, 1996), the data presented here suggest that once oocytes are triggered to progress beyond prophase I, their calcium-releasing machinery eventually becomes competent to generate a normal set of fertilization-induced calcium oscillations. The acquisition of this competence may involve a sensitization of calcium-release mechanisms that in turn results from changes in the functional properties and/or microdistribution of the calcium channel receptors and pumps that regulate calcium flow across the ER membrane (Keizer et al., 1995; Mehlmann et al., 1996; Berridge, 1997; Wagenknecht and Radermacher, 1997). Alternatively, or in addition, a remodeling of the ER throughout the oocyte may place the calcium stores (Stricker, 1996), the data presented here suggest, owing to the opaque nature of these oocytes and/or the particular mode of confocal imaging that was employed. Accordingly, whether the ER aggregates of *C. lacteus* oocytes shift toward a more cortical localization during maturation as has been demonstrated for mammalian oocytes (Mehlmann et al., 1995) is difficult to ascertain based on confocal sections displaying a highly diminished signal in the central ooplasm.

The clumps of ER in mature *C. lacteus* oocytes were termed “microdomains” in accordance with the terminology of Speksnijder et al. (1993), who described ER aggregates distributed from a few micrometers beneath the oolemma to the center of Dil-loaded ascidian oocytes. Subsequently, similar appearing “accumulations” (Mehlmann et al., 1995; Kume et al., 1997) or “clusters” (Shiraishi et al., 1995) of cortical ER have been reported to increase in number during maturation. In addition, the subcortical ooplasm has been shown to contain “lamellar sheets” (Terasaki and Jaffe, 1993) or “spherical shells” (Jaffe and Terasaki, 1994) in unfertilized sea urchin eggs and starfish oocytes, respectively. Thus, although it is unknown if such ER structures are homologous in all species examined, the ER generally forms some sort of aggregates during maturation.

During fertilizations of mature *C. lacteus* oocytes, sperm enter the animal or vegetal hemispheres of oocytes with approximately equal frequency, and the first calcium waves arise from the sperm entry site (Stricker, 1996). Subsequently, however, the onset point of the calcium oscillations tends to shift toward the vegetal hemisphere (Stricker, 1996). In morphometric analyses of microdomain size and density, no marked polarity was observed in mature specimens before or after fertilization. Thus, unlike in mature ascidian oocytes where a vegetal “pacemaker” of accumulated ER initiates repetitive calcium waves (Speksnijder et al., 1993; Speksnijder, 1995), the distribution of ER microdomains cannot clearly explain why the later calcium waves preferen-

**FIG. 12.** The timing of ER microdomain loss is related to cleavage onset. Time-lapse sequence of compressed confocal z sections through 11 mature oocytes undergoing fertilization: (A) just before fertilization; (B) 30 min postfertilization; (C) 100 min postfertilization; (D) 160 min postfertilization; (E) 280 min postfertilization; (F) 17.5 h postfertilization. Note: oocytes that lose their microdomains at the normal 0.75–1.5 h time following fertilization (1) undergo cleavage well in advance of specimens that show a marked delay in microdomain loss (2); specimens that do not lose their microdomains (3) do not cleave. The unmarked oocyte did not have noticeable microdomains before fertilization. Scale bar, 50 μm.
Initially arise in the vegetal half of *C. lacteus* oocytes (Stricker, 1996, 1997), unless some undetected structural and/or functional heterogeneity within the various microdomains helps generate calcium waves more commonly from the vegetal half of mature oocytes. Regardless of their relative position within the oocyte, ER microdomains became clearly visible in DiI-loaded specimens ~2.25 h after removal from the ovary based on repetitive z series taken every 10–15 min. Similarly, time-lapse images of CG-injected maturing specimens examined every 15 s revealed that high-amplitude calcium oscillations started about 2 h postremoval from the ovary. Thus, the onsets of microdomain appearance and high-amplitude oscillation production seem to be correlated, especially given that the serial z-section technique necessarily overestimates the time of initial microdomain appearance, owing to: (i) the relative infrequency of data collection and (ii) the conservative nature of recording onsets as the times when microdomains were clearly evident and not just beginning to form. Such temporal correlations coincide with the view that ER microdomains help to generate high-amplitude calcium oscillations, perhaps by concentrating calcium channel receptors into discrete clusters and thereby facilitating elementary calcium-release events, such as calcium “puffs” or “sparks” (Bootman and Berridge, 1995; Parker et al., 1996; Berridge, 1997). Similarly, the presence of peripheral microdomains may help to explain why calcium waves seem to travel faster around the cortex, as indicated by a concave-shaped wavefront that in turn suggests a more rapid progression along the longer peripheral pathway than occurs via the relatively short central route through the mature oocyte.

**Fertilization of Mature Oocytes Leads to the Disappearance of Their ER Microdomains**

After fertilization, the ER microdomains of mature *C. lacteus* oocytes disappeared and did not reform at least during the subsequent early cleavages that were examined. The loss of the ER microdomains was not simply a nonspecific consequence of oocyte aging, since unfertilized specimens generally retained their microdomains. Moreover, microdomain loss seemed to be linked to normal development because specimens that did not lose their microdomains failed to cleave. Similarly, in cases where the loss of microdomains was delayed, cleavage was also delayed. A similar disappearance of ER microdomains has not been described for other species that produce calcium oscillations upon fertilization. However, the postfertilization structure of the ER has not been documented for DiI-loaded mammalian oocytes, and ascidian oocytes have apparently been monitored for only 25–30 min after fertilization without any microdomain loss being noted (Speksnijder et al., 1993). Alternatively, in sea urchins and starfish which undergo only a single calcium transient at fertilization, the peripheral lamellae or shells of ER disappear directly following fertilization and typically re-form within ~5–15 min after sperm addition (Jaffe and Terasaki, 1993, 1994). In *C. lacteus*, the timing of ER microdomain disappearance correlates well with the termination of the fertilization-induced calcium oscillations because both microdomains and oscillations are no longer evident about ~1 h after fertilization. Given such findings, it will be interesting to determine if: (i) more prolonged analyses of DiI-loaded ascidian oocytes also reveal a reduction in ER microdomains soon after the calcium waves of fertilization.
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cease, and (ii) the termination of oscillations in mouse oocytes at several hours after fertilization (Jones et al., 1995b) is similarly associated with a disappearance of ER aggregates. Although why the ER microdomains of C. lacteus oocytes dissipate when they do remains unclear, previous studies of somatic cells have shown that ER disintegration occurs only after a calcium transient is elicited for a prolonged time (Subramanian and Meyer, 1997). Accordingly, the production of multiple calcium waves for more than an hour may have a similar effect in C. lacteus oocytes.

As summarized in Fig. 13, the findings presented in this study suggest: (i) oocytes become competent to generate normal fertilization-induced calcium oscillations once their ER has matured to form microdomains; and (ii) after facilitating the production of multiple calcium waves, the ER microdomains disappear at least in part because of these repeated calcium waves. To test this hypothesis, experimental manipulations of microdomain appearance and dissolution need to be made to evaluate if the restructurings of the ER observed in this study can be uncoupled from normal calcium dynamics and development.

Regardless of the outcome of such experiments, this investigation documents that reorganizations of the ER are coordinated with the onset and termination of a normal calcium response in C. lacteus. The temporal correlation between ER microdomains and calcium oscillations in turn suggests that the structure of the ER may play an important role in regulating the type of calcium mobilization pattern that is generated during early development.

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