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The Cortical Endoplasmic Reticulum (ER) of the Mouse Egg: Localization of ER Clusters in Relation to the Generation of Repetitive Calcium Waves

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The endoplasmic reticulum (ER) of the mature mouse egg consists of a fine tubular network and pronounced accumulations in the cortex. The ER was visualized both in intact eggs and with *in vitro* preparations of the cortex using the fluorescent lipophilic dye, DiI. Immunofluorescent labeling of the ER in isolated cortical preparations demonstrated that the ER clusters contain inositol 1,4,5-trisphosphate (IP_3) receptors, indicating an important involvement in sperm-induced Ca^{2+} transients, which are triggered by IP_3. We imaged the ER during fertilization and the subsequent Ca^{2+} transients and found that the clusters remained intact throughout this period. Recovery of fluorescence after photobleaching established that the ER clusters are continuous with the reticular ER network and that these structures remain stable and continuous throughout the time of fertilization-induced Ca^{2+} transients; continuity also remained during IP_3 injection. These results indicate that, in contrast to echinoderm eggs, the ER of mouse eggs does not become disrupted when it releases Ca^{2+}at fertilization. The localization and apparent stability of the cortical ER clusters may be important in generating Ca^{2+} oscillations, which are characteristic of fertilized mammalian eggs. Imaging of intracellular Ca^{2+} revealed that Ca^{2+} transients originate in the hemisphere of the egg that contains abundant ER clusters, thus the mouse contains a stable cortical pacemaker responsible for generating Ca^{2+} waves.

Key Words: **endoplasmic reticulum; mouse egg; calcium waves; inositol trisphosphate; calcium stores; fertilization.**

INTRODUCTION

Fertilization of the mammalian egg triggers repetitive Ca^{2+} transients, which are responsible for releasing the egg from metaphase II arrest and causing the exocytosis of cortical granules (Kline and Kline, 1992a). The mechanism by which the fertilizing sperm triggers Ca^{2+} release from intracellular stores is not entirely known; however, it is clear that release of Ca^{2+} from intracellular stores is mediated by inositol 1,4,5- trisphosphate (IP_3) through the opening of IP_3-activated Ca^{2+} channels (IP_3 receptors) on the endoplasmic reticulum (Miyazaki *et al.*, 1992; Xu *et al.*, 1994; Mehlmann *et al.*, 1996). Ca^{2+} release in response to fertilization is substantially greater in the mature mouse or hamster egg than in immature oocytes, indicating that the intracellular Ca^{2+} release mechanisms develop during oocyte maturation (Fujiwara *et al.*, 1993; Mehlmann and Kline, 1994). Enhanced Ca^{2+} release in the mature egg after maturation may depend on a structural reorganization of the endoplasmic reticulum during maturation, as well as an increase in IP_3 receptor number. Evidence in support of these ideas comes from studies of the endoplasmic reticulum using the fluorescent membrane marker DiI and measurement of IP_3 receptor protein. Following oocyte maturation, endoplasmic reticulum (ER) clusters appear in the cortex of mature mouse eggs (Mehlmann *et al.*, 1995). These cortical ER accumulations are located in the region of the egg containing cortical granules and are absent from the cortical granule-free region adjacent to the meiotic spindle (Mehlmann *et al.*, 1995). An increase in immunoreactive mass of the type 1 IP_3 receptor protein suggests that an increase in IP_3 receptor number could also contribute to enhanced Ca^{2+} release in the mature egg (Mehlmann *et al.*, 1996; Parrington *et al.*, 1998; Fissore *et al.*, 1999).

In the present study, we further examine the organization and dynamics of the cortical ER to better understand Ca^{2+}

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oscillations and Ca^{2+} wave generation in the mature mouse egg at fertilization. Dramatic changes in the ER at fertilization have been described for echinoderm eggs (Terasaki and Jaffe, 1991; Jaffe and Terasaki, 1994, Terasaki *et al.*, 1996). The ER of the unfertilized sea urchin egg is a large continuous network. At fertilization, during the single Ca^{2+} rise, the ER becomes transiently discontinuous and ER cisternae become more finely divided. We report here studies of the ER at fertilization of the mouse egg and examine the continuity of the ER during repetitive Ca^{2+} transients. We also examine the role of cortical ER clusters in generating Ca^{2+} waves.

MATERIALS AND METHODS

Preparation of Eggs and Sperm

Eggs were obtained from NSA (CF-1) mice (Harlan Sprague–Dawley) as previously described (Kline and Kline, 1992a; Mehlmann and Kline, 1994). Briefly, eggs were obtained from mice following superovulation and the cumulus cells were removed with 0.3 mg/ml hyaluronidase (Type IV-S). Eggs were cultured in 200-μl drops of minimum essential medium with Earle's salts (Mehlmann and Kline, 1994) or M2 (Kline and Kline, 1992a) under light mineral oil (Fisher Scientific, Pittsburgh, PA) at 37°C. Sperm were obtained from NSA (CF-1) mice and were capacitated in IVF (Whittingham's) medium containing 3% BSA (Kline and Kline, 1992a; Mehlmann and Kline, 1994). Following microinjection of zona-intact eggs, the *zonae pellucidae* were removed with a brief treatment (<1 min) in acid Tyrode's solution (Mehlmann *et al.*, 1996). All observations and fertilization of eggs were made on a microscope warm stage kept at 37°C. For experiments using IVF (bicarbonate-buffered), a laminar flow of 5% CO_2, 95% air was provided over the medium drop, which was kept under mineral oil.

Injection of DiI, Confocal Microscopy, and Fluorescence Recovery after Photobleaching

A saturated solution of DiI [$DiIC_{16}(3)$ (1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) or $DiIC_{18}(3)$ (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate)] (Molecular Probes, Eugene, OR) was prepared in soybean oil (Wesson oil); the solution was stored at room temperature and used over a period of several days. Eggs, held in place with a holding pipette, were injected with DiI using a beveled pipette backfilled with a solution of DiI dissolved in oil (Terasaki and Jaffe, 1993) and connected to a micrometer syringe system filled with Fluorinert FC-70 (Sigma). Approximately 8 pl (about 4% of the egg volume) of DiI-saturated oil was injected. The utility of DiI in labeling the ER has been demonstrated in echinoderm eggs (Terasaki and Jaffe, 1991; Jaffe and Terasaki, 1993, 1994), ascidian eggs (Speksnijder *et al.*, 1993), and mammalian eggs (Mehlmann *et al.*, 1995; Shiraishi *et al.*, 1995). More recently the validity of DiI labeling of the ER was confirmed in experiments comparing its distribution with a probe that targeted the ER by an entirely different mechanism. Similar staining patterns were observed with DiI and with green fluorescent protein (GFP) targeted to the ER of starfish eggs by injecting mRNA coding for a chimeric protein containing a signal sequence and the KDEL ER retention sequence (Terasaki *et al.*, 1996).

DiI-labeled eggs were observed using a laser scanning confocal microscope (Bio-Rad MRC 600 coupled with a Nikon Diaphot). Observations were made with a Nikon Planapo 60× 1.4 NA lens or a Zeiss Neofluar 40× 0.75 NA Ph 2 lens. Confocal images were collected and stored on computer disk or on an optical memory disk recorder (Model TQ-3038F; Panasonic, Secaucus, NJ). During typical observations, the laser was set at full power with a 1% neutral density filter in place and a zoom setting of 2 to 6. For fluorescence recovery after photobleaching (FRAP) experiments, the egg was imaged continuously at 1-s intervals and then a small area of the egg cortex was bleached by increasing the zoom by a factor of 6–12 and scanning for two to four scans using unattenuated laser intensity (neutral density filter removed from the light path). After bleaching, the system was returned to the original zoom and light attenuation. As shown previously (Terasaki *et al.*, 1996), this method is effective in bleaching DiI-labeled ER and monitoring DiI diffusion within the ER.

Calcium Measurements

For confocal experiments, eggs were injected with 30 mM calcium-green dextran using a quantitative direct pressure injection system, as previously described (Hiramoto, 1984; Mehlmann and Kline, 1994). Intracellular concentrations were calculated from the concentration and volume of solution injected, assuming uniform distribution and an egg volume of 205 pl. Each egg was then injected with DiI and imaged on the Bio-Rad confocal system using a dual filter for fluorescein and rhodamine. To prevent movement of eggs during sperm addition and Ca^{2+} measurements, *zona pellucida*-free eggs were lightly attached to a glass-bottom dish treated with Cell-Tak (Becton–Dickinson, Franklin Lakes, NJ).

In experiments to monitor the Ca^{2+} waves with conventional epifluorescence, Fura dextran was injected into the eggs at a final concentration of 17 mM. A ratiometric measurement program was used to record the spatial change in $[Ca^{2+}]_i$ (intracellular free calcium; InCytIm2; Intracellular Imaging, Inc., Cincinnati, OH). The approximate $[Ca^{2+}]_i$ was determined using separate calibration buffers (Molecular Probes) and a ratiometric method. Because of the reliance on external calibration buffers, the Ca^{2+} calibration bar in Fig. 4 is considered only an approximation. In some cases eggs were injected with Fura dextran after initially loading with the membrane-permeant DNA-specific fluorochrome Hoechst 33342 (10 mg/ml). The location of the egg spindle and fused sperm could be visualized even though the excitation and emission wavelengths of Hoechst and Fura overlap; the intensity of the Hoechst spots was greater than the overall fluorescence from Fura. Ratio calibration of Fura is not possible in the presence of Hoechst; however, Ca^{2+} oscillations and waves could be distinguished in the ratio images.

Preparation of Egg Cortices

Mature eggs were injected with DiI as described above. Zona-free eggs were placed on glass-bottom dishes in a buffer similar in ionic composition to the intracellular cytoplasm. The intracellular buffer consisted of 135 mM potassium glutamate, 10 mM Hepes, pH 7.2, 5.0 mM EGTA, 1.0 mM $CaCl_2$, 1.5 mM magnesium acetate, and 10 mM glucose. The $[Ca^{2+}]$ of this buffer is approximately 0.43 nM (Free Calcium Program; Fabiato, 1991). To ensure that the eggs adhered to the glass surface, the dishes were treated with Cell-Tak or washed overnight in Cytoclean (Isolabs, Akron, OH), a tissue culture glassware cleaner, according to the manufacturer's instructions. The eggs were gently compressed with a blunt fire-polished

pipette whose end was just slightly larger than the egg diameter. Gentle compression broke the egg open, leaving a part of the membrane and adjacent cortex attached to the glass. Overlying cytoplasm was gently blown away with a small pipette and the medium was replaced several times with fresh intracellular buffer. All preparations were viewed with a 100× oil immersion lens on an inverted microscope.

Immunofluorescence labeling of egg cortices was performed using either fixed or unfixed cortical preparations, with or without prior injection of DiI. Preparations were fixed in 3.7% formaldehyde and 0.25% glutaraldehyde in intracellular buffer for 5 min and then washed with blocking buffer (intracellular buffer containing 3% BSA). The formaldehyde was prepared from a several-month-old stock of 37% formaldehyde and not directly from fresh paraformaldehyde. The method probably results in a relatively light fixation and may better preserve the membrane structure than a stronger, freshly prepared fixative. Cortices were then incubated with one of the primary antibodies to the type 1 IP_3 receptor for 45 min, rinsed three times for 5 min, and then rinsed in blocking buffer containing 4% goat serum. Finally the preparation was incubated in one of several fluorescent secondary antibodies (see below) for 45 min, washed several times, and viewed, usually in blocking buffer containing 1 mg/ml phenylenediamine to prevent photobleaching during viewing. Unfixed preparations were treated with antibodies in exactly the same manner. Control experiments included treatment with secondary antibodies without prior addition of the primary antibodies.

An affinity-purified, polyclonal antibody, provided by Dr. Barbara Ehrlich (Department of Pharmacology, Yale University), was used to label IP_3 receptors. This antibody, generated in rabbit, was raised against a synthetic peptide comprising the terminal 19 amino acids of the rat type 1 IP_3 receptor (referred to here as the C-19 antibody). The monoclonal antibody 18A10, generated against the mouse type 1 IP_3 receptor, was provided by Dr. K. Mikoshiba (University of Tokyo) (Furuichi *et al.,* 1989).

Secondary antibodies for use with the 18A10 primary antibody were either AMCA (7-amino-4-methylcoumarin-3-acetic acid, succinimidyl ester)-conjugated goat anti-rat or rhodamine-conjugated goat anti-rat. An AMCA-conjugated goat anti-rabbit antibody was used with the C-19 primary antibody. The AMCA conjugates were used for double-label experiments in which the cortices were prepared from eggs previously injected with DiI. Cortices were prepared 1–3 h after DiI injection. The endoplasmic reticulum visualized with DiI was imaged with a rhodamine filter set and the AMCA-conjugated secondary antibodies with a Hoechst filter set. There was no signal when DiI was imaged with the AMCA filter set nor when the AMCA conjugates were imaged with the rhodamine filter set. Significant DiI excitation and emission on the fluorescein channel prevented any double-label experiments with FITC-conjugated secondary antibodies.

RESULTS

The ER and ER Clusters Are Part of a Continuous Network

When DiI was injected into mouse eggs, it diffused throughout the ER network and labeled the cortical ER clusters. The diffusion time course was examined in four experiments; in all cases, substantial labeling of the entire network and cortical clusters occurred within 10 min or less, as illustrated in Fig. 1A. The rapidity of spreading indicates that the ER clusters are part of the continuous ER network, since DiI transfer by membrane trafficking is expected to be much slower (see Discussion). The cortical localization of ER clusters is shown in Fig. 1B.

Continuity of ER clusters with the rest of the ER network was also demonstrated by FRAP. Eggs were injected with DiI and then were locally bleached with unattenuated laser light. Rapid fluorescence recovery of the ER network and cortical clusters was observed in 17 of 18 FRAP experiments performed on separate unfertilized eggs. Substantial relabeling of the ER clusters was observed in confocal images within several minutes after bleaching (Fig. 1C). In one series of 6 experiments, fluorescence intensity of a randomly chosen ER cluster near the center of the bleach area was examined 1 min after the bleach period. Fluorescence intensity recovered by an average of 46% of the original value at 1 min after the bleach (0.46 ± 0.25; $n = 6$, range = 0.13 to 0.80).

The Cortical Endoplasmic Reticulum in Cortical Preparations

Bright ER accumulations were observed in cortical preparations made from DiI-injected eggs. When the preparations were made, the overlying cytoplasm was blown away by pipetting medium across the surface, leaving a 2- to 7-mm-thick cortical segment containing ER clusters overlying the plasma membrane (Figs. 2A and 2B). As shown in Fig. 2B, bright clusters were directly connected to tubular strands of the ER. Often, after the cytoplasm was blown away, some ER tubules and clusters remained attached to each other and rested on the culture dish around the membrane preparation (Figs. 2B and 2C).

ER cortical preparations were routinely made in five separate experiments (5–10 cortices for each experiment). All preparations were similar to the one shown in Fig. 2B, with ER accumulations visible, except in a few cases in which bright clusters were few in number. These may have been cortical preparations made from the cortex in the region adjoining the meiotic spindle, which does not contain clusters (Mehlmann *et al.,* 1995). In some cases, the fine tubules of the ER network vesiculated, although the pattern of large ER clusters over a tubular network (now vesiculated) was still apparent. In other cases, the fine reticulum remained intact. There was some tendency for vesiculation to occur after time in culture. Prolonged culture (several hours) was required for immunofluorescent localization and an example of the vesiculation observed in some specimens under these conditions is shown in Fig. 2E. Nevertheless, the pattern of a reticular network and ER accumulations was almost always observed.

IP_3 Receptors in Cortical Preparations

We focused in this paper on the type 1 isoform of the IP_3 receptor. Western analysis has shown that the type 1 IP_3

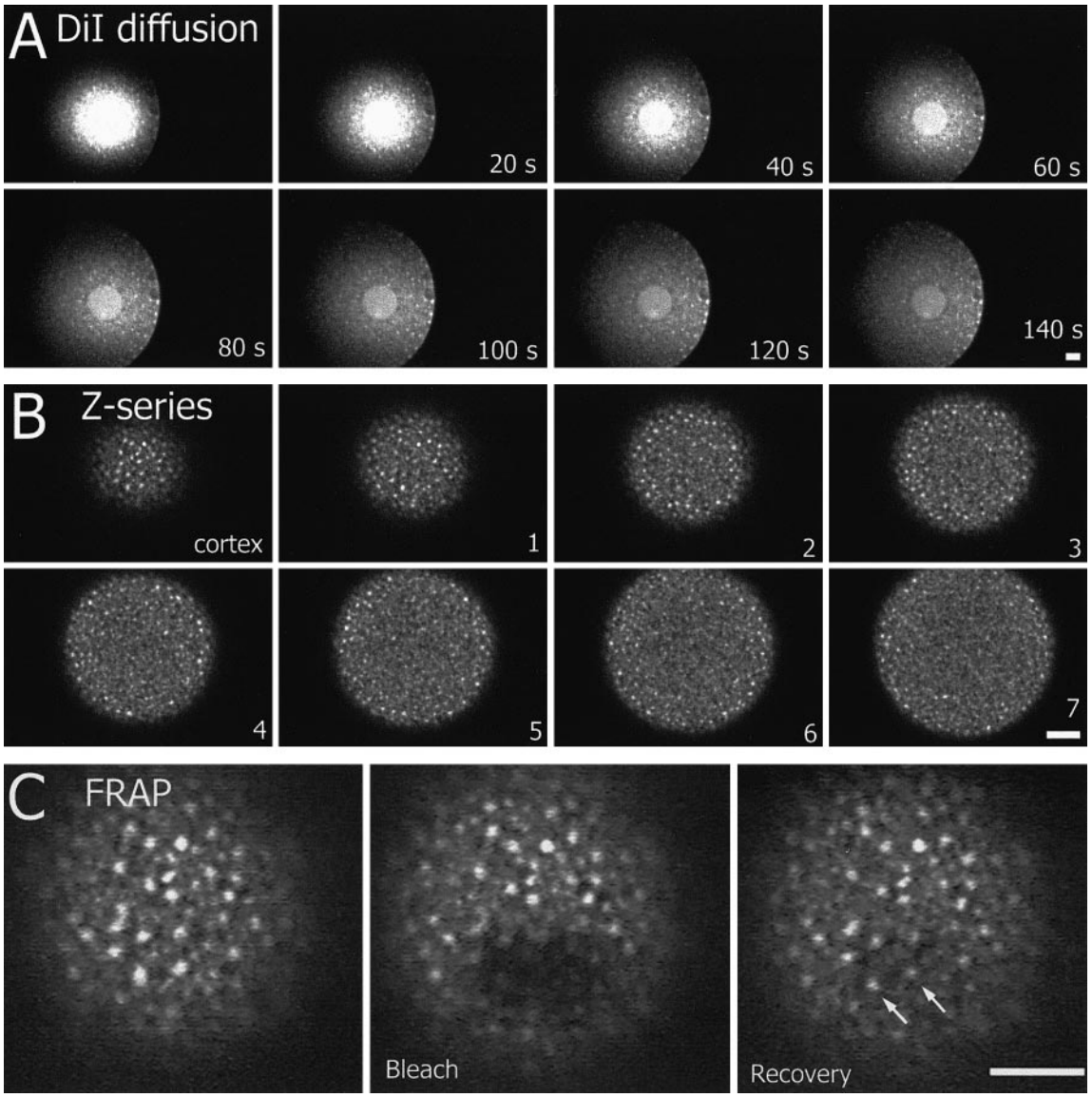

FIG. 1. Cortical ER clusters are labeled by DiI diffusion through the continuous membranes of the ER system. (A) A drop of DiI dissolved in oil was injected into the egg 60 s before the first image in this sequence. Images were collected at the same confocal settings at 20-s intervals. DiI left the oil drop and labeled all of the ER including ER clusters in the cortex within several minutes. (B) A confocal Z series beginning with an image at the cortex and advancing in 7 mm below the surface in 1-mm steps. Large ER accumulations are confined to the cortex and are absent in deeper cytoplasm (apparent in optical sections 3–7). (C) A FRAP experiment demonstrates ER continuity and DiI diffusion after photobleaching. The left image shows the cortex of the same egg as shown in B at higher magnification (confocal zoom 3). The center image is the first full scan after bleaching the lower portion of the egg with unattenuated laser light for four slow scans at zoom 18. The right image shows fluorescence recovery in cortical ER clusters, two of which are indicated by arrows. Bar, 10 mm.

receptor isoform is the predominate IP_3 receptor protein expressed in the mouse egg; the type 2 isoform has not been detected (Parrington *et al.*, 1998; Fissore *et al.*, 1999), while the type 3 isoform was reported to be present in very low abundance, as evidenced by one experiment in which a very faint band was detected by pooling large numbers of eggs (Fissore *et al.*, 1999). Although the type 2 isoform was not detected by Western analysis, Fissore *et al.* (1999) reported that the type 2 receptor could be found and localized by immunocytochemistry. However, the role of the type 2 receptor is unclear because activation and Ca^{2+} release studies have demonstrated that calcium waves are mediated by the type 1 IP_3 receptor. Both egg activation and Ca^{2+} release are completely

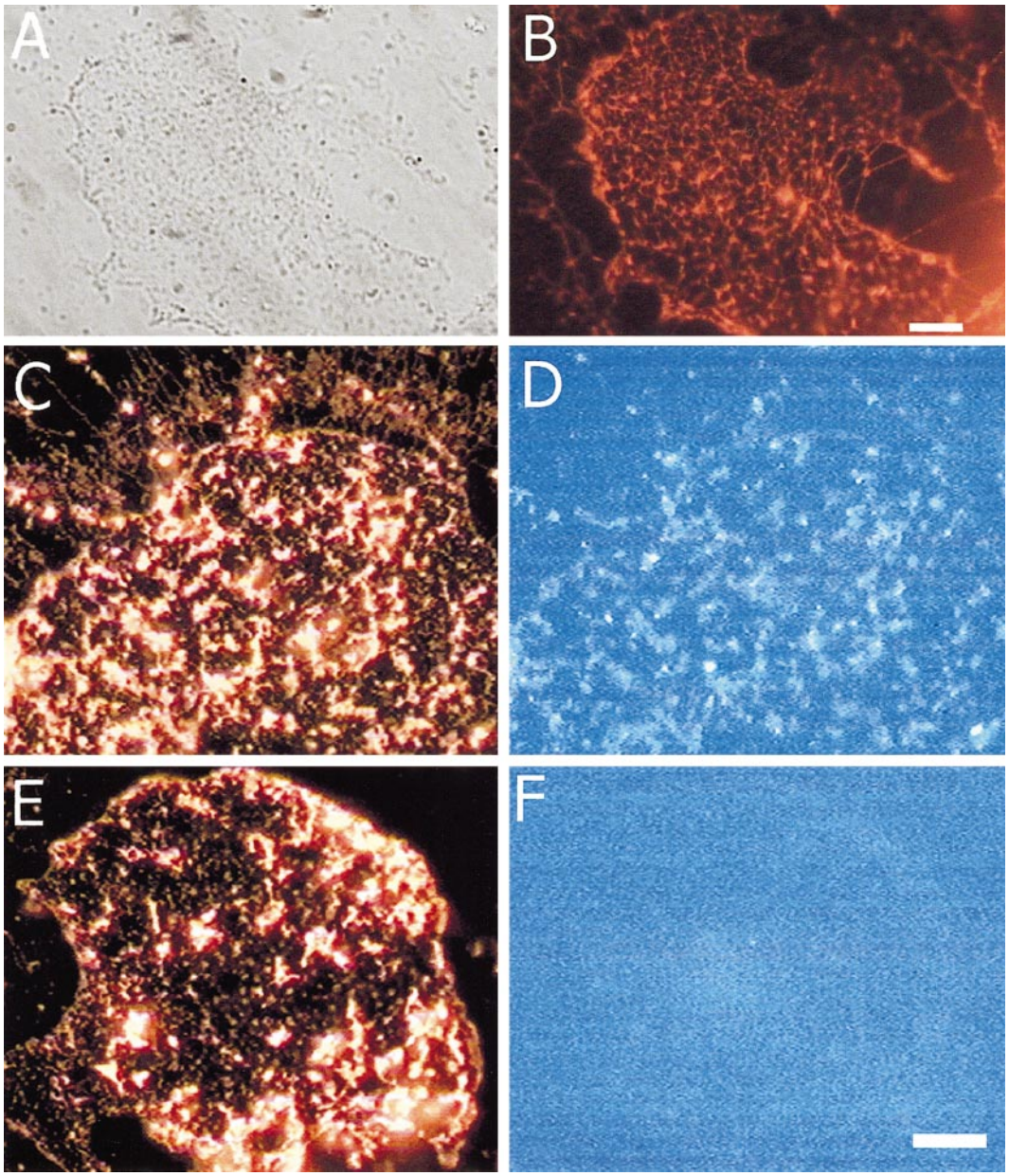

FIG. 2. The cortical endoplasmic reticulum. (A and B) A cortical preparation made 2 h after injection of DiI into a whole egg. The bright-field image is shown in (A) and the DiI-labeled ER in (B). Bright ER clusters and fine tubules are visible. (C and D) Double label of an unfixed cortical preparation showing the DiI-labeled ER (C) and IP_3 receptors labeled with the primary antibody C-19 and the AMCA-conjugated secondary antibody (D). (E and F) Control experiment of an unfixed cortical preparation labeled with DiI (E) and incubated with the secondary antibody, without prior incubation with the primary antibody (F). Bar, 10 mm.

prevented by the function-blocking monoclonal antibody 18A10 against the type 1 IP_3 receptor (Zu *et al.*, 1994; Nakano *et al.*, 1997; Oda *et al.*, 1999).

Several methods were used to label IP_3 receptors in cortical preparations of the ER (see Materials and Methods); fixed and unfixed cortices were labeled with one of several IP_3 receptor antibodies and the appropriate secondary antibody. ER membranes are quite sensitive to fixation and after experimentation with several protocols, it was found that fine reticular ER structure in cortices prepared from DiI-injected eggs was best preserved with 3.7% formaldehyde and 0.25% glutaraldehyde in intracellular buffer (see Materials and Methods).

Both the 18A10 and the C-19 antibodies for the type 1 IP_3

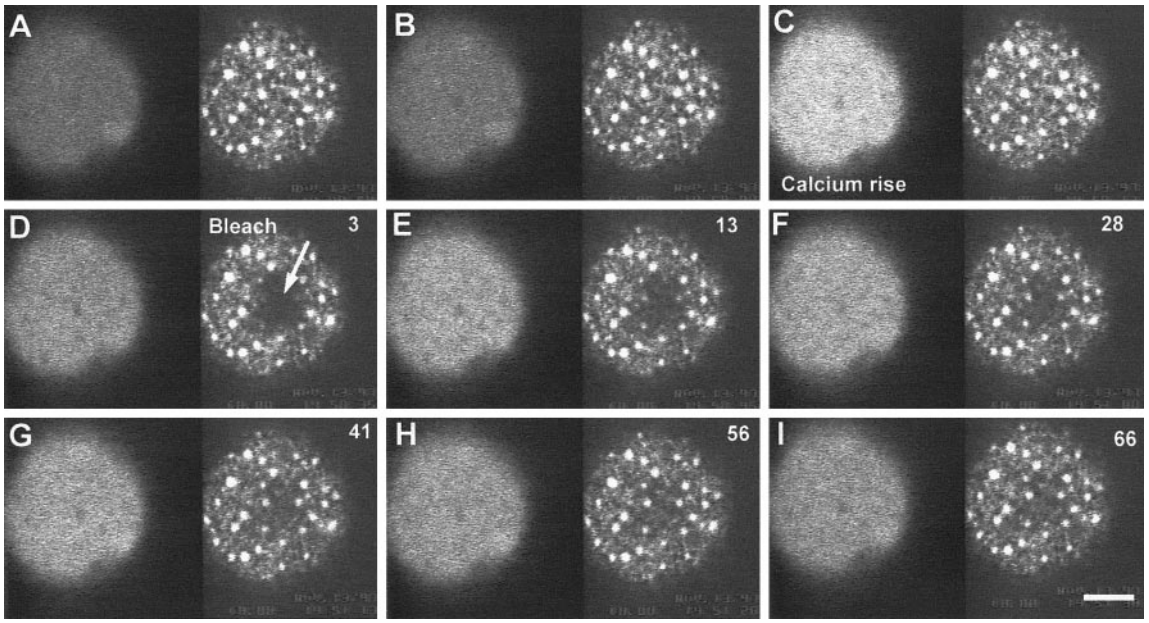

FIG. 3. The ER remains continuous during Ca^{2+} transients. Dual confocal images of the calcium green (left of each image) and the cortical ER (right) in a living egg during fertilization. (A and B) Two images before fertilization. (C) An image just after the beginning of the first Ca^{2+} rise. (D–I). FRAP experiment during the Ca^{2+} rise. (D) 3 s after photobleaching. Substantial recovery of fluorescence (E–I) is seen in images taken 13–66 s after bleaching while Ca^{2+} remains elevated. This result provides evidence that the ER is continuous during periods of high $[Ca^{2+}]_i$ such as the first sperm-induced Ca^{2+} transient.

receptor labeled discrete spots and the pattern of staining was similar to the staining of ER clusters observed in cortices made from DiI-injected eggs (Fig. 2D). Control preparations made without secondary antibodies displayed very low background labeling (Fig. 2F). To confirm that the IP_3 receptor antibodies were labeling the same ER clusters observed with DiI, antibody-labeled cortices were made from eggs previously injected with DiI. A general correspondence between ER accumulations and IP_3 receptors was observed in all doubly labeled cortices. Four experiments were done with DiI, the 18A10 or the C-19 antibody, and an AMCA-conjugated secondary antibody using fixed cortices. One additional experiment was done with DiI, C-19 antibody, and an AMCA-conjugated secondary antibody on unfixed preparations. One of several cortices from this experiment is shown in Figs. 2C and 2D along with the control experiment (Figs. 2E and F). This example is representative of all of the IP_3 receptor labeling experiments with or without DiI labeling. Occasionally IP_3 receptor labeling was seen on single ER tubules; however, in most experiments individual ER tubules were not well labeled (as shown in Figs. 2C and 2D). ER tubules may contain fewer receptors.

The ER Remains Continuous during Calcium Transients

The structure of the ER was imaged at 10-s intervals during fertilization and after the initiation of the sperm-induced Ca^{2+} transients. There was no apparent change in the clusters or in the surrounding ER. In order to determine if there was a change in ER continuity that we were not able to detect by direct imaging, eggs were injected with calcium green and DiI and fertilized and, during or after the first rise in intracellular Ca^{2+}, a FRAP experiment was performed. Figure 3 illustrates a FRAP experiment in which a portion of the cortex was bleached during the first Ca^{2+} transient. The Ca^{2+} rise is marked by the increased calcium green fluorescence (Fig. 3C). Fluorescence recovery occurred within 60 s in ER clusters and in the surrounding reticular ER (Figs. 3D–3I). Five eggs were bleached during the first Ca^{2+} transient and three of these eggs were also bleached in another location after the first Ca^{2+} transient, when the calcium green signal had returned to the original level. In all cases, whether during the period of high $[Ca^{2+}]_i$ or after, the ER clusters remained intact and fluorescence recovery was seen in FRAP experiments.

In two additional experiments, fluorescence recovery after bleaching was demonstrated in one egg both after the first Ca^{2+} transient and during the second, and in the other experiment after seven Ca^{2+} oscillations. All together, these seven experiments demonstrated that high intracellular Ca^{2+} during sperm-induced Ca^{2+} oscillations does not disrupt the ER organization nor cause discontinuity in the ER of the mouse egg.

To further examine the consequences of elevated Ca^{2+}, six eggs were injected with DiI and the ER was examined

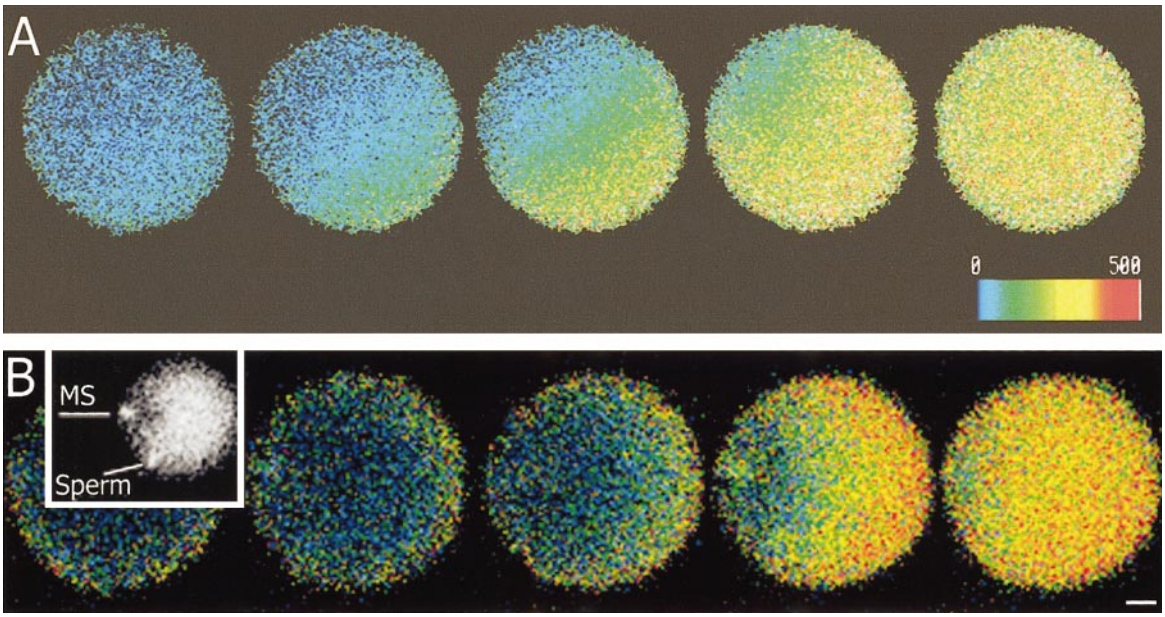

FIG. 4. Ca^{2+} transients consist of Ca^{2+} waves emanating from the hemisphere of the egg containing cortical ER clusters. (A) The third Ca^{2+} transient in a fertilized egg. The wave begins at the lower right. Images in this sequence were taken at intervals of 0.59 s. Use of the ratiometric Ca^{2+} indicator Fura-dextran permits some estimate of the $[Ca^{2+}]_i$ generated during the Ca^{2+} wave. The approximate $[Ca^{2+}]_i$ (nM) is indicated by pseudocolor as shown on the lower right. In this sequence, the orientation of the egg is unknown, but the third Ca^{2+} transient is clearly a wave beginning in one hemisphere. (B) The second Ca^{2+} transient in another egg both injected with Fura-dextran and preloaded with Hoechst to locate the egg chromosomes (MS, for meiotic spindle) and to identify the site of sperm–egg fusion (Sperm). Although the Hoechst fluoresces at the same excitation wavelength as Fura-2 dextran, the intensity of the Hoescht spots was greater than the overall fluorescence from Fura (inset, reduced in size by 1/2, is taken from the raw image collected at an excitation wavelength of 380 nm). The images show successive records taken in 0.67-s intervals. The Ca^{2+} transients begin at the cortex on the right. This is the hemisphere opposite the egg spindle, which contains ER clusters. The site of sperm entry does not determine the origin of secondary waves, since the sperm entered at the lower left. Pseudocolor is proportional to the magnitude of the Ca^{2+} increase, but the ratio measurement cannot be calibrated to external Ca^{2+} buffers because of the presence of Hoechst. Bar, 10 mm

during IP_3 injection. Injection of IP_3 and the subsequent increase in intracellular Ca^{2+} did not alter the organization of the ER. FRAP experiments were done with three of the six eggs. When the ER was locally bleached during the Ca^{2+} rise triggered by injection of IP_3, fluorescence recovery, comparable to that in control experiments, was seen in all three experiments.

Calcium Waves

The first and several subsequent Ca^{2+} waves were recorded in six eggs using conventional epifluorescence and a ratiometric measurement system. In all cases, both the first and the succeeding Ca^{2+} increases proceeded through the egg in a wave-like manner (Fig. 4A). However, closer examination indicated that the origin of the later waves was not related to the origin of the first wave (the site of sperm entry). To clarify wave orientation, in another series of experiments, eggs were initially loaded with Hoechst to identify the location of the meiotic spindle and to identify the site of sperm entry. In seven of nine experiments using Hoechst, the second Ca^{2+} transient was clearly seen to be a wave originating in the hemisphere opposite the meiotic spindle (Fig. 4B). In two of the nine eggs, the orientation of the waves was less clear because the spindle was oriented above the optical equator. These data show that, while the first Ca^{2+} wave begins at the site of sperm–egg fusion, succeeding waves originate in the hemisphere of the egg opposite the meiotic spindle, which we showed previously contains ER clusters (Mehlmann *et al.*, 1995).

Sperm–egg fusion in the mouse may occur anywhere on the surface except in the microvilli-free area overlying the meiotic spindle (Talansky *et al.*, 1991). Therefore, the site of sperm entry will, in some cases, be at the opposite pole from the spindle and both the first and the succeeding waves would appear to arise from the same point (near the site of sperm entry). However, in four of seven experiments, the fertilizing sperm entered nearer the equator or closer to the spindle. In these cases, the first Ca^{2+} wave originated at the site of sperm entry, but all subsequent waves began at the cortex in the hemisphere opposite the spindle.

DISCUSSION

During meiotic maturation, the mouse oocyte develops an enhanced ability to release Ca^{2+} at fertilization (Mehlmann and Kline, 1994). In parallel with this physiological change, there are dramatic changes in the organization of the ER, the organelle which is the primary source of Ca^{2+} released at fertilization. ER accumulations develop in the cortex except in the cortical granule-free and microvilli-free area around the meiotic spindle (Mehlmann *et al.*, 1995).

In this paper, we investigated the dynamics of the ER and Ca^{2+} at fertilization. We found that the ER network and cortical ER clusters appear to be unchanged during fertilization and the following Ca^{2+} transients. Photobleaching experiments indicated that the ER remains continuous during fertilization and the Ca^{2+} transients, as well as following IP_3 injection. By imaging the Ca^{2+} transients, we established that the repetitive transients are Ca^{2+} waves originate in the hemisphere of the egg that contains ER clusters. Immunofluorescent labeling of the egg cortex demonstrated that ER clusters contain IP_3 receptors. Thus, our data support the idea that the ER accumulations, which develop during maturation, serve as a stable cortical pacemaker responsible for generating IP_3-dependent Ca^{2+} waves.

Organization and Stability of the ER

To label the ER, we used the method of injecting a DiI-saturated oil drop into the egg (Terasaki and Jaffe, 1991). The fluorescent dicarbocyanine dye DiI is insoluble in water and diffuses only in the plane of lipid bilayers; the hydrophobic dye transfers to any membrane in contact with the oil drop and spreads through continuous membranes in the cell. This method has been found to label primarily ER in several cell types (see Terasaki and Jaffe, 1991; Jaffe and Terasaki, 1993, 1994), but it does not necessarily label all of the ER nor only the ER. The ER may not be a single continuous network, and since the DiI spreads in continuous membranes, some portions of the ER might not be labeled. DiI can also be transferred between compartments by membrane trafficking, so that DiI-labeled compartments either may be discontinuous parts of the ER or may represent labeling of the Golgi or post-Golgi membranes. However, several lines of evidence indicate that labeling of clusters in the mouse cortex is due to the diffusion of the DiI through the continuous ER membrane and not due to the labeling of Golgi or Golgi-derived membranes.

As evidence that these ER accumulations are part of a continuous ER in the egg, we showed previously that ER clusters could be labeled by DiI even in fixed eggs, which precludes spreading by membrane traffic (Mehlmann *et al.*, 1995). However, in those experiments, DiI spreading was not always complete, possibly because of fixation damage. In the present report, we provide further evidence that the DiI accumulations are continuous with the ER by imaging DiI spreading after injection and during FRAP. These spreading rates are consistent with diffusion in membranes.

Membrane trafficking rates have not been measured precisely until recently. Hirschberg and colleagues used vesicular stomatitis virus ts045 G protein fused to GFP to examine ER to Golgi trafficking. They found a trafficking transfer rate of 2.8% per minute and the fusion protein had a residence time within the ER of 40 min (Hirschberg *et al.*, 1998). This slower rate of membrane trafficking provides further evidence that the labeling of ER clusters by DiI injection is by diffusion of dye within the ER membranes and not due to vesicular membrane trafficking. Continuity of the ER is also suggested by the cortical preparations, in which ER accumulations are closely associated with an ER network in the cortex and are not readily dissociated by mechanical disruption.

DiI-labeled ER was examined during fertilization with parallel imaging of cytosolic Ca^{2+}. The appearance of the ER, as imaged by an oil immersion objective lens (NA 1.4), remained the same during the initial Ca^{2+} transient at fertilization, as well as during repetitive Ca^{2+} transients. The recovery of fluorescence after the localized photobleaching of DiI was rapid, indicating that ER membranes remained continuous during fertilization and during intervals of elevated $[Ca^{2+}]_i$, whether induced by fertilization or IP_3 injection. Therefore it appears that there is no change in ER structure during mouse fertilization. This contrasts with sea urchin and starfish fertilization (Jaffe and Terasaki, 1993; Terasaki *et al.*, 1996), in which large-scale changes in ER appearance occur and in which FRAP experiments indicate a transient disruption of continuity (discussed below).

IP_3 Receptors in the Cortical ER

Immunofluorescence of whole fixed eggs has previously shown that type 1 IP_3 receptors are clustered in the cortex in a pattern similar to the distribution of DiI-labeled clusters (Mehlmann *et al.*, 1996). To provide additional information on the relationship between the ER and the IP_3 receptor clusters, cortical preparations were labeled with antibodies to the type 1 IP_3 receptor. The cortical preparation provides excellent spatial resolution and it is possible to label both the ER and the IP_3 receptors in unfixed as well as fixed preparations. Double-labeling experiments showed that type 1 IP_3 receptors are localized on DiI-labeled ER clusters in cortical preparations. Thus, the cortical ER contains many IP_3 receptors within densely packed ER clusters; this places a substantial IP_3-sensitive Ca^{2+} compartment near the egg membrane, which is the site of sperm–egg fusion and which, as described below, also serves as a pacemaker region for repetitive Ca^{2+} waves.

Calcium Oscillations and Waves

During normal fertilization of mouse eggs, the fertilizing sperm initiates a Ca^{2+} wave that begins at the site of sperm–egg fusion. Following initiation of a Ca^{2+} wave in the cortical region, a wave of increased intracellular Ca^{2+}

moves across the egg to the antipode by a process of Ca^{2+}-sensitized IP_3-mediated Ca^{2+} release (Miyazaki, 1995). Immunocytochemistry of the mouse egg interior demonstrates that type 1 receptors are present in the interior (Parrington *et al.*, 1998; Fissore *et al.*, 1999), indicating that propagation through the interior probably depends on the presence of the type 1 receptors. Type 2 receptors, which appear to be distributed in clusters throughout the egg, (Fissore *et al.*, 1999), could also be involved in wave propagation in the interior and the cortex. The type 2 clusters described by Fissore *et al.* (1999) are similar in size to the clusters of type 1 receptor clusters and ER (Mehlmann *et al.* 1995, 1996). Additional work is needed to determine if the type 2 isoform functions in a significant way in Ca^{2+} wave initiation or propagation.

The initial sperm-induced Ca^{2+} increase is followed by a series of oscillations in $[Ca^{2+}]_i$. Ca^{2+} oscillations have been reported to occur in fertilized eggs of all mammalian species so far investigated, including mouse (Kline and Kline, 1992a), hamster (Miyazaki *et al.*, 1986), pig (Sun *et al.*, 1992), bovine (Fissore *et al.*,1992), rat (Ben-Yosef *et al.*, 1993), rabbit (Fissore and Robl, 1993), and human (Taylor *et al.*, 1993). The function of the Ca^{2+} oscillations following fertilization is not entirely known; however, oscillations persist until pronuclear formation in mouse embryos (Jones *et al.*, 1995). It has been proposed that Ca^{2+} oscillations may be necessary to release the egg from metaphase arrest and to fully inactivate maturation promoting factor and cytostatic factor, which together maintain meiotic arrest at metaphase II, but which must be inactivated for resumption of the cell cycle (Collas *et al.*, 1995; Zernicka-Goetz *et al.*, 1995; Dupont, 1998; Lawrence *et al.*, 1998). It should also be noted that some part of the cell cycle control machinery appears to feed back on the Ca^{2+}-releasing system to regulate its sensitivity, since the Ca^{2+}-releasing system is desensitized during interphase and Ca^{2+} oscillations persist for as long as 18 h if the egg is arrested in the metaphase state with colcemid (see Jones *et al.*, 1995).

We found that Ca^{2+} oscillations begin as waves in the cortical region at the site of ER accumulations. Miyazaki *et al.* (1986) reported that the first few Ca^{2+} transients in the hamster egg originate at the site of sperm–egg fusion and thereafter appear to arise synchronously throughout the egg. However, the temporal resolution may not have been sufficient to resolve the later waves, which traverse the egg in less than 2 s (alternatively, it may be that hamster eggs are unlike mouse eggs). For the mouse egg, Carroll *et al.* (1994) reported that injection of a partially purified sperm extract triggers Ca^{2+} release in mature mouse eggs. Confocal imaging of these eggs demonstrated that repetitive Ca^{2+} transients are wave-like and furthermore, the origin of the waves, after the first wave, shifted to a new location, much like what we show here for fertilization. Carroll and his colleagues also found that the Ca^{2+} oscillations triggered by sperm extract in immature oocytes are characteristically nonpropagating; Ca^{2+} increases in a homogeneous manner throughout the oocyte. The switch to propagating Ca^{2+} waves in mature eggs (Carroll *et al.*, 1994) is likely to be associated with the accumulation of ER in the cortex during oocyte maturation (Mehlmann *et al.*, 1995).

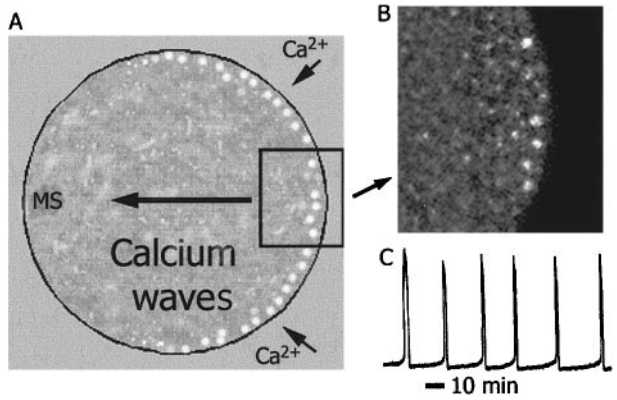

FIG. 5. Ca^{2+} dynamics in the fertilized mouse egg. (A) Diagram illustrating the localization of ER clusters (white spots) in the egg cortex in the hemisphere opposite the meiotic spindle (MS). Following fertilization, a Ca^{2+} influx pathway that is associated with repetitive filling and emptying of a Ca^{2+} store is activated, producing the characteristic repetitive Ca^{2+} waves associated with fertilization. (B) Confocal image of a typical cortical region containing ER clusters. (C) Recording of typical series of repetitive Ca^{2+} transients in a fertilized egg.

The cortical ER and IP_3 receptor localization, together with the observation that Ca^{2+} waves originate in the cortex, may also provide insight into the mechanism by which Ca^{2+} oscillations are generated. Ca^{2+} oscillations are dependent on extracellular Ca^{2+} (Kline and Kline, 1992b). Evidence indicates that the first Ca^{2+} wave at fertilization depletes an intracellular Ca^{2+} store and triggers capacitative Ca^{2+} entry (Kline and Kline, 1992b; McGuinness *et al.*, 1996). The persistent influx of Ca^{2+} is responsible for the repetitive Ca^{2+} transients in the egg, which propagate through the cytoplasm by a process of Ca^{2+}-sensitized IP_3-induced Ca^{2+} release. The accumulation of ER in the cortex of the mouse egg places the Ca^{2+} storage compartment near the plasma membrane (Fig. 5). Although the signal linking store depletion and capacitative entry is not yet known, at least one model predicts a close association between the Ca^{2+} storage compartment and the plasma membrane (Jaconi *et al.*, 1997). It is not yet known if Ca^{2+} entry in the mouse egg is localized or occurs over the entire membrane surface, but the absence of Ca^{2+} waves originating around the spindle region indicates that the cortex containing ER clusters serves as a pacemaker for repetitive Ca^{2+} waves as Ca^{2+} stores are filled and periodically emptied (see Fig. 5).

Relationship to Fertilization in Other Species

With the availability of fluorescent Ca^{2+} indicators and fluorescent dyes to label the ER in living cells, Ca^{2+} and ER

dynamics during fertilization have now been examined in several species. A relationship is becoming apparent between Ca^{2+} and ER dynamics.

Ca^{2+} oscillations occur after fertilization in eggs of mammals, as well as ascidians (Speksnijder *et al.*, 1989, 1990; Sardet *et al.*, 1998), bivalve molluscs (Deguchi and Osanai, 1994), an annelid (Eckberg and Miller, 1995), and a nemertean (Stricker, 1996). The duration of time for Ca^{2+} oscillations is ~30 min for ascidians (McDougall and Sardet, 1995; Speksnijder *et al.*, 1990), ~75 min for a nemertean egg (Stricker, 1996), ~30 min for molluscs (Deguchi and Osanai, 1994), and ~4 h for mouse (Jones *et al.*, 1995). The ER structure has been examined in ascidians (Speksnijder *et al.*, 1993), nemerteans (Stricker *et al.*, 1998), and now mouse and in each, no change in ER structure during fertilization has been observed. This suggests that the ER structure does not change in eggs with Ca^{2+} oscillations at fertilization. The stable organization of the ER may be necessary for generating or permitting long-lasting Ca^{2+} oscillations, which may be important for activation of the egg. Eggs which require a long period of Ca^{2+} stimulation for complete activation (>15 min) may use oscillations in order to avoid prolonged exposure to high Ca^{2+} that is damaging to the cells.

In contrast, there is only a single Ca^{2+} transient at fertilization in frog (Busa and Nuccitelli, 1985), fish (Gilkey *et al.*, 1978), sea urchin (Esien *et al.*, 1984), starfish (Eisen and Reynolds, 1984), hydrozoan (Freeman and Ridgway, 1993), and echiuran eggs (Stephano and Gould, 1997). The time for the Ca^{2+} transient to return close to baseline is ~3–4 min for sea urchin eggs (Shen and Buck, 1993), ~10–15 min for starfish eggs (Stricker *et al.*, 1994), and ~15 min for frog eggs (Nuccitelli *et al.*, 1993). Eggs which only require a short period of Ca^{2+} stimulation (e.g., <15 min) may use the strategy of a single transient (one exception may be the *Chaetopterus* egg in which oscillations are present, but persist for only about 10 min; Eckberg and Miller, 1995). When the ER has been studied in eggs which have only a single Ca^{2+} release, the ER is altered. In sea urchin (Terasaki and Jaffe, 1991; Jaffe and Terasaki, 1993) and starfish (Jaffe and Terasaki, 1994; Terasaki *et al.*, 1996) eggs, striking transient changes in ER appearance correspond to periods of discontinuity. Frog eggs may also fit this pattern; recent observations indicate that ER structure changes drastically during artificial activation (Terasaki, unpublished observations). These observations suggest that transient changes in the ER may somehow prevent oscillations in these species.

In summary, our experiments in mouse eggs indicate that the localization of ER clusters, containing abundant IP_3 receptors, places a Ca^{2+} storage compartment immediately beneath the plasma membrane at the site of sperm–egg fusion. A similar cortical localization of the type 1 IP_3 receptor has now been shown to occur in the human egg (Goud *et al.*, 1999). The importance of the cortical region in initiating the first sperm-induced Ca^{2+} transients was recently demonstrated by experiments in which sperm extract was injected into the egg; Oda and colleagues (1999) have shown that the cortex is substantially more sensitive to IP_3 and to the Ca^{2+}-releasing factor found in sperm extract than is the interior. Their study also showed that the type 1 IP_3 receptor mediates the response to sperm extract, since waves are prevented by the function-blocking monoclonal antibody 18A10. Therefore, the organization of the ER and IP_3 receptors in the cortex favors the rapid production of a Ca^{2+} wave at fertilization. The ER localization also gives rise to a cortical pacemaker region, which is responsible for the generation of the subsequent periodic Ca^{2+} waves. The mouse egg is representative of eggs of a number of species in which a long period of Ca^{2+} stimulation, which may be required for complete activation, is provided by periodic oscillations. The cortical ER remains continuous after fertilization and remains stable during the periods of high $[Ca^{2+}]_i$, permitting periodic Ca^{2+} waves to be generated until the egg completes meiosis.

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