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Reorganization of the Endoplasmic Reticulum during Meiotic Maturation of the Mouse Oocyte

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The endoplasmic reticulum (ER) of live metaphase II mouse eggs and prophase I-arrested oocytes was compared using the fluorescent, lipophilic dicarbocyanine dye, DiI. DiI, dissolved in soybean oil, was microinjected into oocytes and eggs; the dye diffused throughout the cytoplasm to label the ER, which was imaged by confocal microscopy. The mature egg had a fine reticular network of ER throughout the cell and numerous dense accumulations of membrane in the cortex. These ER accumulations, 1–2 μm in diameter, were generally absent deeper in the cytoplasm. A similar staining pattern was observed when the eggs were fixed within 1 min of injection, providing evidence that the cortical accumulations of membrane are part of a continuous ER membrane system, since membrane trafficking could not occur in a fixed egg. Cortical ER accumulations were localized to the same region of the egg as the cortical granules and were not observed in the cortical granule-free region adjacent to the meiotic spindle. In contrast, ER accumulations were rarely found in the cortex of the immature, prophase I-arrested oocyte, but larger and less well-defined membrane clusters were found throughout the deeper cytoplasm of the oocyte. The appearance of ER clusters in the egg cortex following oocyte maturation correlates with an increased ability of the mature egg to release calcium at fertilization. Since the ER is a calcium store, structural reorganization of the ER may be necessary to permit the large release of calcium and resulting cortical granule exocytosis at fertilization.

INTRODUCTION

The endoplasmic reticulum (ER) is a multifunctional organelle divided into continuous subcomponents, including rough ER, smooth ER, and the nuclear envelope (Vertel *et al.*, 1992). The ER is the site of protein synthesis and assembly (Palade, 1975) and lipid synthesis (Davidowicz, 1987; van Meer, 1993). Moreover, the presence of Ca^{2+}-ATPases, Ca^{2+} storage proteins, and specific Ca^{2+} release channels in the ER permits this organelle to perform a crucial role in the regulation of intracellular Ca^{2+}. Direct evidence has been obtained to demonstrate that the ER in eggs serves as a Ca^{2+} store (Han and Nuccitelli, 1990; Terasaki and Sardet, 1991) and that the ER contains inositol 1,4,5-trisphosphate (IP_3) receptors and, in some cases, ryanodine receptors, both of which mediate Ca^{2+} release from the ER (McPherson *et al.*, 1992; Kume *et al.*, 1993; Parys *et al.*, 1994; reviewed in Whitaker and Swann, 1993). A dramatic, transient release of Ca^{2+} from internal stores at fertilization initiates activation of the eggs of many animals, including those of mammals (Jaffe, 1985; Nuccitelli, 1991; Whitaker and Swann, 1993; Freeman and Ridgway, 1993; Miyazaki *et al.*, 1993; Swann and Ozil, 1994).

Immature, prophase I-arrested oocytes of starfish (Chiba *et al.*, 1990), hamster (Fujiwara *et al.*, 1993), and mouse (Mehlmann and Kline, 1994) are less sensitive to IP_3-induced Ca^{2+} release than mature eggs (metaphase I in starfish, metaphase II in mammals). Furthermore, when fertilized, these oocytes release substantially less Ca^{2+} than eggs. These physiological changes suggest that the ER may be modified during oocyte maturation. Examination of fixed oocytes and eggs of frog and mouse has provided some evidence for this (Gardiner and Grey, 1983; Charbonneau and Grey, 1984; Campanella *et al.*, 1984; Larabell and Chandler, 1988; Ducibella *et al.*, 1988b; Stewart-Savage *et al.*, 1991; Kume *et al.*, 1993). In particular, electron micrographs have revealed an increase in the number of membrane vesicles in the cortex of the mouse egg following oocyte maturation; it has been suggested that this increase in cortical membrane vesicles may correspond to a reorganization of intracellular Ca^{2+} stores (Ducibella *et al.*, 1988b).

We used the lipophilic dicarbocyanine dye, DiI ($DiIC_{18}(3)$ or $DiIC_{16}(3)$), and confocal microscopy in living mouse oocytes and eggs to examine the reorganization of the ER during oocyte maturation. The three-dimensional organization of the ER has been successfully studied in living echinoderm eggs by injecting DiI dis-

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solved in soybean oil (Terasaki and Jaffe, 1991, 1993; Jaffe and Terasaki, 1993, 1994). DiI transfers to intracellular membranes that are in contact with the oil drop. Due to the hydrophobic nature of the DiI molecule, DiI diffuses only in continuous membranes, though it can move to and label discontinuous compartments by membrane traffic (vesicular budding and fusion). In living sea urchin eggs, DiI stains cisternae (membrane sheets) in the interior, the nuclear envelope, and a cortical tubular network. This staining closely matches that seen with immunocytochemical labeling using an antibody to a calsequestrin-like protein isolated from sea urchin egg microsomes (Oberdorf *et al.*, 1988; Henson *et al.*, 1989; Terasaki *et al.*, 1991). Also, DiI does not stain yolk granules or cortical granules, membrane-bound organelles that are abundant in the sea urchin egg cytoplasm (Terasaki and Jaffe, 1991). When DiI is injected into fixed eggs, it labels only the cisternae, nuclear envelope, and cortical network, providing evidence that these are part of one continuous ER membrane (Jaffe and Terasaki, 1993) and showing that this method is a useful way to localize ER membranes. Using this method, we find a dramatic change in ER organization associated with mouse oocyte maturation that may account, at least in part, for the enhancement of the Ca^{2+} release mechanism that occurs during oocyte maturation.

MATERIALS AND METHODS

Media and Reagents

Oocytes and eggs were cultured in minimum essential medium with Earle's salts (MEM; Mehlmann and Kline, 1994). MEM and dibutyryl cAMP were obtained from Sigma Chemical Co. (St. Louis, MO). $DiIC_{16}(3)$(1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) and $DiIC_{18}(3)$ (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) were obtained from Molecular Probes (Eugene, OR). Similar results were obtained with each compound, in these studies and in previous studies of ER in the sea urchin egg (Jaffe and Terasaki, 1993); the term DiI refers to either compound. A saturated solution of DiI was prepared in soybean oil (Wesson Oil, Stop & Shop, West Hartford, CT); the solution was stored at room temperature and used over a period of several days. The fixation medium was 1.0% glutaraldehyde and 1.0% paraformaldehyde in 0.1 *M* sodium cacodylate and 0.1 *M* sucrose buffer, pH 7.4.

Preparation of Oocytes and Eggs

Oocytes and eggs from NSA (CF-1) mice (Harlan Sprague-Dawley, Indianapolis, IN) were obtained as previously described (Kline and Kline, 1992; Mehlmann and Kline, 1994). To collect oocytes, ovaries were removed from female mice injected 44–48 hr earlier with pregnant mare's serum gonadotropin (Sigma). The ovaries were repeatedly punctured, in MEM, with a 26-gauge needle to release oocytes. Oocytes, enclosed by cumulus cells, were isolated and cumulus cells were removed by repeated pipetting through a small bore pipette. Fully-grown, germinal vesicle (GV)-intact oocytes, approximately 75–80 μm in diameter, were collected and washed by transfer through several drops of MEM. When prophase I-arrested oocytes were used, 0.1 mg/ml dibutyryl cAMP (dbcAMP) was included in the medium to prevent spontaneous maturation (Cho *et al.*, 1974). dbcAMP was not included in media for experiments using eggs. Eggs were obtained from mice following superovulation and the cumulus cells were removed with 0.3 mg/ml hyaluronidase (Type IV-S, Sigma). Oocytes and eggs were cultured in 200-μl drops of medium under light mineral oil (Fisher Scientific, Pittsburgh, PA) at 37°C.

In experiments with maturing oocytes, prophase I-arrested oocytes were allowed to spontaneously mature after transfer to MEM without dbcAMP. Oocytes matured in this way develop normally to metaphase II eggs and are comparable to eggs matured *in vivo*, since most such *in vitro* matured eggs form blastocysts that develop to term when transplanted into host mothers (Downs *et al.*, 1986). Breakdown of the germinal vesicle usually occurs within 2hr after removing dbcAMP (Mehlmann and Kline, 1994).

Injection of DiI and Confocal Microscopy

Oocytes and eggs, held in place with a holding pipette, were injected with DiI using a beveled constriction 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. Oocytes and eggs were visualized between 1 and 5 hr after DiI injection. All observations of live oocytes and eggs were made on a warm stage kept at 37°C.

DiI-labeled oocytes and eggs were observed using a laser scanning confocal microscope (Bio-Rad Model 600 coupled with a Nikon Diaphot). Observations were made with a Nikon Planapo 60× 1.4 N.A. lens. During observations, the laser was set at full power with a 1% neutral density filter and the confocal aperture was set at three. The distance from the cell surface of each optical section was determined by first focusing on the cell cortex and then focusing into the cell a defined distance using the calibration marks on the fine focus control of the Nikon Diaphot. Most measurements were made at the cortex and at a depth of 5 and 10 μm, but additional images

were collected in some oocytes and eggs at greater depths. Magnification of the image was calibrated with a stage micrometer. Output from the confocal microscope was stored on an optical disk (Panasonic optical disk drive system, Crimson Tech., Cambridge, MA.), and images were photographed from a computer monitor.

RESULTS

ER of the Mature, Metaphase II Egg

Examination of the mature, metaphase II egg with confocal microscopy after DiI staining revealed a fine, reticular network throughout the interior of the cell (Fig. 1). In addition, there were many large, bright clusters of ER present in the cortex immediately beneath the plasma membrane (Fig. 1A). These prominent membrane accumulations, 1–2 μm in diameter, were absent deeper in the cytoplasm of the egg (Figs. 1B and 1C). Of 35 eggs examined, 24 (69%) exhibited very distinct, bright membrane accumulations. The distribution of these ER accumulations was highly polarized, being localized to the cortex that was opposite the meiotic spindle (Fig. 2A). That is, the ER did not accumulate in the cortex of the egg associated with the metaphase II chromosomes and spindle apparatus, the area referred to as the microvilli-free region or cortical granule-free area (Johnson *et al.*, 1975; Nicosia *et al.*, 1977; Longo and Chen, 1985; Ducibella *et al.*, 1988a). In 8 of the 35 eggs, the cortex was more brightly stained than the interior cytoplasm, indicating abundant ER; however, the ER was not organized into distinct, well-defined accumulations. Only 3 of the eggs examined had no ER accumulations or bright staining in the cortex.

The density of cortical ER accumulations in eggs was estimated by counting the number of bright spots in a 20 × 20-μm area of the cortex located away from the meiotic spindle region. For 23 eggs which displayed distinct ER accumulations (as in Fig. 1A), the mean number of accumulations in this area of the cortex was 18 (range 6–30). The size of the accumulations varied, but the diameters were at least 1 μm and were generally 1–2 μm.

To determine whether the spreading of DiI indicated continuity of the ER membrane or whether it might be a consequence of membrane traffic, we injected DiI-saturated oil drops and then fixed the eggs within 1 min after injection, well before the dye would have spread throughout the ER. Of six such eggs, two showed labeling of the ER similar to that seen in live eggs, with bright membrane accumulations (1–2 μm in diameter) in the cortex opposite the meiotic spindle, but not elsewhere (Fig. 3). Dye spreading in the other four eggs was incomplete, possibly as a consequence of fixation damage.

ER of the Prophase I-Arrested Oocyte

In contrast to the mature egg, the cortex of the immature, prophase I-arrested oocyte contained few, if any, well-defined ER accumulations (Fig. 1D). Of 20 oocytes examined in detail, 16 contained only a few bright spots in the cortex and 4 oocytes had no ER accumulations. Scanning deeper into the oocyte revealed the presence of larger, less well-defined membrane accumulations throughout the cytoplasm (Figs. 1E, 1F, and 2B). These accumulations were uniformly distributed and exhibited no polarity. The density of cortical ER accumulations in prophase I-arrested oocytes was estimated, as in eggs, by counting the number of bright spots in a 20 × 20-μm area of the cortex (as in Fig. 1D). For 16 oocytes with ER accumulations, the mean number of accumulations was only 4 (range 1–10). The diameters of the membrane accumulations in immature oocytes were about 1 μm, and they were smaller and less distinct than in mature eggs.

ER of the Prometaphase I Oocyte

Some reorganization of the ER is apparent in oocytes examined soon after germinal vesicle breakdown (GVBD). We examined the ER in oocytes 4–6 hr after dbcAMP was removed from the incubation medium. Such oocytes had undergone GVBD (about 2 hr after dbcAMP removal) and were at prometaphase I. More ER accumulations were present in the cortex of oocytes at this stage of oocyte maturation than were present in the cortex of immature oocytes (Fig. 4). In 18 prometaphase oocytes, the mean number of membrane accumulations in a 20 × 20-μm area of the cortex was 12 (range 7–18). The pattern of cortical DiI staining in maturing oocytes was not as regular as the pattern in most mature eggs, nor was the staining as bright, but it was more like mature eggs than the immature oocytes, which contained few, if any, cortical ER accumulations. The sizes of the accumulations were 1–2 μm, as in eggs. There was more variability in the size of each accumulation in an individual maturing oocyte than there was for a mature egg. At this stage of maturation (4–6 hr after removing dbcAMP), large membrane accumulations, characteristic of the immature oocyte, were still present deeper in the oocyte.

DISCUSSION

Confocal microscopy of live DiI-injected, metaphase II mouse eggs reveals large clusters of ER in the egg cortex; these accumulations of ER are localized in the region of the egg where sperm-egg fusion and cortical granule exocytosis occur and are absent in both the cortical granule-free area near the meiotic spindle and the

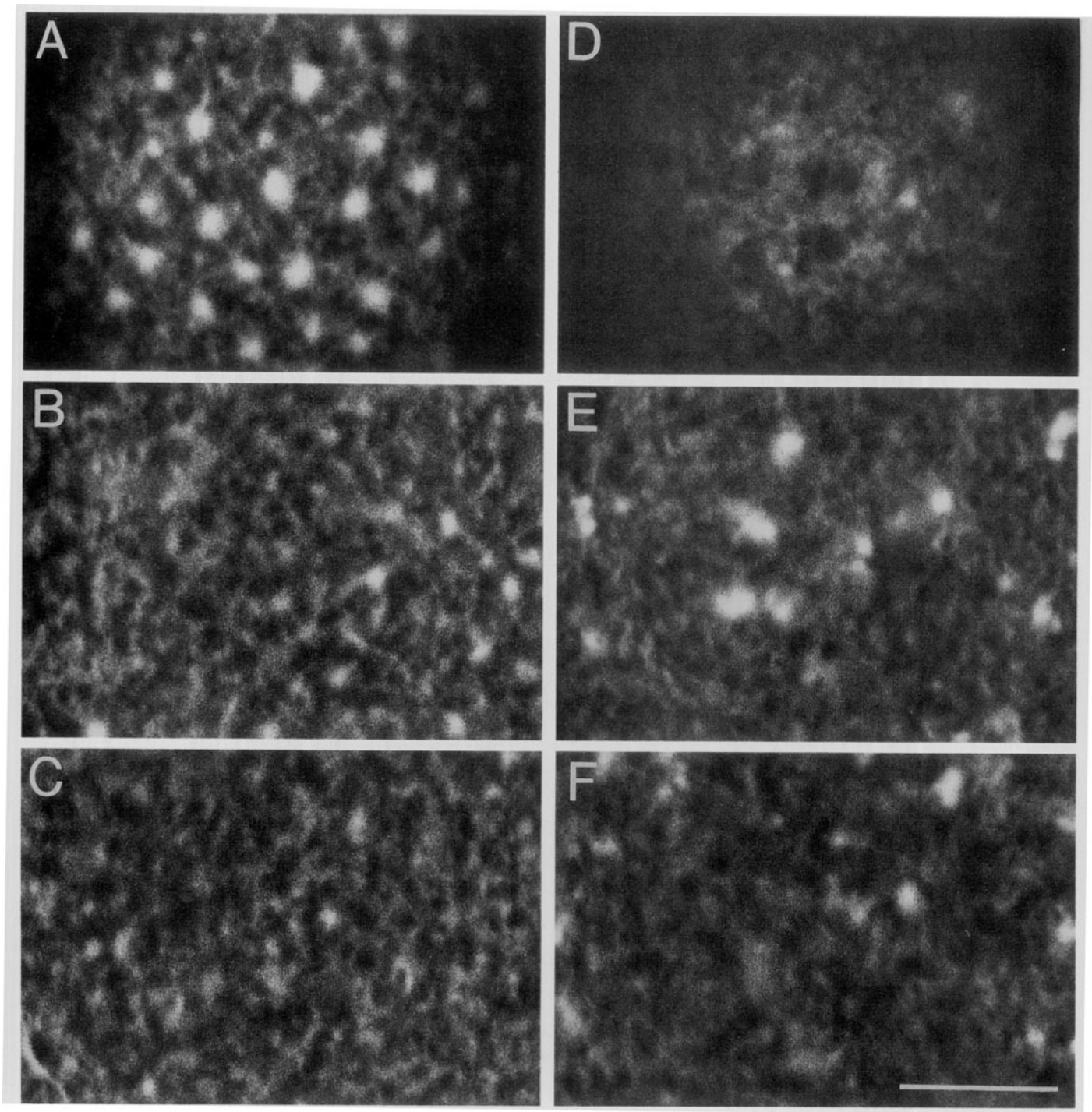

FIG. 1. Endoplasmic reticulum of a metaphase II unfertilized mouse egg and a prophase I-arrested mouse oocyte. Cells were injected with DiI and examined 2.5–3 hr later. (A–C) Confocal sections of an egg at the cortex (A) and at the same location, 5 μm (B) and 10 μM (C) in from the cortex. (D–F) Confocal sections of an oocyte at the cortex (D) and, at the same location, 5 μm (E) and 10 μm in from the cortex. The magnification is the same for all photographs and the bar in (F) represents 10 μm.

deeper cytoplasm. Since the accumulations also become labeled by DiI in fixed eggs, our studies provide evidence that the membranes in the cluster are part of a continuous reticulum. In contrast to the pattern seen in metaphase II eggs, prophase I-arrested oocytes possess few ER clusters in the cortex, while larger membrane accumulations are present throughout the oocyte cytoplasm. Oocytes at prometaphase I of meiosis exhibit an inter-

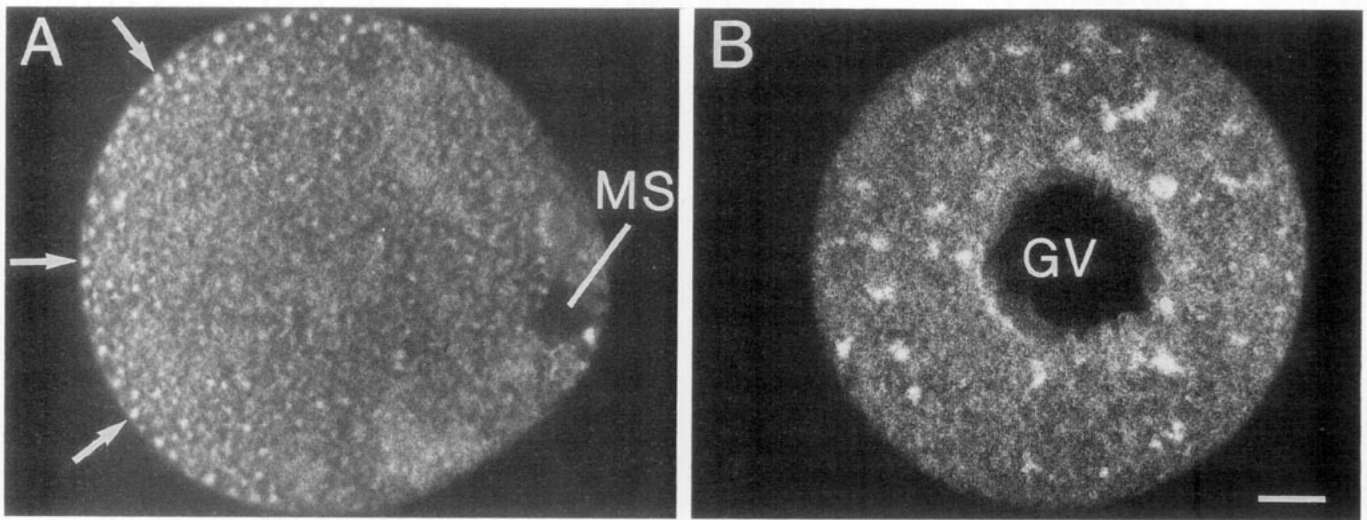

FIG. 2. Endoplasmic reticulum in equatorial sections of a whole egg (A) and oocyte (B), illustrating the difference in the distribution of membrane accumulations in each cell type. The ER accumulations in the egg (A), indicated by arrows, are found primarily in the cortex opposite the meiotic spindle and are not in the cortex overlying or adjacent to the meiotic spindle (MS). Larger, more diffuse accumulations of ER occur throughout the oocyte and display no polarity (B). GV, germinal vesicle. Magnification is the same for both photographs; the bar in B represents 10 μm.

mediate ER organization; the number of cortical accumulations is greater than in immature oocytes, but, as in immature oocytes, ER accumulations are present deep in the cytoplasm. Our observations of ER clusters within the interior cytoplasm of immature oocytes suggest that the cortical ER clusters in the mature egg could arise from migration and condensation of these interior structures in the oocyte.

These observations of ER distribution in live eggs are consistent with previous electron microscopy of fixed mouse eggs. In these studies, the mature egg was found to contain as much as three times the amount of vesicular membrane profiles in the cortex compared to that in the immature oocyte; furthermore, many of these membranes were clustered (Ducibella *et al.*, 1988b). These clusters may correspond to the DiI-labeled accumulations. In the electron micrographs, the membranes appear as vesicles, but this may be due to difficulties in preserving continuous membranes during fixation and processing. The presence of cortical membrane clusters are also suggested by studies using the antibiotic filipin, which labels membrane cholesterol or unesterified sterols. Allworth and Ziomek (1988) reported that filipin labels numerous cortical structures in the mature egg. The identity of these filipin-labeled structures is unknown; their distribution is sparser and less regular than the ER accumulations.

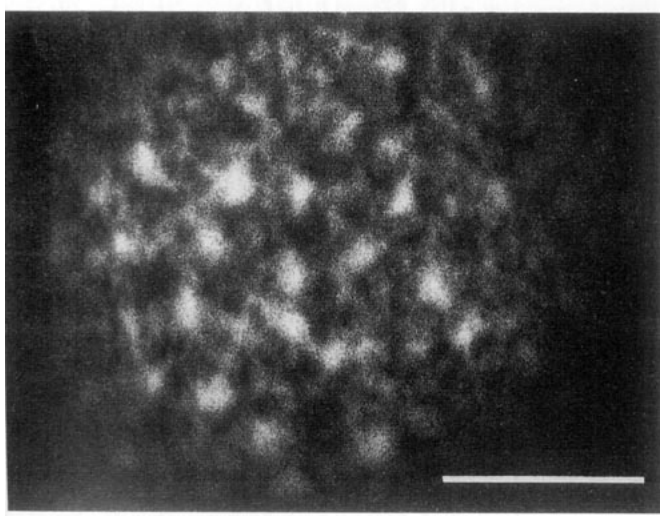

FIG. 3. The pattern of DiI staining in the cortex of an egg which was fixed within 1 min of injecting DiI. Bar represents 10 μm.

A recent study of hamster oocytes and eggs injected with DiI-saturated oil drops also shows changes in ER structure during oocyte maturation (Shiraishi *et al.*, 1995). Although the pattern of changes is not identical in mouse and hamster, both species show a transition from an oocyte with large irregular masses of ER to an egg with a highly organized array of cortical ER clusters, which are absent in the region of the meiotic spindle. The study of hamster oocytes also demonstrates a parallel change in the distribution of IP_3 receptors, seen with immunocytochemistry (Shiraishi *et al.*, 1995).

Polarized ER Distribution in Eggs

ER accumulations were not observed in the cortex of the mature egg adjacent to the meiotic spindle. This dis-

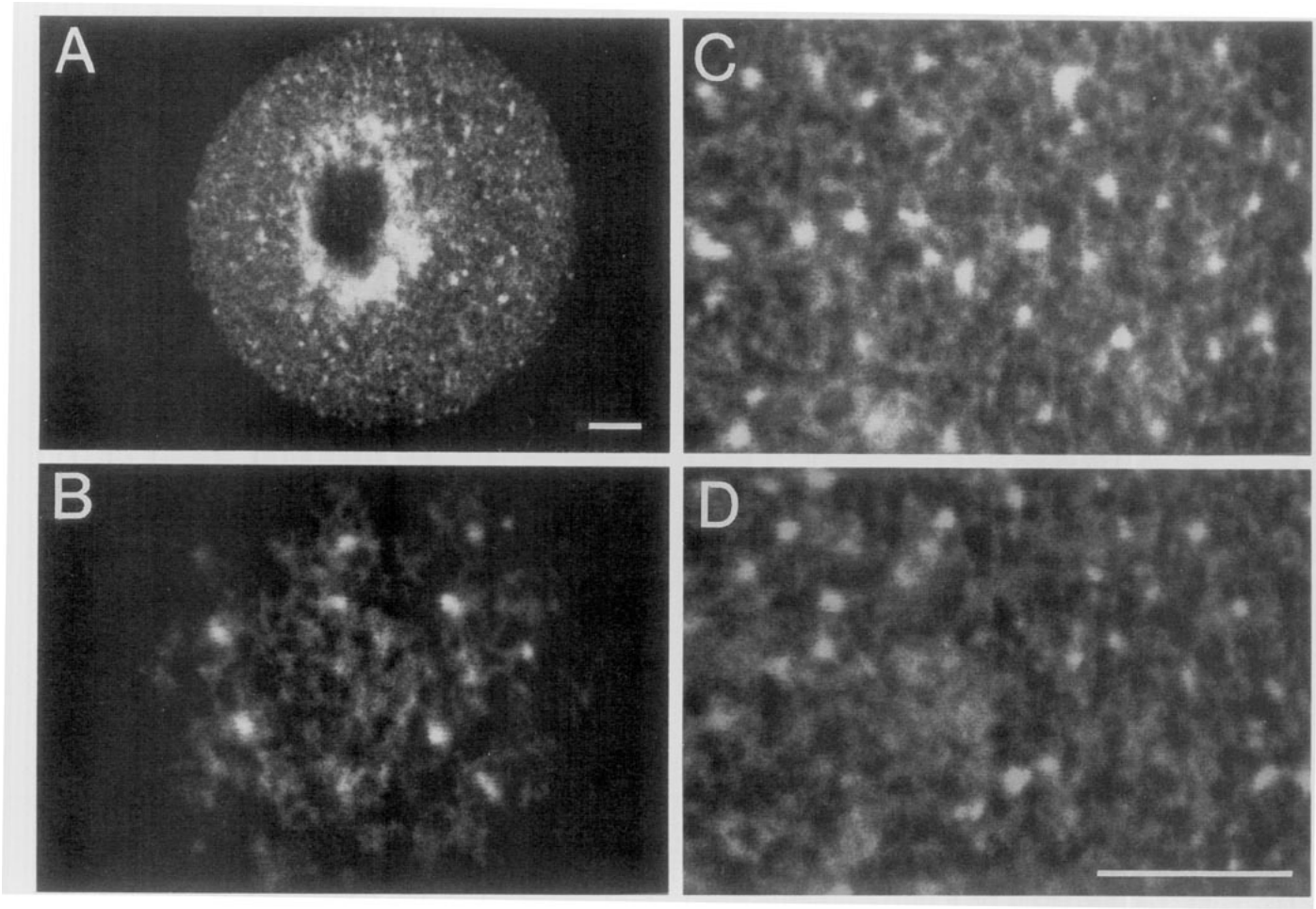

FIG. 4. Endoplasmic reticulum in a prometaphase I oocyte. A prophase I-arrested oocyte was removed from dbcAMP-containing medium to permit spontaneous maturation and was injected with DiI approximately 2 hr later. The oocyte was examined 2–2.5 hr after injecting DiI and about 4 hr after the initiation of oocyte maturation. (A) Section of a whole oocyte, at the level of the former germinal vesicle region, showing the membrane accumulations throughout the cell after germinal vesical breakdown. (B) ER in the cortex of the maturing oocyte. (C) ER at the same location, 5 μm below the cortical image. (D) ER 10 μm below the cortical image. Bars represent 10 μm; magnification is the same for B, C, and D.

tribution correlates with the polarized distribution of microvilli, cortical granules, and surface concanavalin A (con A) binding sites previously reported for mammalian eggs. Microvilli occur over the entire surface of the metaphase II egg, except for the area overlying the meiotic spindle (Longo and Chen, 1985). Similarly, cortical granules are present throughout the egg cortex except in the region near the meiotic spindle (Nicosia *et al.*, 1977; Ducibella *et al.*, 1988a). Con A binding sites are absent on the surface of the egg overlying the meiotic spindle (Johnson *et al.*, 1975). The most common site of sperm-egg fusion is in the area containing microvilli, cortical granules, and con A receptors; sperm rarely fuse with the egg in the microvillar-free area overlying the meiotic spindle (Johnson *et al.*, 1975; Nicosia *et al.*, 1977; Talansky *et al.*, 1991). The localization of ER accumulations to the region where sperm-egg fusion and cortical granule exocytosis take place may be important in generating the large Ca^{2+} transient necessary for activation of egg development at fertilization.

Clusters of ER are also found in the cortex of the egg of the frog, *Xenopus laevis*. These ER clusters are located in both the animal and vegetal hemispheres of the frog egg (Campanella and Andreuccetti, 1977); however, a polarity in ER-membrane junctions was found in the *Xenopus* egg (Gardiner and Grey, 1983). A polarized ER distribution has also been reported to occur in the ascidian egg (Sardet *et al.*, 1992), and it has been linked to polarized Ca^{2+} release (Speksnijder, 1992).

Changes in ER Structure and Ca^{2+} Release Mechanisms during Oocyte Maturation

The structural differences of the ER in mouse oocytes and eggs correlate with the observation that both

sperm- and IP_3-induced Ca^{2+} release is much lower in immature oocytes than in mature eggs (Mehlmann and Kline, 1994). The partial ER reorganization we observe in mouse oocytes after germinal vesicle breakdown (4–6 hr after the onset of maturation) occurs when sperm-induced Ca^{2+} release in the oocyte, as shown by the amplitude and duration of the first Ca^{2+} transient, is nearly the same as in the mature egg. While IP_3-induced Ca^{2+} release in oocytes is normally low, the amount of Ca^{2+} released upon IP_3 injection can be elevated to nearly the same level as in eggs if the IP_3-sensitive stores are first sensitized by treatment with the sulfhydryl reagent, thimerosal (Mehlmann and Kline, 1994). Therefore, the immature oocyte possesses intracellular stores of releasable Ca^{2+} similar in size to Ca^{2+} stores in the egg, but the oocyte is less sensitive to IP_3 and to sperm, which elevates intracellular Ca^{2+} through IP_3 production (Miyazaki *et al.*, 1993). Development of the IP_3-induced Ca^{2+} release mechanism during hamster (Fujiwara *et al.*, 1993) oocyte maturation has also been demonstrated.

Development of the IP_3-induced Ca^{2+} release mechanism during oocyte maturation may involve a change at the level of the IP_3 receptor, such as an increase in receptor number or a change in phosphorylation state of the receptors (Supattapone *et al.*, 1988; Burgess *et al.*, 1991; Quinton and Dean, 1992; Ferris and Snyder, 1992). Alternatively, or additionally, development of the Ca^{2+} release mechanism could be a consequence of a reorganization of the ER. During maturation, the ER becomes more tightly clustered, and the clusters become localized to the cortex. The clustering may be significant in relation to recent evidence that higher cytosolic Ca^{2+} levels increase the sensitivity of the IP_3-sensitive Ca^{2+} release channel to IP_3 (Iino, 1990; Bezprozvanny *et al.*, 1991; Finch *et al.*, 1991). Thus, when IP_3 is generated, more Ca^{2+} may be released from the ER clusters, resulting in locally higher Ca^{2+} levels, which would then result in greater IP_3 sensitivity. Because the phospholipase C that generates IP_3 might be more concentrated in the plasma membrane, localization of ER to the cortex could also be a means for increasing the effective IP_3 sensitivity.

The mechanism by which the ER becomes reorganized during oocyte maturation is not known. Development and organization of the ER in somatic cells is closely associated with the distribution of microtubules (Terasaki *et al.*, 1986; Lee *et al.*, 1989); however, the relationship between the ER and microtubules in meiotic cells has not been examined. Cytoplasmic centrosomes with short microtubule arrays are found in mouse oocytes and eggs (Schatten *et al.*, 1985, 1986; Maro *et al.*, 1985; Messinger and Albertini, 1991). However, the number of cytoplasmic centrosomes is far less than the number of ER accumulations we observe. The papers cited above report a mean of 8–16 centrosomes in metaphase II eggs, compared to about 150–750 ER accumulations (based on counts in 400 μm^2 regions of the cortex and an estimated 10,000 μm^2 area where the ER accumulations are present). For this reason, whether or how these microtubules might participate in reorganizing the ER during oocyte maturation is not clear.

Reorganization of the ER may be a common feature of oocyte maturation in other species as well. Mature *Xenopus* eggs display an extensive cortical ER around the cortical granules that is not present in immature oocytes (Gardiner and Grey, 1983; Campanella *et al.*, 1984; Charbonneau and Grey, 1984; Larabell and Chandler, 1988). Immunolocalization indicates that, during oocyte maturation, the *Xenopus* IP_3 receptor becomes concentrated in patches in the animal hemisphere (Kume *et al.*, 1993; Parys *et al.*, 1994). The elaboration of the cortical ER and the formation of subcortical IP_3 receptor-rich patches coincide with the development during oocyte maturation of the ability of these oocytes to respond to activating stimuli (pricking or exposure to the ionophore, A23187) that initiate Ca^{2+} release and cortical granule exocytosis (Campanella *et al.*, 1984; Charbonneau and Grey, 1984).

Structural changes in the ER also occur during oogenesis of the sea urchin and maturation of the starfish oocyte (Henson *et al.*, 1990; Jaffe and Terasaki, 1994). In the starfish, the structural change involves a transition of the ER from a sheet-like form to a spherical form, in which the ER tightly surrounds yolk platelets (Jaffe and Terasaki, 1994). This change in ER structure may be related to the development during maturation of the ability to release Ca^{2+} in response to IP_3 or sperm (Chiba *et al.*, 1990). In eggs of echinoderms and frogs, as well as the mouse, the geometry of the ER membranes may in some way influence the function of IP_3 receptors and Ca^{2+} release at fertilization.

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