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Redistribution of cytoplasmic components during germinal vesicle breakdown in starfish oocytes

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SUMMARY

The starfish oocyte is relatively clear optically, and its nucleus, termed the germinal vesicle, is large. These characteristics allowed studies by confocal microscopy of germinal vesicle breakdown during maturation in living oocytes. Three fluorescent probes for cytoplasmic components were used: fluorescein 70 kDa dextran, which does not cross the nuclear pore of immature oocytes and probably behaves in the same way as soluble cytosolic proteins, YOYO-1, which was used to localize ribosomes, and DiI which labels the nuclear envelope and endoplasmic reticulum. The first change observable by transmitted light microscopy during maturation is a wrinkling of the germinal vesicle envelope. Several minutes before the wrinkling, the 70 kDa dextran began to enter the germinal vesicle; the ribosomes did not enter during this period. The dextran is likely to be passing through nuclear pores whose size limit has increased but which still exclude ribosomes. At the time of the wrinkling of the germinal vesicle envelope, both 70 kDa dextran and ribosomes entered as a massive wave. The characteristics of this entry indicate that the permeability barrier of the nuclear envelope bilayer has been disrupted. The disruption of the permeability barrier occured in a local region rather than around the entire periphery. Also, the disruption was observed more often on the animal pole side of the germinal vesicle (26/34 oocytes). The endoplasmic reticulum entered the nuclear region more slowly. Cytochalasin B inhibited this movement and also inhibited characteristic endoplasmic reticulum movements seen at high magnification. The effects of cytochalasin indicate that mixing of endoplasmic reticulum with nuclear space is an active process involving actin filaments.

Key words: germinal vesicle breakdown, DiI, dextran, ribosome

INTRODUCTION

The nuclear envelope is a specialized part of the ER that has closed in on itself to form a separate space within the cell volume. The double bilayer of the nuclear envelope is a physical barrier to free movement between nucleus and cytoplasm. Movements across the nuclear envelope occur through nuclear pores; these structures allow free movement of ions and smaller proteins, but require special translocation signals to transport larger molecules (reviewed in Gerace, 1992). In most eucaryotic cells, the nuclear envelope breaks down at the beginning of mitosis, resulting in the mixing of cytoplasmic and nuclear material. During re-formation of the nuclear envelope, it appears that nuclear envelope precursor vesicles bind to individual condensed chromosomes, fuse to enclose each chromosome, then fuse with each other to form one large enclosure (Ito et al., 1981; Lohka and Masui, 1983; reviewed by Wiese and Wilson, 1993). In this way, the chromosomes are contained within the nucleus while organelles and large soluble macromolecules are in the cytoplasm. Procaryotes possess no nucleus, showing that the genetic material does not need to be segregated apart from the rest of the cell. It is not known why eucaryotes are organized in this way, but it must allow more subtle interactions and regulation of the genome (Dingwall and Laskey, 1992).

Starfish oocytes are a useful system for investigating certain aspects of nuclear and cytoplasmic organization. During growth of oocytes, the nucleus is very large and is termed the germinal vesicle. The oocyte is arrested at first prophase of meiosis, and the germinal vesicle breakdown begins soon after application of the maturation hormone 1-methyladenine (Kanatani et al., 1969). By transmitted light microscopy, the former germinal vesicle and cytoplasmic regions slowly become less distinguishable until the interior of the mature egg appears homogeneous. Starfish oocytes are relatively clear optically, allowing observations deep inside the cell using confocal microscopy; this allowed the use of several fluorescent markers of cytoplasmic components to characterise changes in cell organization that occur during germinal vesicle breakdown.

MATERIALS AND METHODS

Bat stars (*Asterina miniata*) were collected at the Bodega Marine Laboratory (Doran Beach, CA). Using a 3.0 mm sample corer (Fine

Science Tools, Foster City, CA), pieces of ovary were removed through a small hole in the dorsal body wall. Oocytes free of follicle cells were obtained by incubating the pieces of ovary in calcium-free sea water on ice, followed by several washes in natural sea water (see Jaffe et al., 1993). Oocyte maturation was stimulated by addition of 1 μM 1-methyladenine (Sigma Chemical Co., St Louis, MO).

Experiments were done at 18-20°C. Oocytes were injected with mercury pipets using methods described elsewhere (Kiehart, 1982; Hiramoto, 1962; Terasaki and Jaffe, 1993).

Fluorescent probes were obtained from Molecular Probes (Eugene, OR). Fluorescein-conjugated 70 kDa dextran and tetramethylrhodamine-conjugated 10 kDa dextran were made as stock solutions of 10 mg/ml in injection buffer (100 mM potassium glutamate, 10 mM HEPES, pH 7). Injections of 1-2.5% of the oocyte volume were made. YOYO-1 was obtained as a stock solution of 1 mM in DMSO. A 100 μM solution was made in injection buffer and 2.5% volume injections were made. YOYO-1 takes relatively long to equilibrate in the oocyte, so experiments were started at least 1 hour after injection. The fluorescence level of YOYO-1 is also lower than that of the fluorescent dextrans, necessitating higher illumination levels. The term DiI refers to either $DiIC_{16}(3)$ (full name: 1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) and $DiIC_{18}(3)$ (full name: 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate). Both dyes stained the oocytes similarly. For injection, a saturated solution of DiI was made in Wesson oil (100% soybean oil; Hunt-Wesson, Inc., Fullerton, CA).

Cytochalasin B was obtained from Sigma Chemical (St Louis, MO) and stored as a stock solution of 1 mg/ml in DMSO.

The experiments were done on three different confocal microscope set ups. In one, the scan head of a Bio-Rad MRC 600 was coupled with an inverted Olympus IMT-2. The data was stored either on the hard disk or on a Sony laser videodisc processor LVS 5000A with recorder LVR 5000A. In another setup, a Bio-Rad MRC 600 was coupled with a Zeiss Axioscope. Images were either recorded on the hard disk or on a Panasonic 2028 OMDR. In the third setup, an Olympus confocal microscope LSM GB200 was coupled with an inverted Olympus IMT-2 similarly equipped as the one described above. Images were stored on the hard disk. Both Bio-Rad confocal microscope setups used a krypton argon laser (with 488 and 568 nm lines), while the Olympus confocal microscope used an argon laser (488 nm line) and a green HeNe laser (540 nm line). The experiments shown in Figs 1-5 were done with the Olympus confocal microscope, with Fig. 1 using an Olympus SPlanApo ×60 NA 1.4 lens and the others using an Olympus DPlanApo ×20 UV NA 0.7 objective lens. Figs 6-8 were done with the Bio-Rad confocal microscope using a Zeiss PlanApo ×40 1.3 NA objective lens on a Zeiss Axioscope.

RESULTS

Transmitted light microscope observations

Fully grown oocytes of the starfish *Asterina miniata* are 180-190 μm in diameter. The oocyte is relatively clear by transmitted light microscopy, and the germinal vesicle is seen as a large oblate spheroid with dimensions of about 60-90 μm. The cytoplasm has a grainy or slightly mottled appearance due to the abundant yolk platelets (1-2 μm in size) while the interior of the germinal vesicle has a clear appearance. The germinal vesicle is located eccentrically, coming to within 5 μm of the plasma membrane at the animal pole where, by definition, the polar bodies are formed (Schroeder, 1985). The centrosomes are often but not always located in this narrow region between the germinal vesicle membrane and plasma membrane (Otto and Schroeder, 1984; Shirai et al., 1990). The germinal vesicle membrane adjacent to the animal pole has large folds in it (Schroeder, 1985). During oocyte maturation, the sharp outline of the germinal vesicle as seen by transmitted light microscopy becomes irregular at about 20-35 minutes after application of 1-methyladenine (the variability is largely a function of the individual animal from which the oocytes are obtained). The wrinkling of the germinal vesicle envelope is the usual reference point for the start of germinal vesicle breakdown (this time is often referred to as GVBD). Within a few minutes of the initial change, the sharp outline disappears, and the clear area of the germinal vesicle region gradually becomes smaller in volume as the yolk platelets and other cytoplasmic components mix with the former germinal vesicle region. By about 40-50 minutes after addition of 1-methyladenine, the cytoplasm of the oocyte appears homogeneous by transmitted light microscopy.

Redistribution of fluorescent dextran

In cultured mammalian cells, fluorescently labeled 10 kDa dextrans penetrate into the nucleus through the nuclear pores, while 70 kDa dextrans do not (Luby-Phelps et al., 1986; Swanson and McNeil, 1987). Similar behavior was seen in immature starfish oocytes; 10 kDa rhodamine-dextran injected into the cytoplasm rapidly spread throughout the cytoplasm and germinal vesicle (Fig. 1A). The average fluorescence intensity is higher in the germinal vesicle than in the cytoplasm. The higher intensity in the germinal vesicle was seen within 2-3 minutes after injection, indicating that the movement across the envelope is rapid. The difference in fluorescence intensity between the cytoplasm and germinal vesicle is at least partially due to dye exclusion by yolk platelets in the cytoplasm; the dye exclusion is particularly evident at higher magnification (Fig. 1B). The 70 kDa fluorescein dextran did not cross the nuclear envelope in immature oocytes, while the cytoplasmic distribution was similar to that seen with 10 kDa rhodamine-dextran.

The 70 kDa fluorescein dextran was used to characterize germinal vesicle breakdown. Two phenomena were noted by time-lapse confocal microscopy of oocytes injected with 70 kDa fluorescein dextran (Fig. 2). First, there was a slow uniform increase in fluorescence of the germinal vesicle lasting for several minutes. Over this period, there was a relatively large net increase in fluorescence in the germinal vesicle (compare Fig. 2a and 2b). At the time corresponding approximately to the start of breakdown by transmitted microscopy (see experiment described in Fig. 7), there was a wave of increased fluorescence that originated at one side and traversed across the germinal vesicle (Fig. 2c-2l). The fluorescence in the germinal vesicle rose rapidly to a level comparable to that seen in the germinal vesicle of immature oocytes injected with 10 kDa dextran (compare Fig. 2l with Fig. 1). The characteristics of this wave entry are consistent with movement through a disruption in the permeability barrier of the double bilayer of the germinal vesicle.

To document the initial slow fluorescence increase before the disruption, the fluorescence intensity level in the germinal vesicle was quantitated. The fluorescence increased for several minutes before the disruption (Fig. 3A). There was no increase in fluorescence in the germinal vesicle in control oocytes that were not exposed to 1-methyladenine (Fig. 3A); this shows that the increase in fluorescence in 1-methyladenine-treated

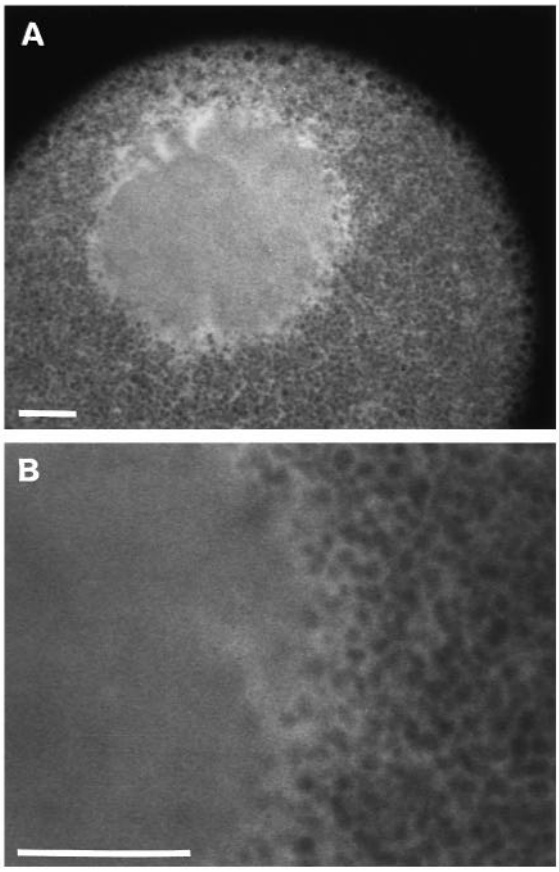

Fig. 1. Confocal fluorescence image of 10 kDa rhodamine dextran in an immature starfish oocyte. After injection, the rhodamine dextran concentration in the oocyte was 250 μg/ml. The oocyte was positioned with its germinal vesicle towards the coverslip, oriented at about a 30° angle from vertical (i.e. the animal pole was towards the coverslip at that angle). The images were obtained with a confocal microscope at a focal plane 30 μm in from the oocyte surface. (A) Lower magnification. The dextran has diffused throughout the cytoplasm and into the germinal vesicle. The average fluorescence intensity in the germinal vesicle is significantly higher than in the cytoplasm. The 10 kDa dextran diffuses rapidly across the nuclear envelope because higher fluorescence intensity in the germinal vesicle was already present in the earliest observations (2-3 minutes post-injection). (B) Higher magnification of same oocyte. At this magnification, it is clear that the yolk platelets exclude the dextran from a large fraction of the cytoplasmic space. This is a major reason for the higher fluorescence intensity in the germinal vesicle compared to the cytoplasm. Bars, 10 μm.

oocytes represents a movement of fluorescent dextran specific to maturation.

The disruption of the germinal vesicle envelope never occurred simultaneously around the entire periphery. Of 34 oocytes (taken from 9 animals), 14 started from a single location (as in Fig. 2), 6 started from a single location followed shortly by a disruption in another location, 6 started from a broader location (one third to one half of the periphery), 7 started from two locations at once, and 1 started from three locations.

The germinal vesicle is eccentrically positioned so that it is closest to the plasma membrane at the animal pole (Schroeder, 1985). The injection chamber contains oocytes oriented in all directions, but it is straightforward to identify oocytes oriented with their animal-vegetal axis parallel to the coverslip, because the focal plane midway through the oocyte is also midway through the germinal vesicle and shows the close apposition to the plasma membrane. In this orientation, the germinal vesicle is seen in side view, and it is possible to determine whether the disruption occurs on the animal half side of the germinal vesicle or the vegetal half. Even though there were several cases with disruptions at two locations, sequential disruptions or broad disruptions, these multiple disruptions all occurred on the same side of the germinal vesicle with the exception of 2 oocytes. The location of the disruption often was in the half of the germinal vesicle towards the animal pole. Of 34 oocytes, the disruption(s) occured 19 times in the animal side of the germinal vesicle, 7 times in the equator, 6 times in the vegetal side, and 2 times simultaneously in locations in the animal and vegetal side. Of the 6 instances of disruption in the vegetal side, 5 came from the same animal.

In the oocyte shown (Fig. 2), the wave front took about 36 seconds to pass across the germinal vesicle distance of 66 μm. The average time for traversal in 6 oocytes was 36±5 seconds.

Redistribution of ribosomes

To observe how another cytoplasmic component redistributes during germinal vesicle breakdown, a dye that probably localizes ribosomes was used. YOYO-1 labels nucleic acids in vitro - it is non-fluorescent when unbound, then when it intercalates into the nucleic acids, it becomes fluorescent (Haugland, 1992). Most of the RNA in the oocyte is rRNA, so that fluorescence in the cytoplasm should represent ribosomes. When injected into starfish oocytes, YOYO-1 labels the nucleolus brightly, the cytoplasm at an intermediate level, and there is a low level of fluorescence in the germinal vesicle. During germinal vesicle breakdown, YOYO-1 entered as a wave (Fig. 4), but in contrast to the fluorescein dextran, there was no slow increase before the wave of entry (Fig. 3B). This was seen in 8 oocytes from 2 animals. In the figure shown, the wave of fluorescence took about 96 seconds to cross the germinal vesicle diameter of 70 μm. The average time for traversal in 6 oocytes was 100±31 seconds. Movement of YOYO-1 is thus slower than the movement of 70 kDa fluorescein dextran, which is consistent with the higher molecular mass of ribosomes (~ 4.2 million daltons).

Redistribution of endoplasmic reticulum

Oocytes were injected with DiI dissolved in oil to label the endoplasmic reticulum (ER) and germinal vesicle membrane (Terasaki and Jaffe, 1991; Jaffe and Terasaki, 1993). The germinal vesicle membrane was seen as a smooth outline with folds near the animal pole (Fig. 5A). Often, though not always in *A. miniata* oocytes, the first change seen is a flattening of the germinal vesicle against the animal pole. This behavior was previously noted in another starfish species, *Pisaster ochraceus*, where the germinal vesicle becomes flattened against the animal pole for a few minutes, then becomes rounder as the breakdown becomes evident by transmitted light microscopy (Stricker and Schatten, 1991). The first change in the germinal vesicle that was always seen was a wrinkling of the smooth boundary of the germinal vesicle (Fig. 5B). For two minutes or so, the outline became less distinct and contracted inward (Fig. 5C). After this, the boundary became brighter and

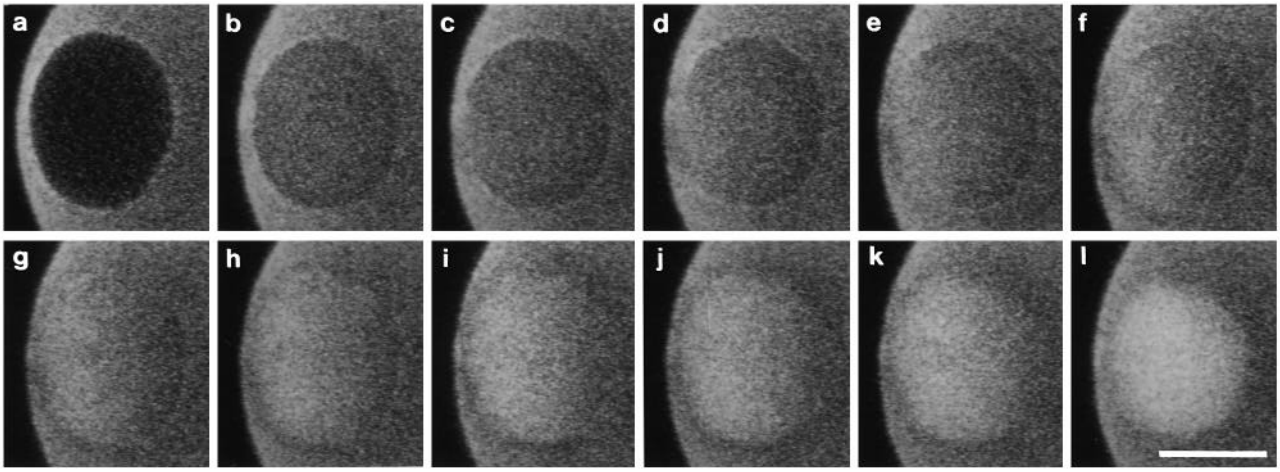

Fig. 2. Distribution of 70 kDa fluorescein dextran during germinal vesicle breakdown. The oocyte was oriented with the animal-vegetal axis parallel to the coverslip. The fluorescein dextran was injected resulting in a final concentration of 100 μg/ml. After 32 minutes for equilibration, 1 μM 1-methyladenine was added. The figure shows images obtained at: (a) 16.0 minutes after 1-methyladenine was added; (b-k) 28.8 to 29.7 minutes in 0.1 minutes intervals; and (l) 31.0 minutes. Images were obtained every 6 seconds from 14.0 minutes after addition of 1-methyladenine. Bar, 10 μm.

more distinct, indicating a change in the organization of ER and germinal vesicle envelope remnants so that the membranes become denser (Fig. 5D). In the next 10 minutes or so, the boundary became thrown into folds and becomes less distinct, as the ER invaded the former region of the germinal vesicle (Fig. 5E,F). About 20 minutes after the breakdown began, the density of ER in the region of the former germinal vesicle was much closer to the density in the rest of the oocyte, and the meiotic apparatus appeared amidst the thick membrane densities near the animal pole (Fig. 5G). In subsequent images, the meiotic apparatus oriented so that it was perpendicular to the plasma membrane and then took part in formation of the first polar body (not shown).

To investigate how the initial changes in nuclear envelope are related to the disruption seen with 70 kDa dextran, double-labeling experiments with DiI and the 70 kDa dextran were done. In 4/4 oocytes from one animal, a change in the germinal vesicle membrane labeled with DiI was observed 10-30 seconds before the wave of fluorescein dextran began (Fig. 6).

It was shown previously that cytochalasin B slows down the mixing of cytoplasm during germinal vesicle breakdown in the starfish *Pisaster ochraceus* (Stricker and Schatten, 1991). Cytochalasin B (1 μg/ml) caused a similar effect on mixing of the ER in *Asterina miniata*. Cytochalasin B did not seem to affect initial stages of germinal vesicle breakdown (i.e. up to the same stage of control oocytes shown in Fig. 5C). However, the movement of ER into the germinal vesicle region slowed down at an intermediate stage so that the mixing was much slower than the control (Fig. 7) (this effect was seen in all oocytes examined in 8 experiments). With longer time (~1.5 hours after the start of germinal vesicle breakdown), the mixing of ER in cytochalasin became more complete.

The ER was observed in time-lapse at high magnification. In control conditions (i.e. no cytochalasin), the ER is relatively stationary in immature oocytes, then starts to move during maturation (L. A. Jaffe and M. Terasaki, unpublished results). The pattern of movement is very similar to that previously described in sea urchin eggs, where the ER undergoes shifting

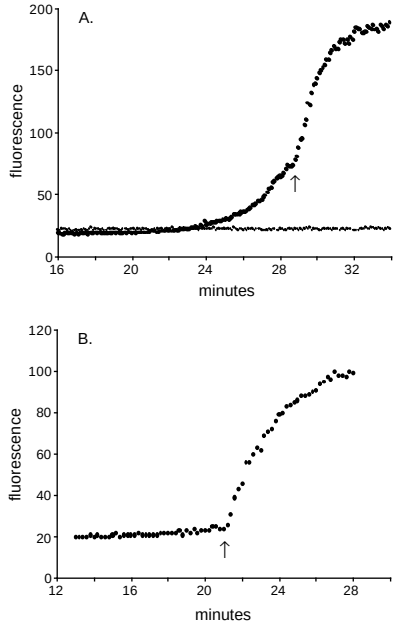

Fig. 3. (A) Graph of fluorescence intensity of 70 kDa fluorescein dextran in the germinal vesicle during maturation. The data was obtained from the series of images shown in Fig. 2, where disruption occurred at about 28.9 minutes (indicated by arrow on the graph). A box approximately 22 μm by 38 μm was positioned inside the germinal vesicle and the fluorescence was quantitated using the confocal microscope software. The control oocyte was injected, then imaged every 6 seconds from 46 minutes after injection, i.e. under the same conditions as the experimental condition without addition of 1-methyladenine. The fluorescence intensity of the cytoplasm was 90-100 (of a maximum of 256). (B) Graph of YOYO-1 fluorescence in the germinal vesicle region during maturation. The data was obtained from the same record shown in Fig. 4, where disruption occurred at about 20.9 minutes (indicated by arrow on the graph). A box approximately 18 μm × 32 μm was positioned inside the germinal vesicle and the fluorescence was quantitated using the confocal microscope software. Compared to the fluorescent dextran, there is no increase in YOYO-1 fluorescence before the wave entry.

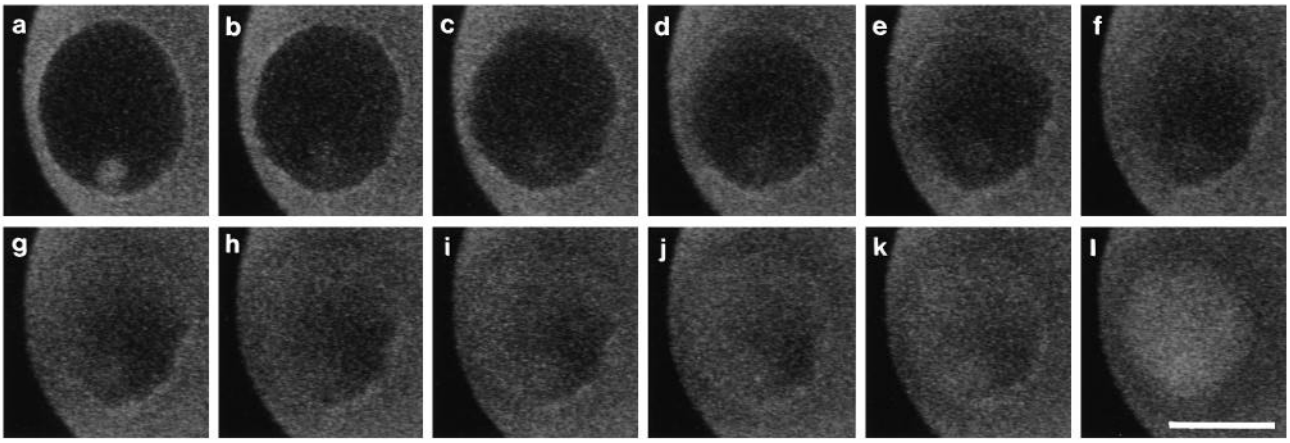

Fig. 4. Distribution of YOYO-1 during germinal vesicle breakdown. YOYO-1 fluorescence probably is mostly due to ribosomes. Final concentration of YOYO-1 after injection was 2.5 μM. The oocyte was oriented with its animal-vegetal axis parallel to the coverslip. The images were taken every 12 seconds from 13.0 minutes after addition of 1 μM 1-methyladenine. The figure shows images taken at: (a) 13.0 minutes; (b-k) 20.8 to 22.6 minutes at 0.2 minutes intervals; and (l) 25.0 minutes. A glancing optical section of the nucleolus is seen in (a), the nucleolus moves out of the optical section in (b). Bar, 10 μm.

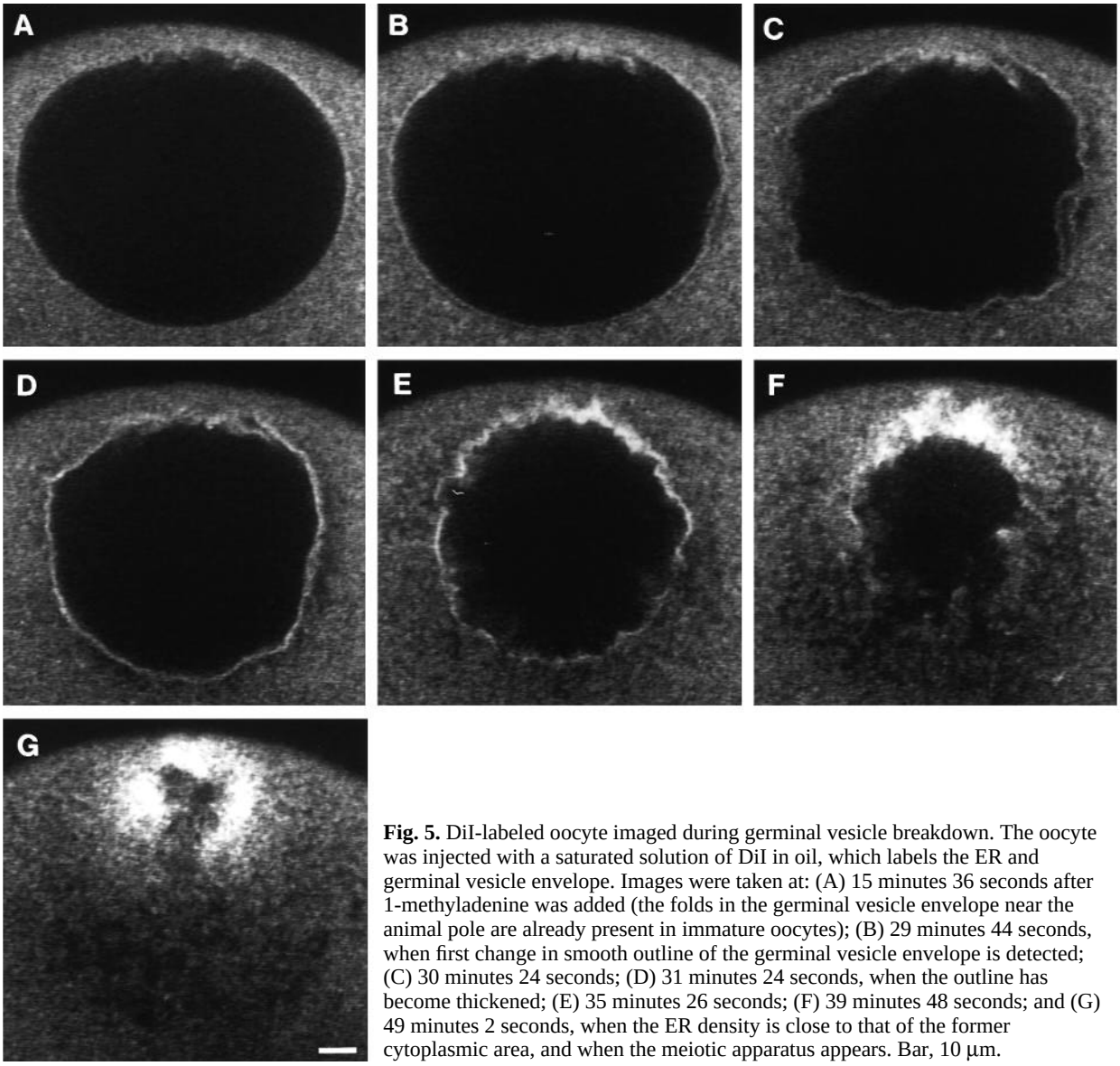

Fig. 5. DiI-labeled oocyte imaged during germinal vesicle breakdown. The oocyte was injected with a saturated solution of DiI in oil, which labels the ER and germinal vesicle envelope. Images were taken at: (A) 15 minutes 36 seconds after 1-methyladenine was added (the folds in the germinal vesicle envelope near the animal pole are already present in immature oocytes); (B) 29 minutes 44 seconds, when first change in smooth outline of the germinal vesicle envelope is detected; (C) 30 minutes 24 seconds; (D) 31 minutes 24 seconds, when the outline has become thickened; (E) 35 minutes 26 seconds; (F) 39 minutes 48 seconds; and (G) 49 minutes 2 seconds, when the ER density is close to that of the former cytoplasmic area, and when the meiotic apparatus appears. Bar, 10 μm.

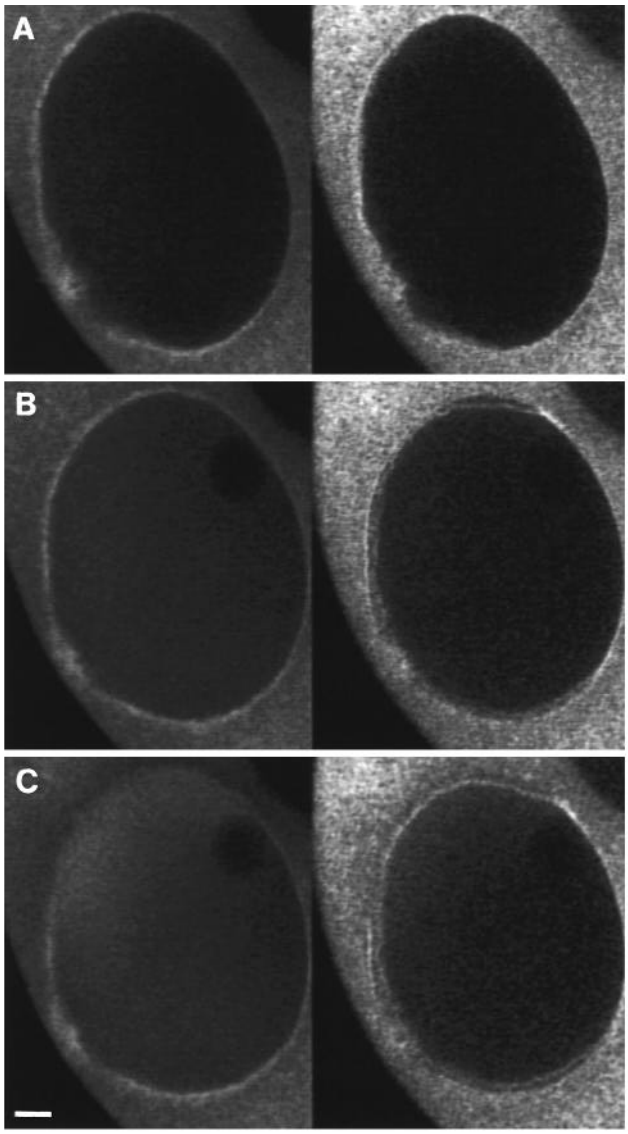

Fig. 6. Double-labeling of DiI- and fluorescein-conjugated 70 kDa dextran. Panels show fluorescein dextran images (left) and DiI images (right). (A) 29 minutes 17 seconds after 1-methyladenine addition, the DiI-labeled germinal vesicle outline is still smooth (right panel). (B) 30 minutes 46 seconds after 1-methyladenine, showing a change in the germinal vesicle outline (right panel) before the wave of fluorescein dextran entry (left panel). (C) 31 minutes 8 seconds, the wave of fluorescein dextran has begun (left panel). The wave comes in from the side rather than the animal or vegetal pole half. Oil droplet is in the upper right corner. The nucleolus appears to exclude fluorescein dextran and is visible as a small dark circle within the germinal vesicle (left panel). Bar, 10 μm.

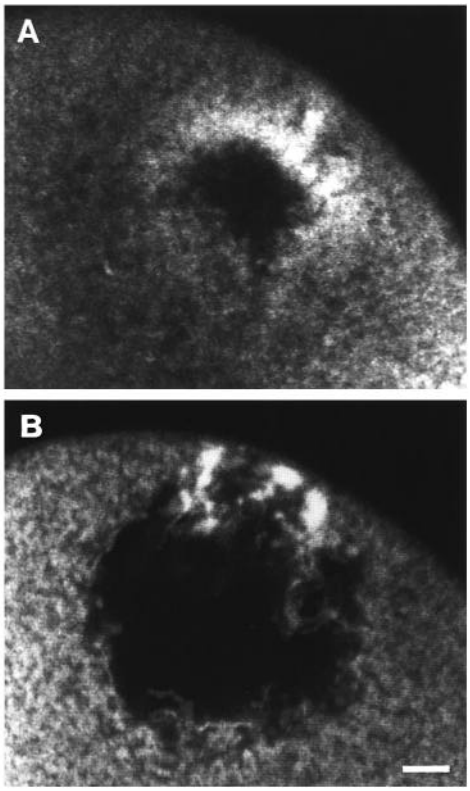

Fig. 7. Inhibition of ER movement into the former germinal vesicle region by cytochalasin. (A) Control oocyte was injected with DiI in oil, then 18 minutes later was changed to 1-methyladenine. This image was taken at 16 minutes 46 seconds after the start of germinal vesicle breakdown. (B) An oocyte, obtained from the same animal as the oocyte shown in A, was injected with DiI in oil, then 13 minutes later was put into 1 μg/ml cytochalasin B. After 5 minutes more, the oocytes were changed to 1-methyladenine. The onset of germinal vesicle breakdown was at 16 minutes; this image was taken 16 minutes 44 seconds afterwards. Bar, 10 μm.

movements in apparently random directions (Terasaki and Jaffe, 1991). In mature eggs treated with cytochalasin observed in time lapse, there was much less movement of the ER. This effect was documented in two ways. In the first way, 10 consecutive scans (3.1 seconds per scan) were averaged from control mature eggs and from cytochalasin B-treated mature eggs (Fig. 8). The averaged image taken from cytochalasin-treated eggs was less blurred, showing that the ER movement was inhibited (11/11 eggs, 3 animals). In the second way, a number for the amount of movement was obtained by using an image processor to form an absolute difference image of two successive images, and then to determine the average intensity of the absolute difference image. By this measurement, the number for the control oocytes was higher than that for the cytochalasin-treated oocyte, giving quantitative documentation of the inhibition of ER movement by cytochalasin (Fig. 9).

The inhibition of movement of ER into the former germinal vesicle region seen at low magnification is likely to be due to this inhibition of movements seen at high magnification. The results suggest that the movement of ER into the germinal vesicle region is an active process that involves actin-dependent movements.

DISCUSSION

The large size of the starfish oocyte germinal vesicle and the relative optical clarity of the oocytes are well suited for studies of changes in organization during germinal vesicle breakdown.

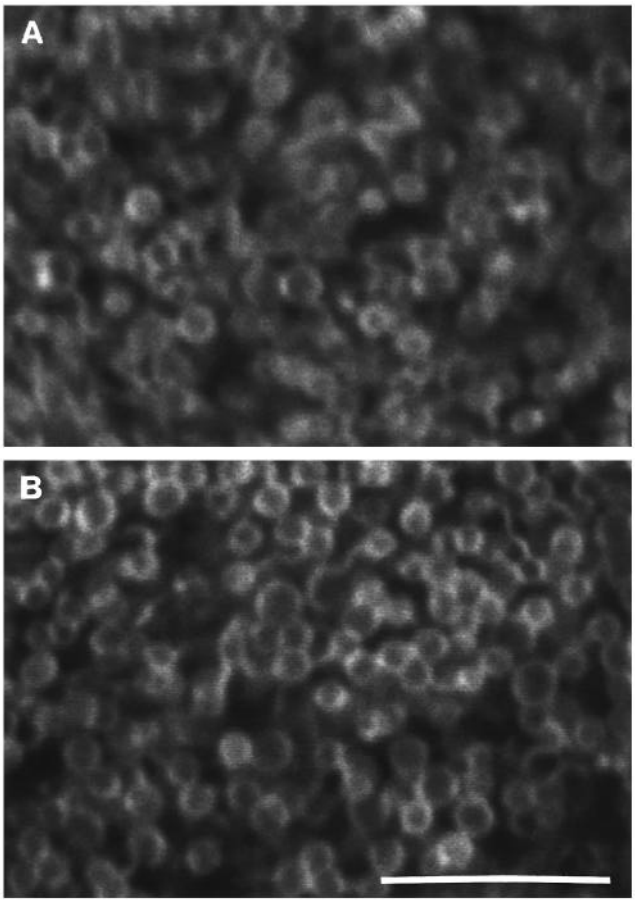

Fig. 8. ER movements at high magnification and their inhibition by cytochalasin B. (A) In control mature eggs, the ER undergoes movements when observed in time lapse at high magnification. In order to document these movements, an average of 10 consecutive scans (3.1 seconds per frame) 8 μm in from the cortex was taken; the ER image is blurred due to the movements occurring during this time. The image was taken 18 minutes after the onset of germinal vesicle breakdown. (B) Cytochalasin B inhibits the ER movements. The oocyte was put into 1 μg/ml cytochalasin B, injected with DiI at 13 minutes, then put into 1-methyladenine at 23 minutes (after oocytes were put into cytochalasin). By transmitted light microscopy, the germinal vesicle began to break down 21 minutes after the change to 1-methyladenine. At 21 minutes after germinal vesicle breakdown, the ER was imaged 8 μm in from the cortex with an average of 10 consecutive scans. The image is less blurred, showing that cytochalasin inhibits ER movements. Bar, 10 μm.

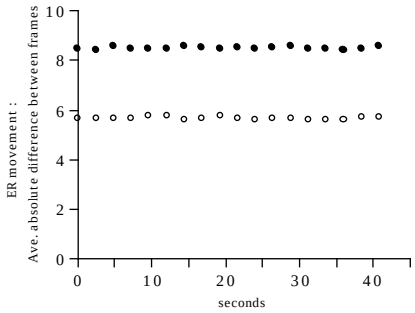

Fig. 9. Quantitative data on the inhibition of ER movements by cytochalasin B. The ER in mature oocytes and in mature oocytes treated with cytochalasin B was labeled with DiI and imaged at similar magnification as shown in Fig. 8. The control oocyte was imaged 44 minutes after 1-methyladenine, while the cytochalasin oocyte was imaged after 36 minutes in 1 μg/ml cytochalasin and 25 minutes after 1-methyladenine. Images were obtained every 2.4 seconds and recorded on an OMDR. As a measurement of how much movement occurred between successive images, the arithmetic difference between successive images was determined. The difference image was made by storing consecutive images in the foreground and background frame buffers in an Image 1/AT image processor (Universal Imaging, West Chester, PA) and then using the absolute difference of foreground and background logic function. This function forms an image consisting of the absolute value of the difference between each corresponding background and foreground pixel. The average intensity of the resulting difference image was determined using the average brightness function. In order to compare control and cytochalasin treated ER, the average intensity of the raw data was determined. The cytochalasin data was approximately 10% higher than the control, so the brightness of each cytochalasin image was reduced accordingly before making the absolute difference calculation. The average intensity of the absolute difference image between consecutive frames was determined over a 43 second period. The higher average absolute difference values for the control oocytes rather than the cytochalasin-treated oocytes indicates that cytochalasin inhibits ER movements.

The use of confocal microscopy along with bright fluorescent probes allowed studies in living oocytes. Three fluorescent probes for cytoplasmic components were used. One probe was a fluorescently labeled dextran, an inert and nontoxic polysaccharide (Luby-Phelps et al., 1986; Swanson and McNeil, 1987). This molecule probably behaves in the same way as soluble proteins of the cytosol. In immature starfish oocytes, 70 kDa dextran did not cross the germinal vesicle boundary while the 10 kDa dextran did cross the boundary. The 70 kDa dextran was used then, as a marker for soluble proteins of the cytoplasm that are too large to pass through the nuclear pores of the immature oocyte. Another fluorescent probe used was YOYO-1 to localize ribosomes. This is the first time that the dye has been used for this purpose. YOYO-1 and its related, parent compound thiazole orange stain DNA as well as RNA, but ribosomal RNA is the predominant nucleic acid species. Both dyes are essentially non-fluorescent until they intercalate into nucleic acids; thiazole orange permeates through the cell membrane while YOYO-1 must be microinjected. Thiazole orange was used previously to stain ribosomes on the cortical ER of isolated sea urchin egg cortices (Terasaki et al., 1991) and, in a preliminary study, to stain ribosomes in cells in culture (Terasaki and Reese, 1991). Thiazole orange has the disadvantage of being reversibly bound to nucleic acids while YOYO-1 is irreversibly bound and may give a brighter signal. The third fluorescent probe for a cytoplasmic component was the long chain dicarbocyanine DiI, which was used to stain the endoplasmic reticulum including the nuclear envelope (Terasaki and Jaffe, 1991; Jaffe and Terasaki, 1993).

By transmitted light microscopy, the first observable change during germinal vesicle breakdown is a wrinkling of the smooth outline of the germinal vesicle. The time at which this occurs is often referred to as the start of germinal vesicle breakdown or simply as GVBD. The 70 kDa dextran, but not ribosomes, begins to enter the germinal vesicle several

minutes before the wrinkling of the germinal vesicle envelope. Then, at the time the wrinkling occurs, there is a massive entry of both dextran and ribosomes into the germinal vesicle as a wave. This entry occurs from a local region rather than from around the entire envelope. This wave entry is probably due to a disruption in the germinal vesicle envelope double bilayer. The evidence for this is the rapid fluorescence increase to a level that corresponds to the level attained by the smaller 10 kDa dextran in immature oocytes (compare Fig. 2l with Fig. 1). If this wave entry is due to a disruption of the bilayer, then it seems likely that the earlier, slow isotropic entry of 70 kDa dextran is through nuclear pores. The nuclear pores normally transport larger molecules only if they have a translocation signal (Gerace, 1992). It is possible that early steps in the disassembly of nuclear pores allow the passage of larger molecules than can normally enter. During this period, the 70 kDa dextran probably does not move as freely through the nuclear pores as the 10 kDa dextran does. The evidence for this is the observation that fluorescence from the smaller dextran becomes higher in the germinal vesicle than in the cytoplasm within 2-3 minutes after injection (Fig. 1) whereas the 70 kDa dextran fluorescence is still lower in the germinal vesicle after 5 minutes (Fig. 2b). Large assemblies, such as ribosomes, which are about 25 nm in size with a molecular mass of 4.2 million daltons, might still be unable to pass freely through the pore during the period before germinal vesicle envelope disruption. This would account for the lack of YOYO-1 fluorescence increase in the germinal vesicle before the wave entry.

These results are likely to have significance for understanding the mechanisms involved in germinal vesicle breakdown. For instance, active $p34^{cdc2}$-cyclinB complex was observed by immunofluorescence studies to enter the germinal vesicle several minutes before the start of germinal vesicle breakdown (Ookata et al., 1992). This complex is in involved in the G_2-M phase transition, and is thought to initiate several changes within the nucleus (Ookata et al., 1992). Since the 70 kDa dextran also begins to enter the germinal vesicle before the start of germinal vesicle breakdown, it seems possible that the ~82 kDa $p34^{cdc2}$-cyclinB complex enters by a non-specific mechanism rather than by specific molecular recognition and transport across the nuclear membrane. If this is the case, it indicates that an alteration in nuclear pore permeability is one of the earliest events in the process of germinal vesicle breakdown.

As shown by the wave entry of dextran and ribosomes, the disruption of the nuclear envelope double bilayer occurs in a local region(s) rather than simultaneously around the entire periphery. It is possible that this is essentially a random process, like the melting of a frozen pond, which does not occur simultaneously over the whole surface but initially in a local region. However, the location of the disruption was from the animal pole side of the germinal vesicle in 26/34 oocytes. It is possible that the location of bilayer disruption is influenced by the location of centrosomes. Since microtubules in immature oocytes have an aster-like distribution centering on the animal pole region, and since two microtubule asters appear in the animal pole region after 1 methyl adenine, it seems likely that the centrosomes are located in the small region between the animal pole and the germinal vesicle (Otto and Schroeder, 1984; Shirai et al., 1990). However, it has also been reported that in some immature oocytes of a population, two astral microtubule arrays are associated with the germinal vesicle membrane away from the animal pole (Otto and Schroeder, 1984). The observed cases of entry from the vegetal pole side might be due to centrosomes located away from the animal pole. To resolve this issue, future experiments should be done to determine the location of centrosomes and germinal vesicle disruption in the same egg. Two possible mechanisms by which centrosomes could affect the location of breakdown are: (1) since the asters begin to grow by 10 minutes after 1-methyladenine (Shirai et al., 1990), microtubules may exert mechanical forces on the germinal vesicle envelope; or (2) activation of enzymes or production of molecular signals in the centrosomal region may diffuse or move along microtubules and cause local disruption of the germinal vesicle membrane.

Both the 70 kDa dextran and ribosomes mix rapidly with the germinal vesicle space in a manner that suggests movement by diffusion following disruption of the nuclear envelope barrier. In contrast, yolk platelets and ER membranes move into the germinal vesicle space much more slowly, and their movements are inhibited by cytochalasin B. In previous studies, it was shown that cytochalasin B inhibits the mixing of cytoplasm without inhibiting the initial breakdown of the germinal vesicle in the starfish *Pisaster ochraceus* (Stricker and Schatten, 1991). These studies used transmitted light microscopy, which primarily detects the presence or absence of yolk platelets in the cytoplasm or nucleus, respectively. By observing DiI-stained ER in *Asterina miniata*, it is now shown that ER is another cytoplasmic component whose mixing is inhibited by cytochalasin B. This effect, seen at low magnification, is paralled by an effect on ER movements that is seen at higher magnification. In time-lapse sequences, the ER is relatively stationary in immature oocytes, but begins to move during maturation (L. A. Jaffe and M. Terasaki, unpublished results). The movements consist of a continuous shifting of position. Cytochalasin B inhibits these movements (Figs 8,9), and it seems likely that this is the basis for its inhibition of the mixing of ER with the germinal vesicle space. Furthermore, it is possible that the function of the normal increase in movements during maturation may be to allow the mixing of the ER with the germinal vesicle. These results show that the ER, as well as yolk platelets, do not move into the germinal vesicle space by diffusion alone, and that the mixing depends on an active process involving actin filaments. Active movements may be required because the continuous membranes of the ER are not able to ‘diffuse’ in the same sense that dextrans can diffuse in the cytoplasm. Alternatively, the nuclear lamina, which remains partially intact after the start of germinal vesicle breakdown (Stricker and Schatten, 1989), may serve as a barrier to movement of ER that must be overcome by the active movements.

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