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Cortical granules of the sea urchin translocate early in oocyte maturation

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SUMMARY

Cortical granules are secretory vesicles poised at the cortex of an egg that, upon stimulation by sperm contact at fertilization, secrete their contents. These contents modify the extracellular environment and block additional sperm from reaching the egg. The role of cortical granules in blocking polyspermy is conserved throughout much of phylogeny. In the sea urchin, cortical granules accumulate throughout the cytoplasm during oogenesis, but in mature eggs the cortical granules are attached to the plasma membrane, having translocated to the cortex at some earlier time. To study the process of cortical granule translocation to the cell surface we have devised a procedure for maturation of sea urchin oocytes in vitro. Using this procedure, we examined the rate of oocyte maturation by observing the movement and breakdown of the germinal vesicle, the formation of polar bodies and the formation of the egg pronucleus. We find that oocyte maturation takes approximately 9 hours in the species used here (*Lytechinus variegatus*), from the earliest indication of maturation (germinal vesicle movement) to formation of a distinct pronucleus. We then observed the translocation of cortical granules in these cells by immunolocalization using a monoclonal antibody to hyalin, a protein packaged specifically in cortical granules. We found that the translocation of cortical granules in in vitro-matured oocytes begins with the movement of the germinal vesicle to the oocyte cell surface, and is 50% complete 1 hour after germinal vesicle breakdown. In the in vitro-matured egg, 99% of the cortical granules are at the cortex, indistinguishable from translocation in oocytes that mature in vivo. We have also found that eggs that mature in vitro are functionally identical to eggs that mature in vivo by four criteria. (1) The matured cells undergo a selective turnover of mRNA encoding cortical granule contents. (2) The newly formed pronucleus begins transcription of histone messages. (3) Cortical granules that translocate in vitro are capable of exocytosis upon activation by the calcium ionophore, A23187. (4) The mature egg is fertilizable and undergoes normal cleavage and development. In vitro oocyte maturation enables us to examine the mechanism of cortical granule translocation and other processes that had previously only been observed in static sections of fixed ovaries.

Key words: cortical granule, oocyte, fertilization, meiotic maturation, *Lytechinus variegatus*

INTRODUCTION

Cortical granule exocytosis is a conserved mechanism in the block to polyspermy at fertilization. Most vertebrates and many invertebrates share this mechanism (Just, 1919; Anderson, 1974; Shapiro et al., 1989; Guraya, 1982). Cortical granules (CG) line the cytoplasmic face of the egg cell surface, and release their contents upon contact by a single sperm. The contents of the cortical granules modify the extracellular environment of the egg to form a biochemical and physical block, which prevents additional sperm from fusing with the egg. When CG exocytosis is inhibited during the fertilization reaction by the use of local anesthetics (Longo and Anderson, 1970; Hylander and Summers, 1981; Decker and Kinsey, 1983) or high pressure (Chase, 1967), the result is a high incidence of polyspermy leading to death of the zygote.

Cortical granule biogenesis begins early in oogenesis in mice and sea urchins, in which it has been most intensely studied. CG contents are believed to transit the Golgi and concentrate in distinct granules, which accumulate throughout the cytoplasm (Anderson, 1968, 1974; Sathananthan et al., 1985). The accumulation of cortical granules in the cytoplasm appears to be linear throughout oogenesis, reaching approximately 8,000 CG in mice (Ducibella et al., 1994) and 15,000 CG in sea urchin (Laidlaw and Wessel, 1994) oocytes. They are morphologically and biochemically distinct from all other secretory vesicles of the oocyte (Anderson, 1974; Shapiro et al., 1989).

Prior to fertilization, cortical granules translocate from the oocyte cytoplasm to the cell surface. In sea urchins, this has been shown both by electron microscopy (Verhey and Moyer, 1967), and functionally by oocytes that are polyspermic because their CG are not yet at the cortex (Runnström and Monné, 1945). In mice (Ducibella et al., 1988, 1994) and starfish (Reimer and Crawford, 1995), cortical granules move to the cortex throughout oogenesis and accumulate at the cell surface. In sea urchins, however, cortical granules translocate to the cell surface in unison sometime prior to formation of the egg (Laidlaw and Wessel, 1994). This translocation appears to be both organelle-selective, since only the cortical granules move to the surface (Verhey and Moyer, 1967), and rapid, because in fixed sections of ovaries containing many stages of oogenesis,

intermediates of cortical granule movement are seldom seen. In sea urchin oocytes, late translocation may be important because, once at the surface, the CG form a complete monolayer of vesicles that would be an impediment to other exocytotic or endocytotic vesicles. For example, the oocyte synthesizes its own vitelline layer and jelly coat late in oogenesis (Harvey, 1956; Niman et al., 1984), so significant exocytosis must occur in the late oocyte. Oocytes also accumulate large stores of yolk, glycogen and other macromolecules by endocytosis throughout oogenesis (Verhey and Moyer, 1967). In starfish and mice the cortical granules are fewer in number, smaller, and do not form a complete lawn like in sea urchins, so perhaps other endocytotis and exocytotic activity continues in these cells in the presence of granules at the cortex.

In this study we show that CG translocate to the cortex early in sea urchin oocyte maturation. We identified this window by developing an in vitro maturation system, which allows us to visualize the maturation process. In addition, we find that oocyte maturation in vitro faithfully replicates other features of in vivo maturation, including the activation of specific genes and selective RNA turnover. In vitro maturation will allow us to explore several aspects of oocyte biology that were not previously accessible.

MATERIALS AND METHODS

In vitro maturation of oocytes

Adult *Lytechinus variegatus* were obtained from Scott's Services and from Sue Decker (both of Miami, FL), and from the Duke University Marine Laboratory (Beaufort, NC).

Females were shed by KCl (0.5 M) injection; ovaries were then removed and minced in calcium- and magnesium-free sea water (CMF; McClay, 1986). After removal of large ovarian fragments by settling, the supernatant containing dissociated somatic cells and oocytes was transferred by pipette to artificial sea water (ASW; Instant Ocean; Aquarium Systems, Mentor, OH) containing 100 μg/ml ampicillin (Sigma, St Louis, MO). A drop of the cell mixture was placed on a slide treated with Gel Slick (AT Biochem, Malvern, PA) and oocytes were isolated by mouth pipette and transferred to either a Kiehart chamber (Kiehart, 1982) or a 96-well Falcon flexible assay plate (Becton, Dickinson, Oxnard, CA) at 22°C, where they matured spontaneously. To test the potential for cortical granule exocytosis, oocytes that matured in vitro overnight were added to ASW containing 10 μg/ml calcium ionophore A23187 (Sigma; stock at 1 mg/ml in DMSO). To minimize damage to the cell surface, the oocytes to be fertilized by sperm were never placed in CMF and were pipetted directly into the assay plate after mincing of the ovary. The oocytes that matured overnight were fertilized with sperm that were acrosome-reacted with egg jelly and diluted in ASW. To determine whether GVBD (germinal vesicle breakdown) could be induced as in starfish, oocytes were placed in 1 μM 1-methyladenine (1-MA; Sigma) in ASW, as reported (Kanatani and Nagahama, 1983).

Visualization of oocytes and immunolocalization of cortical granules

Oocytes and eggs were fixed in 3.7% formaldehyde in ASW for 20 minutes or in ice-cold 100% methanol for 5 minutes. All subsequent incubations and washes were in ASW containing 0.05% Tween 20. Monoclonal antibody 2B7 made to hyalin (G.M. Wessel, L.K. Berg, G. Cannon and D.R. McClay, unpublished data) was used to label cortical granules (1:1 with ASW, 1 hour). Following two washes the monoclonal antibody was detected by incubation for 1 hour in rabbit anti-mouse IgG conjugated to Cy3 (diluted 1:40 in ASW; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Cells were then visualized following washing in ASW. Oocytes were photographed either on a Zeiss Axioplan microscope equipped for epiluminescence or on a Zeiss LSM410 laser scanning confocal microscope. CG translocation was quantitated using high resolution images of oocytes and eggs. To determine the percentage translocation, areas of oocyte approximately 3×6 μm were randomly selected at the cortex and in the cytoplasm (three each); the number of CG within were counted. For each oocyte, the difference between the average number of CG in the cytoplasm and the average number of CG in the cortex was divided by the average number of CG in the cortex. For all oocytes at the same stage, this value was averaged and plotted.

To record the morphology of maturing oocytes, an oocyte in a drop of ASW was photographed at appropriate intervals without a coverslip so as not to interfere with maturation. To visualize chromatin, cells with polar bodies were fixed directly in the 96-well plate with 3.7% formaldehyde in ASW. To label the chromatin, they were then transferred by mouth pipette into a drop of ASW with 0.1 μg/ml Hoechst 33258 (Molecular Probes, Eugene, OR).

In situ RNA hybridization

Oocytes and eggs were fixed in 2% glutaraldehyde and prepared for whole-mount in situ RNA hybridization for hyalin and histone mRNA, as described (Ransick et al., 1993). Digoxigenin-labeled antisense transcripts were synthesized by first linearizing the plasmids with either *Xba*I for hyalin from *Lytechinus variegatus* (clone 6.1; G.M. Wessel, L.K. Berg, G. Cannon and D.R. McClay, unpublished data), or with *Not*I for the tandemly repeated early histones (from *Lytechinus pictus*; Holt and Childs, 1984; subcloned into pBluescript; Stratagene, LaJolla, CA) and then transcribed using T7 RNA polymerase.

RESULTS

The in vitro maturation of a representative, single *L. variegatus* oocyte is shown in Fig. 1. Fig. 1A shows a full-size germinal vesicle-staged oocyte. The first visible sign of oocyte maturation is the migration of the germinal vesicle (GV) to the oocyte cortex (Fig. 1B), which is followed by the breakdown of the GV (Fig. 1C) and the nucleolus (Fig. 1D). Subsequent meiotic divisions result in the formation of one polar body (Fig. 1E), then two (Fig. 1F), and finally in the appearance of the pronucleus (Fig. 1G). We used Hoechst dye to stain the meiotic chromatin in the maturing oocyte to verify the identity of the polar body (Fig. 1H,J). In these cultures approximately 70% of the large oocytes (>100 μm) matured within 56 hours of culture, and they did so spontaneously.

To determine the rate of the maturation process, we cultured populations of full-sized (>100 μm) oocytes and followed the timing of their spontaneous maturation. To define the 'beginning' and 'end' of maturation, we relied on two stages that are apparent by morphology: (1) Germinal-vesicle breakdown (GVBD, when the GV is no longer distinct; Fig. 1C) and (2) pronuclear formation following meiosis (Fig. 1G). Full-size GV-staged oocytes were isolated from a minced ovary and individually placed in a microtiter plate well. At frequent time intervals the stage of maturation was noted. This is summarized in Fig. 2, which shows the cumulative number of oocytes having initiated maturation. The rate at which new oocytes initiate GVBD is relatively constant, and meiosis is complete (as indicated by appearance of the pronucleus) 8-10 hours following GVBD when cultured at 22°C. 1-MA, which stimulates oocyte maturation in starfish, does not significantly change the incidence of GVBD in these sea urchins (data not shown).

To examine the timing of cortical granule translocation,

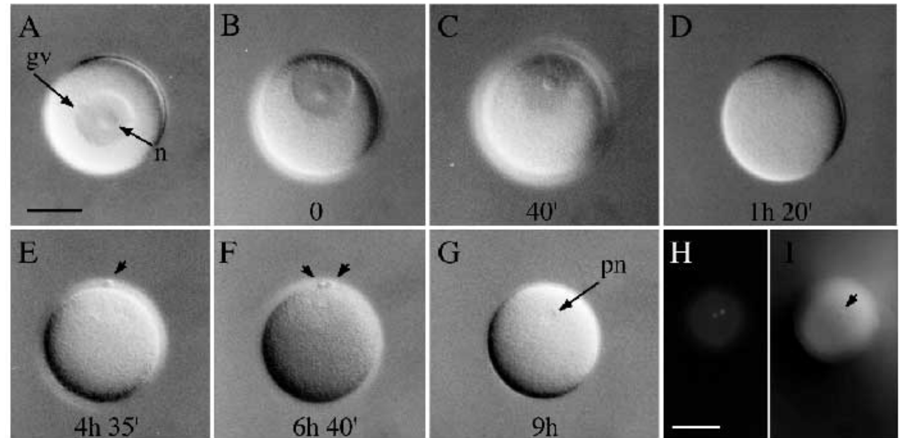

Fig. 1. *L. variegatus* oocyte maturation in vitro. The same oocyte is shown in (B-G) and the timing of its maturation is noted. (A) GV-staged oocyte with large germinal vesicle (gv) and distinct nucleolus (n). (B) GVM; oocyte in which GV has moved to the cortex. (C) Oocyte beginning GVBD (germinal vesicle breakdown). (D) GVBD complete; also note dissolution of nucleolus. (E) First meiotic division complete; note one polar body (arrowhead). (F) Second meiotic division complete; note two polar bodies. (G) Mature egg (100 µm) as indicated by presence of pronucleus (pn, arrow); polar bodies have spontaneously detached. (H and I) Fluorescence and bright field, respectively, of meiotic oocyte with Hoechst-staining chromatin. Bar, 50 µm (A-G), 50 µm (H-I).

oocytes were fixed at three points during in vitro maturation that are easily identified by their morphology under the light microscope: GV (germinal vesicle-staged oocyte; Fig. 1A), GVM (germinal vesicle has moved to the cortex; Fig. 1B) and mature (eggs with a distinct pronucleus; Fig. 1G). In GV oocytes, cortical granules are dispersed evenly throughout the cytoplasm with no accumulation at the cell surface (Fig. 3A). Note the large germinal vesicle void of label. At the time the germinal vesicle has moved to the surface of the cell (Fig. 3B), but before GVBD, approximately 50% of the cortical granules have translocated to the cortex (Fig. 3D). It is difficult to distinguish maturing oocytes prior to GVM in order to determine exactly when CG movements are initiated, due to the lack of a morphological marker prior to GVM, which indicates the begining of maturation, and because the oocytes mature spontaneously. By the time the pronucleus is apparent in these cells, 99% of cortical granules have translocated (Fig. 3D), which is comparable to CG translocation in cells matured in vivo (data not shown). Thus, the machinery for CG translocation is initiated early in maturation, and persists until late in the maturation process. In addition, the result of translocation both in vitro and in vivo is a monolayer of CG at the cortex of the oocyte.

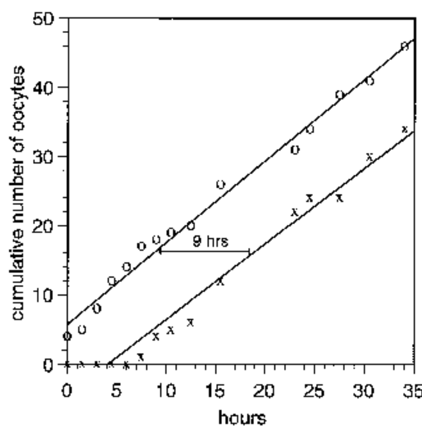

Fig. 2. The rate of the onset of GVBD and the length of maturation. o, the cumulative number of oocytes that began maturation (determined by GVBD, the absence of a distinct germinal vesicle as in Fig. 1C). x, the cumulative number of oocytes that completed maturation (determined by the presence of a pronucleus as in Fig. 1G). The distance on the *x*-axis between the two best-fit lines is the average duration of oocyte maturation, which is about 9 hours.

We also determined whether other features of oocyte maturation occur normally in in vitro cultures. During oogenesis in vivo, mRNAs that encode the proteins of cortical granules accumulate in the cytoplasm of oocytes. The mRNAs of SFE 9, ovoperoxidase, proteoliaisin and hyalin all accumulate during oogenesis to an estimated 0.5-1.0% of the total mRNA population (Laidlaw and Wessel, 1994). However, at some point during oocyte maturation, these mRNAs are degraded so that only low or background levels are detectable (Laidlaw and Wessel, 1994; G.J. LaFleur, M. Laidlaw, J. Harrison, L.K. Berg, S.D. Conner, C. Sommers and G.M. Wessel, unpublished data). In contrast, the early histone genes (H2A, H1, H4 H2B and H3) have an opposite expression pattern. These tandemly arrayed genes are transcriptionally *inactive* in oocytes, but become transcriptionally *active* following the meiotic divisions and pronucleus formation (Venezky et al., 1981). These transcripts remain within the pronucleus until pronuclear breakdown following fertilization.

We tested whether both selective mRNA degradation and selective gene activation occur in the in vitro-matured eggs by using in situ RNA hybridization with digoxigenin-labeled probes. Using anti-sense RNA probes, we found that the hyalin mRNA is undetectable in seven of ten in vitro-matured eggs, which is comparable to that of in vivo-matured eggs (17 of 23; Fig. 4A,B). Conversely, oocytes do not have detectable amounts of histone mRNA, but following maturation in vitro, histone mRNA is readily apparent within the pronucleus of eggs in numbers (12 of 12), comparable to those eggs matured in vivo (four of four; Fig. 4C,D).

Cortical granules are capable of exocytosis following translocation in in vitro-matured oocytes. Although the fertilization envelope does not elevate in oocytes treated by the calcium ionophore A23187, in 51% (15 of 29) of in vitro-matured oocytes activated by A23187, the fertilization envelope rose in varying degrees (Fig. 5A). Although the fertilization envelopes are less uniform than those of activated, freshly shed mature eggs (Fig. 5B), only nine of 21 fertilization envelopes rose in the shed eggs that stayed in culture as long as the in vitro-matured eggs (data not shown). This shows that cortical granules that translocate during in vitro oocyte maturation dock to the plasma membrane appropriately for exocytosis, and that even after extended culture, the vitelline layer is retained or synthesized sufficiently to par-

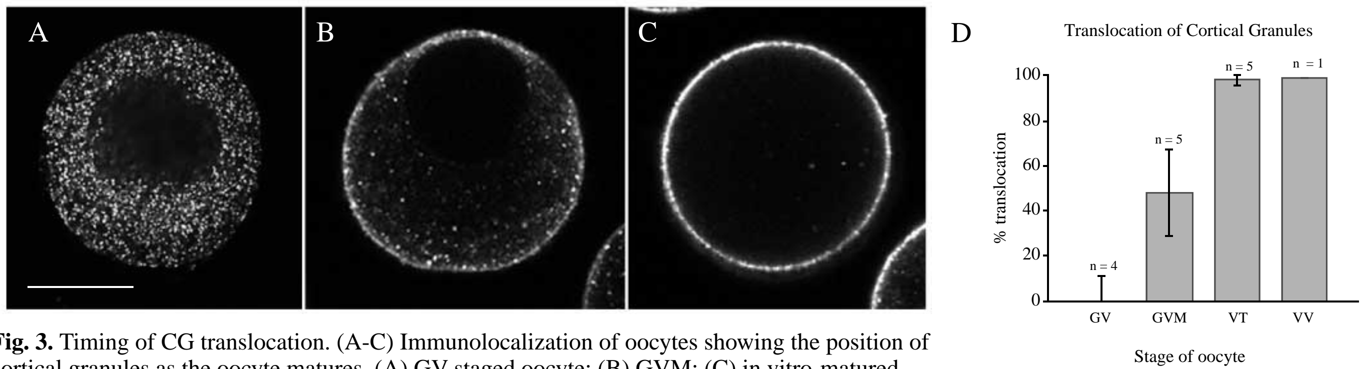

Fig. 3. Timing of CG translocation. (A-C) Immunolocalization of oocytes showing the position of cortical granules as the oocyte matures. (A) GV-staged oocyte; (B) GVM; (C) in vitro-matured oocyte. Cortical granules are labeled with antibody to hyalin. (Bar, 50 μm). (D) Quantitation of CG translocation. Bars indicate average value of *n* oocytes. Error bar indicates ±s.d. GV, oocyte with prominent centrally located germinal vesicle; GVM, oocyte in which the germinal vesicle has moved to the cortex; VT, in vitro-matured egg; VV, in vivo-matured egg.

ticipate in the formation of the fertilization envelope. In vitro- and in vivo-matured oocytes that have been in CMF for an extended period and/or have been transferred several times frequently do not form a fertilization envelope and have a high incidence of polyspermy. We believe that this defect is due to damage to the vitelline layer or to the plasma membrane, which prevents complete elevation of the fertilization envelope. As shown in Fig. 5C, in vitro-matured eggs can be fertilized by acrosome-reacted sperm and will develop normally to plutei. The majority of in vitro-matured eggs that are fertilized will cleave and develop normally if the fertilization envelope rises completely, blocking polyspermy. Thus, by all criteria examined, the in vitro-matured oocytes are identical to those that mature in the ovary.

DISCUSSION

We have shown that oocytes from the sea urchin *L. variegatus* can mature in vitro, independently of the somatic accessory cells of the ovary. Maturation in vitro appears identical to that in vivo, by several criteria. First, the oocytes undergo meiotic maturation and subsequently form two polar bodies. Second, cortical granule translocation is complete (about 99%) and results in a monolayer of vesicles at the cell surface. Third, the translocated CG functionally dock at the plasma membrane, as assessed by their ability to exocytose in response to the calcium ionophore, A23187. Fourth, oocytes that mature in vitro are fertilizable and develop normally to plutei. Finally, the cultured oocytes exhibit the same behavior of selective RNA turnover and gene activation at maturation as those cells in vivo. This ability to faithfully replicate the meitotic maturation process in vitro will be valuable for the study of cortical granule biology and exocytosis.

Using oocytes maturing in vitro we have observed that cortical granule translocation from the cytoplasm to the egg cortex has begun by the time the GV migrates to the cell surface. This timing of translocation is consistent with the observations by Monné and Hårde (1951) and McCulloch (1952) in many sea urchin species that eggs will not fertilize normally until some time after the second meiotic division. This poorly defined period has long been referred to as that of 'cytoplasmic maturation' (Runnström and Monné, 1945; Harvey, 1956). Because the block to polyspermy is not in place in oocytes, blebbing occurs at each point a sperm contacts the cell (Runnström and Monné, 1945; Harvey, 1956; Longo, 1978). Following meiosis, the block to polyspermy gradually becomes more effective, but it is only functional after 'cytoplasmic maturation' (Runnström and Monné, 1945). As summarized by Harvey (1956) and Giudice (1973), fertilization, a complete block to polyspermy and proper development of the sea urchin egg are possible only 5 hours after extrusion of the second polar body. Based on these accounts, we initially believed this 'maturation' period might be the time when CG translocation occurs. Instead, as shown here, translocation is largely complete by the end of meiosis, and the 'maturation' time, referred to earlier, might be required for CG to get other docking or exocytosis machinery in place. Ultrastructural analysis of *Arbacia punctulata* by Longo (1978) shows that

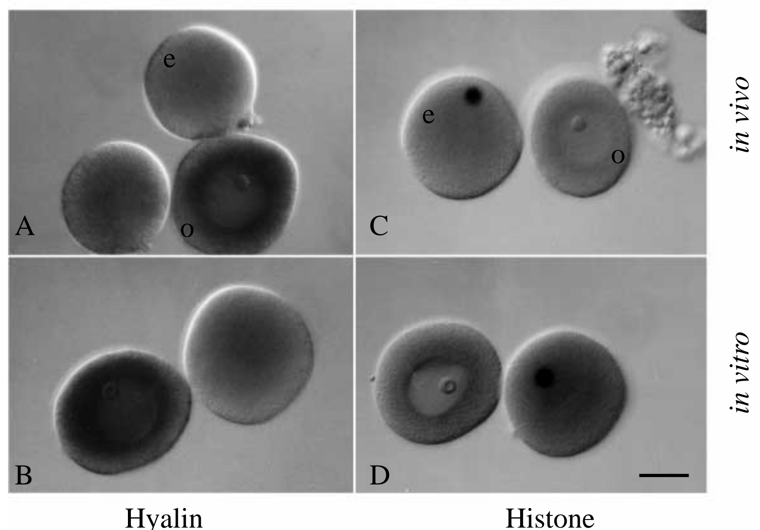

Fig. 4. In situ RNA hybridization showing accumulation of hyalin and histone mRNA. (A,B) Hyalin mRNA is enriched and concentrated around the GV of the oocytes. Hyalin transcripts are largely absent from both in vitro and in vivo-matured eggs. (C,D) Histone transcripts are abundant in the pronucleus of both in vitro- and in vivo-matured eggs, but are not present in oocytes. e, egg; o, oocyte. Bar, 50 μm.

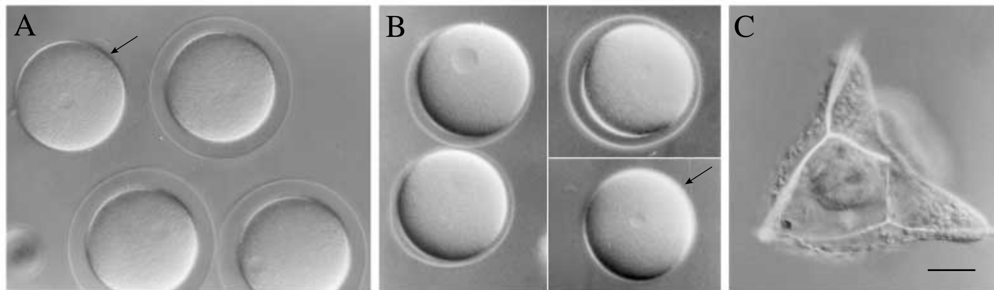

Fig. 5. Activation of eggs with A23187 and pluteus resulting from fertilization of in vitro-matured oocyte. (A) In vivo-matured eggs (immediately after shedding) showing a complete rise of the fertilization envelope. The egg in the upper left corner was not treated with A23187. (B) In vitro-matured oocytes (48 hours after removal from ovary) activated with A23187. Fertilization envelope has risen in three of four eggs. Arrows indicate cells in which fertilization envelopes did not rise. (C) *L. variegatus* pluteus (48 hours), resulting from fertilization of an in vitro-matured egg. The morphology is the same as that of a fertilized egg that has matured in vivo. Bar, 50 µm.

while most CG are at the cortex immediately following meiosis, not all are right at the plasma membrane. The 'maturation' time could also be necessary to establish the signal transduction pathway required to activate the egg and release free calcium from the internal stores within the endoplasmic reticulum (ER; McPherson et al., 1992). In *Xenopus* the ER is absent from the cortex until after polar body emission (Campanella et al., 1984) so alternatively, perhaps the ER of sea urchin eggs is insufficiently 'primed' to regulate calcium fluxes. Immature mouse oocytes cannot undergo CG exocytosis induced by A23187 either (Ducibella et al., 1990), even though many of their cortical granules are at the surface.

During translocation in sea urchin oocytes, nearly 15,000 cortical granules move up to 50 µm through a cytoplasm rich in stored organelles and an extensive ER network (Terasaki and Jaffe, 1991). Because of the size of cortical granules (1 µm) and the speed and distance of their translocation, it is unlikely that diffusion is a mechanism for this movement, since Luby-Phelps et al. (1987) have shown that inert particles greater than 26 nm do not diffuse in a cytoplasm. Under these circumstances, we believe that CG are actively transported to the cell surface, perhaps via cytoskeletal elements. To our knowledge, nothing is known about the mechanism of translocation of cortical granules in any species, but the mechanisms responsible for movement of other vesicles in eggs may provide insight. In the sea urchin *Arbacia punctulata*, pigment granules move to the egg cortex following fertilization, and evidence suggests that this movement is actin-based (Allen et al., 1992). Prior to fertilization, the granules move in a saltatory fashion in random directions. Following fertilization, however, they move directionally to the cortex and this movement is sensitive to cytochalasins (Allen et al., 1992) but not to colchicine (Belanger and Rustad, 1972). Additional support for an actin-based translocation mechanism for CG comes from the observation that spectrin, an actin-binding protein that would provide a means of attachment to microfilaments, is found on CG at the cortex (Bonder et al.,1989; Fishkind et al., 1990a,b) and that the cortex is rich in both filamentous and non-filamentous actin (Bonder et al.,1989; Boyle and Ernst, 1989). Evidence also suggests that CGs at the cell surface are functionally associated with actin, since cytochalasin B causes the normal monolayer of cortical granules at the cell surface to become disorganized (Morton and Nishioka, 1983).

At present, the only suggestion that microtubules may interact with CG comes from the finding that dynamin, a GTPase that binds microtubules, is located on CG (Faire and Bonder, 1993). Alternatively, the cortex of oocytes from sea urchins and starfish have a network of cytokeratin-like filaments, and disappearance of this network in mature eggs (Boyle and Ernst, 1989; Schroeder and Otto, 1991) is temporally correlated with completion of CG translocation Although it is unlikely that these intermediate filaments play a role in the translocation of exocytotic vesicles, they may block CG from reaching the surface prematurely.

Because CG begin translocating to the cortex in sea urchin oocytes at about the same time as does the germinal vesicle, it is possible that they use the same mechanism. Studies of mice, goldfish and frog oocytes all suggest that microtubules (possibly in association with intermediate filaments) maintain the position of the GV in the center of the oocyte; this array is rearranged at maturation, at which point the GV moves to the surface of the oocyte in association with microfilaments (Alexandre et al., 1989; Habibi and Lessman, 1985; Lessman et al., 1988; Lessman, 1987; Lessman and Kessel, 1992; Ryabova et al., 1986). However, in mice and starfish, CG and GV translocations are temporally distinct events. In these oocytes, CG are translocated as they are synthesized throughout oogenesis (Ducibella et al., 1994; Reimer and Crawford, 1995) and the oocyte nucleus does not move to the surface until just prior to meiosis. Thus, were the motors of CG and GV movement the same in these oocytes, additional temporal regulation must be invoked that would not be apparent in sea urchin oocytes.

The oocyte may use two distinct transport systems for cortical granules since, once at the cell surface, the cortical granules adhere to the plasma membrane in a perfect monolayer. No evidence is found of 'double parking' by cortical granules, nor of any empty 'parking spaces'. This suggests that one motor may be used for translocation to the surface, and one for rearrangement once at the surface. We prefer this scenario to one in which each of the (15,000) cortical granules has a predetermined pathway to the surface. We will now be able to address the dynamics of this mechanism using the in vitro maturation protocol following labeling of cortical granules in vitro.

Sea urchin oocytes provide a useful model system for studying CG translocation because there is a mass migration of vesicles during a narrow window of time, oocytes can be microinjected prior to translocation, they mature effortlessly in vitro and they are easily obtained in abundance. The in vitro maturation approach described here will also enable us to study the mechanisms of docking and exocytosis in a system that is synchronous in the stepwise progression for stimulated exocytosis.

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