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Visualization of exocytosis during sea urchin egg fertilization using confocal microscopy

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SUMMARY

A Ca^{2+} wave at fertilization triggers cortical granule exocytosis in sea urchin eggs. New methods for visualizing exocytosis of individual cortical granules were developed using fluorescent probes and confocal microscopy. Electron microscopy previously provided evidence that cortical granule exocytosis results in the formation of long-lived depressions in the cell surface. Fluorescent dextran or ovalbumin in the sea water seemed to label these depressions and appeared by confocal microscopy as disks. FM 1-43, a water-soluble fluorescent dye which labels membranes in contact with the sea water, seemed to label the membrane of these depressions and appeared as rings. In double-labeling experiments, the disk and ring labeling by the two types of fluorescent dyes were coincident to within 0.5 second. The fluorescent labeling is coincident with the disappearance of cortical granules by transmitted light microscopy, demonstrating that the labeling corresponds to cortical granule exocytosis. Fluorescent labeling was simultaneous with an expansion of the space occupied by the cortical granule, and labeling by the fluorescent dextran was found to take 0.1-0.2 second. These results are consistent with, and reinforce the previous electron microscopic evidence for, long-lived depressions formed by exocytosis; in addition, the new methods provide new ways to investigate cortical granule exocytosis in living eggs.

The fluorescence labeling methods were used with the Ca^{2+} indicator Ca Green-dextran to test if Ca^{2+} and cortical granule exocytosis are closely related spatially and temporally. In any given region of the cortex, Ca^{2+} increased relatively slowly. Furthermore, Ca^{2+} began to rise about 8 seconds before the first fluorescent labeling of exocytosis, suggesting that it takes a relatively long time for the Ca^{2+} concentration to reach a threshold value that triggers fusion. No evidence was found for highly localized Ca^{2+} increases that could account for the pattern of individual exocytic events. These results indicate that there is a long lag period between Ca^{2+} rise and cortical granule exocytosis, and that Ca^{2+} and cortical granule exocytosis are not tightly coupled spatially and temporally.

Key words: exocytosis, confocal microscopy, sea urchin egg

INTRODUCTION

During fertilization of sea urchin eggs, a dramatic structural alteration in the egg surface proceeds as a wave from the sperm contact point (Just, 1919). The alteration is due to exocytosis of cortical granules that in turn is triggered by a wave of Ca^{2+} release (Steinhardt et al., 1977; Eisen et al., 1984; Hafner et al., 1988). The cortical granules are present in a dense monolayer underneath the entire cell surface and are about 0.5-1.0 μm in diameter. They contain several molecular species, including mucopolysaccharides, hyalin, proteases, and ovoperoxidase (reviewed by Schuel, 1985; Kay and Shapiro, 1985). Upon fusion, the cortical granule contents interact with the preexisting vitelline envelope to form the fertilization envelope, which acts as a slow block to polyspermy and as protection for the developing embryo.

The evidence that cortical granule exocytosis is initiated by the Ca^{2+} wave is that the Ca^{2+} wave precedes envelope elevation (Eisen et al., 1984; Hamaguchi and Hamaguchi, 1990), Ca^{2+} ionophores cause envelope elevation (Steinhardt and Epel, 1974; Chambers and Hinkley, 1979), injection of Ca^{2+} or agents that release Ca^{2+} cause envelope elevation (Hamaguchi and Hiramoto, 1981; Whitaker and Irvine, 1984; Turner et al., 1986), Ca^{2+} buffers block envelope elevation (Zucker and Steinhardt, 1978; Hamaguchi and Hiramoto, 1981; Mohri and Hamaguchi, 1991; Swann and Whitaker 1986), and the addition of calcium alone to the isolated egg cortex causes fusion of the cortical granules (Vacquier, 1975; Whitaker and Baker, 1983; Vogel et al., 1991).

Although it is well established that Ca^{2+} triggers cortical granule exocytosis, the molecular mechanisms involved are not known. To begin to understand the mechanisms, it would be very useful to simultaneously observe Ca^{2+} concentration and single exocytic events. Here, new techniques using fluorescent dyes and confocal microscopy are developed to observe cortical granule exocytosis, and these methods are used to test how closely exocytosis is coupled spatially and temporally with the Ca^{2+} increase at fertilization. The results indicate that

there is a relatively long lag period between Ca^{2+} rise and cortical granule exocytosis, and that Ca^{2+} and cortical granule exocytosis are not tightly coupled.

MATERIALS AND METHODS

Lytechinus variegatus were collected by Sue Decker (Hollywood, FL) and were kept in an aquarium with artificial sea water. Gametes were obtained by electric shock. Experiments were done at room temperature (22-24°C) using chambers described by Kiehart (1982) (see also Terasaki and Jaffe, 1993). To fertilize the eggs, a 1:1,000 dilution of sperm was added at the entrance of the chamber.

A Bio-Rad MRC 600 laser scanning confocal microscope with a krypton-argon laser coupled with a Zeiss Axioscope was used for microscopy. A Zeiss Planapo 63× 1.4 NA lens was used. The video signal from the confocal scan card was passed through a time date generator (Panasonic WJ 810) and then recorded on a Panasonic 3031F OMDR (optical memory disk recorder). The microscope was set to scan continuously at either the normal or fast 1 rates. The speed of the scan rate was determined by using a stop watch to time 100 scans; the normal scan rate was 1.07 seconds/frame and the fast 1 rate was 0.535 second/frame. To record the images, the record button on the OMDR was pressed manually as the scan reached the bottom of the monitor.

For scanning at more rapid rates, the scan area was set to one quarter size and the fast 2 scan rate was used. With this setting, the scan rate was 0.133 second/frame. A continuous record of the scanned data was recorded on a Sony ED Beta video casette recorder, which was connected to the Bio-Rad video output. Each confocal scan corresponded to 4 video frames, which was advanced using the jog-shuttle dial on the tape recorder.

For data analysis, the output from the tape recorder was captured by a Data Translation QuickCapture frame grabber card in a MacIntosh 2 computer using NIH Image software (from Wayne Rasband, Research Services Branch, NIH, Bethesda, MD).

FM 1-43, rhodamine-dextrans, Texas Red-conjugated ovalbumin, and Ca Green-1 conjugated to 10 kDa dextran were obtained from Molecular Probes (Eugene, OR). Tetramethylrhodamine-labeled dextrans (both 3 kDa and 10 kDa), when dissolved in sea water at 0.3 mg/ml, gave the same results as the Texas Red-ovalbumin. Sea water made hypertonic with dextran (10 kDa, 0.2 osmoles/kg added) inhibits cortical granule swelling (Whitaker and Zimmerberg, 1987) but these concentrations (about 2 g/ml) are about 10,000 times higher than the amounts used for fluorescence imaging. A stock solution of 10 mg/ml Texas Red-ovalbumin was made in PBS. It was diluted to 0.3 mg/ml in sea water. FM 1-43 (molecular mass, 612) was stored as a 2 mM stock solution in methanol at –20°C, and for experiments was diluted to 2 μM in sea water. For double labeling, a 0.2 mg/ml solution of Texas Red-ovalbumin was used with 2 μM FM 1-43 to get a good balance between the two signals. For the double labeling of Ca and exocytosis, eggs were injected with a 5% volume of 1 mM Ca Green-1-dextran (in 100 mM potassium glutamate, 10 mM Hepes, pH 7; cytoplasmic concentration of Ca Green-1-dextran, 50 μM), and 0.3 mg/ml Texas Red-labeled 10 kDa dextran was included in the sea water.

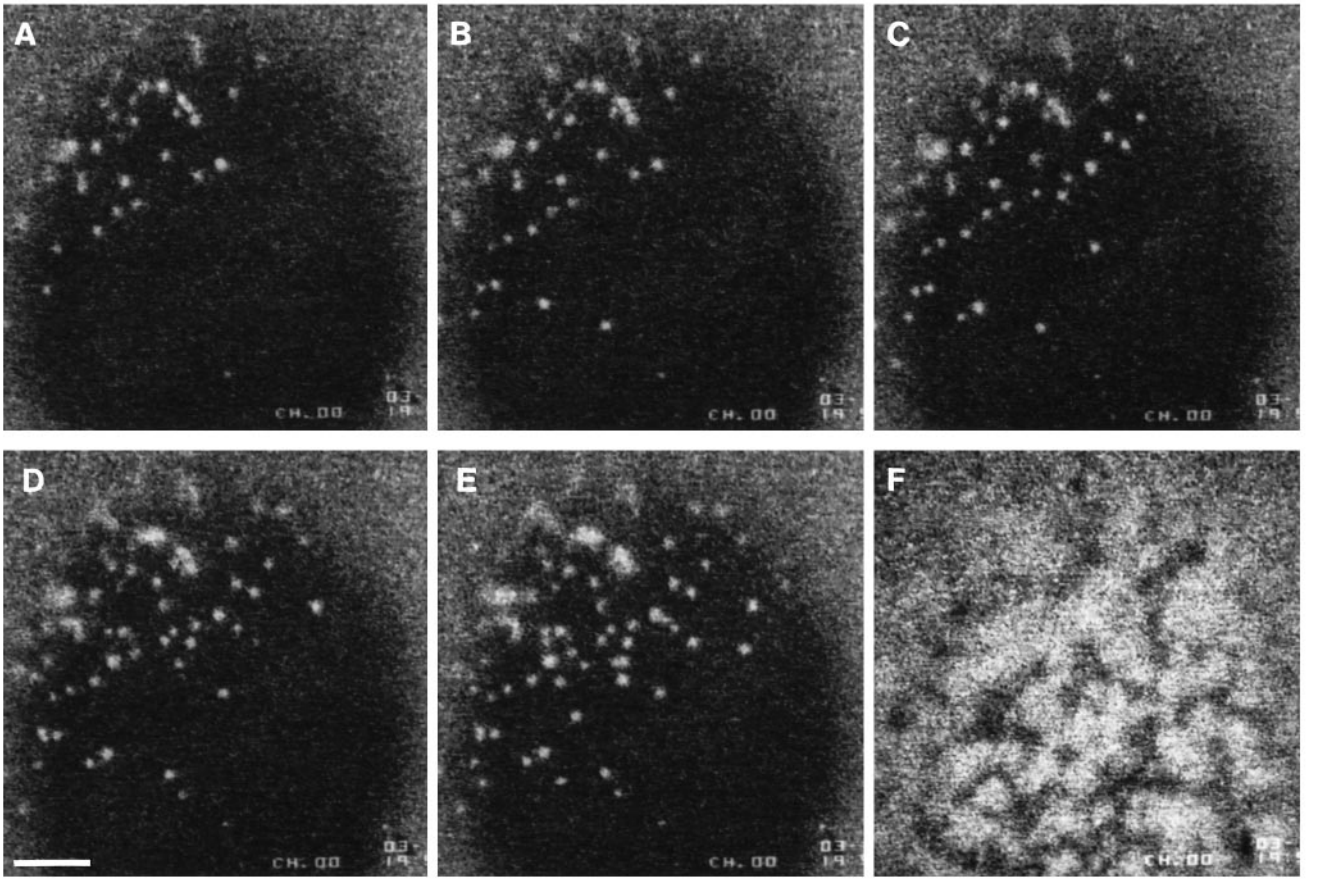


Fig. 1. An egg fertilized in the presence of 0.3 mg/ml Texas Red-ovalbumin in sea water. The microscope objective was focused on the egg surface adjacent to the coverslip. A wave of disks passed across the surface of the egg. The fluorescent marker apparently diffused into depressions in the egg surface formed during cortical granule exocytsosis. Images were obtained every 0.535 second, but only every second image is shown here. (A-E) Successive images, 1.07 seconds apart. (F) Taken 15.9 seconds after last image (E). Bar, 10 μm.

RESULTS

Observations with dextrans and ovalbumin

Eggs from the sea urchin *Lytechinus variegatus* were loaded into observation chambers in the presence of sea water containing inert fluorescent markers (fluorescently conjugated dextran or ovalbumin). The objective lens of a confocal microscope was focused on the surface of the egg adjacent to the coverslip, and the microscope was set to scan continuously at the rate of either ~1 or 2 frames per second. Each completed scan was recorded on an optical memory disk recorder. Several seconds after sperm were added to the chamber, a wave of fluorescent disks passed across the field (Fig. 1). The size of the disks was measured as 1.0 ± 0.1 μm (mean ± s.d., n = 37). The disks continued to appear after the wave of initial disk appearance, so that in any given region, the density of disks increased with time (Fig. 1). This pattern is similar to the pattern of cortical granule disappearance observed by transmitted light microscopy (e.g. see Mohri and Hamaguchi, 1990). About 20-30 seconds after the disks first appeared, the surface had large mottled fluorescent areas (Fig. 1F). A similar wave of disks was observed at fertilization in two other sea urchin species, *Strongylocentrotus purpuratus* (S. Vogel and M. Terasaki, unpublished observations) and *Lytechinus pictus*.

To investigate how rapidly the individual disks appeared, the scan speed was increased to about 8 frames per second. In some instances, faint intermediate fluorescence labeling in one frame preceded brighter labeling at the same location in the next frame (Fig. 2). This suggests that that the time scale for fluorescence labeling is of the order of about 0.1 to 0.2 second.

Observations with FM 1-43

Eggs were also observed in the presence of the fluorescent dye FM 1-43. This water-soluble dye becomes more fluorescent when it is partitioned into a membrane; also, it does not cross membranes (Haugland, 1992; Betz et al., 1992b). During fertilization, there was a similar wave-like appearance, like that seen with the dextrans and ovalbumin, but with two differences. Instead of disks, there were rings and, after a few seconds, dots and lines replaced the rings (Fig. 3). The thickness of the optical section of the confocal microscope under the conditions used was about 0.5-1 μm, and the ring appearance is consistent with staining of the membrane of the former cortical vesicle, where the membrane elements that are perpendicular to the plane of the optical section contribute more signal than the membranes that are parallel. The later dots and lines are consistent with plasma membrane staining of microvilli known to form on the surface after fertilization (Eddy and Shapiro, 1976; Schroeder, 1978). The size of the rings was about 1.2 ± 0.2 μm (n=39). FM 1-43 labeled similar structures during fertilization in eggs of *S. purpuratus* and *L. pictus*.

Texas Red-ovalbumin and FM 1-43 were both put into sea water and imaged simultaneously. In sequences of images taken at ~0.5 second intervals, the disks and rings appeared in the same frame at the same location (Fig. 4). This shows that the two different types of probes are labeling the same structural change in the cell surface.

The cell surface was observed simultaneously with scanning differential interference contrast (DIC) and FM 1-43 at 0.5 second/frame. The appearance of FM 1-43-labeled rings corresponded exactly with the disappearance of cortical granule images in the same location (Fig. 5). Since the disappearance of cortical granules by transmitted light microscopy is thought to be due to exocytosis, this demonstrates that the FM 1-43 labeling is directly associated with exocytosis, rather than endocytosis.

Eggs were injected with a fluorescent dextran (Ca Green-dextran) and imaged simultaneously with rhodamine-dextran in the sea water. The cortical granules excluded the intracellular fluorescent dextran and were clearly visible in negative image. Labeling of cortical granule exocytosis by extracellular dextran corresponded with an enlargement of the negative image (Fig. 6). This indicates that the cortical granule expands during exocytosis.

Relationships of Ca^{2+} and exocytosis

The fluorescent methods allowed an investigation of Ca and single exocytic events. Eggs were injected with the fluorescent Ca^{2+} indicator Ca Green-dextran, and observed simultaneously with Texas Red-labeled dextran in the sea water (Fig. 7). The

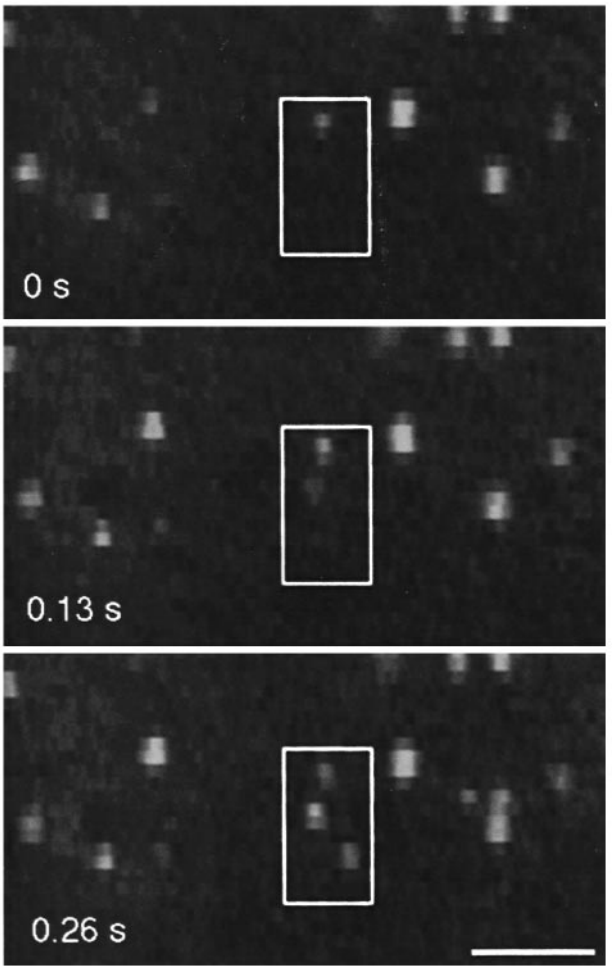


Fig. 2. Rapid scanning of an egg fertilized in the presence of 0.3 mg/ml Texas Red-ovalbumin in sea water. The rate of frame acquisition was 0.134 second/frame. Two disks appeared within the white rectangle during this sequence. The upper disk can been faintly at 0.13 second and more brightly at 0.26 second. This indicates that the time scale for fluorescent ovalbumin to enter the cortical granule is about 0.1 to 0.2 second. Bar, 5 μm.

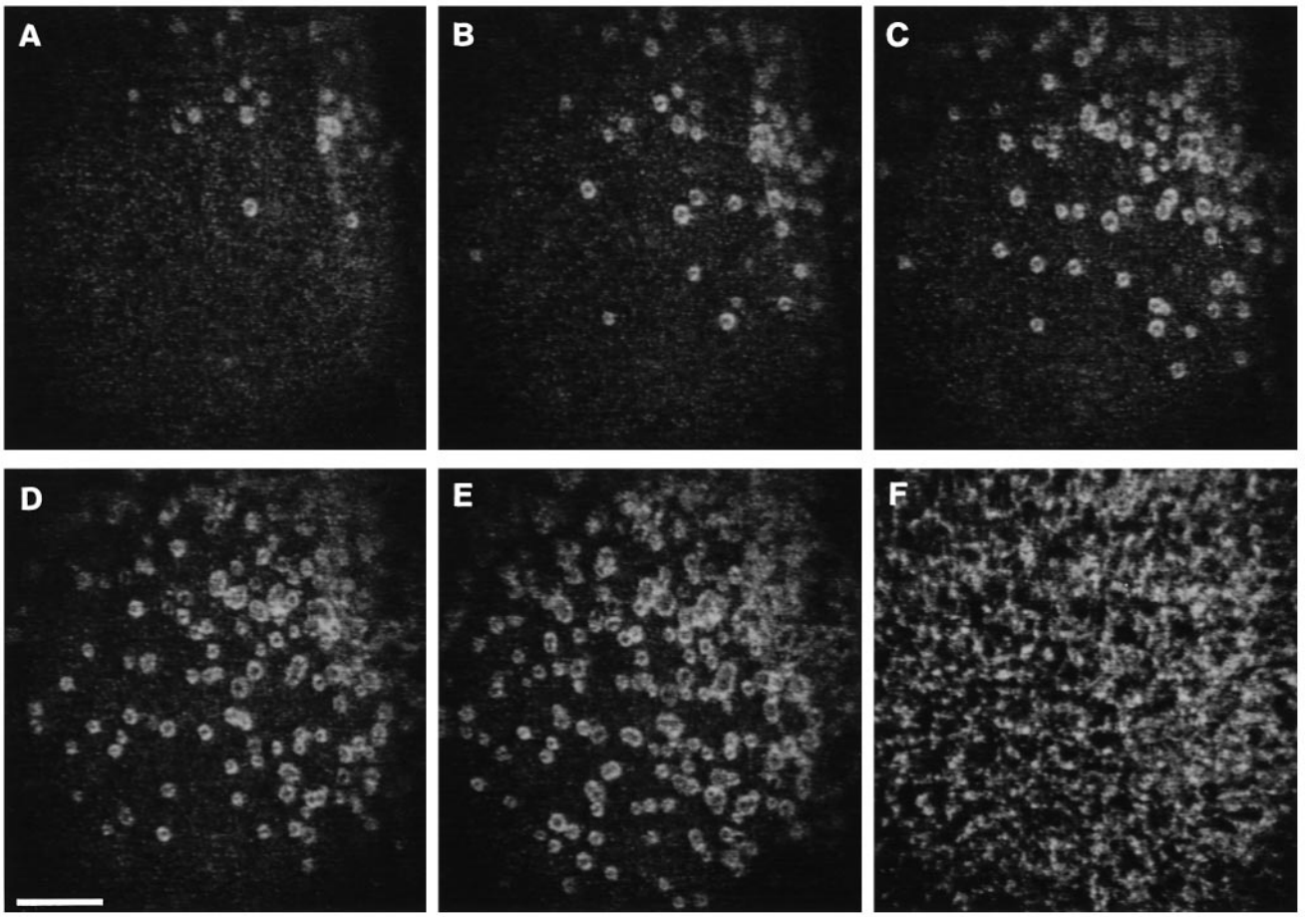


Fig. 3. An egg fertilized in the presence of 2 μM FM 1-43 in sea water. The microscope objective was focused on the egg membrane adjacent to the coverslip. A wave of rings passed across the surface of the egg. This membrane-staining dye apparently labeled the membrane boundary of the depressions in the cell surface and appears as a ring in optical section. (A-E) Successive images obtained at 1.07 second intervals. (F) Taken 23.3 seconds after the last image. Bar, 10 μm.

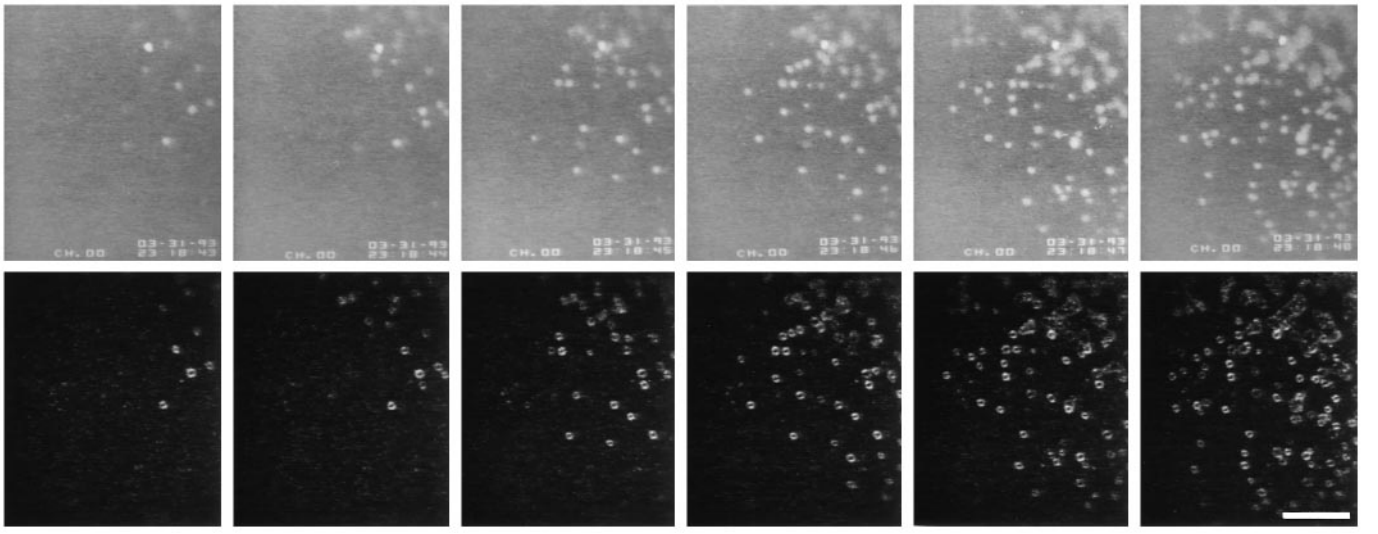


Fig. 4. An egg fertilized in the presence of 0.2 mg/ml Texas Red-ovalbumin and 2 μM FM 1-43. The disk and ring labeling were coincident, showing that the two markers label the same structures. In some of the coincident images, the disks appear to be fully labeled while the FM 1-43 label is dim, then becomes fully labeled in the next image. This suggests that FM 1-43 is slightly slower in labeling the depressions formed during cortical granule exocytosis. The images were obtained at 1.07 second intervals. Bar, 10 μm.

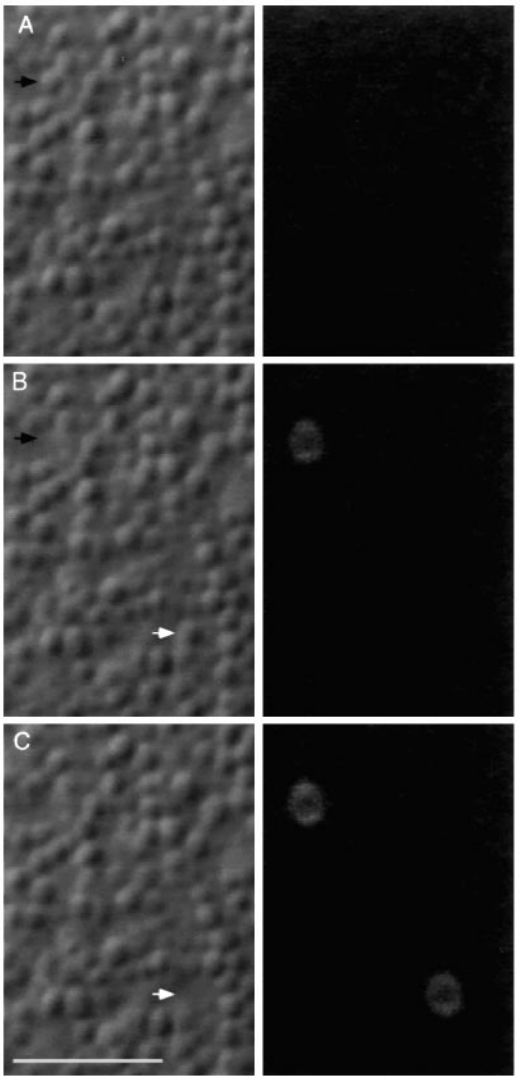


Fig. 5. Simultaneous scanning differential interference contrast and FM 1-43 labeling of exocytosis. The cortical granules were visible in the transmitted light image (left column). A ring appeared in both the middle and lower panels of the FM 1-43 fluorescence images (right column). The appearance of the rings corresponded with the disappearance of the image of the cortical granule in the same location (black and white arrows). Images were obtained at 0.535 second intervals. Bar, 5 μm.

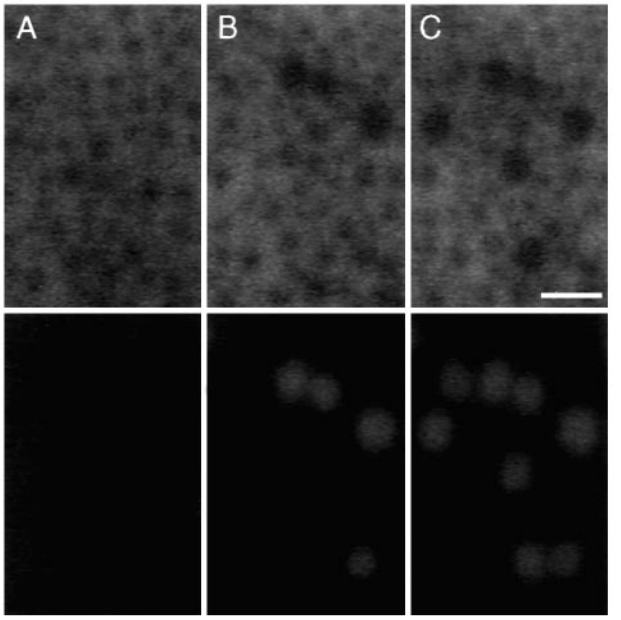


Fig. 6. Expansion of cortical granules at exocytosis. The egg was injected with Ca Green-dextran, which diffused throughout the cytosol. The space occupied by the cortical granules excluded the dye, so that the cortical granules were seen in negative image (upper panels). Rhodamine-dextran was included in the sea water and imaged simultaneously (lower panels) with the Ca Green-dextran during fertilization. When exocytosis of a cortical granule was labeled by rhodamine-dextran, there was a simultaneous enlargement of the negative image. This indicates that the cortical granule expands during exocytosis. Also shown in this figure, there is no localized increase in Ca Green-dextran fluorescence in the region of cortical granules that undergo exocytosis. This is evidence that the apparently random pattern of exocytosis is not due to localized increases in Ca^{2+}. The images were obtained at 1.07 second intervals. Bar, 2 μm.

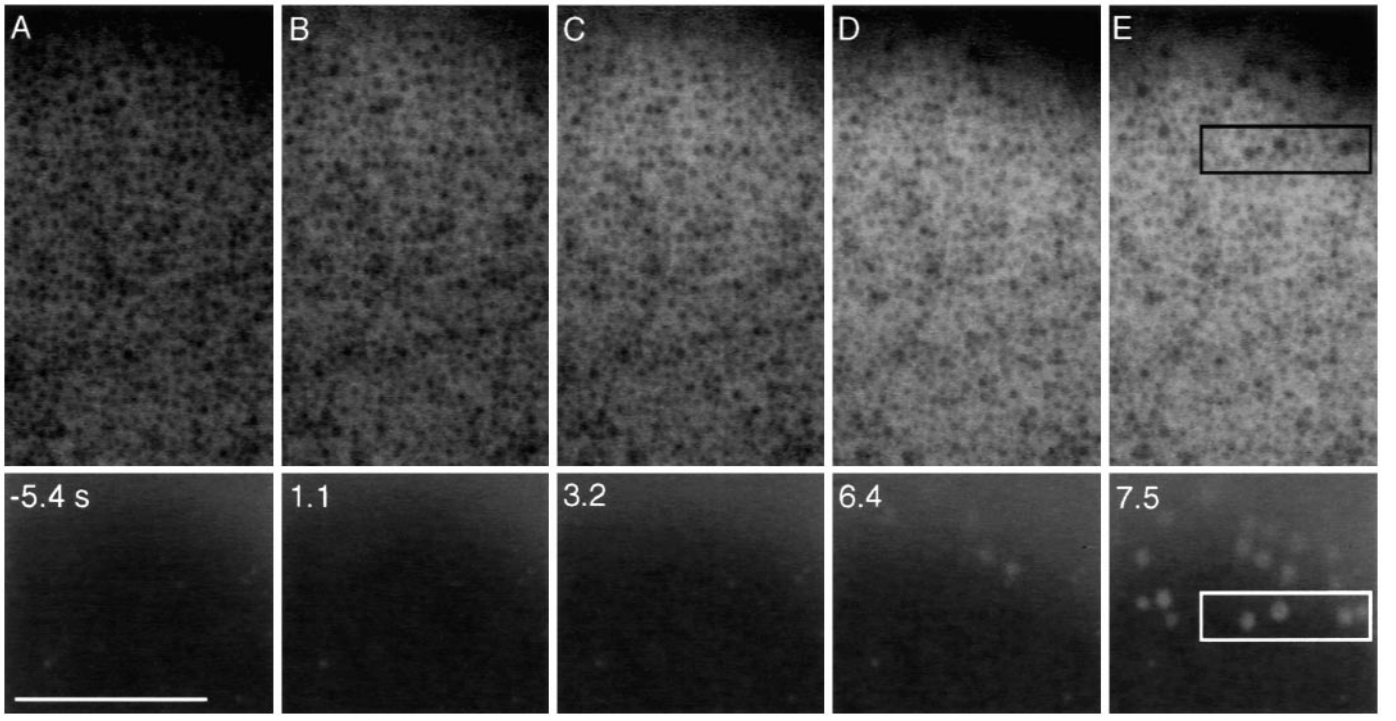
indicator detected the Ca^{2+} wave that passed across the egg surface. At any given location, the Ca^{2+} concentration increased relatively slowly. Also, at any given location, exocytosis of cortical granules began a long time after the first increase in Ca concentration was detected in that region. By monitoring the Ca Green-dextran fluorescence and exocytosis in a small region (e.g. see Fig. 7), the time interval between the first detected increase in Ca indicator fluorescence and the first labeling of exocytosis was 8.3 ± 2.0 seconds (n = 6 eggs).

It is possible that local variations in Ca^{2+} concentration account for the distribution of cortical granule exocytosis. The variations could result from rapid release of Ca^{2+} from local regions of the ER that, for instance, have a higher density of Ca^{2+} release channels. In these recordings, and in recordings at higher magnification (e.g. Fig. 6; n = 2 eggs), there was no apparent local Ca^{2+} increase around cortical granules that underwent exocytosis. This is evidence against the possibility that spatial variations in release of Ca^{2+} from the ER account for the seemingly random distribution of cortical granule exocytosis.

DISCUSSION

The cortical granules were discovered by Moser (1939) using bright-field transmitted light microscopy. At fertilization, the cortical granules disappear, as shown by this and other transmitted light microscopy techniques. The pattern of disappearance is wave-like, but the individual cortical granules seem to disappear in a random manner (e.g. see Mohri and Hamaguchi, 1990). That is to say, there is a considerable variation in the time of disappearance of neighboring cortical granules, so that the pattern resembles that made by raindrops from an advancing storm.

When fertilized eggs are examined by thin-section electron microscopy, deep openings are visible (e.g. see Endo, 1961). Scanning electron microscopy showed depressions in the cell surface that seemed to correspond with cortical granule exocytosis (Eddy and Shapiro, 1976). Depressions were also seen



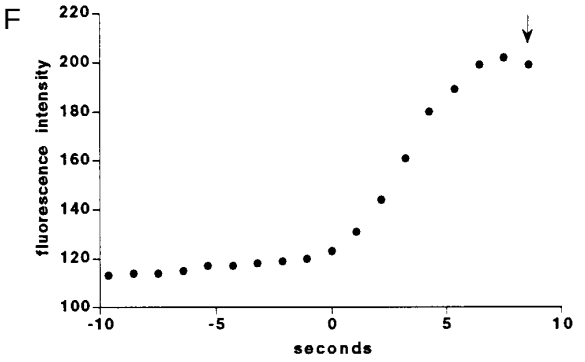


Fig. 7. An egg injected with Ca Green-dextran and fertilized in the presence of 0.3 mg/ml extracellular Texas Red-dextran (10 kDa) to discover the relationship of Ca^{2+} increase (upper panels) and exocytosis (lower panels). The scan rate was 1.07 seconds per frame. The images are at: A, –5.4 seconds, before the Ca began to increase; B, 1.1 seconds, after the first increase in Ca^{2+} was seen at the edge of the egg; C, 3.2 seconds; D, 6.4 seconds, the first labeling of exocytosis occurs; and E, 7.5 seconds; measurements for the graph were made in the rectangle shown (size = 2.5 μm × 9 μm). (F) Graph of fluorescence from the Ca^{2+}-sensitive indicator Ca Green-dextran in the rectangle. 0 second was set as the frame before the fluorescence began to increase. The first labeling of exocytosis in the rectangle was observed at the time indicated by the arrow, which is 7.5 seconds after the first frame in which Ca Green fluorescence increased. The fluorescence starts to decrease at the last time point, but the cytosolic Ca^{2+} has not necessarily reached its maximum. Displacement of dye from the optical section by the expansion of the cortical granules reduces the fluorescence signal so that the measured fluorescence intensity in the rectangle is no longer directly related to the cytosolic Ca^{2+}. Bar (A-E) 10 μm.

in freeze-fracture studies of rapidly frozen eggs (Chandler and Heuser, 1979). At low magnification, there was a wave front consisting of sparse round depressions in the cell surface; behind this wave front was a shallow gradient of increasingly dense events (Chandler and Heuser, 1979).

Exocytosis is a rapid process, and it is not known how well chemical or physical fixation preserves transient structures. However, the depressions seen by electron microscopy suggest that the former boundary of the cortical granule does not immediately flatten out during the process of exocytosis. To reconcile these depressions with the disappearance of cortical granules by light microscopy, it seems that the cortical granules disappear because their contents have dispersed and there is no longer a large refractive index difference between cortical granule and cytoplasm. In hypertonic sea water, the cortical granules remain visible by light microscopy after membrane fusion; this is thought to be because the cortical granule contents do not disperse in these conditions (Zimmerberg and Whitaker, 1985; Whitaker and Zimmerberg, 1987; Chandler et al., 1989; Merkle and Chandler, 1991).

Fluorescent markers dissolved in the sea water are used here as markers for new spaces that become accessible to the extracellular milieu at fertilization. The markers appear to label depressions resulting from cortical granule exocytosis. In the presence of fluorescent dextran or ovalbumin in the sea water, a wave of disks was observed. The disk appearance is consistent with these markers diffusing and filling a depression. In the presence of the membrane probe FM 1-43, a wave of rings passed across the surface. FM 1-43 is a water soluble dye that becomes more fluorescent when it is partitioned into a membrane; also, it does not cross membranes (Haugland, 1992; Betz and Bewick, 1992, 1993; Betz et al., 1992a,b; Ryan et al., 1993). FM 1-43 is likely to diffuse into a depression and then partition into and label the newly formed plasma membrane boundary of the depression where it appears as a ring in optical section. Double-label experiments showed that the membrane probe labeling is coincident to within 0.5 second time resolution with the fluorescent dextran or ovalbumin labeling. The correspondence of FM 1-43 labeling with the disappearance of the cortical granule by transmitted light microscopy is the key

observation for interpreting the fluorescence labeling; this observation demonstrates that the fluorescent probes are labeling a structure associated with exocytosis.

The results are consistent with, and also reinforce the electron microscopic evidence for, long-lasting depressions formed by cortical granule exocytosis. Since the fluorescence methods are used on living cells, they provide additional information, such as the timing for diffusion of extracellular molecules into the depression, a direct comparison of this diffusion with the change in refractive index and expansion of the cortical granule, and in conjunction with Ca^{2+} indicators, information about local Ca^{2+} concentration and single exocytotic events.

These fluorescence methods should be useful tools for characterizing structural changes at the cell surface in living cells. The methods can be used to investigate exocytosis involved in repair of membrane wounds (R. A. Steinhardt, personal communication) and to characterize endocytosis during fertilization (T. Whalley, M. Terasaki and S. S. Vogel, unpublished). By using a photomultiplier tube to quantitate the total fluorescence, FM 1-43 can also be used as a measure of the membrane addition following cortical granule exocytosis (D. Carroll and L. A. Jaffe, personal communication).

Relationship of calcium and exocytosis

It is accepted that Ca^{2+} triggers cortical granule exocytosis, but little is known about how it does so. A simple hypothesis is that Ca^{2+} and exocytosis are tightly coupled spatially and temporally. According to this hypothesis, a certain Ca^{2+} concentration activates the fusion mechanism, which rapidly (within milliseconds) causes exocytosis; it can also be proposed that the triggering Ca^{2+} concentration is the same for all cortical granules, and that the fusion machinery, once activated, takes the same amount of time for all cortical granules.

The fluorescence methods for visualizing exocytosis, in conjunction with the fluorescent calcium indicator Ca Green-dextran, provide the means to investigate whether such a relationship exists. Previous work had shown the Ca^{2+} wave at fertilization as a smoothly graded increase in Ca^{2+} concentration (e.g. see Eisen et al., 1984; Hafner et al., 1988; Hamaguchi and Hamaguchi, 1990; Shen and Buck, 1993). At any given location, the Ca^{2+} concentration rises slowly to reach a maximum level. With the simple relationship of Ca^{2+} and exocytosis, there should be a moving band of synchronized exocytic events. This is not the case, since the pattern of cortical granule exocytosis consists of an initial wave of sparse events, after which cortical granules continue to exocytose for many seconds (Endo, 1952; Chandler and Heuser, 1979; Mohri and Hamaguchi, 1990; Figs 1, 3). It is possible though, that the apparently smoothly graded increase in Ca^{2+} at fertilization observed in previous studies may actually consist of a wave-like progression of highly localized increases in Ca^{2+}. The source of Ca^{2+} is probably the ER, which is present as a tubular network at the cortex (Henson et al., 1989; Terasaki et al., 1991; Terasaki and Jaffe, 1991), and the ER may release Ca^{2+} non-uniformly. A non-uniform pattern of Ca^{2+} increase may have been missed in previous studies using aequorin, a technique that has relatively low resolution, or in those using conventional wide-field microscopy to image fluorescent indicators at relatively low magnification. If there are localized Ca^{2+} increases, a direct relationship would allow us to predict individual exocytic events that correspond to those local Ca^{2+} increases.

With the new fluorescence methods and confocal microscopy, the rise in Ca^{2+} was first detected an average of 8 seconds before the first fluorescence labeling of exocytosis. The Ca^{2+} increases slowly and the lag period suggests that the ER releases Ca^{2+} slowly, so that it takes a relatively long time for the Ca^{2+} concentration to reach a threshold value that triggers fusion. In addition to the lag period, a uniform cytoplasmic increase in Ca^{2+} was found rather than localized increases surrounding exocytotic events. There is a reservation that there may be very localized or rapid transient Ca^{2+} increases that were not detected by the indicator or imaging system used. With this qualification, the results rule out a tight coupling of Ca^{2+} and exocytosis.

One possible explanation for the lack of a tight coupling is that there is variability in the threshold Ca^{2+} concentration that activates the fusion machinery associated with the cortical granules. Since the Ca^{2+} concentration rises slowly, the scattered appearance of exocytic events could be simply due to a random distribution of cortical granules with different sensitivity to Ca^{2+}. Another possible explanation is that the time interval between the Ca^{2+} activation of the fusion machinery and membrane fusion is relatively long. An average time interval of the order of seconds (with variability also of the order of seconds) would give rise to the observed relationship. In support of this, there is evidence for at least one step between Ca^{2+} activation and membrane fusion from experiments with isolated cortex preparations in which lysophosphatidylethanolamine inhibits a process in fusion after Ca^{2+} binding (Vogel et al., 1993). It should be interesting in the future to correlate the accumulating data on Ca^{2+}-triggered fusion on isolated sea urchin egg cortices (Shafi et al., 1994) with experiments in whole eggs that are made possible by these new fluorescence techniques.

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