An increase in surface area is not required for cell division in early sea urchin development

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Abstract

Cell division requires an increase in surface area to volume ratio. During early development, surface area can increase, volume can decrease, or surface topography can be optimized to allow for division. While exocytosis is thought to be essential for division [Mol. Biol. Cell 10 (1999), 2735; Proc. Natl. Acad. Sci. USA 99 (2002), 3633], exocytosis doesn’t always yield an increase in surface area [Proc. Natl. Acad. Sci. USA 79 (1982), 6712]. We used multiphoton laser scanning microscopy, fluorescence spectroscopy, and electron microscopy to monitor membrane trafficking, surface area, volume, and surface topography during early sea urchin development. Despite extensive membrane trafficking monitored by FM 1-43 fluorescence, we find that the net surface area of the embryo does not change prior to the eight-cell stage. During this period, embryo volume decreases by 15%, and microvilli disappear from interior facing membrane segments. Thus, the first three cell divisions utilize residual membrane liberated by decreasing cytoplasmic volume, and reducing microvilli density on interior facing membranes. Only after the eight-cell stage was a net increase in FM 1-43 fluorescence from the embryo surface detected. Our data suggest that compensatory endocytosis is downregulated after this developmental stage to yield an increase in surface area for cell division.

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Introduction

In sea urchin eggs, fertilization causes the release of calcium from intracellular stores (Shen, 1995; Steinhardt et al., 1977). Elevation of the intracellular calcium concentration triggers a massive exocytotic event called the cortical reaction. Approximately 15,000 predocked cortical vesicles fuse with the plasma membrane, secrete their contents, double the egg surface area (Jaffe et al., 1978; McCulloh, 1985), and thus construct the fertilization envelope, a protective barrier around the egg that helps prevent polyspermy (Jaffe and Cross, 1986; Miyake and McNeil, 1998). Within minutes of the cortical reaction, calcium influx through agatoxin-sensitive voltage-gated calcium channels initiates a form of compensatory endocytosis that retrieves the newly inserted cortical vesicle membranes and thus compensates for the increase in cell surface area (Vogel et al., 1999). Because cortical vesicle membrane is rapidly and efficiently retrieved by compensatory endocytosis, the cortical vesicle membrane pool will not be available to allow for the changes in surface area to volume ratio prescribed for the forthcoming cell divisions.

If embryonic surface area increases to allow for cell division, there must be other forms of exocytosis responsible for delivering additional membrane to the cell surface. Alternatively, embryos might use other mechanisms to change their surface area to volume ratio. Fertilization is known to trigger the formation of an axial cortical actin network (Terasaki, 1996) that is involved in transporting endosomes away from the cell surface (Ikebuchi et al., 2001). This same actin network is also thought to bring secretory vesicles to the cell surface following the cortical reaction (Alliegro and McClay, 1988). Microfilaments also play a key role in bringing cortical granules to the cell

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surface prior to fertilization (Wessel et al., 2002). Syntaxin, a protein thought to be required for membrane fusion, is required for cell division (Conner and Wessel, 1999), suggesting that exocytosis is required for cell division. Furthermore, vesicles containing hyaline are targeted to the cleavage furrow, where the hyaline is deposited prior to cell division (Shuster and Burgess, 2002). It is not known whether exocytosis is critical for cell division because the contents of these vesicles are required, or alternatively, vesicular membrane components might be needed for cell division.

We were interested in determining whether other forms of vesicle fusion and endocytosis are occurring during early development, and in learning how the embryo integrates membrane trafficking events to allow for cell division. Our strategy was to use the fluorescent steryl dye FM 1-43 to monitor exocytotic and endocytotic activity from 30 min postfertilization, a time when cortical vesicle exocytosis and their subsequent endocytotic retrieval has reached completion but before the first cell division, to the 32-cell stage. FM 1-43 readily partitions into both aqueous and lipidic environments. Its utility for membrane trafficking studies arises from the fact that FM 1-43 is only fluorescent when in a lipidic environment. Furthermore, FM 1-43 always leaves a membrane from the same leaflet that it enters (Betz et al., 1996). Thus, the outer leaflet of the plasma membrane of embryos placed in a buffer containing FM 1-43 will initially fluoresce (Terasaki, 1995), and the extent of this initial fluorescence should be proportional to the cell surface area. With time, and prolonged incubation in FM 1-43, an increase in fluorescence should be observed if a cell has exocytotic activity.

Materials and methods

Obtaining and handling gametes

Sea urchins were obtained from: Marinus (Long Beach, CA; Strongylocentrotus purpuratus), Charles Hollahan (Santa Barbara, CA; S. purpuratus), and Jennifer M. Keller (Beaufort, NC; Lytechinus variagatus) and maintained in artificial sea water (ASW) in marine aquaria. ASW is routinely prepared by dissolving “Instant Ocean” (Aquarium Systems, Mentor, OH) per the manufacturer’s directions and adjusting the osmolarity to 1000 ± 10 mOsm and pH to 8.0. For experiments, ASW was prepared by using the formula of the Marine Biological Laboratory (Woods Hole, MA): 425 mM NaCl, 9 mM KCl, 9.3 mM CaCl₂, 19.9 mM MgCl₂, 25.5 mM MgSO₄, 2.1 mM NaHCO₃. Eggs and sperm were obtained as previously described (Vogel et al., 1999).

Multiphoton laser scanning microscopy

A Zeiss 510 NLO laser scanning microscope coupled to a Mira Ti: sapphire laser (Coherent, Santa Clara, CA) was used to excite FM1-43 or tetramethylrhodamine fluorescence. Emission from these probes was collected through a 535 to 590-nm band pass filter. Sea urchin eggs were attached to polylysine-treated coverslips and placed in a microscope perfusion chamber. Eggs were fertilized by perfusion with a 1:2000 dilution of sperm.

Assays for measuring FM1-43 fluorescence

Sea urchin eggs were fertilized with sperm (0.5 µl/ml) in a single pool, and aliquots of the fertilized eggs were added to wells of a low fluorescence microtiter dish in triplicate for each time point. FM 1-43 (final concentration of 4 μM) was added to the wells at set intervals to measure changes in the embryo surface area and membrane flux. Fluorescence (excitation: 480, emission 580) was determined immediately (“Surface Area”) and over a 15-min incubation period (“Exocytosis Rate”) on a Molecular Devices (Sunnyvale, CA) Spectramax Gemini XS fluorometer. Cell-stage number was monitored by light microscopy.

Measuring and quantifying embryo volume by multiphoton microscopy

Sea urchin eggs were attached to polylysine (1 mg/ml)-treated slides and placed in a microscope perfusion chamber and fertilized. Then, 1-, 2-, 4-, and 8-cell-stage embryos were perfused with 100 μM tetramethylrhodamine dextran (3000 mol. wt.; Molecular Probes, Eugene, OR) and imaged immediately by multiphoton microscopy. Individual data sets contained approximately 125 one-micron spaced optical sections and were used to measure the tetramethylrhodamine dextran occluded volume of the embryo. The volume for each embryo was measured by counting the number of black voxels (i.e., 3D pixels with no tetramethylrhodamine fluorescence summed over all Z-sections) per embryo.

Electron microscopy and analysis

After reaching various cell number stages, eggs were fixed at 4°C overnight in 1% glutaraldehyde in ASW and postfixed in 1% OsO₄ in MBLASW for 60 min at 4°C. The fixed eggs were washed 5 min each in series of ASW diluted with deionized water solutions of 95, 75, 50, 25, 10, and 0% ASW to gradually reduce the osmolarity, and then they were dehydrated in a graded ethanol series (70–100%) and propylene oxide. The eggs were embedded in Epon. Thin sections (700 Å) were cut and stained with alcoholic uranyl acetate and lead citrate and examined in a JEOL 1010 transmission electron microscope. Digital images were recorded with Advanced Microscopy Techniques Corp. software. (Kodak Megaplus 6.11 camera). The analysis of microvilli density was performed on 2 sets of electron micrographs. In the first, electron micrographs were taken of randomly selected 1-, 2-, 4-, and 8-cell stages at a magnification of 25,000×. Only the external surface of the
plasma membrane (the surface facing the fertilization envelope) was photographed. The number of microvilli attached to the plasma membrane were determined and expressed as microvilli per micron. In the second analysis, electron micrographs were taken of 1-, 2-, 4-, and 8-cell-stage embryos at a magnification of 5000×. The lower magnification was required because the area photographed was selected on the basis of having exterior and juxtaposed (segments where blastomeres appose each other) membrane segments (except in 1-cell stages, which were taken to insure the change in magnification did not affect the analysis). In some cases, in the 4- and 8-cell stages, these micrographs also contained interior plasma membrane segments (segments determined to be interior to the juxtaposed membrane segments in the 4- and 8-cell stages). The length of the plasma membrane segment (corrected for magnification) and the number of microvilli attached to the plasma membrane were determined and expressed as microvilli per micron. The results of the exterior segments were combined with previous data. The total number of microvilli counted per cell stage per segment analyzed ranged from 91 to 157 in the 1- to 8-cell stages except on interior segments where only 1 or 2 were observed per cell stage.

Results

Multiphoton imaging of FM1-43-labeled embryos

To determine whether there is any change in the distribution of FM 1-43 fluorescence in developing sea urchin embryos, we imaged fertilized eggs in artificial seawater (ASW) containing FM 1-43. Eggs were attached to polylysine-treated glass cover and mounted in a microscope perfusion chamber. Next, eggs were perfused with a 1:2000 dilution of sperm in ASW, and at 30 min postfertilization, eggs were perfused into ASW containing 4 μM FM 1-43. A laser scanning microscope coupled to an ultra-fast modelocked Ti:sapphire laser was used to image FM 1-43 fluorescence in developing sea urchin embryos. A time series of FM 1-43 fluorescence and scanning differential interference contrast (DIC) images were collected over a period spanning the 1-cell to 32-cell stages (Fig. 1). Initially, FM 1-43 fluorescence was observed on the plasma membrane and in the egg cortex. With time, we observed punctate fluorescence throughout the cytoplasm but occluded from nuclei and the blastocele. This suggested that, with prolonged incubation, FM 1-43 is internalized by endocytotic mechanisms. Labelling of the fertilization envelope was also observed, but this staining remained static with time. Both fluorescence and DIC images revealed that there was no obvious perturbation of cell division due to prolonged incubation in FM 1-43. When Ti:sapphire laser excitation was switched from modelocked to a continuous wave mode, FM 1-43 fluorescence disappeared while the DIC image remained (data not shown). This suggests that FM 1-43 fluorescence was excited by a multiphoton event. To verify 2-photon excitation, we plotted the log of excitation power against the log of FM 1-43 fluorescence in a sample of isolated sea urchin egg membranes (data not shown). A line with a slope of 1.7 fit this data over a broad range of laser power. Thus, FM 1-43 excitation at 820 nm is primarily a 2-photon absorption event. To confirm that FM 1-43 is internalized into endosomes during this time period, fertilized eggs were incubated in FM 1-43 between 30 and 60 min postfertilization. Embryos were washed with ASW to remove extracellular FM 1-43 and placed into ASW containing trypan blue to quench avid extracellular FM1-43 fluorescence.
staining of the fertilization envelope. Internalized FM 1-43 fluorescence was imaged by 2-photon microscopy (Fig. 2). Consistent with endocytotic retrieval, numerous small fluorescent subcortical puncta, typically smaller than 1 µm in diameter, were observed.

**Embryo surface area does not change between the 1- and 8-cell stages**

To determine whether there is any net change in embryo surface area during development, we measured the instantaneous FM 1-43 fluorescence of developing sea urchin embryos using a 96-well fluorometer. By measuring FM 1-43 fluorescence within seconds of its addition to embryos, we obviate many of the interpretation difficulties caused by internalization of dye. We fertilized batches of eggs and added FM 1-43 to aliquots at various times during development. Fluorescence was measured within 30 s of FM 1-43 addition (Fig. 3, black filled circles). The instantaneous FM 1-43 fluorescence did not change over a 5-h period spanning the 1-cell to 8-cell stages of development. This suggests that the surface area of the developing embryo does not change during this period. A 42% increase was observed between the 8- and 32-cell stages, indicating an increase in surface area. Tight junctions form after the 8-cell stage (Andreucetti et al., 1987), which might limit FM access to the entire surface of the embryo. Because tight junctions require calcium, we placed embryos in calcium-free ASW to remove this potential barrier (Fig. 3, red filled squares). In calcium-free ASW, a 64% increase in fluorescence was observed between the 8- and 32-cell stages; fluorescence again remained constant between the 1- and 8-cell stages. We conclude that net embryo surface area does not change during the first 3 cell divisions, but increases after the 8-cell stage.

A static instantaneous FM 1-43 signal between the 1-through 8-cell stages could indicate the absence of any exocytotic and endocytotic activity in the embryo. Alternatively, it is possible that the embryo does express exocytotic activity during this period but membrane insertion into the cell surface is compensated for by perfectly matched endocytosis. This seems more plausible because we have already demonstrated that endocytosis does occur during this period. To discriminate between these two possibilities, we monitored the change in FM 1-43 fluorescence for each of the individual points used to measure the instantaneous FM 1-43 signal (Fig. 3, open circles and squares). In these experiments, the change in FM 1-43 fluorescence was measured between the instantaneous FM 1-43 signal and after

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Fig. 2. Eggs (*S. purpuratus*) were perfused with seawater containing 4 µM FM 1-43 at 30 min after fertilization and incubated in FM 1-43 for 30 min at 15°C. Eggs were next washed with seawater to remove any noninternalized FM 1-43. Finally, eggs were perfused into seawater containing trypan blue (1 mg/ml) to quench residual FM 1-43 tightly associated with the surface of the embryo. An optical section of two-photon excited FM 1-43 fluorescence (Green; left panel) and the DIC transmitted image (center panel) were collected with the focal plane set to pass through the equator of the developing embryo. The internalized FM 1-43 was excited with 820 nm mode-locked laser pulses through a Zeiss C-Apochromat 63× 1.2 NA W objective. The right panel shows the FM 1-43 fluorescence merged with the DIC image. Yellow arrow indicates the plasma membrane (PM); blue arrow indicates the fertilization envelope (FE). Size bar is 10 µm.
15-min incubation for each time point. Regardless of which stage embryos were analyzed, or having calcium in the seawater, the FM 1-43 signal always increased by approximately 150 fluorescent units following the addition of the dye. This suggests that the rate of exocytosis was constant over this period, and is indicative of constitutive exocytotic activity. Because the embryonic surface area remained constant through the 8-cell stage, and there is continuous exocytotic activity, we are forced to conclude that membrane added by exocytosis is compensated for by endocytosis. Following the 8-cell stage, surface area increases yet the exocytotic rate remained constant. Thus, the increase in surface area after the 8-cell stage must result from a decrease in endocytotic activity.

Embryo volume decreases during early development

Excess surface area generated after the 8-cell stage can be used for subsequent cell divisions. Because surface area is preserved prior to the 8-cell stage, we expect that either cytoplasmic volume decreases, and/or the embryo surface topography is optimized between the 1- and 8-cell stages to allow for division. Multiphoton microscopy was used to determine whether embryos decrease their volume during early development. Staged embryos were placed in ASW containing the membrane impermeant dye tetramethylrhodamine dextran (TMR-Dex; 3000 mol. wt.). Because the plasma membrane excludes this dye, we used the embryos dye-occluded volume to follow volume changes between

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**Fig. 3. Changes in embryo surface area and exocytotic rate during development.** Sea urchin eggs (*S. purpuratus*) were fertilized in a single pool. Embryos were placed in calcium-free ASW (red) or normal ASW (black) at 60 min postfertilization. At 60-min intervals after fertilization, FM 1-43 was added to aliquots at a final concentration of 4 μM to measure the instantaneous fluorescence. Fluorescence was determined immediately (within 30 s). Fifteen minutes after the addition of FM 1-43, the fluorescence was again determined to calculate the change in fluorescence, an indicator of exocytotic rate. Cell-stage number was monitored by light microscopy. All points are mean ± SEM, n = 6.
the 1- and 8-cell stages. Serial Z-section fluorescent and DIC images were collected and used for 3-D reconstruction (Fig. 4A). In this orthogonal view of 2-cell-stage embryos, TMR-Dex is occluded from the cytoplasmic volume of blastomeres, but penetrates past the fertilization envelope. Dye occluded voxels were counted and normalized mean values plotted as a function of cell stage (Fig. 4B). Embryo volume decreased by 15% over the first 3 cell divisions.

Electron microscopic analysis of microvilli during the first three cell-divisions

The existence of microvilli (MV) on the embryo cell surface (Burgess and Schroeder, 1977; Chandler and Heuser, 1981; Eddy and Shapiro, 1976; Schroeder, 1978) can introduce errors in estimated embryo volume. Furthermore, if MV density decreases with development, membrane used to cover these protrusions may be released for use in cell division. To test whether the size and/or number of MV decreases between the 1- through 8-cell stages, we fixed staged embryos and imaged the exterior surface of blastomeres using transmission electron microscopy (Fig. 5A). MV were observed protruding from the plasma membrane of blastomeres at all stages. Obvious changes in the number and size of MV were not observed. Next, we imaged MV on three unique surfaces of the developing blastomeres. The exterior surface of the plasma membrane facing the fertilization envelope, the surfaces juxtaposed between blastomeres from the 2- through 8-cell stages, and interior segments in 4- and 8-cell embryos facing the void space where the blastocele ultimately forms (Fig. 5B). While the size and density of MV did not appear to significantly decrease on the exterior facing and juxtaposed surfaces, they were almost completely absent from interior facing membrane segments. We quantified the density of MV as a function of cell stage on these three different surfaces (Fig. 5C). MV density remained constant on exterior segments (0.38 MV/μm) and was slightly reduced at juxtaposed surfaces (0.33 MV/μm). MV were largely absent from interior facing segments (0.02 MV/μm).

Discussion

Exocytotic activity is thought to provide excess surface membrane to allow developing embryos to undergo cell division (O’Halloran, 2000; Shuster and Burgess, 2002; Straight and Field, 2000). To be utilized towards this end, vesicular membrane inserted into the embryo surface by exocytosis must remain on the surface. While cortical vesicle exocytosis transiently doubles the surface area of the newly fertilized egg (Jaffe et al., 1978), we have shown that newly inserted cortical vesicle membrane is rapidly retrieved into the cytoplasm by compensatory endocytosis (Smith et al., 2000; Vogel et al., 1999; Whalley et al., 1995). Thus, the cortical vesicle membrane pool is not available to allow for the changes in surface area to volume ratio prescribed for forthcoming cell divisions.

Other pools of vesicles are also known to undergo exocytosis at various times following egg activation (Alliegro and McClay, 1988; Chestkov et al., 1998; Shuster and Burgess, 2002). It is not known whether the membranes of these vesicles are also subsequently retrieved by compensatory endocytosis. Multiphoton imaging of FM 1-43 fluorescence and fluorescence spectroscopy of FM 1-43 labeled sea urchin embryos suggests that new membrane is continuously added to the embryo surface at a constant rate between the 1- and 32-cell stages. Imaging also indicates that endocytic uptake of FM 1-43 also occurs. Thus, it is possible that the membrane inserted into the embryo surface
by these forms of exocytosis is also being compensated for by endocytotic membrane retrieval. We measured the instantaneous FM 1-43 fluorescence of sea urchin embryos between the 1- and 32-cell stages to determine if and when a net increase in embryo surface area is detected. We found that the instantaneous FM 1-43 fluorescence remained static between the 1- and 8-cell stages. If FM 1-43 has free access to the entire surface of the embryo during this period, our
data would indicate that the surface area of the embryo does
not change between the 1- and 8-cell stages. Four lines of
reasoning argue that FM 1-43 does indeed have free access
to the embryo surface between the 1- and 8-cell stages.
First, it is known that tight junctions in sea urchins do not
form until after the 8-cell stage (Andreuccetti et al., 1987).
Second, electron microscopy of juxtaposed surfaces of an
8-cell-stage embryo shows that there is ample space be-
tween adjacent blastomeres for dye penetration (Fig. 5).
Third, removing calcium from the seawater, an experimen-
tal manipulation known to break down tight junctions, did
not alter the instantaneous FM1-43 fluorescence between
the 1- and 8-cell stages (Fig. 3). Finally, if dye access was
limited due to cell divisions, we would expect a decrease in
the rate of FM 1-43 labeling with development, and this was
not observed (Fig. 3). Another potential problem is that our
fluorescence assay might not be sensitive enough to detect
changes in fluorescence resulting from changes in embryo
surface area. This too seems unlikely because the same
assay did detect changes after the 8-cell stage, as well as
changes resulting from a 15-min incubation in FM 1-43
containing ASW. Thus, we conclude that the embryo sur-
face area does not change between the 1- and 8-cell stages.

It is surprising to find that the surface area of the embryo
does not increase during the first 3 embryonic cleavages.
Net membrane insertion by vesicle fusion is a simple mech-
anism to increase the embryo surface area to volume ratio
in preparation for cell division. Furthermore, it is known
that exocytotic vesicle fusion does occur in the early stages
of sea urchin development (Alliegro and McClay, 1988;
Shuster and Burgess, 2002). Nonetheless, our experiment
using FM 1-43 to monitor embryo surface area indicates
that only after the 8-cell stage is a net increase in surface
area detected. Because the exocytotic rate was constant
from the 1- to 32-cell stages, we conclude that the mainte-
nance of cell surface area during the first 3 cell divisions is
not caused by a lack of cell growth or exocytotic activity.
Rather, our experiments suggest that cell surface area ho-
meostasis results from a strong coupling of embryonic exo-
cytotic and endocytotic activity. After the 8-cell stage, it
seems that embryos can uncouple exocytosis from compen-
satory endocytosis and thus increase their surface area for
division. One possible explanation for a transition from a
tight to a loose coupling of exocytotic and endocytotic activities is that vesicular membranes inserted into the cell
surface during the first 3 cell divisions might have compo-
nents, such as channels or receptors, whose actions would
be detrimental to development if they remained on the
embryo surface.

Without a net increase in surface area during the first
three cell divisions, it remained unclear how an early em-
byro manages to change its surface area to volume ratio for
cell division. We investigated two possible mechanisms:
first, embryos might decrease their volume, or second, they
might optimize surface features, such as MV structure or
density to provide excess membrane. We found using mul-
tiphone imaging of a membrane impermeant dye, that the
volume of the embryo does decrease by 15% between the 1-
and 8-cell stages. While this is a potential source for excess
surface membrane for cell division, a 15% decrease in
embryo volume was less than the decrease predicted by
modeling (64%), assuming the 1-cell-stage embryo is a
smooth taut sphere and surface area conservation is main-
tained throughout the first 3 divisions. Egg membranes,
however, are not taut or smooth. Thus, it is likely that, in
addition to the 15% volume decrease observed, excess
membrane might be generated by optimization of surface
topography. Using electron microscopy, we found that the
structure and density of MV remain constant on exterior
facing and juxtaposed surfaces of developing embryos. In
contrast, interior facing surfaces of blastomeres, formed
following the second cell division, were devoid of MV.
While two mechanisms, a decrease in embryo volume and a
reorganization of cell surface topography, have been iden-
tified that participate in providing excess surface area for
cell division, further study is required to determine their
relative contributions to the process.

Thus, while exocytosis might deposit vesicle cargo in-
volved in early cytokinesis (Conner and Wessel, 1999;
Shuster and Burgess, 2002), a net increase in surface area
was not observed until after the 8-cell stage. For division,
early embryos utilize residual membrane flaccidity, de-
crease cytoplasmic volume, and reduce MV density on
interior facing membranes.

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