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Calcium-Responsive Contractility During Fertilization in Sea Urchin Eggs

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Abstract

Fertilization triggers a reorganization of oocyte cytoskeleton, and in sea urchins there is a dramatic increase in cortical F-actin. However, the role that myosin II plays during fertilization remains largely unexplored. Myosin II is localized to the cortical cytoskeleton both prior to- and following fertilization, and to examine myosin II contractility in living cells, *Lytechinus pictus* eggs were observed by time-lapse microscopy. Upon sperm binding, a cell surface deflection traversed the egg that was followed- and dependent on the calcium wave. The calcium-dependence of surface contractility could be reproduced in unfertilized eggs, where mobilization of intracellular calcium in unfertilized eggs under compression resulted in a marked contractile response. Lastly, inhibition of myosin II delayed absorption of the fertilization cone, suggesting that myosin II not only responds to the same signals that activate eggs, but also participates in the remodeling of the cortical actomyosin cytoskeleton during the first zygotic cell cycle.

Keywords

fertilization; myosin II; sea urchin; cytoskeleton; calcium

Introduction

Fertilization sets in motion a series of structural and physiological changes in the egg that initiates the developmental program in animal cells. In echinoderm eggs, sperm binding induces a bifurcated signaling cascade that both blocks further sperm binding and reactivates protein synthesis and the cell cycle (Runft et al., 2002). One of the central initiating events following sperm binding in all animal oocytes is the propagation of a calcium wave across the cell that induces cortical granule exocytosis and contributes to the activation of Protein Kinase C (PKC) (Stricker, 1999). And while the exact molecular mechanisms by which the calcium wave is initiated remains an area of active investigation (Kuo et al., 2000; Jaffe et al., 2001; Voronina and Wessel, 2004), it is clear that cytoskeletal remodeling is one downstream consequence of the sperm signal and calcium wave.

The egg cortex in unfertilized echinoderm- and ascidian eggs is comprised of a thin layer of cortical actin filaments, short microvilli, and unpolymerized actin monomers (Schroeder, 1979; Henson and Begg, 1988; Spudich et al., 1988; Bonder et al., 1989; Sardet et al., 2002). Following sperm binding, the egg cortex undergoes a remodeling of the actomyosin cytoskeleton that includes a rapid elongation of microvilli and thickening of the cortical shell (Vacquier, 1981; Bonder and Fishkind, 1995). This remodeling is initiated at the site of

sperm binding where a cone of actin filaments rapidly elongates to engulf the sperm head (Schatten and Schatten, 1980; Tilney and Jaffe, 1980; Cline and Schatten, 1986). A wave of cortical actin polymerization then spreads over the surface of the egg by two minutes post-fertilization (Cline and Schatten, 1986; Yonemura and Mabuchi, 1987). Although actin polymerization follows the calcium wave, the increase in actin polymerization has been closely tied to the rise in intracellular pH that follows PKC activation (Begg and Rebhun, 1979; Carron and Longo, 1982). However, the regulatory molecules that respond to cytoplasmic alkalinization or the potential roles of rho family GTPases such as Rac1 or Cdc42 in modulating actin polymerization have not been identified.

Myosin II (MII) represents one of the most intensely studied contractile proteins, and its regulation during cell division and morphogenesis in nonmuscle cells remains an active area of investigation. However, relatively little is known about myosin's role during fertilization. In ascidian oocytes, a cortical contraction follows the calcium wave across the eggs surface that reorganizes the subcortical cytoplasm and confines the germ plasm into a constricted pole (Sawada and Osanai, 1985; Speksnijder et al., 1990; McDougall et al., 1995; Roegiers et al., 1995; Roegiers et al., 1999). In mouse oocytes, both MIIA and IIB isotypes are expressed in oocytes, and can be found localized in the cortex adjacent to the meiotic spindle, polar body, and fertilization cone following fertilization (Simerly et al., 1998). Further, expression of nonphosphorylatable mutants of myosin regulatory light chain resulted in defects in meiotic progression, fertilization cone retraction and cytokinesis (Simerly et al., 1998). Together, these studies suggest that myosin II activation may represent a very early cytoskeletal-remodeling event during development.

Studies of myosin II in echinoderm eggs have focused primarily on the role of myosin II during cell division. Studies in starfish eggs were the first to define the essential role of nonmuscle MII in force generation during cytokinesis (Mabuchi and Okuno, 1977; Kiehart, 1982). Myosin II contractile activity can be detected in both egg extracts (Kane, 1980; Kane, 1983) and isolated cortices (Walker et al., 1994), and biochemical studies of MII phosphorylation have implicated both heavy chain- and regulatory light chain- based regulation during cell division (Mabuchi and Takano-Ohmuro, 1977; Chou, 1986; Yabkowitz and Burgess, 1987; Larochelle and Epel, 1993; Mishima and Mabuchi, 1996; Totsukawa et al., 1996; Komatsu et al., 1997; Walker et al., 1997; Shuster and Burgess, 1999). However, a role for myosin II during fertilization in echinoderms is less well established. Biophysical studies of eggs during fertilization noted a dramatic rise in cortical stiffness during fertilization (Mitchison and Swann, 1955; Hiramoto, 1970), but these studies could not discriminate whether these changes were due to an increase in cortical contractility or the increase in cortical actin. Similarly, early video analyses of fertilization noted a deformation in the cell surface originating at the point of sperm entry (Hamaguchi and Hiramoto, 1980; Eisen et al., 1984), but it could not be determined whether the surface deflections were an actual contractile event or a secondary effect of the fertilization envelope's separation from the plasma membrane.

Several lines of evidence have implicated a role for myosin II contractility as one of the earliest cytoskeletal remodeling events of development, and we took advantage of the optical clarity, physiological- and physical malleability of sea urchin eggs to further characterize myosin II contractility during fertilization. Time-lapse analysis of fertilization using DIC optics revealed the presence of a sperm-induced contractile wave that was dependent on myosin II activity and the calcium wave. This contractile response could be replicated by mobilizing calcium in unfertilized eggs, suggesting that myosin II-based contractility was directly responsive to calcium. Lastly, inhibition of myosin II activity during- and immediately following fertilization slowed retraction of the fertilization cone. Together, these results suggest that myosin II activation is the initial cytoskeletal remodeling

event during development, and whose activity is required for further cytoskeletal remodeling during the first cell cycle.

Materials and Methods

Reagents

Unless noted otherwise, all chemicals were purchased from Sigma Co (St. Louis, MO). (+/-)-Blebbistatin, Adenophostin A, Ionomycin, U73122, and U73343 were purchased by Calbiochem (La Jolla, CA). Calcium Green dextran (mw 10,000), DMNB-caged fluorescein dextran, NPE-caged Inositol (1,4,5) trisphosphate (IP₃), NPE-caged cyclic ADP-ribose (cADPR), and rhodamine dextran (mw 10,000) were purchased from Molecular Probes (Eugene, OR).

Embryo culture, microinjection and manipulation

The sea urchins *Lytechinus pictus* and *Strongylocentrotus purpuratus* were obtained from Marinus, Inc (Long Beach, CA), and maintained in artificial seawater (ASW) at 15°C. Gametes were obtained by intracoelemic injection of 0.5 M KCl. The egg jelly was removed by washing the eggs in calcium-free seawater and passage through 150 µm Nitex membranes, and in some cases, vitelline envelopes were removed from unfertilized eggs by a brief treatment with 1M Urea or by incubation in ASW containing 10 mM DTT for 10 minutes. For all live cell experiments, a minimum of 10 recordings were taken for each condition.

To visualize changes in cortical contractility, eggs were placed under compression (Yoneda, 1984; Asano and Mabuchi, 2001). Dejellied- and demembranated eggs were placed in protamine sulfate coated, glass-bottomed 35 mm culture dishes (World Precision Instruments, Inc, Sarasota FL), and the seawater was carefully removed and replaced with FC-40 fluorocarbon oil as previously described (Sluder et al., 1999). Cells compressed under in this manner were flattened to a thickness of approximately 15-28 µm, and remained viable for up to 8 hours at 15°C.

For injection experiments, cells were injected using a Parker-Hannifin Picospritzer II pressure injection system prior to fertilization or flattening under FC-40 oil. To visualize calcium transients in living cells, unfertilized eggs were injected with 2 mM Calcium Green dextran (MW 10,000) in injection buffer (10 mM HEPES, 150 mM Potassium aspartate, pH 7.0) to a final concentration of 20 µM. To mobilize calcium in cells under compression, 25 µM DMNB-caged Fluorescein dextran, 2mM NPE-caged Inositol 1,4,5-trisphosphate or 2 mM NPE-caged cADP-ribose (Molecular Probes, Eugene OR) were injected into unfertilized cells in injection buffer in the presence or absence of Calcium Green dextran to image the resultant calcium transient at an injection volume of approximately 0.5% of cell volume. Cells were then flattened under fluorocarbon oil as described above. Ten seconds following the initiation of image acquisition, cells were exposed to a 1 sec pulse of UV light to uncage the injected compounds.

Calcium ionophore treatment

Unfertilized eggs were treated with 1 M urea for ~2 minutes to remove the jelly coat and fertilization envelope, washed in calcium-free seawater (CFSW), and flattened under fluorocarbon oil. 0.1 % DMSO or 50 µM Ionomycin diluted in ASW was loaded into pulled capillary pipettes and a 20-50 pl dose was applied directly to the surface of the flattened cell (Smith et al., 2000).

To detect changes in myosin light chain phosphorylation in response to ionophore treatment, eggs were treated with either 0.05% DMSO or 1 μ M Ionomycin and samples were collected at time points following ionophore addition. Samples were resolved by SDS-PAGE, transferred to Immobilon membranes, and probed with rabbit anti-phospho-Serine19 myosin light chain antibodies (Cell Signaling Technologies, Beverly MA). Blots were stripped and reprobed with monoclonal anti- α tubulin antibodies (Sigma Co, St. Louis MO) as a loading control. Blots were imaged using a Biorad Chemidoc XRS system (Hercules, CA), and bands were quantified using Quantity One- and Image J software.

Imaging acquisition and processing

All live cell experiments were performed on an inverted Carl Zeiss Axiovert 200M DIC- and epifluorescence microscope equipped with a computer-driven Uniblitz (Vincent Associates, Rochester, NY) and optical fluorescence shutters to control bright field and epifluorescence light sources, respectively. The temperature was maintained at 15°C using a Brook Industries heating and cooling stage (Lake Villa, IL). DIC- and fluorescence images were recorded using a 12-bit AxioCam CCD camera driven by Axiovision 4.2 and figures were prepared using ImageJ, and Adobe Photoshop 6.0.1 software.

Immunofluorescence and confocal microscopy

0.1% DMSO carrier control- or blebbistatin-treated eggs were fertilized, stripped of their fertilization envelopes and settled on protamine-coated coverslips. Cells were then fixed and permeabilized in 40 mM PIPES, 1 M glycerol, 5 mM EGTA, 5 mM MgCl₂, 0.5% Triton X-100, and 3.7% formaldehyde pH 6.9 and fixed for 3 hours at 4°C. Cells were then washed, blocked with 3% BSA in PBS, and processed for actin and DNA localization with Alexafluor 546-phalloidin and Hoechst 33342 (Molecular Probes, Eugene, OR), respectively. Images were acquired using an Olympus Fluoview confocal microscope at the Central Microscopy Facility at the Marine Biological Laboratory. To analyze F-actin and myosin organization in *S. purpuratus*, eggs were fertilized, stripped of their fertilization envelopes, and fixed and then permeabilized in according to Wong (Wong et al., 1997). Eggs were then analyzed for F-actin and myosin II with Rhodamine phalloidin and anti-myosin II-(Yabkowitz, 1987) antibodies, respectively. Primary antibodies were detected with Alexafluor-labeled secondary antibodies, and observed by wide-field epifluorescence microscopy.

Results

Myosin II organization in sea urchin eggs

It has been previously demonstrated that myosin II, as well as a light chain kinase activity, are present and active in isolated cortical cytoskeletons from unfertilized eggs (Walker et al., 1994; Walker et al., 1997), and the fraction of cortical myosin II increases significantly by the time of first cleavage (Walker et al., 1994). To examine myosin localization during fertilization, sea urchin eggs were fixed at various times prior to- or following insemination, and processed for actin- and myosin II localization. Unfertilized eggs contain a thin shell of both F-actin, mostly in the form of short microvilli (Figure 1, Panel A). Following sperm binding, a well-characterized wave of actin filament polymerization swept across the egg, resulting in a well-developed cortical shell by three-minutes post-fertilization (Figure 1, Panel B). Myosin II distribution largely mirrored F-actin (Figure 1, Panels C and D), but was not enriched in the growing fertilization cone (not shown). Additionally, there was a population of MII-labeled linear elements beneath the 1 μ m cortical shell and aligned perpendicularly to the membrane (Figure 1, Panel C) that was devoid of F-actin and diminished following fertilization (Figure 1, Panel D). While the exact identity of these

structures was unclear, myosin II localization confirmed earlier biochemical studies indicating that myosin II was present in the cortex prior to fertilization (Walker et al., 1994).

A wave of myosin II contractility follows the calcium wave during fertilization

Classic biophysical studies of the sea urchin egg cortex during fertilization noted a sharp, transient increase in cortical stiffness following sperm binding (Mitchison and Swann, 1955; Hiramoto, 1970). However, from these studies it was unclear whether the observed changes in cortical stiffness resulted from an increase in cortical contractility or from the increase in cortical F-actin that accompanies the increase in cytoplasmic pH (Begg and Rebhun, 1979; Carron and Longo, 1982). To examine sperm-induced surface contractile activity in sea urchins, *Lytechinus pictus* eggs were fertilized and followed by time-lapse microscopy. Within 8 seconds of sperm binding, a slight ripple occurred at the sperm entry point (Figure 2, Panel B, asterisk) that developed into a broad flattening at the site of sperm entry that propagated as a wave across the egg surface (Figure 2, Panel B arrow). When displayed as a kymograph, the contractile wave could be seen not only as a deflection of the surface, but also by the displacement of yolk platelets and pigment granules as the wave progressed across the cell (Figure 2, Panel D, bracket). While the degree of deformation was variable from cell to cell, sperm-induced surface deflections were consistently detected in all cells observed where the site of sperm entry was clearly visible (n= 45).

The deformation of the cell surface traveled across the egg concomitantly with the elevation of the fertilization envelope (Figure 2), but it was unclear whether the alteration of the cell surface was due to a change in the contractile state of the cortex or was a secondary effect of the envelope separating from the plasma membrane. To discriminate between these possibilities, unfertilized eggs were pretreated with Dithiothreitol (DTT) or 1M Urea (not shown) to prevent formation of the fertilization envelope (Figure 3). While the marked flattening of the cell surface observed in eggs with an intact fertilization envelope (Figure 3, Panel D) was not as evident in DTT-treated eggs, a contractile wave was clearly observed traversing the cell (Figure 3, Panel B'). Sperm binding resulted in a contractile wave that initiated at the point of sperm entry (Figure 3, Panel B', black bracket) and traveled across the cell surface, ending in a small ingression of the membrane after the wave had traversed the egg diameter (Figure 3B', white bracket). Similar results were obtained with urea-treated eggs, but urea treatment was less efficient in stripping the fertilization envelope (not shown). To confirm that the contractile wave was due to myosin II, and not some other membrane activity (such as cortical granule release), cells were treated with blebbistatin, a small molecule inhibitor of nonmuscle myosin II ATPase activity (Straight et al., 2003). Recent studies in nonmammalian species revealed that blebbistatin blocks polar body formation in *Spisula* oocytes (Pielak et al., 2004), inhibits scallop- and *Dictyostelium* myosin II (Limouze et al., 2004; Shu et al., 2005), and cytokinesis in sea urchin eggs at the same concentrations that block myosin II in cultured cells (not shown). To inhibit myosin II function during fertilization, eggs were pretreated with 100 μ M blebbistatin for 30 minutes, and the contractile response was monitored following sperm binding (Figure 3, Panels C and C'). Blebbistatin-treated eggs fertilized normally, and in cells not treated with DTT, greater than 92% of eggs had elevated fertilization envelopes (n=300). However, as shown in Figure 3C', minor deflection could be detected following sperm binding, but no surface contractile activity could be detected traversing the cell (n= 15). Together, these results suggest that the deformations observed during fertilization were due to a transient wave of myosin II contractility, and was not a secondary effect of the fertilization envelope separating from the cell membrane.

The early events of fertilization in echinoderm eggs include a rapid membrane depolarization (Jaffe, 1976; Chambers and de Armendi, 1979; Whitaker and Steinhardt, 1983) and a "cortical flash" of free calcium (McDougall et al., 1993; Shen and Buck, 1993),

both of which precede the initiation and propagation of the calcium wave (Jaffe et al., 2001). Earlier descriptions of surface activity ascribed the cell deformation to the membrane depolarization (Eisen et al., 1984). However, the apparent contractile wave traversing the egg closely paralleled the elevation of the fertilization envelope, raising the possibility that the contraction was following the calcium wave. To determine whether there was a causal relationship between the calcium wave and the contractile activity following fertilization, eggs were pretreated with the phospholipase C inhibitor U73122, which has been shown to block the calcium transient in sea urchin eggs (Lee and Shen, 1998; Leckie et al., 2003), its inactive analog U73343, or Blebbistatin. The spatio-temporal relationship between the calcium wave and membrane surface activity following fertilization was imaged using the calcium indicator Calcium Green-dextran, and kymographs were generated from the resulting image stacks. As shown in Figure 4 (Panel A), eggs pretreated with the inactive analog U73343, a noticeable deflection in the cell surface was detected within 10 seconds from the earliest detectable rise in Calcium Green fluorescence signal. As the calcium transient crossed the egg (from left to right), a second more visible deflection of the cell surface followed the increase in Calcium Green fluorescence. In contrast, suppression of PLC activity with U73122 inhibited both the calcium wave and the contractile response (Figure 4B). In Blebbistatin-treated eggs, neither the initial surface deflection nor the sustained contraction could be detected (Figure 4C), although the calcium transient progressed normally. Together, these results suggest that the myosin II-dependent deflection of the cell surface closely followed- and was dependent on the calcium wave following sperm binding.

Induction of a myosin II contractile response in cells under compression

The cytoskeleton of a spherical cell is isotropic, and subtle changes in contractile activity are difficult to detect by microscopy alone. To further examine the calcium-responsiveness of unfertilized eggs, an assay was developed where changes in cortical contractility could be simply detected by monitoring resistance to compression. Compression has been used to monitor contractile changes in echinoderm eggs during cell division (Yoneda, 1984; Hiramoto, 1990; Rappaport, 1996) as well as in unfertilized eggs treated with the phosphatase inhibitor calyculin A (Asano and Mabuchi, 2001). Unfertilized eggs stripped of their fertilization envelopes were settled onto glass-bottomed culture dishes, and the seawater was rapidly replaced with a gas-permeable fluorocarbon oil that has been routinely employed to compress dividing eggs (Figure 5A) (Sluder et al., 1999). The weight of the oil typically compressed eggs to a thickness of 15-28 μm , and fertilized eggs flattened in this manner remained for 8 hours (not shown). When flattened eggs were injected with 1 mM Adenophostin A, a potent IP_3 receptor agonist (Sato et al., 1998; Bird et al., 1999), cells began to round up within 10 seconds of injection, as evidenced by a retraction of the cell margins (Figure 5, Panels C' D', and E'). Alternatively, direct mobilization of intracellular calcium by local application of 50 μM ionomycin to the cell margin induced a strong contractile response in both unfertilized- and fertilized eggs (Figures 6B' and C', respectively), whereas application of 0.1% DMSO in seawater (Figure 6A and A') had no effect. Lastly, the contractile response occurred in both the presence (Figure 6) and absence (not shown) of Ca^{++} in the seawater, suggesting that mobilization of internal Ca^{++} stores was sufficient to induce a contractile response.

Injection of IP_3 receptor agonists induced a strong contractile response (Figure 5), and together with the local application of calcium ionophore (Figure 6), suggested that the cortical cytoskeleton could directly respond to a calcium stimulus in the absence other sperm-induced signals. In an effort to better temporally resolve the initiation of a calcium transient and the subsequent contractile response, unfertilized eggs were injected prior to flattening with caged-Inositol 1,4,5-triphosphate or caged cADP-ribose along with Calcium

Green dextran. Cells were then flattened, and then exposed to a single pulse of UV light to uncage the injected reagents (Figure 7). While the UV flash was able to uncage a control substance (caged fluorescein dextran, Figure 7, Panel A), there was no contractile response from UV exposure alone (Figure 7, Panel A). However, uncaging either caged IP₃ or cADP resulted in both an increase in intracellular calcium and a contractile response (Figure 7, Panels B and C). Simultaneous mapping of the calcium transient and the contractile response indicated that contractile response followed the initial calcium transient on average by 4 seconds for IP₃ and 7 seconds for cADP-ribose. From these results, it appeared that the cell cortex could respond within 5 seconds of stimulation, which agreed with the earliest contractile responses observed in spherical, fertilized eggs (Figure 4).

Mobilization of intracellular calcium either using adenophostin, ionomycin or caged compounds resulted in a rapid contractile response (Figures 5-7), suggesting that calcium alone may be responsible for the contractile wave following sperm binding. Nonmuscle myosin II is regulated in part by light chain phosphorylation (Bresnick, 1999), and to determine whether egg activation resulted in an increase in light chain phosphorylation, unfertilized eggs were treated with ionomycin, and analyzed for serine 19 phosphorylation by immunoblotting (Figure 8). Consistent with earlier *in vivo* labeling studies (Walker et al., 1997), a basal level of regulatory light chain phosphorylation could be detected in whole cell lysates from untreated eggs (Figure 8, Lane 2). Ionomycin treatment resulted in detectable increases in serine 19 phosphorylation by one minute post-activation, with a 2.3-fold increase after five minutes (Figure 8, Lane 6). Possibly due to the transient nature of the contractile wave (approx. 15 seconds) or the differential regulation of soluble- and cytoskeletal myosin II (Shuster and Burgess, 1999), we were unable to detect a similar increase in myosin light chain phosphorylation following fertilization in whole cell lysates (not shown).

Inhibition of Myosin II affects cytoskeletal remodeling following fertilization

Time-lapse microscopy of both spherical and manipulated cells suggested that there was a calcium-induced, myosin II-dependent contractile response during egg activation (Figures 1-7). To determine whether the increase in myosin II activity during fertilization plays an immediate- or downstream role in cytoskeletal remodeling during the first cell cycle, eggs were pre-incubated in blebbistatin or DMSO carrier control, fertilized and monitored over time for the major morphological characteristics of the first cell cycle (Figure 9). Blebbistatin treatment had no effect on sperm binding, cortical granule release (as evidenced by elevation of the fertilization envelope) or engulfment of the sperm head or tail (not shown). However, blebbistatin-treated eggs retained their fertilization cones long after the structure had been absorbed in controls. Examination of F-actin organization following fertilization revealed that by 20 minutes post-fertilization, microvilli were prominent in both control- and blebbistatin-treated eggs (Figure 9, Panels C and D). However, by 10 minutes post-fertilization control eggs had absorbed the fertilization cone, and by 20 minutes only 3% of cells had a detectable fertilization cone (n=330 cells). In contrast, 83% of blebbistatin-treated cells still had a detectable fertilization cone (Figure 9, Panels B and D).

Discussion

Egg activation has been an intense focus of study for over a century, and while differences have been noted amongst the different metazoan groups (Stricker, 1999), all eggs and oocytes undergo a similar set of responses following sperm binding. A mobilization of intracellular calcium, establishment of a block to polyspermy, resumption of the cell cycle and reorganization of the cortical cytoskeleton all occur to differing degrees within the first minutes following insemination. Echinoderm eggs have proven an invaluable experimental system for studying fertilization and early development, and we have taken advantage of the

optical clarity as well as the physical and physiological malleability of sea urchin eggs to study the activation of myosin II during fertilization. Using time-lapse microscopy, we describe a myosin II-dependent contractile wave that supports and extends the classic biophysical studies of cortical stiffness during fertilization (Mitchison and Swann, 1955; Hiramoto, 1970). Moreover, this contractile response could be replicated by mobilizing calcium in unfertilized eggs, suggesting that calcium was responsible for activating myosin II activity following fertilization. Lastly, inhibition of myosin II activity retarded the retraction of the fertilization cone. Together, these results suggest that the fertilization-induced activation of myosin II represents another downstream effector of the sperm-egg interaction that participates in the remodeling of the actin cytoskeleton.

Cytoskeletal remodeling during the first zygotic cell cycle

In comparison to somatic cells, the cortical actomyosin cytoskeleton of unfertilized eggs and oocytes lends relatively little structural integrity to the cell. Ultrastructural analyses identified two populations of actin in the cortical cytoskeletons of unfertilized sea urchin eggs: a filamentous fraction and an amorphous layer of unpolymerized actin (Spudich, 1992; Bonder and Fishkind, 1995). F-actin was found primarily in the form of short microvilli, with a sparse population of actin filaments lying directly beneath the membrane that often terminated at the microvillar base (Henson and Begg, 1988). Localization studies also described a 1 μm layer subjacent to the microvilli that was immunoreactive with anti-actin antibodies, but not with fluorescent phallotoxins (Spudich et al., 1988; Bonder et al., 1989), and this population of actin appeared to be associated with cortical granules. Myosin II is also localized in the cortical cytoskeleton of unfertilized eggs (Figure 1), and as shown in Figures 2-7, a contractile response could be induced by mobilizing intracellular calcium within seconds of activation, suggesting that myosin II was able to generate force against this relatively disorganized cortical actin cytoskeleton. It is possible that new actin filament polymerization is being stimulated by calcium mobilization, but all data to date in both eggs and acrosomal processes points toward cytoplasmic alkalinization as the major stimulus for actin polymerization (Tilney et al., 1978; Begg and Rebhun, 1979; Carron and Longo, 1982). A detailed ultrastructural analysis of myosin II organization in the egg cortex similar to what has been performed in sea urchin coelomocytes, fish keratocytes, and mammalian fibroblasts (Verkhovsky and Borisy, 1993; Svitkina et al., 1997; Henson et al., 1999) will better resolved the linear structures found in unfertilized egg (Figure 1, Panel C) as well as clarify how myosin II generates contractile force against this relatively unorganized substrate.

We detected no visible effects on the actin polymerization in blebbistatin-treated cells, which is consistent with earlier findings of cells with compromised myosin II activity (DeLozanne, 1987; Knecht, 1987; Honer, 1988; Straight et al., 2003). However, we did find a significant delay in the absorption or retraction of the fertilization cone. Derived from microvilli directly adjacent to the bound sperm head, the fertilization cone rapidly elongates to engulf the sperm nucleus (Tilney and Jaffe, 1980; Cline and Schatten, 1986). In *Lytechinus pictus*, the fertilization cone grows to approximately 10 μm with retraction beginning by 9 minutes post-fertilization (Terasaki, 1996). However, in blebbistatin-treated cells, the fertilization cone remains visible for more than 20 minutes post insemination (Figure 9, Panel D), and in 28% of cells examined, the fertilization cone was still detectable 30 minutes post-insemination. Similar results were observed in mouse eggs injected with nonphosphorylatable mutants of myosin regulatory light chain, where both polar body extrusion and fertilization cone absorption were inhibited (Simerly et al., 1998). Live cell analyses of actin filament dynamics in sea urchin eggs revealed that actin in the fertilization cone undergo a retrograde-like transportation from the cell cortex towards the cell interior (Terasaki, 1996), which is consistent with the involvement of a + end-directed actin motor.

Moreover, studies of retrograde transport in coelomocyte lamellapodia (Henson et al., 1999; Henson et al., 2003) as well as actin dynamics in the contractile ring (Guha et al., 2005; Murthy and Wadsworth, 2005) have also implicated myosin II in the regulation of actin filament transport and turnover. Taken together, it appears that one role for myosin II in early development may include the turnover of the earliest actin filament structures.

Regulation of contractility in eggs and oocytes

Nonmuscle Myosin II is regulated by both heavy and light chain phosphorylation (Bresnick, 1999), and microinjection of nonphosphorylatable mutants of regulatory light chain leads to errors in fertilization cone retraction in mouse oocytes and cytokinesis (Simerly, 1998). A low level of myosin regulatory light chain phosphorylation has been detected in unfertilized eggs *in vivo*, and in isolated cortical cytoskeletons, a light chain kinase activity can also be detected (Walker et al., 1997). Moreover, phosphatase inhibitor treatment of unfertilized eggs induces increased light chain phosphorylation and a strong contractile response, lending further support to the notion that there exists a basal light chain kinase activity prior to fertilization (Tosuji et al., 1992; Asano and Mabuchi, 2001). Our results indicate that the contractile wave follows the calcium transient at fertilization (Figure 4), and that mobilization of calcium alone was sufficient to induce a contractile response (Figures 5-7). Ionomycin-activation of unfertilized eggs resulted in an increase of serine 19 phosphorylation (Figure 8), but after normalization there was only a 2.3-fold increase over DMSO controls. This may be due to the fact that only a small fraction of myosin II is cytoskeletal in eggs and blastomeres (~10%), and soluble- and cortical populations are regulated differentially (Shuster and Burgess, 1999). Thus, examination whole cell lysates may underestimate regulatory events at the cell cortex. The Ca^{++} /calmodulin-dependent myosin light chain kinase is a likely candidate to mediate myosin II activation in unfertilized eggs (Bresnick, 1999), but studies using established MLCK- or Rho-kinase inhibitors gave equivocal results (not shown), so we cannot eliminate the possibility that Rho-kinase is not involved in the activation myosin II following fertilization. Indeed, Rho GTPase has been localized to cortical granules in unfertilized eggs (Cuellar-Mata et al., 2000), and inhibition of Rho with C3 transferase inhibits cortical granule translocation to the cortex and exocytosis during fertilization (Covian-Nares et al., 2004). Further experimentation will further discriminate the respective roles of Ca^{++} - and Rho-dependent functions in cytoskeletal remodeling during fertilization.

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Figure 1. Actin- and myosin organization prior to- and following fertilization in sea urchin eggs
 Unfertilized *Strongylocentrotus purpuratus* eggs (UF, Panels A, C and E) or eggs 3 minutes post-fertilization were fixed and processed for F-actin (Panels A and B) and myosin II (Panels C and D) localization. Prior to fertilization F-actin is confined to short microvilli at the cell surface (Panel A), whereas myosin II was found both at the surface and on linear elements beneath the cortical shell (Panel D). Bar 10 μ m.

Figure 2. Surface contractile activity during fertilization

Lytechinus pictus eggs were fertilized and followed by time-lapse DIC microscopy, with images acquired every second. Panels A, B and C correspond to frames 1, 19, and 26, respectively. Panel A represents the unfertilized egg four seconds prior to sperm binding (denoted as SP on Panel D), with the white bar denoting the area cropped from the image stack to generate the kymograph in Panel D. Panel B illustrates the contractile wave (arrow) as it moved from the point of sperm entry (asterisk) across the egg, with the cortex relaxing as the wave passed, and returning to its original spherical shape (Panel C; Bar, 25 μ m). The contractile response to sperm binding is illustrated in kymograph form in Panel D, where the area denoted by the bar in Panel A was cropped from the image stack and converted into a montage, with the site of sperm binding on the left. Note that the contractile wave can be seen not only by the retraction of the cell margin, but also by the displacement of granules in the cytoplasm, as denoted by the bracket. Bar, 25 μ m.

Figure 3. The sperm-induced contractile wave occurs in the absence of the fertilization envelope
L. pictus eggs were stripped of their fertilization envelopes by treatment with 10 mM DTT and were followed through fertilization by time-lapse microscopy. Images were acquired at one frame per second, and fertilized eggs were oriented with the sperm entry point on the left. The white bars in Panels A-C denote the section cropped from each image stack to generate the kymographs in Panels A'-C', with each cropped section containing the sight of sperm binding oriented on the left. Time series were run for 40 seconds, with $t=0$ denoting the time of sperm addition. Note that while cytoplasmic granules and the nucleus remained stationary in unfertilized eggs (Panels A and A'), a contractile wave could be clearly seen following sperm binding (Panels B and B') by both an ingression of the cell margin as well as the movement of cytoplasmic granules that initiated at the sight of sperm binding (black bracket) and terminated at the opposite pole a (white bracket). In contrast, no surface deflection or granule movement was observed in the presence of the myosin II inhibitor (Panel C and C'). Bar (Panel C), 50 μm . Bar (Panel C'), 25 μm .

Figure 4. Myosin II contractility follows the calcium wave during fertilization

L. pictus eggs were pre-incubated presence of 10 μ M 73343 (Panel A), 10 μ M 73122 (Panel B) or 100 μ M blebbistatin (Panel C) for 15 minutes. Cells were then injected with Calcium Green dextran, fertilized, and images were acquired every second with $t=0$ denoting the approximate time of sperm binding. A slice was cropped from each image stack to generate a kymograph where the site of sperm entry is oriented on the left. Bar, 25 μ m.

A

B

Figure 5. Detection of a calcium-dependent contractile response in cells under compression

A. Unfertilized eggs were briefly treated with 1M urea to remove the vitelline envelope before settling onto protamine-coated, glass-bottomed culture dishes. Seawater was removed and replaced with Fluorinert FC-40 fluorocarbon oil (denoted by the green bar), flattening the cell from a diameter of $\sim 110\ \mu\text{m}$ to $15\text{--}28\ \mu\text{m}$. When myosin II is activated in the cortical cytoskeleton, resistance to compression increases and cell diameter decreases as the cell rounds up.

B. A'-D'. 1 mM Adenophostin A was injected into a flattened egg (at $t=0''$), and the contractile response was followed by time-lapse DIC microscopy at 1 frame/second. The cell margin at the time of injection is denoted by a red circle. Images shown in A'-D' correspond to time 0, 10, 40, and 60 seconds post-injection respectively. Bar, $50\ \mu\text{m}$. D' A kymograph was assembled using a horizontal slice through the image stack (Shown as a white bar in A'), and assembled with Time 0 at the top. Note the retraction of the cell margin over time. Bar, $50\ \mu\text{m}$.

Figure 6. Local application of calcium ionophore induces a contractile response in unfertilized- and fertilized eggs

Unfertilized (A and B) or fertilized (C) eggs were flattened under fluorocarbon oil. A pipette containing either 0.1% DMSO (A) or 50 μ M Ionomycin (B and C) was positioned adjacent to the cell margin, and approximately 25 μ l was applied to the side of an unfertilized egg. Images were acquired every second, and sequences were started 5 seconds prior to application of 0.1% DMSO or Ionomycin. Bar, 50 μ m. Kymographs (A'-C') were generated by cropping the area shown by the white bar from the image stack, and assembled with time 0 at the top, and the arrow denoting the time of drug application. Note that while application of seawater resulted in no contractile response (A'), 50 μ M Ionomycin applied to unfertilized eggs (Panel B') resulted in a strong contractile response. A similar response was observed when ionomycin was applied to fertilized eggs in interphase of the first cell cycle (C'). (C) Bar, 25 μ m; (C') Bar, 10 μ m.

Figure 7. Mobilization of intracellular calcium stores induces a contractile response in flattened, unfertilized eggs

L. Pictus eggs were injected with either a caged-fluorescein alone (Panel A), 2mM caged Inositol 1,4,5 triphosphate and Calcium-Green dextran (Panel B), or 2mM caged cyclic ADP-ribose and Calcium-Green dextran (Panel C). Cells were flattened and imaged using DIC- and fluorescence microscopy, acquiring images every two seconds. 20 seconds into the sequence, cells received a single pulse of UV light to uncage the compounds. Kymographs were generated by cropping the area denoted by the white bar from the image stacks. Arrows denote the time of the uncaging. While the UV flash efficiently activated the caged fluorescein (Panel A), there was no contractile response to UV light alone. In contrast, uncaging of either IP_3 or cADP-ribose (Panels B and C) resulted in both a rise in intracellular calcium levels and a contractile response as evidenced by a retraction of the cell margins. Bar, 50 μm .

Figure 8. Ionophore-activation induces myosin regulatory light chain phosphorylation
 Unfertilized eggs were treated in the presence of 0.1% DMSO (Lane 2) or 1 mM Ionomycin for 30 seconds (Lane 3), 1 (Lane 4), 2 (Lane 5), or 5 (Lane 6) minutes. Samples were resolved on a 4-15% gradient SDS-PAGE gel, along with a sample of purified myosin II (Lane 1, M) that is phosphorylated by a contaminating kinase activity (Yabkowitz, 1987). Membranes were then probed with rabbit anti-phosphoserine19 myosin light chain antibodies, and equal loading was determined with anti-tubulin antibodies.

Figure 9. Inhibition of Myosin II activity delays retraction of the fertilization cone

Eggs were treated with either 0.1% DMSO (Panels A and C) or 100 μ M blebbistatin (Panels B and D) for 20 minutes prior to fertilization. Following addition of sperm, eggs were stripped of their fertilization envelopes, settled onto protamine-coated coverslips, and fixed 10- and 20 minutes post-fertilization. Cells were then processed for F-actin (red), and DNA (blue) localization and confocal microscopy. Note the presence of the fertilization cones in blebbistatin-treated cells (Panels C and D). Bar, 20 μ m.