pH REGULATES THE POLYMERIZATION OF ACTIN IN THE SEA URCHIN EGG CORTEX

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ABSTRACT

The state of actin in the isolated cortex of the unfertilized sea urchin egg can be controlled by experimentally manipulating the pH of the isolation medium. Cortices isolated at the pH of the unfertilized egg (6.5–6.7) do not contain filamentous actin, while those isolated at the pH of the fertilized egg (7.3–7.5) develop large numbers of microvilli which contain bundles of actin filaments. Cortices that are isolated at pH 6.5 and then transferred to isolation medium buffered at pH 7.5 also develop actin filaments. However, the filaments are not arranged in bundles and microvilli do not form. Although the cortical granules in cortices isolated at pH 6.5 discharge at a free Ca^{++} concentration of ~10 μm, actin polymerization is not induced by increasing the Ca^{++} concentration of the isolation medium. These results suggest that the increase in cytoplasmic pH which occurs following fertilization induces the polymerization of actin in the egg cortex.

KEY WORDS actin polymerization · calcium · cortex · fertilization · microvilli · pH · sea urchin egg

Many forms of actomyosin-based cell motility are transitory events which require the precise temporal and spatial control of the assembly and disassembly of the contractile apparatus. One way in which this control could be exerted is through the localized regulation of the polymerization and depolymerization of actin. Such a mechanism has been postulated to explain a variety of types of motility, including amoeboid movement (24, 25), changes in cell shape (10), cytokinesis (17, 18), phagocytosis (23), and platelet contraction (2). The relationship between the control of actin polymerization and cell motility has been studied most extensively by Tilney and his co-workers in the formation of the acrosomal process in echinoderm sperm (26–30). Before the acrosomal reaction, actin is localized in the periacrosomal cap in a nonfilamentous (profilamentous) state and is inhibited from polymerizing through its association with control proteins (26). This cap of profilamentous actin can be induced to polymerize into a bundle of acrosomal filaments by experimentally raising the intracellular pH (28), which presumably releases actin from its associated proteins (26, 27).

We have studied the changes which occur in the organization of actin in the sea urchin egg cortex at the time of fertilization. Immediately following fertilization, the surface topography of the egg changes dramatically. The short microvilli, 0.2–0.3 μm long, which cover the surface of the unfertilized egg rapidly elongate (7, 15, 19, 21). Johnson et al. (12) and Shen and Steinhardt (20) have reported that there is also an increase in cytoplasmic pH during this period of microvillar elongation. These observations suggested to us that, as in the extension of the acrosomal process, the increase in cytoplasmic pH which accompanies fertilization might result in the polymerization of cortical actin, which in turn would cause the extension of the microvilli.

We have tested this hypothesis by examining

the effects of pH upon the state of actin in cortices isolated from unfertilized eggs of the sea urchin *Strongylocentrotus purpuratus*. The results reported here demonstrate that actin is present in the unfertilized egg cortex in a nonfilamentous form and that it can be induced to polymerize by isolating cortices at a pH of 7.3 or above. The resulting organization of actin filaments resembles that in normally fertilized eggs. The organization of actin in the fertilized egg cortex and its relationship to the mechanism of cytokinesis will be the subject of a following paper (Begg, Morell, and Rebhun, manuscript in preparation).

MATERIALS AND METHODS

The shedding of eggs from the sea urchin *S. purpuratus* was induced by the intracoelomic injection of 0.5 M KCl. The eggs were washed once in Ca^{++}/Mg^{++}-free sea water (0.5 M NaCl, 27 mM KCl, 2 mM EDTA, pH 7.8) to remove the jelly coat. De-jellied eggs were then washed three times in Marine Biological Laboratory (MBL, Woods Hole, Mass.) formula artificial sea water (6) and stored at 4°C in artificial sea water.

Cortex Isolation

Cortex isolation medium consists of 0.35 M glycine, either 0.1 M piperazine-*N*-*N'*-bis(2-ethane sulfonic acid) (PIPES) or 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) buffer, 5 mM EGTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, and contains 0.5 mM phenylmethylsulfonyl fluoride and 0.1 mg/ml soybean trypsin inhibitor to reduce proteolysis. PIPES buffer was used for pHs between 6.0 and 7.1 and HEPES buffer for pHs between 7.2 and 8.0. There was no detectable effect of buffer composition on cortical actin polymerization. Identical results were obtained when PIPES was substituted for HEPES buffer at higher pHs. However, because of its higher pK (7.55), HEPES was routinely used for the isolation of cortices in concentrated egg suspensions between pH 7.2 and 8.0. The pH of the isolation medium was adjusted with NaOH or HCl at the temperature at which it was to be used. The rational for the development of the cortex isolation medium will be presented in a forthcoming paper (Begg, Morell, and Rebhun, manuscript in preparation).

Eggs were prepared for cortex isolation by washing once in Ca^{++}/Mg^{++}-free sea water to remove divalent cations and once in isolation medium at 0–4°C. Washed eggs were resuspended in fresh isolation buffer as a 5% suspension and were gently homogenized in a Teflon pestle homogenizer until >98% of the eggs were lysed (12–20 passes). Cortices were sedimented at 200 *g* for 5 min in 15-ml conical plastic centrifuge tubes. The pellet consisted of two layers: a lower layer containing intact eggs and some cortices, and an upper layer containing only cortices. The upper pellet layer was pipetted off and washed three times in isolation buffer.

Myosin S-1 Decoration

Isolated cortices were decorated with myosin S-1 in isolation medium as described previously (1).

Ca^{++} Treatment

Cortices isolated at pH 6.5 were incubated for 15 min at 24°C in isolation medium buffered at pH 6.7 containing 1 mM EGTA, 1 mM $MgCl_2$, and varying concentrations of Ca^{++}. The free Ca^{++} concentration was estimated using an apparent association constant of $1.32 \times 10^6\ M^{-1}$ for EGTA at pH 6.7, calculated by the method of Raaflaub (16). The total concentration of Ca^{++} added for any desired free Ca^{++} concentration was calculated from the formula: $Ca_t^{++} = E_t[K_aCa_f^{++}/1 + K_aCa_f^{++}] + Ca_f^{++}$, where Ca_t^{++} = total $[Ca^{++}]$, E_t = total [EGTA], K_a = EGTA association constant, and Ca_f^{++} = free $[Ca^{++}]$.

SDS-Urea Polyacrylamide Gel Electrophoresis

Samples of isolated cortices were prepared for electrophoresis by boiling for 2 min in Laemmli sample buffer (14) containing 50 mM dithiothreitol. Boiled samples

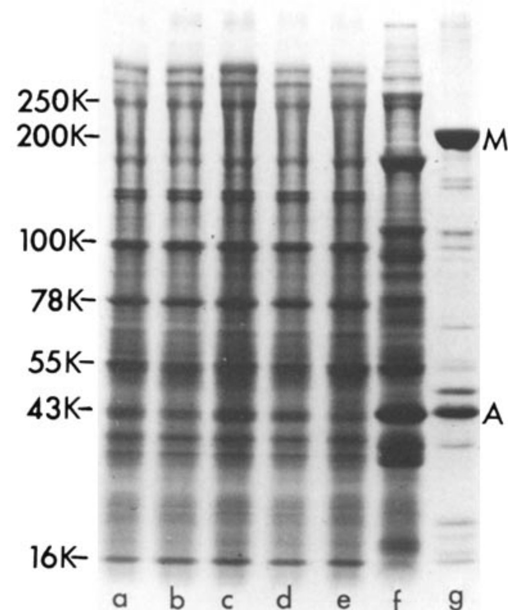

FIGURE 1 SDS-urea polyacrylamide gel of cortices isolated from unfertilized eggs at various pHs. (*a*) pH 6.5, (*b*) pH 7.0, (*c*) pH 7.5, (*d*) pH 8.0, (*e*) isolated at pH 6.5 and then transferred to isolation medium at pH 7.5, (*f*) cortices isolated from fertilized eggs at pH 7.5, (*g*) rabbit skeletal muscle standard. 25 μg of protein were loaded onto each well. The electrophoretic pattern of proteins from unfertilized cortices isolated at pHs ranging from 6.5 to 8.0 are essentially identical. *M*, myosin; *A*, actin.

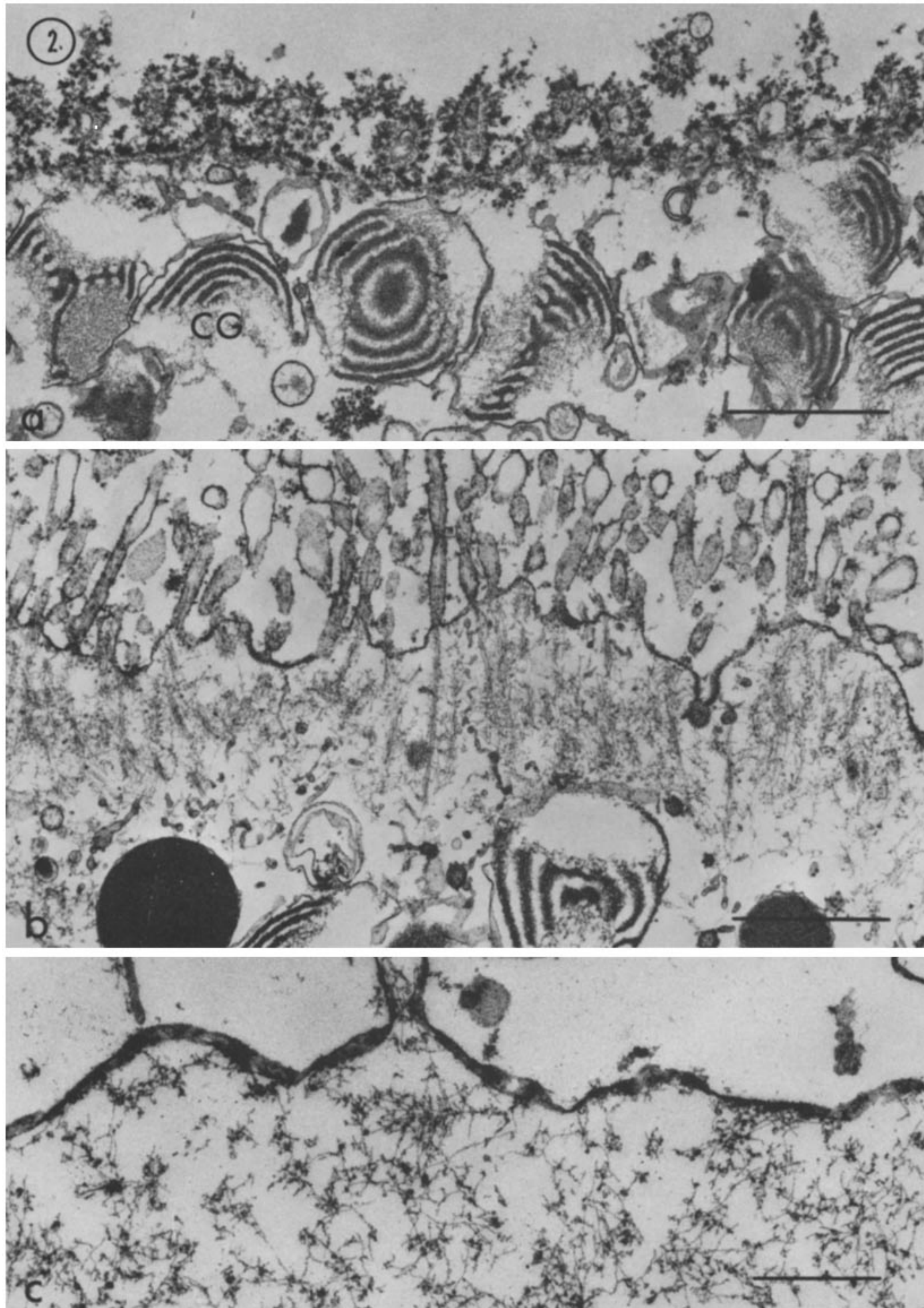

FIGURE 2 Cortices isolated from unfertilized eggs at different pHs. (*a*) Isolated at pH 6.5. Short microvilli project from the surface of the cortex while cortical granules (*CG*) are associated with the cytoplasmic face of the plasma membrane. Actin filaments are absent. The vitelline membrane appears as a dense granular layer which coats the external face of the cortex. Bar, 1.0 μm. × 25,000. (*b*) Isolated at pH 7.5. The microvilli have elongated and contain bundles of actin filaments which extend down into the cortex. The cortical granules are displaced away from the plasma membrane. Bar, 1.0 μm. × 24,000. (*c*) Isolated at pH 6.5 and transferred to pH 7.5 isolation medium after isolation. Actin filaments develop but form a network rather than bundles. The plasma membrane occasionally appears folded, but does not contain microvilli. Bar, 0.5 μm. × 40,000.

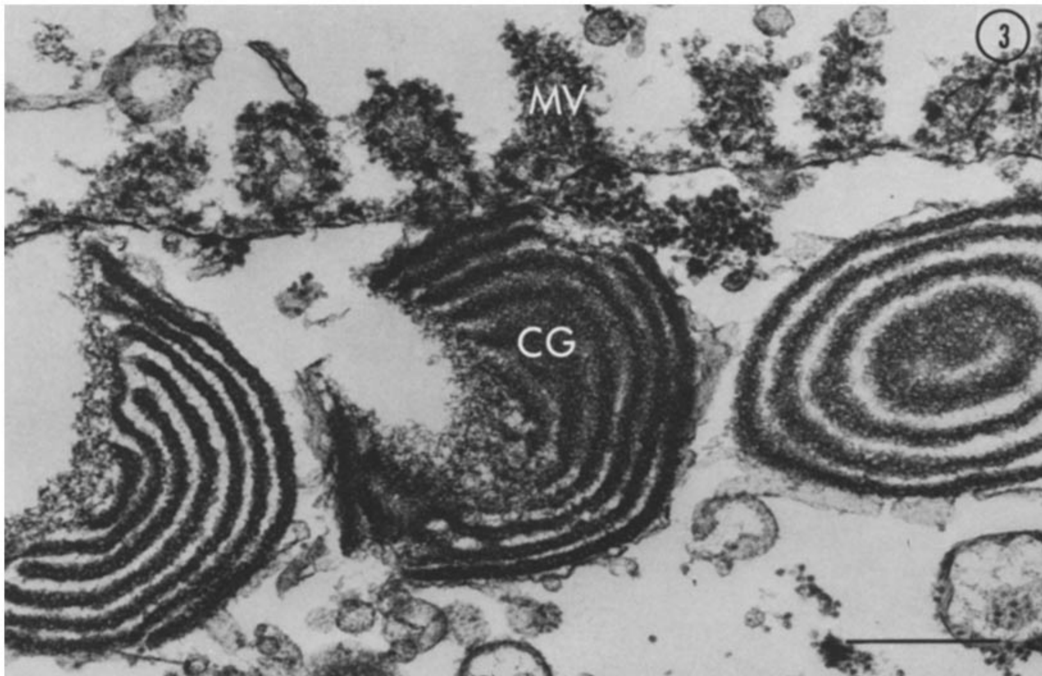

FIGURE 3 Higher magnification of unfertilized cortex isolated at pH 6.5. There is no evidence of filamentous actin. The cortical granules (*CG*) appear tightly associated with the plasma membrane, and short microvilli (*MV*) are clearly visible. Bar, 0.5 μm. × 42,000.

FIGURE 4 Cortex isolated from a fertilized *S. purpuratus* egg at pH 7.5. Elongated microvilli which are characteristic of the fertilized cortex extend from the outer surface. Bundles of actin filaments, similar to those seen in Fig. 2*b*, project from the bases of the microvilli into the cortex. Bar, 0.5 μm. × 46,000.

were electrophoresed on 4–16% linear gradient slab gels by the method of Binder and Rosenbaum (3).

Electron Microscopy

Samples of isolated cortices were fixed in the presence of tannic acid according to the method of Begg et al. (1), dehydrated through a graded series of ethanol, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and were examined with either a Hitachi HU-11E electron microscope operated at an accelerating voltage of 75 kV or a Philips 200 electron microscope operated at an accelerating voltage of 60 kV. The microscopes were calibrated with a 54,800 lines/in. replica grating.

RESULTS

Effects of pH on the Isolated Cortex

Cortices can be isolated from unfertilized *S. purpuratus* eggs at pHs ranging from 6.0 to 8.0, although below pH 6.5 the cytoplasm does not disperse well. SDS-gel electrophoresis reveals that actin is a major component of cortices isolated at pHs between 6.5 and 8.0 (Fig. 1). However, the morphological state of actin in isolated cortices varies dramatically over this pH range. Cortices isolated at a pH close to that of the unfertilized

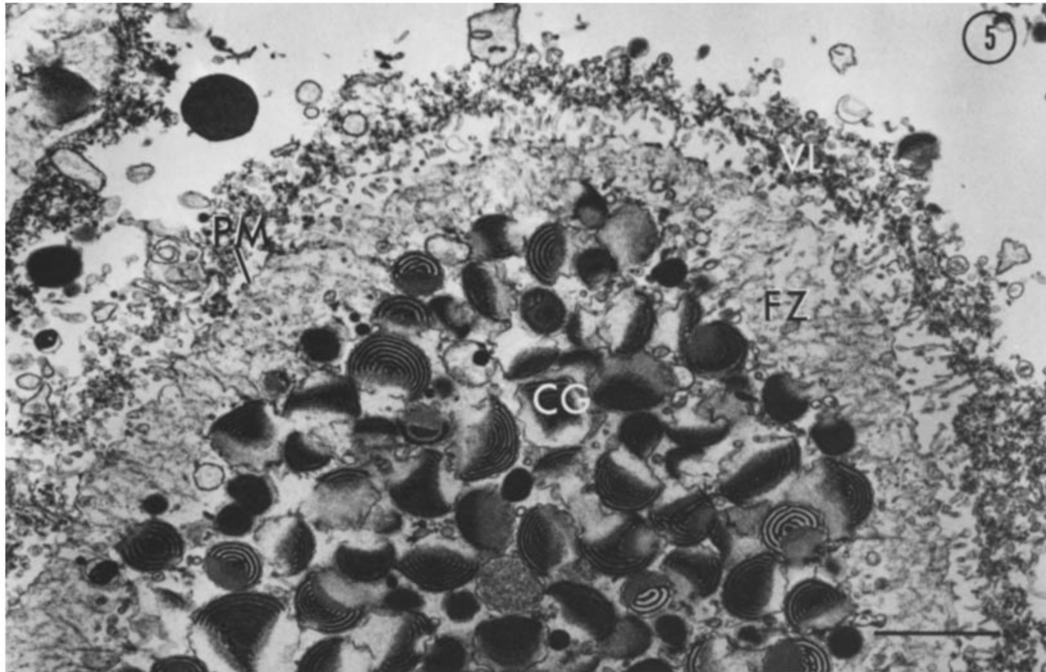

FIGURE 5 Low magnification view of unfertilized cortex isolated at pH 7.5. The vitelline layer (*VL*) has been partially degraded during isolation and appears as a dense granular layer overlying the plasma membrane (*PM*). Directly beneath the plasma membrane is a zone of radially oriented filament bundles (*FZ*). The cortical granules are displaced away from the plasma membrane and form a dense mass in the center of the isolated cortex. Bar, 2.0 μm. × 8,600.

egg (6.5–6.7) do not contain filamentous actin and exhibit morphological features which are characteristic of the unfertilized egg (Figs. 2*a* and 3). Typical cortical granules attach to the cytoplasmic face of the plasma membrane, and short microvilli, 0.1–0.3 μm long, cover the entire surface of the cortex. The vitelline membrane becomes partially degraded during isolation and appears as a dense, granular coating on the outer surface of the cortex. This disruption of the vitelline layer may be due to the presence of dithiothreitol in the isolation medium (9).

In contrast to cortices isolated at acid pH, those isolated at a pH near that of the fertilized egg (7.3–7.5) contain large numbers of 60- to 70-Å actin filaments (Fig. 2*b*). Except for the presence of intact cortical granules, these cortices are essentially indistinguishable from those isolated from fertilized eggs (Fig. 4). Their surface is densely populated with long microvilli which contain highly organized bundles of actin filaments (Fig. 2*b*). The cortical granules are displaced away from the plasma membrane and form a dense mass in the center of the isolated cortex (Fig. 5). Myosin S-1 decoration demonstrates that the cortical actin filaments have a uniform polarity, with the arrowhead complex pointing away from the tips of the microvilli (Fig. 6). The same polarity has been reported for microvillar actin filaments in the fertilized egg (1, 4).

Cortices that have been isolated at pH 6.5 form dense arrays of actin filaments when transferred to fresh isolation medium buffered at pH 7.5 (Fig. 2*c*). However, instead of developing as organized bundles, these filaments form a network beneath the plasma membrane. In addition, the plasma membrane fails to develop microvilli.

Once actin filaments have formed in the isolated cortex, they become insensitive to pH and cannot be depolymerized by decreasing the pH of the isolation medium. Actin filaments in fertilized egg cortices are also unaffected by the pH of the isolation medium (Begg, Morell, and Rebhun, manuscript in preparation).

The isolated cortex contains a large number of protein components in addition to actin. However, there is no detectable difference in the electrophoretic pattern of proteins in cortices isolated at pHs

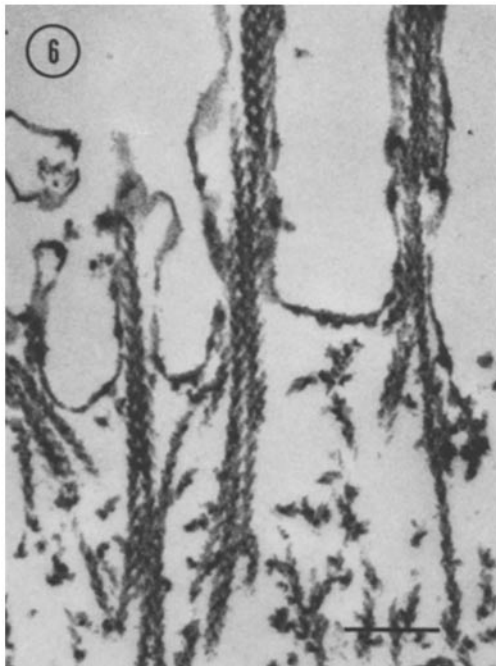

FIGURE 6 S-1-decorated actin filaments in an unfertilized cortex isolated at pH 7.5. The filaments display a uniform polarity, with the arrowheads pointing away from the tips of the microvilli. Bar, 0.2 μm. × 65,000.

between 6.5 and 8.0 (Fig. 1). Cortices isolated from fertilized eggs appear to contain a greater concentration of actin than those isolated from unfertilized eggs (Fig. 1), suggesting that in addition to the cortical profilamentous actin, cytoplasmic actin may participate in the formation of cortical actin filaments following fertilization.

Effects of Ca^{++} on the Isolated Cortex

One of the first consequences of fertilization in the sea urchin egg is the release of Ca^{++} from an intracellular store (22). Because this increase in cytoplasmic Ca^{++} has been shown to initiate a number of early developmental events (8), we investigated the possibility that increased levels of free Ca^{++} rather than the increase in intracellular pH caused the in vivo polymerization of cortical actin filaments. Cortices isolated from unfertilized eggs at pH 6.5 were exposed to concentrations of 0.5, 1, 5, 10, 25, 50, and 100 μM Ca^{++} by transferring them to isolation medium at pH 6.7 which contained Ca^{++}-EGTA buffers. Actin filaments were not observed at any of the Ca^{++} concentrations used. However, cortical granule breakdown occurred at a free Ca^{++} concentration of $\sim$10 μM (Fig. 7).

DISCUSSION

The results reported in this paper demonstrate that actin is associated with the unfertilized sea urchin egg cortex in a nonfilamentous form and that it can be induced to polymerize in the isolated cortex by increasing the pH of the isolation medium. These results suggest that the increase in cytoplasmic pH which accompanies fertilization induces the polymerization of cortical actin and, furthermore, that this actin polymerization may participate in the process of microvillar elongation. This suggestion is supported by the observation that microvillar elongation in vivo occurs over the same time interval during which the cytoplasmic pH increases (7, 20). In addition, the increase in pH which is required for the in vitro polymerization of cortical actin is close to the in vivo change in cytoplasmic pH which has been reported to accompany fertilization (12, 20).

Several proteins have been identified which appear to bind to G-actin in the cell and prevent its polymerization. Tilney (26) has described two proteins with mol wt of 250,000 and 230,000 daltons which are associated with nonfilamentous actin in the periacrosomal cap of echinoderm sperm. In addition, Carlsson et al. (5) have isolated a 16,000-dalton protein from spleen that complexes with actin and inhibits its polymerization. We hypothesized that pH might influence the binding of similar proteins to actin in the sea urchin egg cortex. These proteins would bind to actin at the pH of the unfertilized egg, thereby inhibiting its polymerization, and dissociate at the pH of the fertilized egg, permitting the actin to polymerize. However, we were unable to detect any differences in the electrophoretic pattern of proteins from cortices isolated at pHs between 6.5 and 8.0. This observation suggests that if the polymerization of actin results from the pH-induced dissociation of regulatory proteins from actin subunits, then these proteins must be integral components of the cortex and are not simply released into the isolation medium. Alternatively, the polymerization of cortical actin may be directly affected by pH. However, this does not seem to be a likely possibility, because the rate of polymerization of purified actin decreases in going from acidic to alkaline pH (13).

Changes in the intracellular concentration of Ca^{++} have also been proposed as a mechanism for controlling actin polymerization (18, 24). Because fertilization of the sea urchin egg results in a rapid increase in the concentration of cytoplasmic Ca^{++}

(22), we considered the possibility that Ca^{++} release, rather than the change in cytoplasmic pH, might cause the polymerization of cortical actin. However, free Ca^{++} concentrations between 0.5 and 100 μM failed to produce any detectable effect upon actin in the isolated, unfertilized egg cortex. It is unlikely that this lack of response to Ca^{++} results from an alteration in the calcium sensitivity of the isolated cortex because cortical granule breakdown occurs at a free Ca^{++} concentration of ~10 μM, a value which agrees closely with that obtained by Steinhardt et al. (22) for *Lytechinus pictus* eggs. In addition, when the cytoplasmic pH of unfertilized *L. pictus* eggs is increased by treatment with NH_4Cl (20), the microvilli elongate (15) and the eggs activate in the absence of Ca^{++} release (29). These results argue that Ca^{++} does not normally function in the control of actin polymerization in the sea urchin egg cortex. This conclusion is supported by the observation of Tilney et al. (28) that, while Ca^{++} is necessary for the fusion of the acrosomal vesicle membrane with the plasma

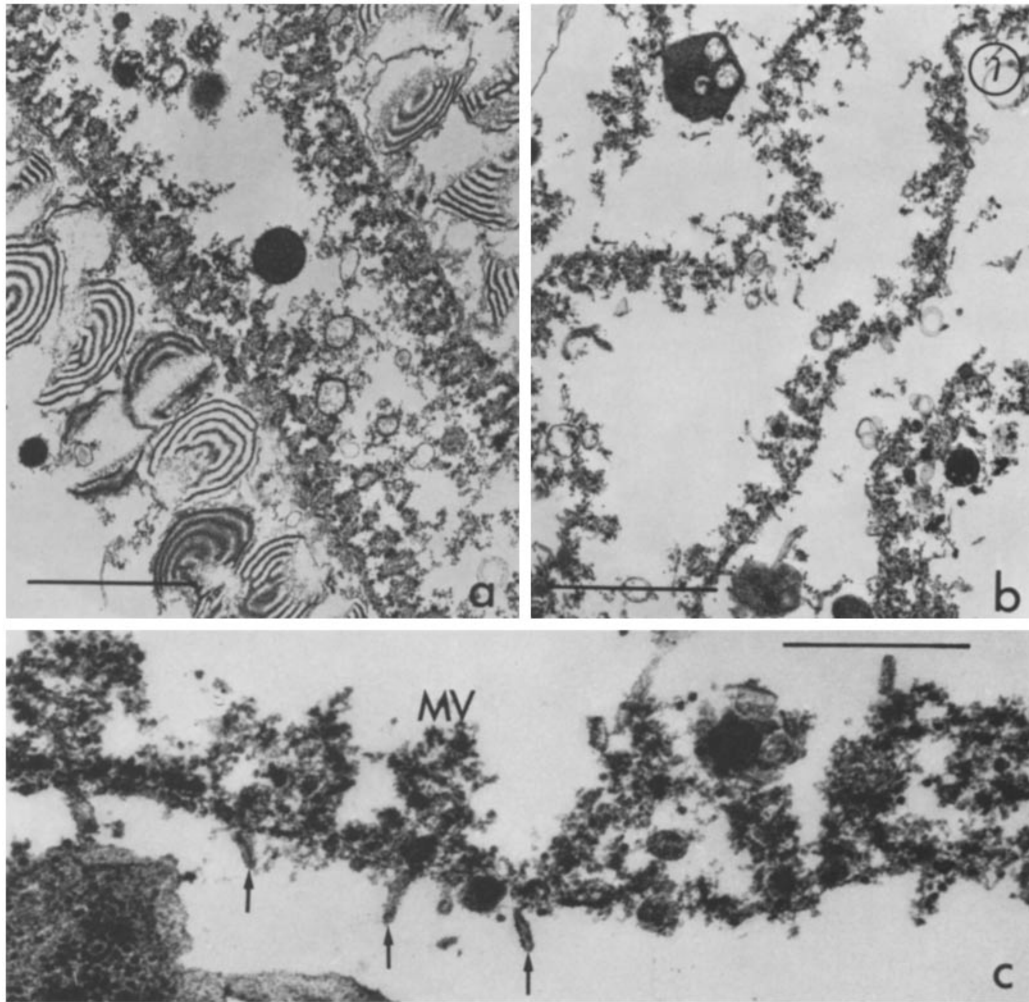

FIGURE 7 Effects of Ca^{++} on the isolated, unfertilized cortex. (*a*) Portions of two cortices isolated at pH 6.5 and then incubated for 15 min at 24°C in isolation medium at pH 6.7. The cortices show the morphology typical of the unfertilized cortex at acid pH. (*b*) Cortices isolated at pH 6.5 and incubated for 15 min at 24°C in pH 6.7 isolation medium containing 50 μM free Ca^{++}. The cortical granules have broken down, but actin filaments are not present. Bar, 2.0 μm. × 11,700. (*c*) Higher magnification of cortex shown in Fig. 7 *b*. No actin filaments are visible. Note the membrane protrusions (arrows) where cortical granules were attached previously. *MV*, microvillus. Bar, 0.5 μm. × 50,800.

membrane in echinoderm sperm, it is not required for the polymerization of acrosomal actin.

Schroeder (19) has reported that the elongation of egg microvilli occurs in two stages: an initial elongation which accompanies fertilization and a secondary elongation which occurs midway between fertilization and the first cleavage division. Although we have shown that the first stage of microvillar elongation is probably caused by a pH-induced polymerization of cortical actin, it is unclear what controls the second stage. It may result from a second period of actin polymerization, but if so, it is unlikely that this is induced by a further increase in pH, because available evidence suggests that the pH of the egg cytoplasm remains constant following its initial increase at fertilization (11, 20). Alternatively, the second stage of microvillar elongation might be caused by the further extension of pre-existing actin filament bundles which were formed during the initial stage of pH-induced actin polymerization.

This report and the previous study by Tilney et al. (28) demonstrate that relatively small changes in pH can induce actin polymerization, suggesting that changes in intracellular pH may be an important control mechanism in regulating the assembly of actin filaments for various aspects of cell motility. At the present time it is unclear what regulates the depolymerization of actin in the cell. A decrease in cellular pH may permit the reassociation of regulatory proteins with F-actin, resulting in depolymerization, or the actin filaments may be disassembled by a mechanism entirely different from that which controls their assembly.

We wish to thank Thomas Keller for originally suggesting the idea of testing the effects of pH on the isolated cortex. This work was supported by National Science Foundation grant PCM 75-17790 A04 to L. I. Rebhun and by American Society Postdoctoral Fellowship PF-1089 to D. Begg.

Received for publication 21 May 1979, and in revised form 2 July 1979.

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