Cortical Actin Filaments Form Rapidly during Photopolarization and Are Required for the Development of Calcium Gradients in Pelvetia compressa Zygotes

Rongsun Pu, Michele Wozniak, and Kenneth R. Robinson 1
Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907-1392

Previous research has shown that cortical gradients of cytosolic Ca\(^{2+}\) are formed during the photopolarization of Pelvetia compressa zygotes, with elevated Ca\(^{2+}\) on the shaded hemisphere that will become the site of rhizoid germination. We report here that the marine sponge toxin, latrunculin B, which blocks photopolarization at nanomolar concentrations, inhibited the formation of the light-driven Ca\(^{2+}\) gradients. Using low concentrations of microinjected fluorescent phalloidin as a tracer for actin filaments, we found that exposure to light induced a striking increase in actin filaments in the cells as indicated by an increase in fluorescence. The increase was quantified in the cortex, where it was most apparent, and the fluorescence there was found to increase by about a factor of 3. This increase in cortical phalloidin fluorescence was inhibited by latrunculin B at the same concentration required to inhibit Ca\(^{2+}\) gradient formation and photopolarization. The distribution of the increasing phalloidin fluorescence was uniform with respect to the developing rhizoid–thallus axis during the formation of the axis, and no intense patches of fluorescence were observed. After germination, fluorescence suggestive of an apical ring of actin filaments was seen near the rhizoid tip. Finally, inhibitor studies indicated that myosin may be involved in the photopolarization process.

Key Words: cell polarity; calcium gradients; actin; latrunculin B; Pelvetia compressa.

INTRODUCTION

The emergence of pattern during embryonic development depends on polarity present in the egg or the zygote. Typically, at least one axis of polarity is established during oogenesis and is then elaborated during embryogenesis. The Drosophila egg, for example, possesses both an anterior–posterior axis and a dorsal–ventral axis in the form of localized cytoplasmic and membrane components. While the powerful tools of molecular genetics have revealed many important aspects of egg polarity, the physiological mechanisms that are involved in creating and maintaining orderly gradients in the egg remain poorly understood, in part because these gradients arise gradually in the relatively inaccessible environment of the ovary.

Exceptions to this general pattern are the eggs of various species of the fucoid algae, Fucus and Pelvetia. When the eggs of these algae are released into the swirling intertidal waters and fertilized, they have no apparent polarity other than the faint and easily overcome one of the point of sperm entry. While a number of imposed gradients can polarize these cells, unilateral blue light readily dominates other gradients and is presumably the natural polarizing agent. In response to unilateral light, the zygotes organize an axis, which is later expressed by the localized germination of the rhizoidal bulge. After the rhizoidal region extends by tip growth, two highly unequal cells with quite different fates are created by a cell division plane that is perpendicular to the axis defined by the rhizoidal growth. One cell becomes the thallus of the adult organism while the other becomes the root-like holdfast, the rhizoid, which attaches the algae to the rocks. Thus, these algal cells are a valuable system for the study of the genesis of polarity as the main axis of the adult organism is formed in a free-living cell and the orientation of that axis can be readily controlled by the experimenter. Because the zygotes stick tenaciously to almost any substrate, the orientation of the axes relative to the fixed marks on the dish is not disturbed by manipulations such as the exchange of the surrounding seawater.

1 To whom correspondence should be addressed. Fax: (765) 494-0876. E-mail: ken@video.bio.purdue.edu.
The zygotes are densely pigmented and absorb or scatter more than 95% of the impinging unilateral blue light across the diameter of the cells, and they respond to this self-created light gradient by germinating from the darkest pole (Robinson, 1996). Brief exposure to unidirectional light is sufficient to efficiently polarize a population of zygotes and the direction of the tentative axis can be changed up to the time of germination by exposure to a second light from a different direction (reviewed Robinson et al., 1999).

Two cellular components have been shown to be involved in the conversion of a light gradient to a morphological gradient in these algal cells, the actin cytoskeleton and Ca\textsuperscript{2+}. Some years ago, Quatrano (1973) and Nelson and Jaffe (1973) showed that cytochalasin B blocked photopolarization, albeit at fairly high concentrations. More recently, photopolarization has been shown to be highly sensitive to cytochalasin D and especially to latrunculin B (Hable and Krof, 1998; Robinson et al., 1999), although the negative finding of Love et al. (1997) in this regard should be noted. Efforts to visualize the distribution of actin filaments in the early stages of the photopolarization process using chemically fixed material have not revealed asymmetries (Brawley and Robinson, 1985; Krof et al., 1989), but there are significant problems with aldehyde fixation including the artifactual disruption of filaments (Miller et al., 1996) and increased autofluorescence. Alessa and Krof (1999) introduced fluorescent phalloidin into living cells by saponin permeabilization and reported the existence of dense patches of F-actin some 25 μm in diameter that redistributed to the future rhizoidal pole during photopolarization.

The first indication of a role for Ca\textsuperscript{2+} in photopolarization came from tracer flux studies that showed that the permeability of the plasma membrane of the shaded (future rhizoid) pole of \textit{Pelvetia} zygotes was about five times greater than the Ca\textsuperscript{2+} permeability of the illuminated side (Robinson and Jaffe, 1975). This polar difference in permeability declined with the approach of germination. It was subsequently found that dark-grown zygotes responded to external gradients of calcium ionophore by germinating from the regions that were exposed to the highest ionophore concentration (Robinson and Cone, 1980), consistent with the view that the formation of an intracellular Ca\textsuperscript{2+} gradient, with elevated Ca\textsuperscript{2+} at the future germination site, is an essential step in the polarization process. Because of the unfavorable optical properties of the fucoid zygotes (optical density and autofluorescence), only recently has there been experimental confirmation of the existence of cytosolic Ca\textsuperscript{2+} gradients associated with photopolarization. Using the long excitation wavelength calcium indicator, Calcium Crimson, ratioed against Rhodamine B, it was shown that a gradient of cytosolic Ca\textsuperscript{2+} developed within 1 h of exposure to unilateral blue light during the photosensitive period, with elevated Ca\textsuperscript{2+} in the cortex of the shaded hemisphere (Pu and Robinson, 1998). This asymmetry increased with continued light exposure and reached a peak after 2 h at which time cortical Ca\textsuperscript{2+} in the shaded hemisphere was two- to threefold greater than in the illuminated hemisphere. The difference in cortical Ca\textsuperscript{2+} between the two hemispheres then declined and was nearly absent at germination, despite continued unilateral illumination. This pattern of change in cortical Ca\textsuperscript{2+} paralleled the pattern of changes in membrane Ca\textsuperscript{2+} permeability revealed by the tracer flux measurements.

We wish to understand the process by which the activation of photoreceptors on one side of the \textit{Pelvetia} zygote leads to Ca\textsuperscript{2+} influx and elevated cortical Ca\textsuperscript{2+} on the opposite side. Clearly, the direct regulation of calcium channels by the photoreceptors cannot be involved, but rather there must be some more gradual signal transduction process that appears to involve an increase in cGMP (Robinson and Miller, 1997). There is some indirect evidence that calcium channels are redistributed rather than coordinately opened on one side of the zygote and closed on the other side (Robinson and Jaffe, 1975), and we have presented a model that incorporates that idea (Robinson et al., 1999). According to the model, the calcium channels are immobilized in the dark by links to the actin cytoskeleton. Upon unilateral illumination, the model proposes that the links to F-actin are broken, perhaps by depolymerizing the actin, allowing the channels to diffuse into the shaded hemisphere where they are reanchored to the still-intact actin filaments. Given the striking sensitivity of the photopolarization process to actin filament inhibitors, we have investigated the effects of latrunculin B on the formation of Ca\textsuperscript{2+} gradients in response to unilateral light. We have also determined the distribution of cortical actin filaments during photopolarization by microinjecting low concentrations of fluorescent phalloidin into the zygotes. This method reveals a pattern that is quite different from that reported by Alessa and Krof (1999). Our results are also not consistent with the passive diffusion-trapping model described above, leading us to consider the alternative possibility that calcium channels are redistributed actively by a myosin-powered process.

MATERIALS AND METHODS

Preparation of Material

Mature \textit{Pelvetia compressa} (formerly known as the species \textit{P. fastigiata}, now proposed to be transferred to a new genus, \textit{Silvetia} (Serrão et al., 1999)) fronds were collected near Santa Cruz, or Monterey, California, and shipped on ice by the collector via overnight express. Fronds were dried with absorbent paper towels and stored in monolayers in loosely capped containers at 4°C. The next day, the fronds were rinsed overnight at 1°C. The next day, the fronds were rinsed overnight at 1°C. The next day, the fronds were rinsed once more, this time in filtered natural seawater (NSW; Marine Biological Laboratory, Woods Hole, MA) and positioned near a unilateral white light source of 40 to 90 μmol photons m\textsuperscript{-2} s\textsuperscript{-1}. The next day, the fronds were rinsed with absorbent paper towels and stored as described above, leading us to consider the alternative possibility that calcium channels are redistributed actively by a myosin-powered process.
Zygotes were filtered through a 125- or 190-μm Nitex nylon filter shortly after fertilization and put either in the dark or back under the original light source until use. For imaging experiments, the zygotes were moved into dim red light after 15 min in the dark and filtered through a 190-μm Nitex nylon filter at the time of fertilization. For assessing the effects of various treatments upon photopolarization, the zygotes were filtered at 45 min after fertilization. At 6 h after fertilization, NSW was replaced by NSW containing various concentrations of putative inhibitors. The dishes were placed in front of a light box so that each dish received a white light intensity of about 50 μmol photons m⁻² s⁻¹. After 90 min of unilateral light exposure, the zygotes were rinsed three times in NSW to wash off the test solution and then returned to the dark. The next day, the orientation of the rhizoids with respect to the light direction of 200 zygotes in each dish was determined. Percentage photopolarization was calculated by using the formula (number of rhizoids growing away from the light – number of rhizoids growing toward the light)/2. In each experiment duplicate dishes were scored and the percentage photopolarization for the experiment was the mean of the two dishes. Each experiment was repeated at least three times and the overall mean of the percentage photopolarization was calculated.

The test solutions used were latrunculin B (Lat B) and 2,3-butanedione-2-monoxime (BDM). Lat B (Calbiochem) was dissolved in NSW and kept at a stock concentration of 50 nM at 70°C. BDM (Sigma) was dissolved in NSW just before its use.

**Microinjection**

Quantitative microinjection of Pelvetia zygotes was performed as described in Pu and Robinson (1998). To minimize cytoplasmic leakage, micropipettes were silanized the day before use by drying them at 160–220°C in a metal chamber for at least 1 h or overnight, and then baking with N,N-dimethytrimethylsilylamine (Fluka Chemical Corp., Milwaukee, WI) at the same temperature for 15–30 min. Zygotes were injected within 2 h after fertilization, when they had little cell wall, low turgor, and low sensitivity to external polarizing cues. Lat B was prepared as a 1.0 mM stock solution in dimethyl sulfoxide. Working solutions of Lat B at micro- and nanomolar concentrations were prepared by serial dilution of the stock solution in NSW. Calcium Crimson dextran (CCdex) and Alexa 568-conjugated phalloidin (AlexaPh) were supplied by Molecular Probes, Inc. (Eugene, OR) and dissolved in 200 mM KCl, 5 mM Hepes (pH 7.0) to achieve a final concentration of 2.5 mM for CCdex and 6.6 μM for AlexaPh.

**Fluorescence Imaging of P. compressa Zygotes**

For fluorescence imaging, zygotes, selected to have a diameter of 95–98 μm, were grown under dim red light and injected with 1.2 to 1.7 pl of 2.5 mM CCdex to reach a final cytoplasmic concentration of 13 to 18 μM. For AlexaPh imaging, zygotes were injected with 11.5 to 15.6 pl of 6.6 μM AlexaPh. Since the injection amount represented 5.5 to 6.6% of the cytoplasmic volume, the final cytoplasmic concentration of the AlexaPh was 360 to 440 nM. More than 90% of the cells that were injected under these conditions germinated normally. After microinjection, the zygotes were left under the dim red light in the injection chamber until close to 5.5 h after fertilization, at which time they became firmly stuck to the glass coverslip on which they grew. Fluorescence imaging of Pelvetia zygotes was performed essentially as described previously (Pu and Robinson, 1998), using the LaserSharp image acquisition program (version 3.0) installed on an upright Bio-Rad MRC-1024 Laser Scanning Confocal Imaging System. A refrigerated circulating water bath was utilized throughout the imaging process to maintain the cells at 16 ± 1°C. CCdex and AlexaPh were both excited by the 568-nm line of the krypton–argon laser installed on the microscope. The monitored emission wavelength for both indicators was 598 nm with a bandwidth of 40 nm. In conjunction with the Leitz 25× water immersion objective lens (numerical aperture 0.60), the settings on the confocal system were scan speed slow, filter Kalman n = 1, box size 512 × 512, zoom 2.80, and laser power 10% (for CCdex) or 30% (for AlexaPh). For CCdex imaging, the gain setting on the photomultiplier tube was adjusted so that only a few pixels of the entire image reached the saturation value of 255. For AlexaPh imaging, the gain was kept constant at a value of 1200 since the output was overall far below the saturation level. Polarizing blue light (about 10 μmol photons m⁻² s⁻¹) was directed on the cells perpendicular to the optical axis at the indicated times.

The cortical 5 μm of the cytoplasm just underneath the plasma membrane was analyzed for average pixel values of AlexaPh fluorescent intensity using the "linescan" function of the MetaMorph software program as described above for CCdex and RBdex imaging. Only those zygotes that germinated on the same schedule as the uninjected controls were included in the analysis. To compare the relative fluorescence intensity of the shaded and the illuminated hemispheres, for individual zygotes at each time point of imaging, the average fluorescence intensity of the shaded hemisphere and that of the illuminated hemisphere were obtained and their ratio was calculated. Grand means of the ratio were obtained over several zygotes imaged independently. To compare the relative fluorescence intensity at different time points after unilateral light exposure was begun, for individual zygotes, the average relative fluorescence intensity of the entire cortex just prior to unilateral blue light exposure was considered to be 1.0. Means of the relative fluorescence intensity over several zygotes at the same time point of imaging were calculated.

**RESULTS**

**Latrunculin B Inhibits Normal Photopolarization**

In view of the uncertainty of the effect of Lat B on photopolarization (Hable and Kropf, 1998; Love et al., 1997), we exposed P. compressa zygotes to 90-min pulses of unilateral white light in the presence of various concentrations of Lat B. This light treatment resulted in a photopolarization of about 55% (always between 50 and 60%) in controls that were not exposed to Lat B. At a concentration of 1 nM, Lat B reduced photopolarization to less than half of the control level (Fig. 1, filled circles) and higher concentrations abolished normal photopolarization altogether. Remarkably, Lat B at 10 nM produced a significant reversal of the normal direction of photopolarization; that is, the zygotes tended to germinate toward the light source (relative photopolarization of −0.21 ± 0.03). In these experiments, Lat B was present only during the 90-min light exposure and the treated cells recovered and germinated normally, albeit with some delay.
Latrunculin B Prevents the Formation of Cytoplasmic Ca\(^{2+}\) Gradients during Photopolarization

To test the hypothesis that actin filaments were the anchors for plasma membrane Ca\(^{2+}\) channels responsible for the formation of cytoplasmic Ca\(^{2+}\) gradients, CCdex was microinjected into newly fertilized Pelvetia zygotes, and the pattern of cytoplasmic Ca\(^{2+}\) was followed during photopolarization in the presence of 5 nM Lat B. It was possible to use a nonratiometric assay for Ca\(^{2+}\) because the zygotes show no cortical optical asymmetry during the first few hours of photopolarization, as determined by imaging injected Rhodamine B dextran (Robinson and Pu, 1998). Images of a CCdex-injected, Lat B-treated zygote are shown in Fig. 2. Prior to unilateral blue light exposure (Fig. 2A), there was no apparent asymmetry in the imaged zygote. After 1 h (2B) and 2 h (2C) of blue light exposure, the usual cytoplasmic Ca\(^{2+}\) gradient (elevated Ca\(^{2+}\) on the shaded side) did not form, as indicated by the uniform CCdex fluorescence, and an apparent reversed gradient is evident in Fig. 2C. After Lat B was washed out, a small cytoplasmic Ca\(^{2+}\) gradient was established within 2 h of continued light exposure (Figs. 2D and 2E), and the CCdex fluorescence asymmetry persisted until germination had occurred (2H and 2I), when increased fluorescence brightness was seen at the site of germination in these nonratiometric images.

Quantitative analysis of five zygotes photopolarized in the presence of 5 nM Lat B is summarized in Fig. 3. There was little initial asymmetry prior to photopolarization at 5.5 h. In response to unilateral blue light, a faint, but statistically significant, asymmetry in the reverse of the usual direction arose, and after 2 h of light exposure, the average fluorescence intensity on the illuminated hemispheres was about 5% more than on the shaded hemispheres. This is quite different from the situation in the absence of Lat B, in which the CCdex fluorescence from the shaded hemispheres averages 20% more than that from the illuminated hemispheres (Pu and Robinson, 1998). When Lat B was washed out at 7.5 h after fertilization, a small asymmetry in the usual direction was reestablished during the next 4 h. Due to the optical asymmetry of the cells that arises shortly before germination (Pu and Robinson, 1998), the fluorescent asymmetry seen in Figs. 2H and 2I was not entirely due to the influence of Ca\(^{2+}\) and so those images are not included in the analysis in Fig. 3.

Actin Filament Reorganization Occurs during Photopolarization

In view of the striking effects of actin filament inhibitors on photopolarization and Ca\(^{2+}\) gradient formation, we have investigated the distribution of actin filaments during the early stages of polar axis formation. Chemical fixation increases the already significant autofluorescence of these cells and also has the potential to distort actin filament distribution; therefore we chose to microinject trace amounts of the actin filament-binding toxin phalloidin.
Miller et al. (1996) have shown that low cytoplasmic concentrations of phalloidin did not interfere with pollen tube growth, and they were able to follow actin filament dynamics in phalloidin-injected pollen tubes using confocal microscopy. Utilizing a similar approach, we injected the long excitation wavelength fluorescent toxin Alexa 568 phalloidin into Pelvetia zygotes. Although injections that resulted in greater than 500 nM cytoplasmic concentration of AlexaPh usually delayed germination, 360 to 440 nM AlexaPh in the cytoplasm did not interfere with the normal development of Pelvetia zygotes and also provided adequate signal above the autofluorescence of the cells. This concentration of phalloidin is an order of magnitude less than the concentration that has been shown to be required to significantly alter the distribution of actin between the monomeric and the polymerized state (e.g., Butler et al., 1998). Zygotes that were injected with AlexaPh and developed on the same schedule as the uninjected controls were used in the following analysis. Pelvetia zygotes in general were tolerant of the repetitive scanning of 30% of the maximal laser power when three to four serial images were taken every hour. Autofluorescence accounted for approximately

---

**FIG. 2.** The effect of Lat B on the formation of Ca$^{2+}$ gradients during photopolarization. Shown are pseudocolor images of a zygote that was injected with CCdex. Image A was taken at 5.5 h AF, just before blue polarizing light was turned on and just before Lat B (5 nM final concentration) was added. Images were then obtained at 1-h intervals. Lat B was washed out immediately after image C was obtained, 2 h after unilateral light exposure was begun. In the absence of Lat B, the maximal Ca$^{2+}$ gradient forms in response to unilateral blue light in 2 h, with high Ca$^{2+}$ on the shaded side (Pu and Robinson, 1998). In this case, image C shows no fluorescence intensity gradient in the normal direction.
point.

4, and 5 h are not significantly different from the "Just before" Lat B, the same concentration that blocked the formation of degradation or sequestration inside the cells. The increase be resolved currently. The result could be due to phalloidin indication of a change in actin filament distribution cannot light exposure. Whether this decline represents a genuine decline in cortical fluorescence. As 10 μM Lat B is 3 orders of magnitude greater than the concentration that blocks photopolarization and Ca\(^{2+}\) gradient formation, and 2 orders of magnitude greater than the measured equilibrium dissociation constant of Lat B binding to maize pollen G-actin (Gibbon et al., 1999), we assume that it was sufficient to disrupt all or nearly all of the actin filaments. We therefore regard the remaining fluorescence as originating from autofluorescence and from dispersed AlexaPh not associated with actin filaments. The value of the remaining fluorescence was then subtracted from the initial measurements of cortical fluorescence that were used to generate the filled circles of Fig. 5 and a new set of corrected values was obtained. These background-corrected values are shown in Fig. 5 (open triangles) and it is seen that the cortical AlexaPh fluorescence associated with actin filaments increases by 2.7-fold following 2 h of unilateral blue light treatment.

At each subsequent time point of imaging, the relative cortical AlexaPh fluorescence of each zygote was measured and the means of the relative fluorescence of all zygotes were calculated. When the average pixel values of the shaded hemisphere and the lighted hemisphere from individual zygotes were analyzed (Fig. 6), it was found that the ratios did not vary significantly from 1.0 until 2 h after beginning light exposure. We also did the same analysis on the background-corrected data shown in Fig. 5, and the results were not different from the analysis on the uncorrected measurements (data not shown).

Growing Rhizoids Contain a Distinct Pattern of AlexaPh Fluorescence

When thin optical sections were taken of growing rhiz-oids after germination had occurred (Fig. 7), it was observed that AlexaPh fluorescence increased at the growing rhizoid (7A and 7B) and that diffuse fluorescence formed around the nucleus. This diffuse nuclear fluorescence became continuous with the apical fluorescence, and distinct bright sub-apical spots were present (Figs. 7C–7E). Serial scanning
images of the zygotes indicate that those bright spots were present throughout the subapical region of the rhizoid, suggesting that the distinct spots formed a ring structure in the continuously extending rhizoid. The diffuse fluorescence, presumably indicating loose actin bundles, became more compact and the subapical actin ring increased in brightness (7D and 7E). In the uninjected control zygote (7F), imaged with the same settings as the injected cell, the autofluorescence was weak and only the outline of the zygote was barely visible.

BDM Inhibits Photopolarization

In view of the finding that actin filaments assemble uniformly rather than disassemble locally during photopolarization, as predicted by our model, we have considered the alternate possibility that the creation of calcium gradients depends on an active actin/myosin system. BDM disrupts the interaction of actin and myosin with low affinity, but apparently high specificity, and interferes with myosin-driven processes in a number of biological systems (May et al., 1998; Ruchhoeft and Harris, 1997; Zhao et al., 1995). In our hands, BDM rapidly abolishes the characteristic streaming known to be myosin-dependent in lily pollen tubes at a concentration of 75 mM (unpublished observation of K.R.R.). In the case of Pelvetia zygotes, we found that BDM reduces photopolarization by 50%, compared to controls, at a concentration of 10 mM and completely abolishes photopolarization under our standard conditions at 40 mM (Fig. 1, triangles).

DISCUSSION

Our results confirm that actin filaments are necessary for photopolarization of Pelvetia zygotes and that a very low
Actin Polymerization and Calcium Gradients

Concentration, about 5 nM, of the marine sponge toxin, Lat B, abolished photopolarization. This is in agreement with the findings of Nelson and Jaffe (1973), Quatrano (1973), and Hable and Kropf (1997), but in disagreement with Love et al. (1997). Furthermore, our data indicate that higher concentrations of the toxin caused a significant negative photopolarization; that is, the rhizoids tended to form on the side of the cells that faced the light source. Negative photopolarization was also reported by Hable and Kropf (1998) at 30 nM Lat B.

We have previously shown that exposure to unilateral light caused the development of a cortical gradient of Ca\(^{2+}\), with elevated Ca\(^{2+}\) at the shaded, future rhizoidal pole (Pu and Robinson, 1998). Lat B, at a concentration that blocked photopolarization, blocked the formation of the usual Ca\(^{2+}\) gradients, and when the toxin was washed out, Ca\(^{2+}\) gradients in the normal direction formed in response to continued unilateral illumination. A small but significant reversed Ca\(^{2+}\) gradient formed in the presence of Lat B. This may underlie the negative photopolarization that was induced by Lat B and gives support for the idea that the ultimate determinate of the axis in these cells is a Ca\(^{2+}\) gradient. It is also consistent with the possibility that there is a second photosystem that is detected only if the normal dominant system is compromised. This hypothesized second photosystem would not be dependent on F-actin to form a Ca\(^{2+}\) gradient and might involve the direct regulation of calcium channel gating by the photoreceptor molecules.

The use of trace amounts of microinjected fluorescent phalloidin showed that treatment with unilateral blue light induced a substantial increase in cortical fluorescence, about 2.5-fold after background correction. This increase was inhibited by 5 nM Lat B. Somewhat surprisingly, the increase in cortical AlexaPh fluorescence was not significantly asymmetric initially; that is, it occurred uniformly along the rhizoid-thallus axis. Only after 2 h of light treatment was a significant asymmetry detected in the shaded hemisphere and the difference between the two hemispheres did not exceed 10% until the time of overt germination. This is consistent with earlier experiments that examined the distribution of phalloidin and phallacidin in aldehyde-fixed zygotes of P. compressa (Brawley and Robinson, 1985) or F. distichus (Kropf et al., 1989). Both of those papers reported no obvious asymmetries in F-actin distribution during the early stages of the photopolarization process. It is important to note that a significant cytosolic Ca\(^{2+}\) gradient is evident after 1 h of exposure to polarizing light (Pu and Robinson, 1998), so an asymmetry in cortical actin filaments is not involved in the initial stages of the generation of the Ca\(^{2+}\) gradient.

We also observed an increase in perinuclear fluorescence brightness and it too appeared to be initially uniform with respect to the axis defined by the unilateral light. Further analysis of this interesting phenomenon was not attempted because it is difficult to image the interior of these highly opaque cells, even with confocal microscopy.

The concentration of LatB that blocked the light-induced increase in cortical actin filaments and also completely inhibited photopolarization (5 nM) did not depolymerize the actin filaments that were present in the dark-grown cells, as determined by the amount of AlexaPh fluorescence (Fig. 5). A considerably higher concentration (10 \(\mu\)M) was required to reduce the AlexaPh fluorescence to a minimum. It may be that the process of actin filament formation is more sensitive to Lat B than existing filaments or that two populations of actin with different sensitivities to Lat B are involved. A related situation has recently been reported to occur in maize pollen tubes (Gibbon et al., 1999). There it was found that 5 nM Lat B blocked pollen tube growth even though the equilibrium dissociation constant for Lat B binding to maize pollen G-actin was found to be 74 nM. Gibbons et al. (1999) offer as one explanation the possibility of “the presence of a small population of LATB-sensitive actin filaments [that] is critical for maintenance of tip growth.”

Alessa and Kropf (1999) introduced rhodamine phalloidin into P. compressa zygotes by permeabilizing the cells with saponin for 1 h. They reported that large (25 \(\mu\)m), intense patches of actin filaments were present in the cortex of young zygotes and that the patches became localized to the rhizoidal pole during photopolarization. In no case did we
detect such patches of actin with microinjected phalloidin. One possible explanation for the results of Alessa and Kropf (1999) is that they permeabilized the cells in an artificial seawater containing 9 mM Ca$^{2+}$ as well as 450 mM Na$^{+}$. As permeabilization sufficient to allow the phalloidin to enter must necessarily allow massive movements of other small molecules and ions across the plasma membrane, the cytosolic Ca$^{2+}$ will have gone to extremely high levels from its normal concentration of about 300 nM. Perhaps this induced the polymerization and aggregation of actin into the patches that they observed. In our hands, saponin permeabilization in seawater resulted in extremely poor survival of zygotes and survivability was considerably increased if the permeabilization was done in a cytoplasmic-like solution (unpublished observations of R.P. and K.R.R.). In any case, microinjection of fluorescent phalloidin never resulted in images that showed intense patches of fluorescence, even when the confocal microscope was used to optically section the injected cells. We think that microinjection is a gentler, if more tedious, method than permeabilization for introducing phalloidin and is more likely to give results that accurately reflect the state of actin filaments in the zygotes. We have found that microinjection of Pelvetia zygotes with up to 6% of the cellular volume is followed by normal development of more than 95% of the injected cells, if the injected solution itself is not inhibitory (Pu and Robinson, 1998, Table 1). Thus, the introduction of materials into the cytoplasm by microinjection is nonperturbing.

The lack of cortical asymmetry in actin filament distribution during the early stages of light exposure raises questions about the manner of the filaments’ participation in photopolarization. No significant asymmetry was detected until 2 h after the polarizing light was turned on. This is in contrast to the cortical Ca$^{2+}$ asymmetry, which is significant within 1 h of initiating unilateral light exposure and is fully developed by 2 h (Pu and Robinson, 1998). Our previous model in which actin filaments were imagined to anchor calcium channels differentially on the shaded and illuminated sides, leading to trapping of the channels on the shaded side, is not supported by the data presented here, as

**FIG. 7.** Pseudocolor images of growing rhizoid of an Alexa 568 phalloidin-injected zygote (A–E) and an uninjected germinated zygote (F). Images A–E were obtained from 11.5 until 15.5 h AF at 1-h intervals, during which time the rhizoid grew continuously. Image F shows the negligible autofluorescence of the zygotes under conditions identical to those used for A–E.
that model requires an asymmetry in actin filament distribution to precede the Ca\(^{2+}\) asymmetry. In response to unilateral light, actin filaments form in the entire cortical region of the zygotes, with little or no axial asymmetry. One possibility is that the actin filaments act as a passive scaffold and the directionality of the photopolarizing event is supplied by some other agent. An alternate possibility is that the actin filaments do have a polarity that is determined by the light gradient and that polarity determines the direction of movement of plasma membrane calcium channels, or channels present in vesicles, to the future rhizoidal pole. The inhibitory effect of BDM on photopolarization suggests a role for an actin/myosin interaction.

We confirm earlier reports of a condensation of actin filaments, as indicated by greater AlexaPh brightness, near the site of germination at about the time of germination. We also confirm Alessa and Kropf’s (1999) report of the formation of a subapical ring of actin filaments near the rhizoidal tip, again as indicated by AlexaPh brightness. The apparent paucity of actin filaments at the rhizoidal apex is reminiscent of the situation in growing pollen tubes, in which filaments are rare at the growing tip (Kost et al., 1998; Miller et al., 1996). Alessa and Kropf (1999) suggest that the ring may function to establish the boundary between the actively growing domain of the rhizoid and nongrowing subapical domain. The ring may act to keep calcium channels localized to the tip of the rhizoid.

In summary, our results reveal that the actin cytoskeleton is quite dynamic during the polarization process and that the formation of cortical actin filaments is necessary for the development of cytosolic Ca\(^{2+}\) gradients, which, in turn, are necessary for the functional polarization of the zygotes. The question of how the actin filaments interpret the illumination gradient and impart directionality to calcium channel distribution remains open, but we suggest that there is a global polarity to the actin filaments and that the directional motor molecule, myosin, actively determines the distribution of calcium channels and other important morphological determinants.

ACKNOWLEDGMENTS

We thank David Epel (Hopkins Marine Station, Stanford University) and Henrik Klbak (Monterey State College) for their help in locating Pelvetia collectors. This research was supported by the National Science Foundation (IBN-9972975).

REFERENCES


Received for publication October 6, 1999
Revised January 10, 2000
Accepted March 12, 2000