Cytochalasin Treatment Disrupts the Endogenous Currents Associated with Cell Polarization in Fucoid Zygotes: Studies of the Role of F-Actin in Embryogenesis

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ABSTRACT We determined the distribution of F-actin in fucoid (*Pelvetia, Fucus*) embryos with nitrobenzoxadiazole-phallacidin, and studied the effect of cytochalasin upon the endogenous currents associated with cell polarization by using the vibrating probe. F-actin is not localized at the presumptive rhizoid immediately after experimental induction of the polar axis with a light gradient; however, a preferential distribution of F-actin develops at the presumptive rhizoid by the time the position of the polar axis is fixed. F-actin continues to be localized at the tip of the rhizoid after germination, except during cytokinesis, when the furrow is the only brightly staining region of the embryo. Incubation with cytochalasin can result in either an enhanced or a diminished pool of F-actin in the embryonic cortex (see Results).

Cytochalasin D (100 μg/ml) significantly reduces the inward current at the rhizoid pole (n = 11) after a 2.5-h incubation. This drop is concentration dependent and occurs within ~30 min at 100 μg/ml and ~60 min at 10 μg/ml. Cytochalasin treatment eliminates the pulsatile component of the current. Preliminary results suggest that 100 μg/ml cytochalasin D prevents development of inward current at the presumptive rhizoid but does not completely delocalize this locus if added after photopolarization. We conclude that microfilaments are required for the establishment and maintenance of the pattern of endogenous currents observed during early embryogenesis. This suggests a new model for axis formation and fixation.

Cell polarization is an essential aspect of development. The fucoid algae have been especially favorable systems for studies of this process because the unfertilized egg is radially symmetric, and the polar axis is established many hours after fertilization. The polar axis can be induced by unilateral illumination and reoriented numerous times before ~10 h postfertilization (see review by Quatrano, reference 36). This first phase of cell polarization, during which the axis remains labile, is called axis formation. When the axis can no longer be reoriented (at ~10 h postfertilization), axis fixation is said to have occurred. Germination occurs several hours afterwards. Recent work has demonstrated that the earliest phase of axis formation is associated with the appearance of inward electrical current at the presumptive site of germination (30). Part of this current is calcium carried (40), and a critical role for calcium has been demonstrated by ionophore experiments as well (39). Other studies have indicated that actin microfilaments probably mediate some step of polarization, because zygotes cannot be polarized when incubated with cytochalasin (8, 35).

Important interactions may occur during polarization between localized, endogenous current and the cytoskeleton in these zygotes, but these have been impossible to elucidate without a way to localize actin. A presumptive actin fraction was isolated from *Fucus* zygotes (37), but microfilaments have not been observed in ultrastructural studies (7, 9), nor has it been possible to localize actin with heavy meromyosin (6). Large quantities of intracellular phenolics (38) are generally held responsible for these difficulties; the phenolics are released from membrane-bound vesicles during many proce-

THE JOURNAL OF CELL BIOLOGY · VOLUME 100 APRIL 1985 1173–1184
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dures, and they bind exogenous and endogenous protein.

We have used nitrobenzoxadiazole (NBD)-[1] phallacidin (2) to stain F-actin, and the vibrating probe to study the effect of cytochalasin upon the endogenous currents. Our results (below) suggest that microfilaments are required for the establishment and maintenance of the pattern of endogenous currents observed during polarization of fucoid zygotes. The new model we propose for cell polarization hypothesizes (*a*) that microfilaments are required for both formation and fixation of the axis through distinct processes, and (*b*) that an important aspect of axis fixation is incorporation of membrane-containing ion channels at the rhizoid pole vis à vis localized secretion from the Golgi complex.

MATERIALS AND METHODS

Collection and Culture of Material: *Fucus vesiculosus* and *F. distichus* were obtained from Nahant, MA, and Bar Harbor, ME: *Pelvetia fastigiata* was obtained from Pacific Grove, CA. Unless noted otherwise, chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

We obtained batches of synchronously developing zygotes by standard methods (19, 36). They were cultured in seawater at 15°C until use with constant unilateral light (32 $\mu E/m^2$ per s) from a bank of fluorescent lights. Zygotes were grown on coverslips to which they were firmly attached by secretion (i.e., of cell wall constituents) 5 h postfertilization. Coverslips were marked so that the presumptive axis induced by the light gradient was known during subsequent processing of zygotes. Under our culture conditions, germination of *Pelvetia* occurred ~12 h postfertilization. Jaffe (20) found that ~50% germination had occurred by 16 h postfertilization when zygotes were grown at 15°C and exposed to 1 h of 100 ftc white light. Germination occurs as early as 9 h postfertilization (30) when zygotes are cultured (15°C) with continuous (unilateral) illumination (125 ftc) supplemented by light from the microscope (passed by a 546-nm interference filter).

Cytochalasin D (CD) or dihydrocytochalasin B (H_2CB) was used in a dimethyl sulfoxide carrier (1%) at 50–100 μg/ml to block photopolarization, as described by Quatrano (35). Before being used to collect new data, each batch of cytochalasin was bioassayed by repetition of Quatrano's experiment.

Localization of F-Actin: NBD-phallacidin (Molecular Probes, Junction City, OR) was prepared as a methanol stock solution. Aliquots were dried under a stream of air and reconstituted in phosphate-buffered saline (PBS) at 150 ng/ml. Controls were stained with a solution of phalloidin (unlabeled) and NBD-phallacidin (50:1). Phalloidin binds irreversibly to F-actin, and, in excess, will displace NBD-phallacidin, which binds reversibly to F-actin (2).

Zygotes were fixed for 30 min with 3% paraformaldehyde in PBS (pH 7.4) containing 4% polyethylene glycol, 3% NaCl, 1% bovine serum albumin and 1 mM EGTA, then permeabilized with saponin (0.01–0.1%) in this buffer for 2–3 min. After a rinse in buffer, zygotes were transferred to PBS (pH 7.4) containing 3% NaCl. We eliminated the NaCl in 25% steps over a period of 10 min. Several drops of NBD-phallacidin or phalloidin/NBD-phallacidin (50:1) were added to specimens on coverslips, and these were incubated in a moist chamber. After staining was done for 30 min, the coverslips were rinsed in PBS and mounted in PBS (pH 7.8)/glycerol (1:1) for observation. Zygotes <5 h old were used as suspensions. The dense eggs quickly sank to the bottom of staining tubes and were transferred between solutions with a pipette.

We photographed specimens with Kodak TRI X film and a Leitz 40 × oil objective (numeric aperture 1.30), using an exposure of 30 s. We push-processed the TRI X film with Kodak HC-110 developer (12 min at 20°C using dilution B). However, the specimens in Figs. 5 and 6 were photographed by the use of a Leitz 40 × dry objective (numeric aperture 1.00) with an exposure time of 1 min, and the film was not push-processed. Control and experimental figures in each plate were printed identically, but, between plates, a longer development during printing was sometimes necessary to produce figures that accurately reflected our microscopic observations. (see legend to Figs. 11–13).

We blocked the red autofluorescence of the chloroplasts by viewing and photographing specimens through an interference filter (Pomfret Research Optics Inc., Stamford, CT) that passed only light of 510–540 nm. The yellow autofluorescence of other materials (e.g., phenolics) could not be filtered and was not reduced when polyvinylpyrrolidone or caffeine (12, 26) was added to any step of the staining procedure. Since we observed increased autofluorescence after the saponin treatment, despite the addition of materials to quench phenolics, we suspected proteolysis and adjusted buffers with EGTA, etc. EGTA did reduce the autofluorescence, but 5 mM EGTA was no more effective than 1 mM. Several protease inhibitors (α-macroglobulin, leupeptin, and antipain) were added to buffers, as was dithiothreitol, but none of these materials reduced the autofluorescence. Met-embryos (8) of *Fucus* were less autofluorescent than normal embryos, but elimination of free sulfate in this way had no effect upon *Pelvetia*. In general, autofluorescence was less a problem in experiments in which *Pelvetia*, rather than *Fucus*, was used. There was considerable seasonal variability in material, however.

Material was embedded in JB-4 (Polysciences, Inc., Warrington, PA) and HPMA (SPI Supplies, West Chester, PA) water-soluble resins after a similar fixation (3% paraformaldehyde, PBS, pH 7.4, 3% NaCl), step-rinse in PBS to eliminate the NaCl, and ethanol dehydration. Sections were cut on dry glass knives with a MT-2 ultramicrotome and mounted on slides for staining.

Vibrating Probe Studies: Jaffe and Nuccitelli (21) have described the extracellular vibrating probe. The platinum black electrode (15–30 μm in diameter) was vibrated normal to the cell with a gap of 25 μm between the cell surface and the electrode. Zygotes were cultured and measured in an artificial seawater medium of high resistivity (68 $\Omega \cdot$cm), as devised by Nuccitelli (30). This medium contains only 40% of the normal concentration of sodium and magnesium in seawater; osmotic strength is maintained by mannitol. Our measurements were made at pH 8. Specimens were exposed to 26 $\mu E/m^2$ per s light from the illumination system of the inverted microscope, but this was filtered through a 546 nm interference filter so that the axis would not be affected. Polarization of the zygotes of these algae depends upon blue light (18).

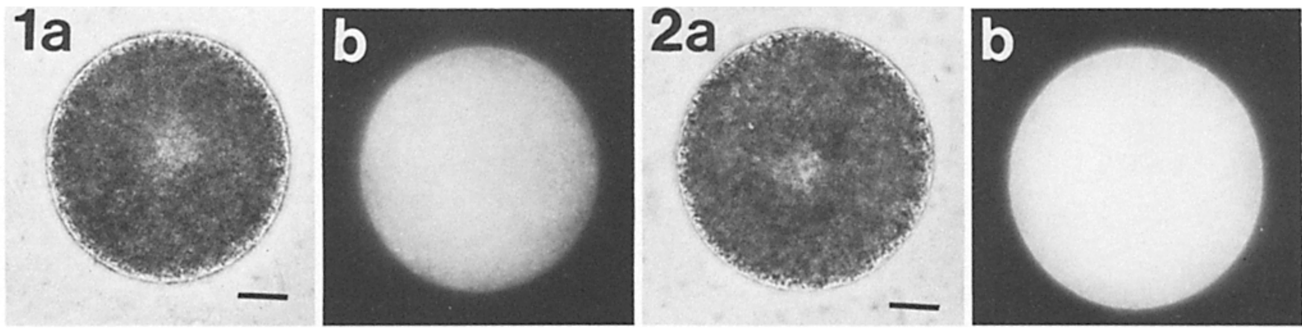
RESULTS

Localization of F-Actin

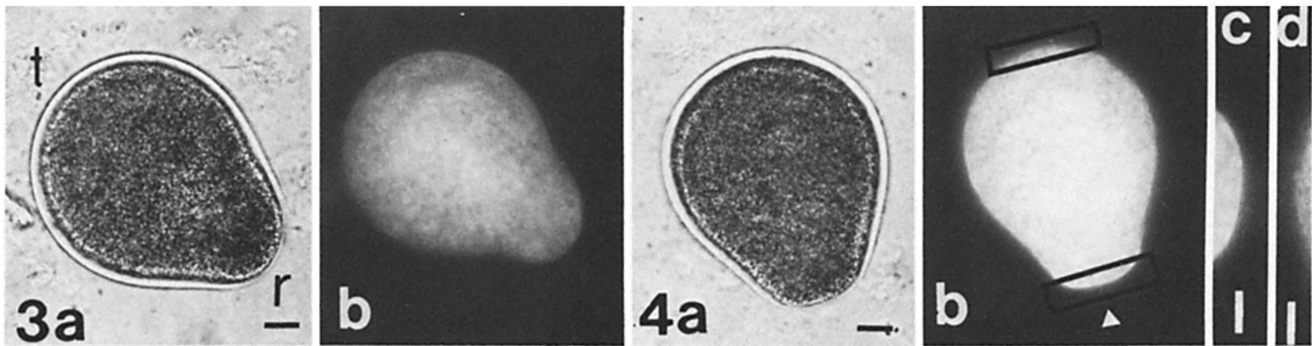
We stained zygotes and embryos of *F. vesiculosus* and *P. fastigiata* with NBD-phallacidin to find whether asymmetries of F-actin occur in association with developmental asymmetries. These studies elucidated only the cortical distribution of F-actin; the shape, size, density, and autofluorescence of the fucoid egg made it impossible to infer what the internal pool of F-actin may be from study of permeabilized whole mounts. We embedded material in several water soluble resins (HPMA, JB-4) and attempted to stain semithin sections with NBD-phallacidin without success. Autofluorescence was a significant problem (see Materials and Methods); however, the in-focus margin of the cell stained brightly with NBD-phallacidin, and asymmetries in staining were apparent as development proceeded. Background autofluorescence was not increased by exposure to phalloidin/NBD-phallacidin (50:1). Most of the results we present here are for *Pelvetia*; however, we obtained essentially identical results with *Fucus*.

We observed significant changes in the cortical distribution of F-actin during early embryogenesis. In contrast to controls (Fig. 1, stained with NBD-phallacidin and an excess of unlabeled phalloidin), the cell margin is well-defined in zygotes (Fig. 2) stained solely with NBD-phallacidin. Excess, unlabeled phalloidin was used as a control (see Materials and Methods) to show binding specificity and integrity of the fluorochrome-phallacidin moiety. For ~2 h postfertilization, the F-actin (Fig. 2) in the cortex was distributed evenly (compare Fig. 2*b* with a control in Fig. 1*b*). This uniformity contrasts with the asymmetric pattern of F-actin observed in the cortex of germinated embryos (control, Fig 3*b*; Fig. 4, *b–d* [*Pelvetia*]; control, Fig. 5*b*; Fig. 6*b* [*Fucus*]). A thin border of F-actin is usually found throughout the cortex, but localization occurs preferentially at the tip of the rhizoid. Several hours after germination, the first cell division occurs, and F-actin is localized along the cleavage furrow at this time (Fig. 7). After completion of cytokinesis, the partition region is unstained (not shown). There appears to be little F-actin at the rhizoid pole during cytokinesis, since this region is rarely stained by NBD-phallacidin at this time.

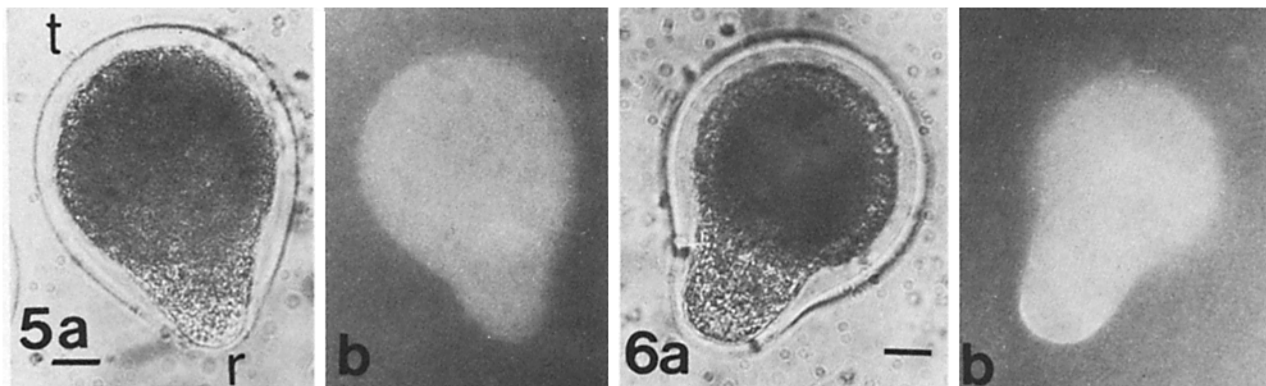
[1]*Abbreviations used in this paper:* CD, cytochalasin D; H_2CB, dihydrocytochalasin B; NBD, nitrobenzoxadiazole.



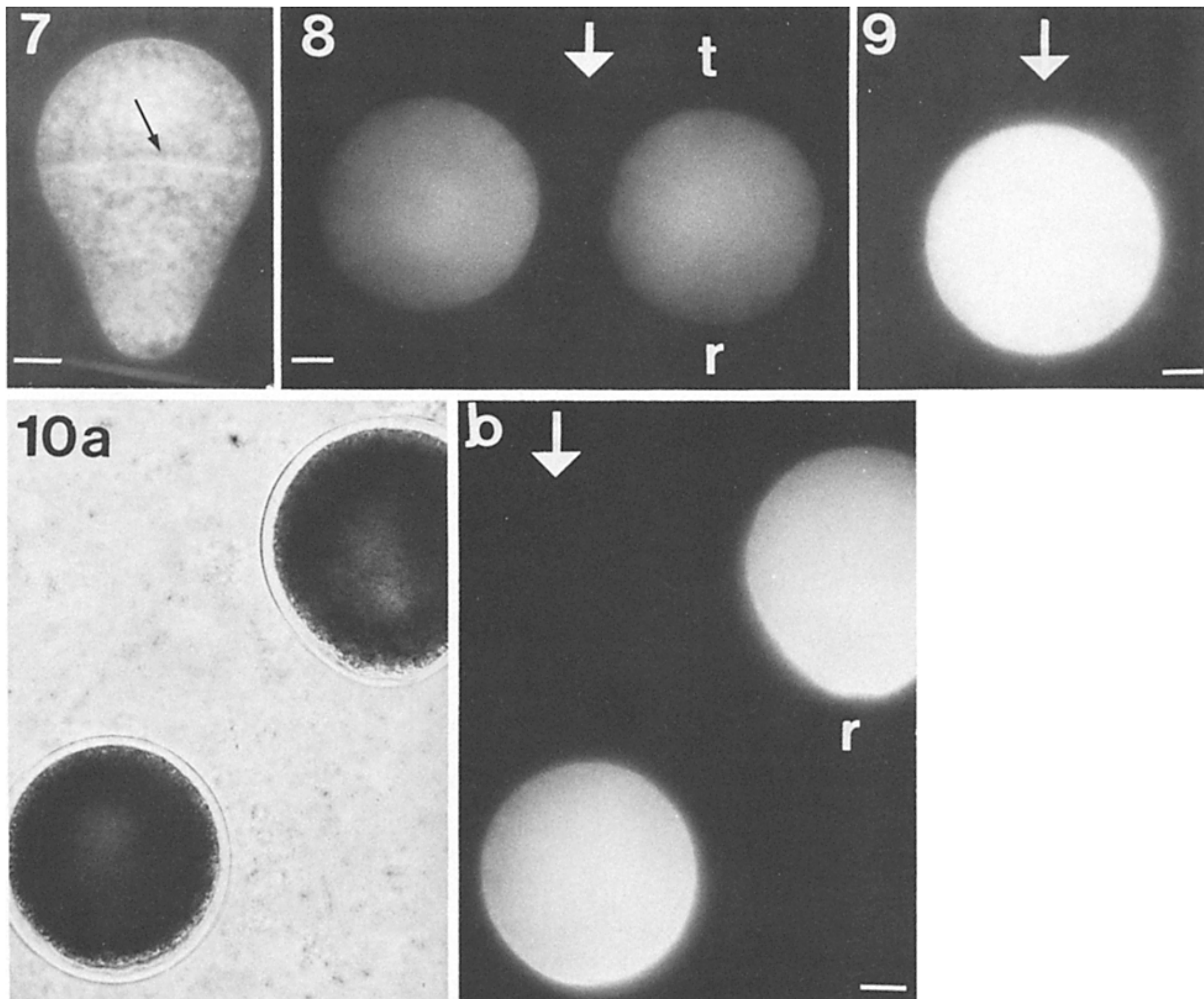
Figures 1 and 2 (Fig. 1) 2-h old zygote of *P. fastigiata* stained with a solution of NBD-phallacidin/phalloidin (1:50) after fixation with formaldehyde and saponin treatment (see Materials and Methods). (*a*) Bright-field. (*b*) Fluorescent. Control for Fig. 2. Note that the cortex (observe at cell margin) is indistinct as compared with Fig. 2. Treatment with NBD-phallacidin/phalloidin (1:50) did not increase background fluorescence as compared with cells similarly fixed, but left in buffer (not shown). Bar, 20 μm. × 340. (Fig. 2) 2-h zygote of *P. fastigiata* stained with NBD-phallacidin. (a) Bright-field. (b) Fluorescent. Note the sharp definition of the cortex adjacent to the plasmalemma in Fig. 2*b* as compared with Fig. 1*b*. Autofluorescence was a significant problem (see Materials and Methods) but did not mask NBD-phallacidin staining of the cortex. Bar, 20 μm. × 340.



Figures 3 and 4 (Fig. 3) Germinated embryo of *P. fastigiata* (19 h old). Corresponding bright-field (*a*) and fluorescent (*b*) micrographs. This embryo was stained with NBD-phallacidin/phalloidin (1:50) as a control. The margin (i.e., the cortex) of the cell is indistinct including both rhizoid (*r*) and thallus (*t*) poles. Bar, 20 μm. × 250. (Fig. 4) Germinated embryo of *P. fastigiata* (19 h old). Corresponding bright-field (a) and fluorescent (*b*) micrographs. This embryo was stained with NBD-phallacidin. The cortical cytoplasm of the rhizoid pole (arrowhead) immediately adjacent to the plasma membrane is brightly fluorescent; the thallus pole is only slightly brighter than the control (Fig. 3*b*). This is also demonstrated by *c* and *d*, equivalent areas at higher magnification of the rhizoid pole (*c*) and thallus pole (*d*) of the embryo in *b*. Although the entire embryo is bright, we infer information on only the cortical distribution of F-actin from the micrograph; the pear-shape, size, density, and autofluorescence of fucoid embryos make it impossible to observe the internal pool of F-actin from permeabilized whole mounts. (*a* and *b*) Bar, 20 μm. × 250. (*c* and *d*) Bar, 10 μm. × 500.



Figures 5 and 6 (Fig. 5) Germinated embryo of *F. vesiculosus* (20 h old). *a* and *b* are corresponding bright-field and fluorescent micrographs, respectively. Control is stained with NBD-phallacidin/phalloidin (1:50). Note that the margin of the cell is indistinct at both rhizoid (*r*) and thallus (*t*) poles. Bar, 20 μm. × 300. (Fig. 6) Germinated embryo of *F. vesiculosus* (20 h old). Corresponding bright-field (*a*) and fluorescent (*b*) micrographs stained with NBD-phallacidin. The cortex of the cell at the rhizoid pole is stained brightly by NBD-phallacidin. The thallus pole is indistinct although brighter than that of the control (Fig. 5*b*); compare with Fig. 4*b* (NBD-phallacidin–stained *Pelvetia* with similar localization of F-actin). Bar, 20 μm. × 300.



FIGURES 7–10 (Fig. 7) *P. fastigiata* embryo (23 h old) near completion of first cell division. NBD-phallacidin stains the cytokinetic furrow (arrow). The plane of focus is on the surface of the embryo rather than at the margin of the cell. During cytokinesis, the rhizoid pole rarely stains with NBD-phallacidin. Bar, 20 μm. × 425. (Figs. 8 and 9) Zygotes of *P. fastigiata*. (Fig. 8) Control (NBD-phallacidin/phalloidin [1:50]). (Fig. 9) NBD-phallacidin stained. These zygotes were photopolarized by unilateral light 5–6.5 h postfertilization and then fixed with formaldehyde and stained. Note that the presumptive thallus pole (*t*) stains as brightly as the presumptive rhizoid pole (*r*). The orientation of the zygotes with respect to the light gradient is known precisely because they were firmly attached by cell wall material on marked cover slips before photopolarization and remained attached during processing. The arrow indicates the direction of the light gradient. Bar, 20 μm. × 300. (Fig. 10) *P. fastigiata* (11 h old). Matching bright-field (*a*) and fluorescent (*b*) micrographs. Embryos were photopolarized (arrow indicates direction of light gradient). Note NBD-phallacidin staining of the presumptive rhizoid (ungerminated cell on left) and rhizoid pole (cell on right, *r*). Bar, 20 μm. × 300.

Axis formation and fixation occur before germination and, with respect to potential interactions of the cytoskeleton with endogenous electrical current, the distribution of F-actin at these times is especially significant. When zygotes were fixed with formaldehyde immediately after photopolarization (5–6.5 h postfertilization), the presumptive thallus pole stained as brightly with NBD-phallacidin as did the presumptive rhizoid pole (Fig. 8, control; Fig. 9). We observed a slight asymmetry in some zygotes, but, in these cases, the greater amount of F-actin was found in the illuminated hemisphere (i.e., thallus pole). We know the orientation of stained cells with regard to the photopolarization precisely because zygotes remained firmly attached to marked coverslips during processing (see Materials and Methods). By the time (~10 h postfertilization) at which the axis is established permanently, a substantial localization of F-actin occurs in the cortex of the presumptive rhizoid (Fig. 10).

Effect of Cytochalasin upon F-Actin Distribution

CD and H_2CB were used at a concentration of 100 μg/ml to determine the effect of these microfilament inhibitors on the distribution of F-actin in the embryo. These concentrations are much higher than those used to study actin-mediated events in animal cells, but plant cells appear to be relatively impermeable to cytochalasin. For example, in *Vaucheria*, some cell streaming continues for more than an hour in the presence of 50 μg/ml of cytochalasin B (4), and the rate is 50% of the normal rate after 20 min of treatment. Streaming recovers when cytochalasin is washed out. In fucoid algae, cytochalasin B (50–100 μg/ml) reversibly inhibits photopolarization and, by blocking transport of Golgi vesicles to the rhizoid from perinuclear Golgi bodies, inhibits localized secretion of the polysaccharide fucoidan (8, 27, 29, 35). Cytochalasin will not disorganize a previously induced axis, how-

ever, at least not during a subsequent 8-h incubation (35). When treated with 100 μg/ml of cytochalasin from 8 h postfertilization, mitosis occurs, as does cytokinesis (slowly). Delocalized growth is also observed; cytochalasin B- and CD-treated embryos have larger diameters than controls (6, 8).

Cytochalasin had various effects upon the distribution of cortical F-actin, but these seemed due at least partially to the type of cytochalasin used. Both CD and H_2CB are considered to be quite specific inhibitors of actin (e.g., see reference 1). When embryos that had germinated were treated for 2–3 h with either of these cytochalasins at 100 μg/ml, a preferential localization of F-actin at the rhizoid pole was still observed in ~20% of stained embryos. However, the entire cortical pool of F-actin increased in ~80% of embryos ($n = 50$, but thousands of embryos were scanned to confirm this data) treated with H_2CB (100 μg/ml; Fig. 11; compare Figs. 4*b*, 6*b*, and 10*b*). This was sometimes observed with CD treatment; however, exposure to CD frequently produced an overall reduction in cortical F-actin (not shown). These different effects of H_2CB and CD may be genuine. Different cytochalasins produce distinctive types of microfilament disruption in other cells (42). It is also possible that H_2CB and CD induce the same sequence of events but at quite different rates.

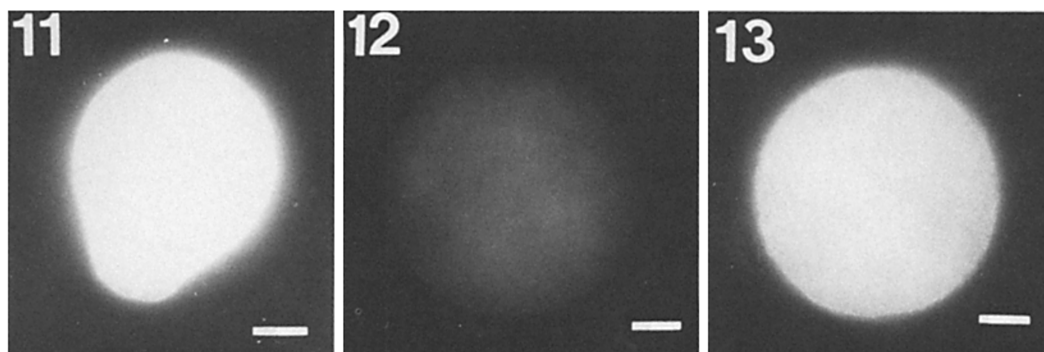
When zygotes were photopolarized in the presence of cytochalasin D and incubated with CD through 18 h postfertilization, the embryonic cortex appeared to contain little F-actin (Fig. 12). The pool of F-actin throughout the cortex was enhanced by H_2CB treatment under identical circumstances (Fig. 13). Whether enhanced or diminished, the distribution of F-actin was symmetric in the cortical cytoplasm after cytochalasin treatment.

Effect of Cytochalasin upon Endogenous Electrical Currents

We treated zygotes and embryos with CD (100 μg/ml) in several experiments to assess its effects upon the endogenous electrical currents associated with embryogenesis. The endogenous currents observed during embryogenesis in *Pelvetia* have two components. A pulsatile current appears primarily after germination (31, 32) and may be involved in osmotic regulation (33). The pulses have a rather constant shape and a duration of about 100 s, but the amplitude ranges from 0.38 to 1.25 $\mu A/cm^2$ at the center of the probe's vibration (~50 μm from the rhizoid tip). Although the pulsatile current accounts for only ~5% of the total current through the zygote during the first few hours after germination, the frequency of the pulses increases; such pulses account for ~25% of the total current at the two-cell stage.

The component of the current that is most evident in early embryogenesis is the steady current. The amplitude of the steady current varies from 0.38 to 0.75 $\mu A/cm^2$, but it changes 100 to 1,000 times more slowly than the pulse current (31). A region of steady, inward current is detected as early as 30 min postfertilization, and steady current is measured at the presumptive rhizoid ~40 min after the zygote is placed in a unilateral light gradient (30). The average inward current is 0.06 $\mu A/cm^2$ (at the probe) during early embryogenesis; it approximately doubles at germination.

To assess whether cytochalasin treatment affects the endogenous currents, we used embryos that had germinated 0–2 h before our initial measurements. An examination of such effects in young zygotes is important but would be a significantly more difficult starting place for such experiments; the current density is much lower at that time than in germinated embryos (30). We studied 11 different embryos in four similar experiments (Table I). In each experiment, the mean current density for each embryo was calculated from recordings made (over ~10 min) before and after incubation with CD (100 μg/ml) for 2.5 h. In three of the four experiments, a significant difference ($\alpha = 0.05$, two-tailed Mann-Whitney test) was found in the density of inward current at the rhizoid pole after incubation in CD (Table I, Fig. 14, *a–c*). Controls of ages comparable ($t_{0+2.5\,h}$) to those of the CD-treated embryos had higher current densities than did the preliminary values (t_0) for treated embryos (Table I, Fig. 14*d*). Portions of the recordings (e.g., see Fig. 14*c*) were of short duration and distinctly higher amplitude; these are interpreted as pulses of current. We observed pulses during the preliminary measurements from 2 of 11 embryos; we observed one pulse after CD treatment (Table I). Pulses were observed in three of seven controls ($t_{0+2.5\,h}$), confirming earlier observations that they are more common by this time (31). Pulse current densities were not included in the calculations shown in Table I.



FIGURES 11–13 (Fig. 11) *P. fastigiata* embryo treated with H_2CB (100 μg/ml) for 2 h beginning 16 h postfertilization, then fixed and stained with NBD-phallacidin. The cortical cytoplasm is evenly and brightly stained after H_2CB treatment. Controls with 1% dimethyl sulfoxide (not shown) were identical to those in Fig. 3*b*. Bar, 20 μm. × 300. (Figs. 12 and 13). *P. fastigiata* embryos photopolarized 7–9 h postfertilization and treated with cytochalasin beginning 7 h postfertilization. (Fig. 12) Treated with CD (100 μg/ml). (Fig. 13) H_2CB (100 μg/ml). The cortex (Fig. 13) is evenly and brightly stained in contrast to that of the CD-treated embryo (Fig. 12). The embryos are spherical because cytochalasin prevents germination. (Compare with Figs. 4, 6, and 10.) Figs. 11–13 were photographed and the film was processed as described in Materials and Methods; however, longer exposure times were used during printing than for Figs. 4, 6, and 10.

Next, we investigated the rate at which a drop in current could be detected. Medium was simultaneously added and withdrawn from the small dish containing the specimens, by the use of 2 Pasteur pipettes. Two or more volumes were exchanged to yield a final concentration of 100 μg/ml of CD. The current density fell after ~30 min (Fig. 15). There was not a significant difference in current density for one embryo, but the experiment was terminated after only 60 min of CD treatment. The graph of this experiment (Fig. 15*a*) suggests that the current had dropped. Measurements made before CD treatment of a second embryo were significantly different ($\alpha = 0.001$, two-tailed Mann-Whitney) from those obtained after the first 30 min of incubation with CD (i.e., from 33 to 124 min after addition of inhibitor, Table II). Pulses of current were observed during only the first 30 min of CD treatment, probably before significant penetration of the cytochalasin. The effect of a lower concentration of CD (10 μg/ml) was also assessed (Table II, Fig. 15*b*). After 60 min of preliminary observation in the standard medium, CD (10 μg/ml) was added. We observed one pulse after the addition of CD; however, this occurred only 11 min after addition of the inhibitor, probably before significant penetration of the cytochalasin. We made measurements for 167 min and added fresh medium after 78 min. When data for the first 60 min of CD treatment are compared with data for the 60-min period before the addition of CD, no significant difference is observed. However, there is a significant difference ($\alpha = 0.005$, two-tailed Mann-Whitney) between the control current density and measurements made from the period 60–167 min after CD addition. Some inward current through the rhizoid was still evident when the experiment was terminated, but the amount was small. Thus, CD causes an inhibition of the endogenous electrical currents of young embryos with a concentration-dependent rate.

Finally, we tested the effect of CD on the establishment of a polar axis. Zygotes were incubated in 100 μg/ml of CD beginning 6 h postfertilization and photopolarized with unilateral light from 6–7.5 h postfertilization. This treatment

TABLE I

Effect of Cytochalasin upon Endogenous Current at the Rhizoid Pole

		Current density		
Experiment	Initial age t_0	At t_0	At t_0 + 2.5 h after exposure to CD (100 μg/ml)	Untreated (t_0 + 2.5 h)
	h		μA/cm^2	
1	14	0.59 ± 0.19 (n = 2)	0.27 ± 0.09 (n = 3)	0.64 ± 0.16§ (n = 3)
		0.59 ± 0.19 (n = 2)	0.15 ± 0.05 (n = 3)	1.01 (n = 1)
		0.55 ± 0.13* (n = 2)	0.06 ± 0.05 (n = 3)	1.01 (n = 1)
				1.01 ± 0.16 (n = 3)
2	11	0.18 ± 0.09 (n = 3)	0.24 ± 0.19 (n = 4)	0.50 ± 0.22 (1) (n = 3)
		0.51 ± 0.24 (n = 3)	0.26 ± 0.02 (n = 3)	
		0.19 ± 0.02 (n = 2)	0.14 ± 0 (n = 3)	
3	13	0.70 ± 0.43 (1)‡ (n = 5)	0.15 ± 0.10 (n = 4)	1.18 ± 0.88 (1) (n = 3)
		0.85 ± 0.50 (n = 3)	0.24 ± 0.07 (n = 4)	0.80 ± 0.73 (1) (n = 5)
			0.10 ± 0.09 (n = 4)	
			0.15 ± 0.05 (n = 3)	
4	12	0.48 ± 0.25 (n = 4)	0.40 ± 0.41 (1) (n = 7)	
		0.17 ± 0.14 (n = 4)	0 ± 0 (n = 3)	
		0.84 ± 0.77 (1) (n = 4)	0.10 ± 0.10 (n = 6)	

Inward current through the rhizoid of young, germinated embryos declined after exposure to CD (100 μg/ml) for 2.5 h. Values for 11 different embryos obtained from 4 separate batches of eggs (i.e., 4 different experiments) are presented. Values for current density at t_0 and t_0 + 2.5 h are significantly different ($\alpha = 0.05$) by a two-tailed Mann-Whitney test for pooled data from each experiment except experiment 2. The number of pulses of current observed through each embryo appears parenthetically. Note measurements in experiment 3 at t_0 + 2.5 h for two additional embryos exposed to CD (100 μg/ml), for which no pretreatment values are available. The sample size, n, is the number of successive data points at 2-min intervals in each recording, excluding pulses and reference positions. The electrode was moved between a position close to the specimen and a reference position at about 1-min intervals.

* See Fig. 14*a*.

‡ See Fig. 14*c*.

§ See Fig. 14*d*.

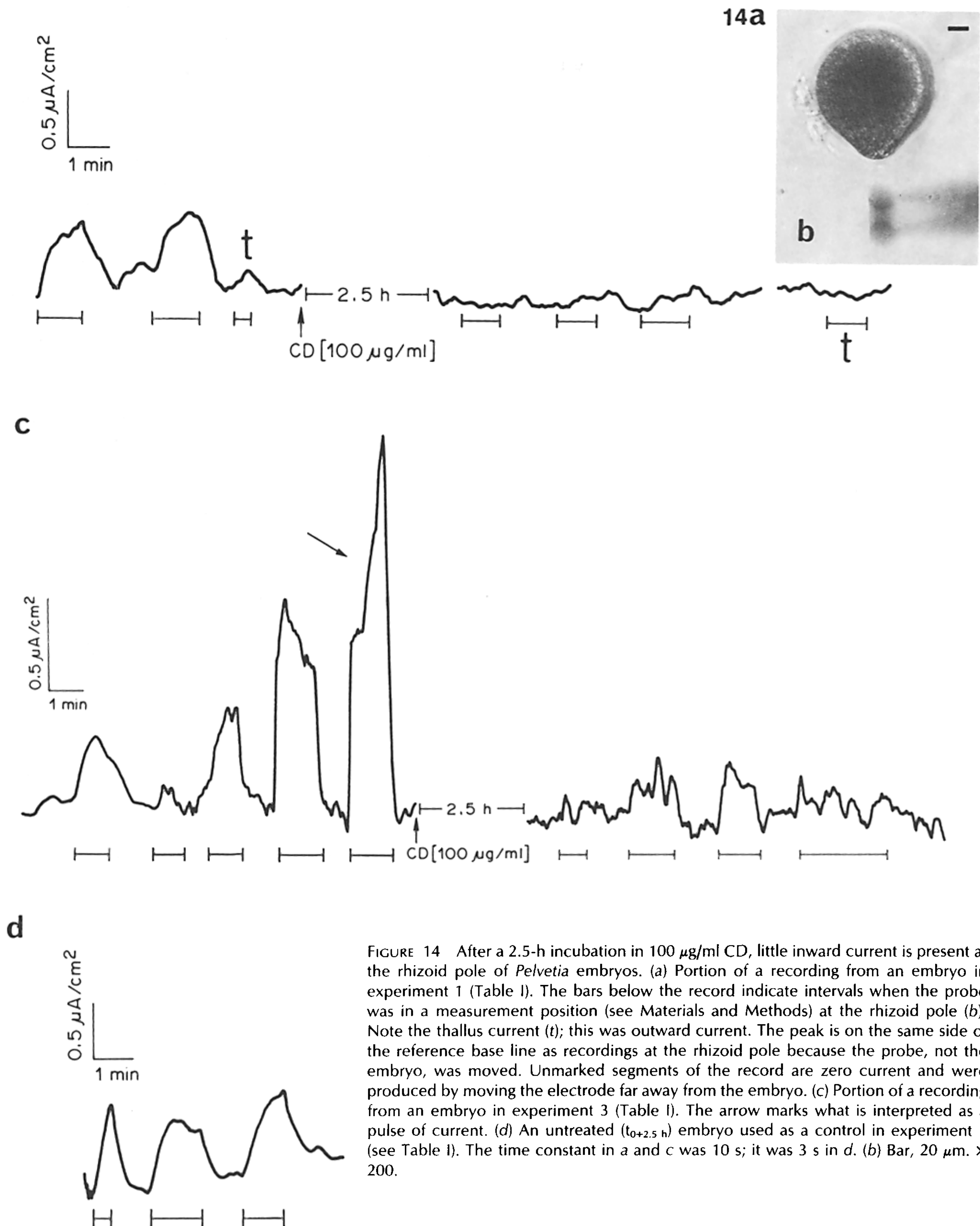


FIGURE 14 After a 2.5-h incubation in 100 μg/ml CD, little inward current is present at the rhizoid pole of *Pelvetia* embryos. (*a*) Portion of a recording from an embryo in experiment 1 (Table I). The bars below the record indicate intervals when the probe was in a measurement position (see Materials and Methods) at the rhizoid pole (*b*). Note the thallus current (*t*); this was outward current. The peak is on the same side of the reference base line as recordings at the rhizoid pole because the probe, not the embryo, was moved. Unmarked segments of the record are zero current and were produced by moving the electrode far away from the embryo. (*c*) Portion of a recording from an embryo in experiment 3 (Table I). The arrow marks what is interpreted as a pulse of current. (*d*) An untreated ($t_{0+2.5\ h}$) embryo used as a control in experiment 1 (see Table I). The time constant in *a* and *c* was 10 s; it was 3 s in *d*. (*b*) Bar, 20 μm. × 200.

regime prevents both establishment of a polar axis and germination (35). When we examined zygotes with the vibrating probe 17 h postfertilization, we detected no inward current (0 ± 0 μA/cm^2, n = 3 embryos, each represented by 3–4 min of recording) near the pole that had been shaded during photopolarization (Fig. 16). However, when 100 μg/ml of CD was added to zygotes after photopolarization (6–7.5 h), a small region of inward current (0.06 ± 0.04 μA/cm^2, n = 1) could be detected at the presumptive rhizoid pole when measured 16 h later (Fig. 17*a*). Note that mitosis had occurred (Fig. 17*b*). Thus, CD appeared to prevent the establishment of inward current in response to photopolarization but did not

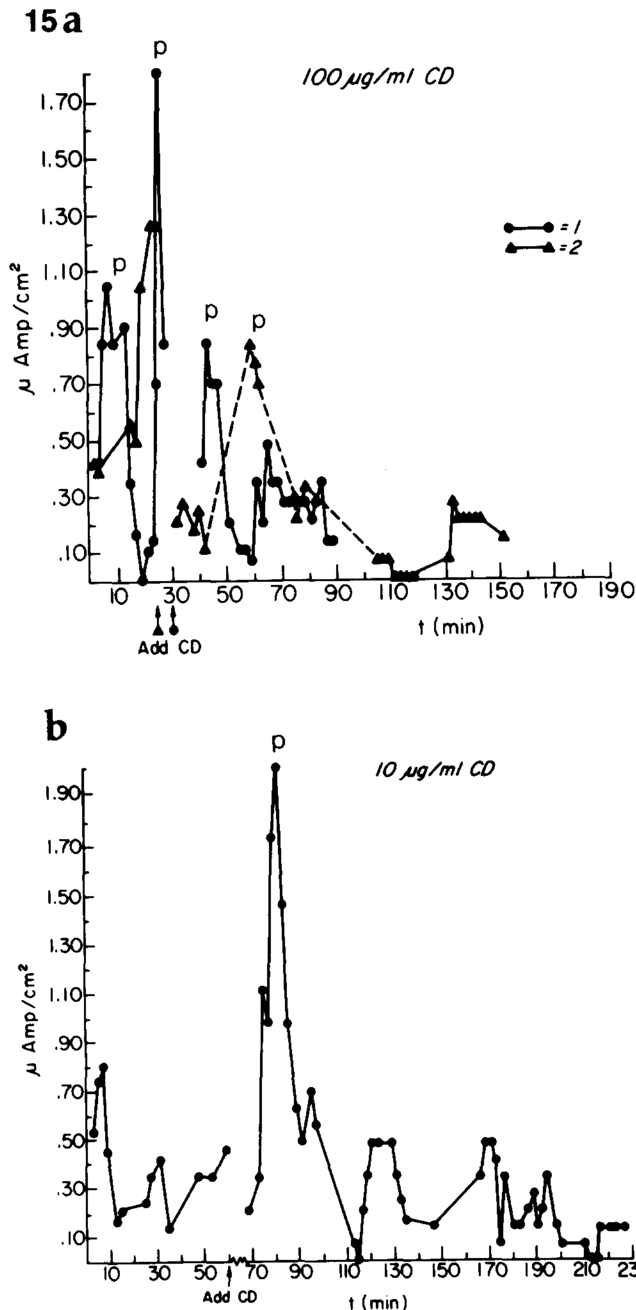


FIGURE 15 Plots of current density (microamperes per centimeter²) vs. time (minutes) for embryos treated with 100 μg/ml of CD (*a*) and 10 μg/ml of CD (*b*) after a pretreatment period in which control recordings were made. Pulses (*p*) were observed during the first 30 min of 100 and 10 μg/ml of CD. The current density varied little after this time and decreased as compared with the pretreatment periods. (see Table II).

completely disorganize the inward current observed by others at the presumptive rhizoid immediately after photopolarization. We consider these data to be preliminary because of the small sample size ($n = 3$; controls, however, never had a 0 ± 0 μA/cm² current density) and because of the elapsed time between photopolarization and our assay of the CD effect. We chose this assay period to permit comparison with our data on germinated embryos (Tables I and II). To confirm the inhibiting effect of CD on the appearance of endogenous currents during photopolarization, studies should be made as zygotes are polarized.

DISCUSSION

Our results demonstrate that cortical F-actin is localized preferentially at the tip of the rhizoid in fucoid embryos. This localization also occurs at the presumptive rhizoid (i.e., before germination), but it is not apparent (i.e., with NBD-phallacidin) until several hours after axis induction. CD decreased the inward current found at the rhizoid pole and eliminated the pulsatile component of the current. The rate of inhibition was concentration dependent. Preliminary data suggest that 100 μg/ml of CD prevents the appearance of inward current at the presumptive rhizoid but does not eliminate this locus if added after photopolarization. These results support the importance of microfilaments in axis formation and fixation, including effects upon the pattern of endogenous current.

F-Actin Localization

The preferential localization of F-actin at the rhizoid of germinated embryos was expected because Golgi vesicles containing fucoidan are transported from perinuclear Golgi bodies to the rhizoid tip via a cytochalasin-sensitive process (8). Continued studies (e.g., ultrastructural immunochemical studies) of actin distribution within the embryo may demonstrate a transcellular network of microfilaments between the rhizoid pole and the perinuclear area, not evident here because of fluorescent quenching by overlying organelles. A surprising aspect of our data, however, is that the rhizoid-associated F-actin is not apparent until about the time at which the axis is fixed. Photopolarization did not produce a redistribution of F-actin to the cortex of the shaded hemisphere during the time when the axis was labile (i.e., subject to multiple reorientation by altered light gradients). Indeed, zygotes fixed with formaldehyde and stained immediately after photopolarization may appear to have slightly more F-actin in the cortex of the illuminated hemisphere. These observations suggest that F-actin has two spatially distinct distributions during early embryogenesis that correspond to discrete roles in polarization of axis establishment and axis fixation.

Further studies (e.g., with microinjected 5-iodoacetamidofluorescein-actin, ultrastructural immunochemial analysis, etc.) are required to clarify the anomalous pattern of F-actin induced by cytochalasin treatment. Little F-actin remained in the cortex of a few embryos after 2 h of treatment with 100 μg/ml of H_2CB but in most embryos (~80%), there was an overall enhancement. With treatment with 100 μg/ml of CD there was sometimes an enhancement but more often a slow depolarization of the cortical F-actin. Several previous studies suggested that the actin cytoskeleton of plant cells may be relatively insensitive to disruption by cytochalasin B and even enhanced by the inhibitor (10, 16, 41). However, recent reexamination of one of these plant systems (*Chara*) using NBD-phallacidin demonstrated that substantial disruption was produced by cytochalasin B (28).

Although the effect of cytochalasin upon the distribution of F-actin is complex and requires further study, a change in the normal distribution of F-actin always occurs in embryos treated with cytochalasin. These effects correlate with the effect of cytochalasin upon endogenous currents.

Endogenous Current

The substantial reduction in endogenous current through the rhizoid after CD treatment is especially significant to

Table II
Rate of Decline of Inward Current after CD Treatment

Concentration of CD	Current density before treatment	Number of pulses	Current density after treatment	Number of pulses
μg/ml	*μA/cm²*		*μA/cm²*	
100 (embryo 1)*	0.49 ± 0.36 (0–25 min) [n = 11]	2	0.25 ± 0.11 (55–89 min) [n = 18]	0 (55–89 min)
100 (embryo 2)*	0.66 ± 0.38 (0–25 min) [n = 6]	1	0.21 ± 0.21 (63–154 min) [n = 24]	0 (63–154 min)
10‡	0.40 ± 0.20 (0–61 min) [n = 13]	1?	0.22 ± 0.14 (129–229 min) [n = 27]	0 (129–229 min)

The effect of CD upon inward current through the rhizoid of young, germinated embryos is not immediate and is concentration dependent (see Fig. 15). Comparisons of current density before and after addition of CD were made by use of measurements taken from the record at successive 2-min intervals (including pulses but not those times when the probe was in a reference position) to total (n) samples over the time course of the experiment. Measurements from embryo 1 are not significantly different before and after incubation; the experiment was ended prematurely because a drop appeared to have occurred (see Fig. 15*a*). There is a significant ($\alpha = 0.001$, two-tailed Mann-Whitney test) decrease in current density after incubation of embryo 2 when data from the pretreatment period are compared with data from 63–154 min after addition of 100 μg/ml of CD (first 30 min of treatment time were deleted due to penetration considerations; see Results). Values at 10 μg/ml of CD (deleting first 60 min of recording after CD addition) are significantly different ($\alpha = 0.005$, two-tailed Mann-Whitney test) from pretreatment values. A single pulse was observed during the first 30 min of CD treatment in each of these records. These embryos were 16 h old at t_0.

* See Fig. 15*a*.

‡ See Fig. 15*b*.

additional interpretation of the roles of microfilaments during embryogenesis. However, growth and current amplitude are positively correlated (31; and unpublished data), so one important consideration is whether the effect of cytochalasin is a gross inhibition of growth, as compared with effects upon channel position within the membrane. Although we have no data to indicate whether the rates of growth are identical in cytochalasin-treated and control embryos, we do know that delocalized growth continues. Embryos treated with cytochalasin are of larger diameter (wider but not as long) than controls (compare Figs. 70–83 with 100–103 in reference 6). Also, mitosis and cytokinesis, including slow furrowing, occur during continuous incubation with 100 μg/ml of cytochalasin. Comparatively little fucoidan secretion occurs, and none of it is localized (8, 29). The diameter and ultrastructural appearance of the cell wall are similar to those observed in met-embryos (6, 8), so secretion of wall components other than fucoidan may be normal.

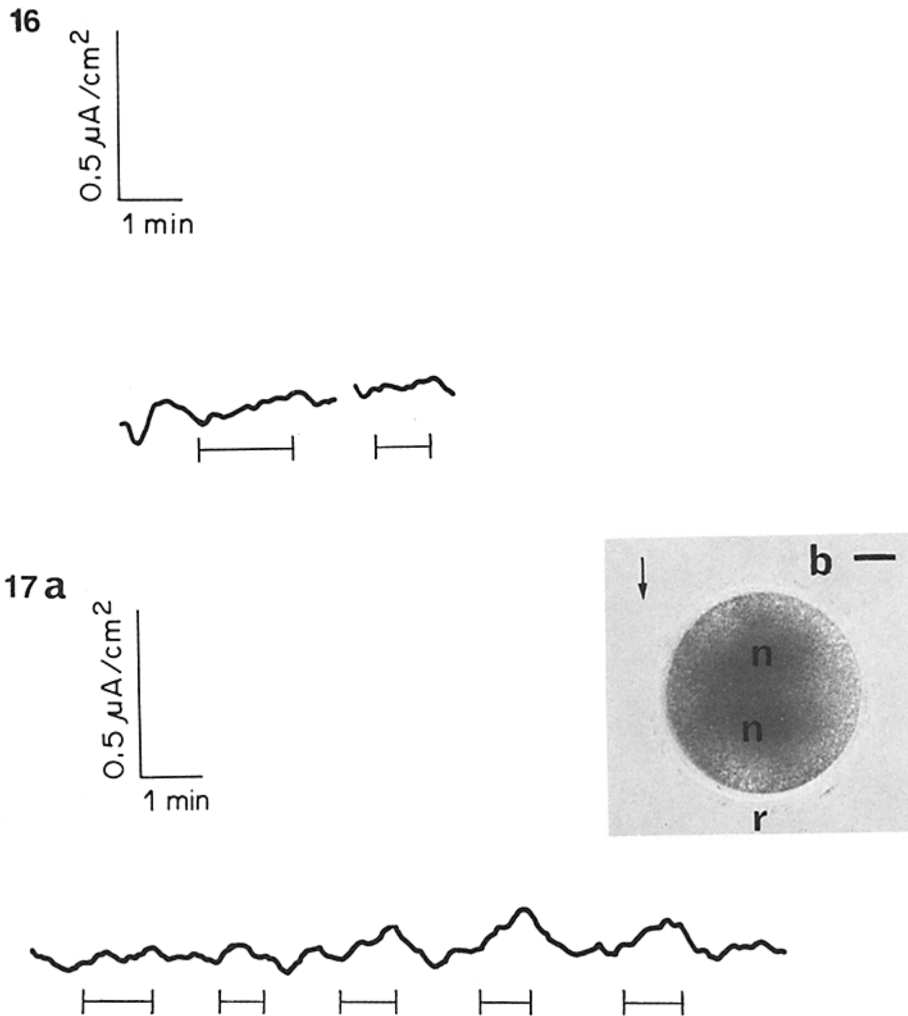
Since CD significantly reduces the amplitude of the endogenous current, it is possible that microfilaments regulate the pattern of endogenous currents observed during embryogenesis. An important question is whether both pulsatile and steady components of the current are sensitive to cytochalasin, because the pulsatile component is not prominent until some hours after germination. The pulsatile current may be involved in axis fixation, since a few pulses are observed before germination; however, only the steady current is associated with axis formation (30). Recall that different axes may be formed sequentially for ~10 h postfertilization, but that the axis present at ~12 h postfertilization becomes fixed (reviewed in reference 36). Our designation (Table I) of components of the current as steady vs. pulsatile is somewhat tentative because of the intermittent sampling between a position near the rhizoid pole and in a reference position. Entrained pulses can produce a record that appears to be steady current (31); however, it seems unlikely that similar current amplitudes would have been recorded from so many different embryos if pulsatile rather than steady current were being measured. While there is some possibility that we have confused small pulses and steady current, a substantial number of short, high current density recordings (i.e., pulses) were easily distinguished from the base steady current in control embryos. These pulses were eliminated by cytochalasin treatment.

What Is the Role of the Pulsatile Current and How Does Cytochalasin Inhibit It?

Nuccitelli and Jaffe (31) inferred that the pulsatile current involves many ion channels or pumps in a membrane patch. Since pulsing did not occur in calcium-free seawater, they also hypothesized that the pulsatile current was stimulated by calcium entry and carried by chloride efflux, serving an osmotic function (33).

The effect of cytochalasin upon the pulsatile component of the current is not immediate, so blocking of a channel or some other rapid effect of the inhibitor upon the membrane can be eliminated in considering its mode of action. The rate at which pulses disappear (*Pelvetia*) is compatible, however, with the inhibition by cytochalasin of movement of Golgi vesicles to the rhizoid pole (in *Fucus*; reference 8) where their contents (i.e., fucoidan) will be localized in the cell wall. Cytochalasin does not prevent the sulfation of fucoidan, and Golgi vesicles accumulate near the hypertrophied dictyosomes when vesicle transport is inhibited by cytochalasin (8).

Our results, in concert with those of Nuccitelli and Jaffe (33) and Brawley and Quatrano (8), lead us to hypothesize that (*a*) the pulsatile component of the current depends upon fusion of Golgi vesicles at the rhizoid tip, and (*b*) that this secretion is a major pathway for addition of calcium or other ion channels to the membrane and may be part of axis fixation. We believe that Golgi vesicle secretion at the presumptive rhizoid may be important in axis fixation vis à vis concentration of ion channels because of a feedback effect upon the network of microfilaments. Edelman (14) and Penman et al. (e.g., references 15 and 24) have suggested that cytoskeletal networks can trigger metabolic activation. Evidence to support such hypotheses is accumulating. H_2CB disorganizes microfilaments in 3T3 cells and prevents insulin



FIGURES 16 and 17 (Fig. 16) Record from an embryo photopolarized (6–7.5 h postfertilization) in the presence of 100 μg/ml of CD and cultured in CD until 17 h postfertilization. At 17 h postfertilization, no inward current was detected at the presumptive rhizoid pole or at points 30° from the pole. The bars below the record indicate intervals when the probe was in a measurement position (see Materials and Methods) at the rhizoid pole. Unmarked segments of the record are zero current and were produced by moving the electrode far away from the embryo. The time constant was 10 s. (Fig. 17) (*a*) A small region of inward current was detected at the presumptive rhizoid pole (r) of an embryo (*b*) to which 100 μg/ml of CD was added after photopolarization (6–7.5 h postfertilization). The record was made 16 h after addition of CD. Note that mitosis had occurred (*n*, nuclei). The arrow indicates the direction of the light gradient used during photopolarization. The time constant was 10 s. This is the only embryo we have examined under this set of experimental conditions. (*b*) Bar, 20 μm. × 225.

or serum stimulated DNA synthesis (25). In several different cells, a specific association of mRNA with the cytoskeleton that appears necessary for translation has been described (11, 22). Jeffery (23) reviews the importance in this regard of a sub-plasma membrane lamina that is associated with the yellow crescent of ascidian eggs. Axis fixation in fucoid algae may require enough calcium influx to localize a substantial microfilament network to trigger a similar metabolic activation and, thereby, unequivocal determination of rhizoid vs. thallus poles. Quatrano (34) found that RNA synthesis required for rhizoid formation in *Fucus* occurred 1–5 h postfertilization, but, significantly, translation of the mRNA did not occur until 9–13 h postfertilization. This is the time at which we have observed substantial NBD-phallacidin staining of the rhizoid pole (e.g., Fig. 10).

A discussion of the met-embryo (8, 13, 17) is relevant to our hypothesis that calcium channels are present in membranes of Golgi vesicles and that localized addition of these may be important in axis fixation. Met-embryos are zygotes grown in an artificial seawater without free sulfate but containing 10 mM methionine. Such fucoid embryos are photopolarized effectively and germinate (13), but they do not become attached because fucoidan is not secreted at the rhizoid. Hogsett and Quatrano (17) used fluorescein isothiocyanate ricin to demonstrate that fucoidan is not present in cell walls of met-embryos either as the sulfated or unsulfated polymer.

Hogsett and Quatrano's experiments (17) suggested that localized secretion of fucoidan is not required for axis fixation. However, it is possible that a slight asymmetry in secretion of Golgi vesicles exists in met-embryos and that this is required for axis fixation. The dictyosomes of met-embryos appear inactive and, compared with normal organelles, produce few Golgi vesicles, but some are present (see Fig. 8 in reference 8). The absence of sulfate appears to depress fucoidan synthesis, and hence the activity of the dictyosomes, but some vesicle

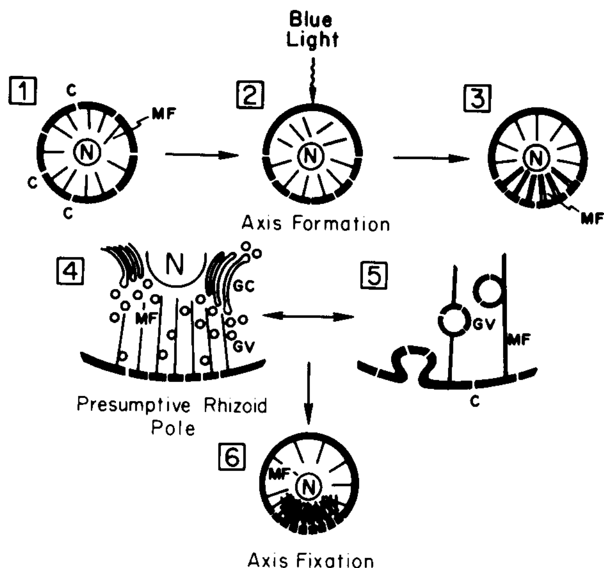


FIGURE 18 A hypothetical model for the interaction of endogenous current and the cytoskeleton during axis formation and fixation (see Discussion). *N*, nucleus; *MF*, microfilament; *C*, ion channel; *GV*, Golgi vesicle; *GC*, Golgi complex. (*1*) The radially symmetric zygote after fertilization. (*2*) Microfilaments become disorganized in the illuminated hemisphere during axis formation, allowing channel localization to occur through the membrane into the shaded hemisphere; this is not a capping phenomenon. (*3*) The calcium portion of the current causes microfilament polymerization at the shaded pole. (*4*) Blow-up of presumptive rhizoid pole showing Golgi vesicle localization along microfilament networks. (*5*) Calcium and other ion channels are localized at the presumptive rhizoid pole via Golgi vesicles. (*6*) As more channels are localized to the presumptive rhizoid, still more microfilaments are induced there. This confers long-term memory because the numerous MFs now present form a tight network. Final determination may result via localization/translation of mRNA by the cytoskeletal network.

production and transport continue. Met-embryos germinate 25 ± 5 h later than normal embryos, and the rhizoids often have a smaller diameter than normal embryos (8). When met-embryos are pulsed with $^{35}SO_4$, secretion of fucoidan is nearly random after a 2 h chase when rhizoid and thallus poles are compared; this suggests that there is a delocalized network of microfilaments (8). Although we believe that the various experiments on met-embryos (e.g., reference 17) eliminate secretion of fucoidan as a requisite for axis fixation, even a tiny asymmetry and low rate of Golgi vesicle secretion could provide the asymmetry in ion channels hypothesized to be necessary for axis fixation. The delay in germination of met-embryos as compared with controls would be explained by the low rate of asymmetric secretion. A test of this hypothesis must be made by finding a marker for Golgi vesicle secretion in fucoid embryos that is membrane specific rather than product related.

Axis Formation

Localized inward current appears at the presumptive rhizoid within 40 min of unilateral illumination (30). The delay between onset of illumination and appearance of this current suggests that a rather slow redistribution and/or localization of ion (e.g., calcium) channels occurs, not a differential opening of evenly distributed ion channels. Robinson and Jaffe (40) explicitly proposed redistribution of calcium channels. Nuccitelli (30) suggested that these channels might be localized by self-electrophoresis within the plasma membrane, but definitive data on the distribution and maintenance of ion channels in these embryos are still lacking.

Quatrano's discovery (35) (that 50–100 μg/ml of cytochalasin prevents formation of the axis when present during photopolarization but will not disorganize a previously induced axis) poses the question of how microfilaments are involved in axis formation. We have assayed the effects of cytochalasin upon appearance of inward current during photopolarization many hours after the light pulse (i.e., when controls had germinated). Nevertheless, we detected some inward current at the presumptive rhizoid of an embryo to which cytochalasin was added after photopolarization, but no current could be detected at the shaded poles of embryos photopolarized in the presence of cytochalasin. These preliminary data are evidence for a direct role of microfilaments in organizing ion channels during the polarization process. Our NBD-phallacidin results indicate, however, that a microfilament-mediated localization of ion channels is not through a capping type of movement of the cytoskeleton and membrane proteins (channels) to the presumptive rhizoid. The failure of 17 h of 100 μg/ml of CD after photopolarization to delocalize fully the current at the presumptive rhizoid fits well with Quatrano's results (35) and is additional evidence for low mobility of ion channels in the membrane.

Interactions between the cytoskeleton and electrical current have been analyzed in only one other system, *Vaucheria* (3–5). When filaments of *Vaucheria* are illuminated with a small spot of blue light, the bundles of microfilaments in the cortex are disrupted; cytoplasmic streaming stops, and organelles such as mitochondria and chloroplasts are trapped. Outward current is observed from this region, and some experiments with the protonophore carbonyl-cyanide-m-chlorophenylhydrazone (CCCP) suggest that it is proton carried. Blatt et al. (5) suggest that a localized change in cytoplasmic pH, resulting from the efflux of hydrogen ions, may be responsible for the microfilament disruption. It is possible that axis formation in fucoid zygotes may involve similar effects of blue light upon cortical, membrane-associated actin. Future work on axis formation must address the early steps of polarization including the identity of the fucoid photoreceptor.

New Model for Axis Fixation and Formation

This report provides strong support for the involvement of microfilaments in cell polarization. We have suggested that microfilaments participate in both axis formation and axis fixation, and we suggest a model (Fig. 18), described as follows, for these processes.

(*a*) Microfilaments in the fucoid zygote selectively immobilize components of the plasma membrane, including some ion channels. In response to appropriate (blue light) irradiation, the conformation of F-actin in the illuminated hemisphere is altered in some way that allows calcium channels to move out of this region and to aggregate on the shaded side of the zygote. These events connote axis formation.

(*b*) Inward current is observed at the presumptive rhizoid pole because of channel aggregation at the shaded side of the zygote.

(*c*) The inward current is partially calcium carried, and it stimulates F-actin localization at the rhizoid pole.

(*d*) Membranes of Golgi vesicles contain calcium channels.

Addition of this membrane to the rhizoid (presumptive rhizoid?) causes additional microfilament polymerization, which in turn causes more Golgi vesicle transport to this region. This amplification step is essential for axis fixation.

(*e*) The network of F-actin at the rhizoid pole extends into the cytoplasm and triggers metabolic activation (see above and references 14, 15, and 24), e.g., localization of essential polarizing agents such as mRNA that irreversibly determine the rhizoid. It is required for axis fixation.

Our model explains the effect of cytochalasin upon polarization, and it provides a mechanism (i.e., addition of ion channels at the rhizoid via Golgi vesicle membranes) for the doubling in steady current observed at germination (30) when Golgi secretion at the rhizoid becomes pronounced. Embryos incubated in cytochalasin would have weak current (as observed) according to our model because, whereas vesicles are produced at normal or higher-than-normal rates, a low and delocalized secretion of Golgi vesicles occurs. Our model predicts that met-embryos should have few pulses of current and a weak current overall, because few Golgi vesicles are produced (8) and therefore, little Golgi membrane is incorporated at the rhizoid pole. Nuccitelli and Jaffe (33) report that sulfate deletion had no effect upon the pulsatile current, either of enhancement or suppression. However, embryos were not preincubated in sulfateless medium and measurements were made over maximum periods of 30 min (Nuccitelli, R., personal communication). The effect upon Golgi vesicle activity would probably require several hours of preincubation. Finally, our model predicts that monoclonal antibodies made to Golgi membranes might include one for the calcium channel, hence an agent that might block polarization. Investigation of (*a*) the effect of cytochalasin upon the appearance of steady current during photopolarization, (*b*) actin-plasma membrane interactions, especially during photopolarization, and (*c*) patterns of mRNA distribution within the zygote and embryo will be important tests of this model. It is clear that at a final level the process of cell polarization is sensitive to cell cycle control because, in contrast to axis formation, the time of axis fixation is fairly constant.

We are particularly grateful to Dr. Robert Stump who made and inserted most of the electrodes used in this work. We thank Drs. Diana Myles, Richard Nuccitelli, and Michael Sheetz for their comments on earlier drafts of the manuscript and Dr. Janet Oliver for helpful discussions during the course of this work. We appreciate the typing assistance of Ms. Sherrie Hughes. Mr. Howard Small (Small Machine & Design, Bloomfield, CT) made a gliding microscope stage that greatly facilitated our studies. We also appreciate the helpful suggestions of two anonymous reviewers.

This work was supported by a grant from the National Science Foundation (PCM 7926715) to Dr. Robinson and by a Science Scholar's appointment (to Dr. Brawley) at the Bunting Institute (Radcliffe College).

Received for publication 21 March 1984, and in revised form 30 October 1984.

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