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Rongsun Pu · Kenneth R. Robinson

The involvement of Ca^{2+} gradients, Ca^{2+} fluxes, and CaM kinase II in polarization and germination of *Silvetia compressa* zygotes

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Abstract Previous work has shown that distinct Ca^{2+} gradients precede and predict the loci of germination of the zygotes of the brown alga, *Silvetia compressa* (J. Agardh) E. Serrão, T.O. Cho, S.M. Boo et Brawley, that are polarized by unilateral blue light. We show here that dark-grown *S. compressa* zygotes also form cytosolic Ca^{2+} gradients prior to germination and then germinate from the site of elevated Ca^{2+} . In no case did germination occur without a prior formation of a Ca^{2+} gradient. Using the self-referencing Ca^{2+} -selective probe, we measured highly localized influx of Ca^{2+} during photopolarization, indicating that extracellular stores supply at least some of the Ca^{2+} needed to construct a gradient. Finally, we find that germination was inhibited by a bath-applied inhibitor of calcium/calmodulin-dependent kinase II (CaM kinase II), KN-93 (but not by its inactive analog, KN-92), and by an injected inhibitory peptide for the kinase. KN-93 did not interfere with the photopolarization of the zygotes, consistent with the view that calmodulin is not involved in the initial response to light. The KN-93 results indicate that the requirement for active CaM kinase II for germination ends about 2 h before overt germination. We conclude that Ca^{2+} gradients, generated in part by localized calcium entry from the seawater, are an essential part of the process of polarity development and expression in these cells, regardless of the nature of the external cue that directs the orientation of the axis. Calmodulin and CaM kinase II are involved in interpreting (but not in establishing) the calcium gradient, allowing germination to occur at the site of elevated calcium, but CaM kinase II appears not to be involved in the initiation of germination.

Keywords Calcium gradient · Calcium/calmodulin-dependent kinase II · Cell polarity · *Pelvetia* · Self-referencing calcium probe · *Silvetia*

Abbreviations AIP: CaM kinase II inhibitory peptide · CaM: kinase II calcium/calmodulin-dependent kinase II · CCdex: Calcium Crimson dextran · MLCK: myosin light-chain kinase · RBdex: Rhodamine B dextran

Introduction

The understanding of how polarity arises in early development remains incomplete. Most eggs, by the time they are competent to be fertilized, already have at least one major axis established in nascent form. In *Drosophila*, for example, the anterior–posterior and dorsal–ventral axes are represented in the egg by a complex pattern of localized mRNA and proteins that is created during oogenesis, and the physiological events that underlie the creation and maintenance of these asymmetries are not well understood. However, in the case of the fucoid algae such as *Silvetia compressa* (formerly called *Pelvetia fastigiata*, see Serrão et al. 1999) and species of *Fucus*, the eggs are radially symmetric at the time of fertilization and they develop their primary rhizoid–thallus axis during the ensuing 5–10 h. These events, including germination at the rhizoidal end, occur before first cell division and direct the plane of first division, which is perpendicular to the rhizoid–thallus axis. The relatively short time for axis formation and the accessibility of the cells allow experiments with the fucoid zygotes that cannot be done easily with other organisms.

A potent determinant of polarity in fucoid zygotes is unilateral blue light, which induces the nascent rhizoid to form on the shaded side, away from the light source. The point of sperm entry leaves a trace about which the cells can organize an axis in the dark (Hable and Kropf 2000), but this faint polarity can be easily

R. Pu · K.R. Robinson (✉)
Department of Biological Sciences,
Purdue University, West Lafayette, IN 47907, USA
E-mail: ken@video.bio.purdue.edu
Fax: +1-765-4940876

Present address: R. Pu
Department of Biological Sciences,
Kean University, Union, NJ 07083, USA

overcome by unilateral light as well as other external asymmetries. There is evidence that the photoreceptor is an opsin-like protein associated with retinal (Robinson et al. 1998; Gualtieri and Robinson 2002). It is known from inhibitor studies that the actin cytoskeleton plays an essential role in the photopolarization process (Hable and Kropf 1998; Pu et al. 2000). Pu et al. (2000) reported a significant increase in cortical actin filaments in response to blue light, and they found the filaments to be symmetrically arrayed around the cell. This distribution of actin filaments in the young zygotes was quite different from that reported previously (Alessa and Kropf 1999), in which actin patches moved to the future rhizoidal pole. The differences in the techniques used may explain the contrasting results and are discussed in Pu et al. (2000).

A second important participant in the photopolarization process is the formation of a cytosolic gradient of Ca^{2+} , with elevated Ca^{2+} at the developing rhizoidal pole (Pu and Robinson 1998). The development of the Ca^{2+} gradient in response to light requires actin polymerization (Pu et al. 2000). If the cells are grown in the dark in the presence of an external gradient of calcium ionophore, they polarize in response to the imposed gradient and form their rhizoids on the portions exposed to the higher concentration (Robinson and Cone 1980), indicating a causal role for Ca^{2+} gradients in the polarization process. The mechanism by which Ca^{2+} gradients might act in the polarization process is not known. One obvious possible target is the ubiquitous calcium-binding protein, calmodulin. There is considerable evidence that calmodulin is involved in germination and rhizoidal growth, based on both inhibitor studies and antibody microinjection (Love et al. 1997; Pu and Robinson 1998), although the role of calmodulin in photopolarization is controversial (Robinson 1996; Love et al. 1997; Robinson et al. 1999).

Significant gaps remain in our understanding of the physiology of calcium and the mechanism of formation of calcium gradients in polar axis determination. There are a number of external vectors that will polarize fucoid zygotes in the absence of light, including gradients of temperature or K^+ , flow, electrical fields, the nearby presence of another zygote or even a bit of thallus (Jaffe 1968). It is not known if Ca^{2+} plays the same essential role in the response to these vectors as it does in photopolarization. It is also not understood how the activation of photoreceptors on one side of the zygote leads to Ca^{2+} entry on the opposite side. It has been suggested that there is a rearrangement of Ca^{2+} channels in the plane of the plasma membrane in response to light, but evidence for such a mechanism is lacking. Finally, little is known of the downstream targets of Ca^{2+} during photopolarization. We have addressed some aspects of these issues in the experiments described here.

Materials and methods

Preparation of material

Mature fronds of *Silvetia compressa* (J. Agardh) E. Serrão, T.O. Cho, S.M. Boo et Brawley were collected near Santa Cruz, or Monterey, California and shipped to us on ice via FedEx. After being dried with absorbent paper, fronds were stored in monolayers in loosely capped containers at 4 °C in low humidity. Under such conditions, sufficient amounts of viable gametes could be obtained for up to 2 weeks. Gametes were obtained by a light-to-dark treatment (Jaffe 1954). Briefly, the fronds were placed in flat-bottomed glass vials in filtered natural seawater (NSW; Marine Biological Laboratory, Woods Hole, Mass., USA) near a unilateral white light source of 40–90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ overnight at 16 ± 1 °C, and were rinsed thoroughly with NSW the next day and transferred to a beaker in the dark for 15 min. Zygotes were then filtered through a 125- or 190- μm Nitex nylon filter shortly after fertilization, arbitrarily taken as 30 min after the fronds were first placed in the dark. Zygotes were kept in the dark or dim red light until use.

Microinjection

Microinjection pipettes with a tip size of approximately 0.5 μm were made from 100-mm-long, 1.0-mm-outer-diameter, no-filament glass capillaries (Drummond Scientific Co., Broomall, Pa., USA) pulled on a vertical pipette puller (Sutter Instrument Co.; model P-30). Before microinjection, micropipettes were first dried at 160–220 °C in a metal chamber for at least 1 h or overnight, and then baked with a small amount of *N, N*-dimethyl-trimethylsilylamine (Fluka, Milwaukee, Wis., USA) at the same temperature for 15–30 min. This silanization process minimized cytoplasmic leakage during microinjection.

Before injection, holding chambers were constructed according to (Kiehart 1982), using a piece of Scotch double-sided tape as a spacer between two glass coverslips to create a chamber for loading zygotes. The chamber had a height slightly more than the diameter of the zygotes (95–97 μm) and was non-disturbing to their normal development. Between 20 and 40 zygotes were loaded into each chamber by capillary action and positioned appropriately with a micropipette.

A high-pressure microinjection system was utilized to allow quantification of injection volume (Kiehart 1982; Speksnijder et al. 1989; Miller et al. 1994). Our system (Pu and Robinson 1998) consisted of a microinstrument holder and a horizontal micropositioner, a micrometer syringe and tubing filled with fluorinert oil (FC-40; Sigma) for generating high pressure. Injection volumes were calculated by injecting oil columns of various lengths into seawater, measuring the volume of the oil droplet, and constructing a standard curve. The volume of zygotes was also estimated in order to calculate the final cytoplasmic concentration of the injected solution (Pu and Robinson 1998). Zygote injections were done in NSW within 2 h after fertilization. After injection, fresh seawater was added frequently to the chamber to compensate for evaporation. After a few hours, the chamber was carefully transferred to a large, covered plastic petri dish filled with NSW and placed in the dark. Germination was scored from 12 to 20 h after fertilization.

Preparation of calcium/calmodulin-dependent kinase II (CaM kinase II) inhibitory peptide (AIP), myosin light-chain kinase inhibitory peptide (MLCK inhibitor), KN-92 and KN-93

The CaM kinase II AIP and MLCK inhibitor (both from BIOMOL Research Laboratories, Plymouth Meeting, Pa., USA) were made fresh as 1.0 mM stock solutions in 200 mM KCl, 5 mM HEPES

(pH 7.0). KN-93 (2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)amino-N-(4-chlorocinnamyl)-N-methylbenzylamine) and KN-92 (2-[N-(4-methoxybenzenesulfonyl)amino-N-(4-chlorocinnamyl)-N-methylbenzylamine, phosphate) were purchased from Calbiochem (San Diego, Calif., USA) and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and stored frozen in small aliquots until used. Solutions of KN-92 and KN-93 in seawater were always made freshly just before use. Controls received the same amount of DMSO as cells treated with 10 μ M KN-92 or KN-93.

Fluorescent imaging of zygotes injected with Calcium Crimson dextran or Rhodamine B dextran

Calcium Crimson dextran (10 kDa; CCdex) and Rhodamine B dextran (10 kDa; RBdex) were from Molecular Probes (Eugene, Ore., USA). Both were dissolved in 200 mM KCl, 5 mM Hepes (pH 7.0) at a working concentration of 2.5 mM. Only those zygotes that did not exhibit overt initial asymmetry in fluorescent brightness and also germinated on schedule with non-injected control zygotes were chosen for image analysis. Zygotes were grown in the dark and microinjected with either 1.2–1.7 pl of CCdex or 1.0–2.0 pl of RBdex within 2 h after fertilization. Images were obtained with the LaserSharp image acquisition program installed on an upright Bio-Rad MRC-1024 Laser Scanning Confocal Imaging System with a Leitz $\times 25$ water immersion objective lens (N.A. 0.60). For both CCdex and RBdex imaging experiments, excitation wavelength was 568 nm, and emission wavelength was 598 nm with a bandwidth of 40 nm. Settings on the confocal system were as follows: scan speed “slow”, Kalman $n = 3$, box size 512 \times 512, zoom 2.80, laser power 3% or 10% for CCdex images and 1% for RBdex images. The “gain” setting for the photomultiplier tube was adjusted so that only a few pixels over an entire image reached the maximum value of 255. Cells were maintained at 16 ± 1 °C throughout the imaging process by a refrigerated circulating water bath. For image analysis, obtained “.pic” files were exported as “.tif” files to the MetaMorph software program (Universal Imaging Corporation, version 2.0). An 8-bit mask was constructed to subtract background from the images when necessary. For zygotes, the cortical 5% (just less than 5 μ m) of the cytoplasm just underneath the plasma membrane was analyzed for average pixel values of fluorescent intensity using the “linescan” function of the MetaMorph software program. The “linescan” function scanned along the selected area just beneath the circumference of each cell, obtaining an ordered set of numbers each of which corresponded to the average pixel value of a small region measuring approximately 17 \times 1 pixels. One set of such numbers was obtained for each individual CCdex and RBdex image. For each time point of imaging, five sets of numbers were obtained for the five CCdex-injected zygotes and averaged. Another five sets were obtained for the five RBdex-injected zygotes and also averaged. The final averaged pixel values of five CCdex zygotes were then divided by the corresponding averaged pixel values of five RBdex zygotes. The ratios were then divided into two equal sections, with one section containing the future germination site at its center. Means and SE were calculated for both sections for each time point of imaging.

Ion-selective probe measurements

The principle of operation of the self-referencing ion-selective (Seris) probe has been described elsewhere (Smith et al. 1994). In brief, the system consists of an ion-selective microelectrode that is translated stepwise between two points in the extracellular space near the plasma membrane of a cell. If there is locally a net flux of the sensed ion into or out of the cell, a concentration gradient of the ion will be produced and this gradient will be detected as a voltage difference between the two points. Measurement of the gradient allows the calculation of the ion’s flux through the plasma membrane.

In this case, micropipettes with tip diameters of 3 μ m were pulled from 1.5-mm O.D. glass capillary tubes, dried overnight at 180 °C, and then silanized for 30 min at 180 °C. A micropipette was then backfilled with a short (1–2 mm) column of 100 mM CaCl₂ buffered

with 10 mM Hepes at pH 7.0. Pressure from a syringe was applied to force the aqueous solution to the tip of the silanized pipette. The tip of micropipette then was inserted into a calcium ionophore cocktail (Calcium Ionophore–Cocktail A; Fluka) and a short (15–20 μ m) column of cocktail was allowed to flow into the tip, displacing the saline. An electrode holder made contact with the saline solution via a chloridized silver wire and the holder allowed attachment to the amplifier’s headstage. The calibration of the electrodes was done by moving between solutions of artificial seawater containing 0.1, 1 or 10 mM Ca²⁺. The electrodes that were used gave at least a 28 mV change per 10-fold change in Ca²⁺ concentration.

The electrodes were positioned near a cell so that the closest approach to the cell surface was about 5 μ m; thus the electrodes were moved stepwise between positions 5 μ m and 15 μ m from the cells’ surfaces. The zygotes were illuminated with unilateral blue light provided by an array of blue-light-emitting diodes (LED). The LED array could be positioned on either side of a zygote with respect to the electrode so that the electrode could measure either at the illuminated pole or the shaded pole of the cells. Small changes in the location of the electrode measuring position were accomplished by rotating the stage of the microscope; in those cases, the light source was also rotated so that there was no change in the direction of the light with respect to the zygote.

The acquisition of one differential measurement took about 2.5 s. Rapid changes in net flux, that is, large changes in less than 1 min were never observed. Thus, a 20-point rolling average of the data was done, and the points shown in Fig. 5 are 20-point averages at 10-min intervals.

The averaged voltage differences, Δv , were converted to fluxes, J , by the equation

$$J = 2.3D C_{ave} \Delta v s^{-1} (\Delta x)^{-1}$$

where D is the diffusion coefficient of Ca²⁺ (1.1×10^{-5} cm² s⁻¹), C_{ave} is the average Ca²⁺ concentration in the medium (1.0 mM), s is the response of the electrodes to a 10-fold change in Ca²⁺ concentration (typically 29 mV) and Δx is the translational distance over which Δv was measured (10 μ m). In addition, the empirically determined efficiency of the electrodes was considered. The calcium electrodes require about 5 s to reach 90% of their maximum response to a changed Ca²⁺ concentration, while the electrodes were in each measuring position for only 1 s. We have previously determined that under these conditions, the electrodes detect only 65% of the actual Ca²⁺ gradient (Messerli et al. 1999). Using these considerations, a measured difference of 1.0 μ V represents a flux of 1.3 pmol cm⁻² s⁻¹.

Determination of photopolarization

The degree of photopolarization of zygotes exposed to unilateral white light (90 μ mol photons m⁻² s⁻¹) was determined 22–26 h after fertilization by scoring the locus of the rhizoid with respect to the direction of the light. Each cell was scored as having its rhizoid emerge either on the hemisphere facing the light source or away from the light source. Two hundred cells were scored in each sample and the percent polarization was calculated by subtracting the number of rhizoids emerging on the illuminated hemisphere from the number emerging from the shaded hemisphere and dividing that difference by 2, then multiplying by 100. The resultant value could, in principle, vary from +100% to –100%, with +100% representing complete polarization away from the light source and 0 representing random growth. The typical response of *S. compressa* zygotes in these conditions to light pulses of varying duration from 30 min to 150 min has been published previously (Robinson and Miller 1997).

Results

As *Silvetia compressa* zygotes will polarize and germinate in complete darkness, we wished to examine the

formation of Ca^{2+} gradients during dark polarization. In the absence of any external gradient, the zygotes germinate randomly in three dimensions, apparently from the point of sperm entry. To reliably predict the site of germination and thus ease the problem of selecting a focal plane for imaging, the well-documented group effect was used. At the usual pH of seawater (8.0–8.2), zygotes germinate toward each other if the cells are within one cell diameter, thus restricting the plane of germination to the horizontal. Newly fertilized zygotes of similar diameter (95–97 μm) were selected and some were injected with CCdex (final concentration, 13–18 μM) while others were injected with RBdex (final concentration, 13–20 μM). After injection, zygotes were aligned into closely spaced rows. Under these conditions, it was observed that a zygote would usually germinate in the general direction of its nearest neighbor, with a slight offset toward the second-nearest neighbor. In some cases, the dark-grown zygotes did not germinate from the site closest to an adjacent cell but rather from some other region. If the site of germination was in the horizontal plane, we could still analyze the loci of any Ca^{2+} gradients with respect to the site of germination.

Because the zygotes do not firmly stick to the substrate until about 5 h after fertilization, it was not possible to transfer the cells from the injection chamber to the observation chamber and begin imaging until then. The first images were taken at 5.5 h after fertilization. Only those zygotes that germinated with a similar time course as non-injected or KCl-injected controls were analyzed. Figure 1 is a series of pseudocolor images of a CCdex-injected zygote grown continuously in the dark, starting at 5.5 h after fertilization. Figure 2 shows a similar set of images of an RBdex-injected zygote that shows nearly uniform fluorescence brightness until just after germination (Fig. 2H). Germination of both zygotes as well as most of the controls occurred between 13 and 14 h after fertilization, approximately 2 h later than zygotes grown under continuous illumination. During the first 2 h of imaging (Fig. 1B, C) the brightest fluorescence was at the injection site, which is apparent from a small amount of cytoplasmic leakage. Later, this brightest fluorescence dissipates and a second zone of elevated cytosolic Ca^{2+} forms at the eventual site of germination. Figure 3 is another example of a CCdex-injected cell. In this case, there was already a localized region of elevated Ca^{2+} , perhaps from injection damage, that did not dissipate. As in all cases that were observed, germination finally occurred from the region of elevated Ca^{2+} .

The results of the Ca^{2+} distribution in dark-grown cells are summarized in Fig. 4, in which fluorescence distribution in cells injected with CCdex was ratioed against those injected with RBdex. This somewhat awkward method of ratioing one set of images against another from different cells is a consequence of the vexing autofluorescence of fucoid zygotes and was discussed in detail previously (Pu and Robinson 1998). In the near absence of an initial asymmetry ($P=0.067$,

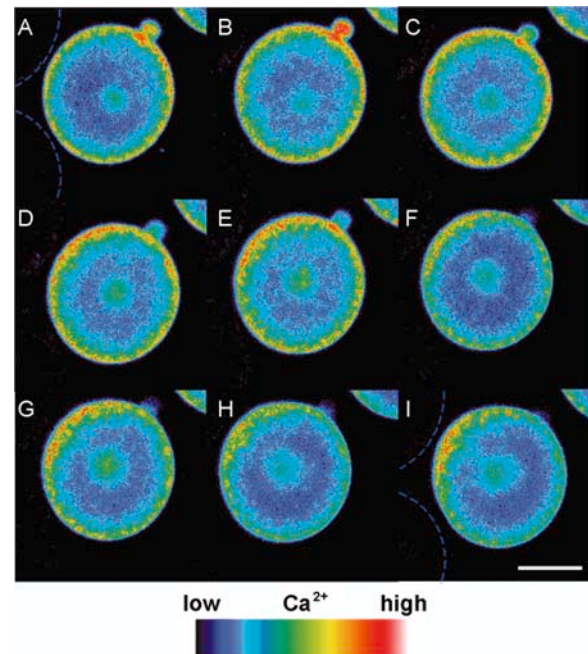


Fig. 1A–I Pseudocolor images of a *Silvetia compressa* zygote injected with Calcium Crimson dextran and grown in continuous dark. Time 0 was defined as just prior to polarization, at 5.5 h after fertilization. From A through I, the images were obtained at 0, 1, 2, 3, 4, 5, 6, 7 and 8 h, by which time the zygote had germinated. Part of another injected zygote is visible on the upper right corner. Dashed outlines indicate the two non-injected zygotes. Bar = 50 μm

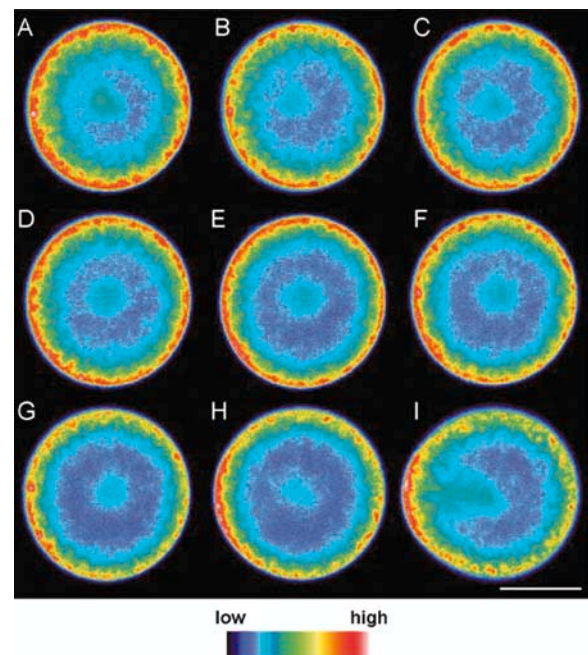


Fig. 2A–I Pseudocolor images of an *S. compressa* zygote injected with Rhodamine B dextran and grown in continuous dark. Time 0 was defined as just prior to polarization, at 5.5 h after fertilization. From A through I, the images were obtained at 0, 1, 2, 3, 4, 5, 6, 7 and 8 h, by which time the zygote had germinated toward a nearby zygote on its left. Bar = 50 μm

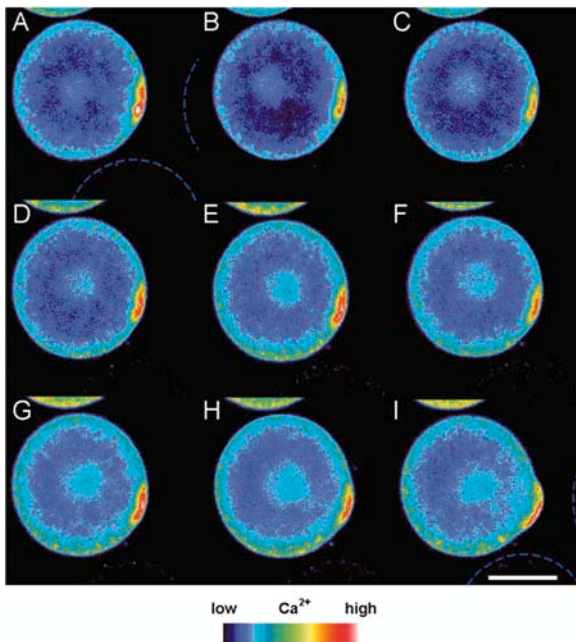


Fig. 3A–I Pseudocolor images of an *S. compressa* zygote injected with Calcium Crimson dextran. The zygote was grown in continuous dark after injection. From **A** through **I**, the images were obtained at 1-h intervals starting from 5.5 h after fertilization (**A**), and the zygote germinated by 13.5 h after fertilization (**I**). Part of another injected zygote is visible on the upper left corner. Dashed outlines indicate the two non-injected zygotes. Bar = 50 μm

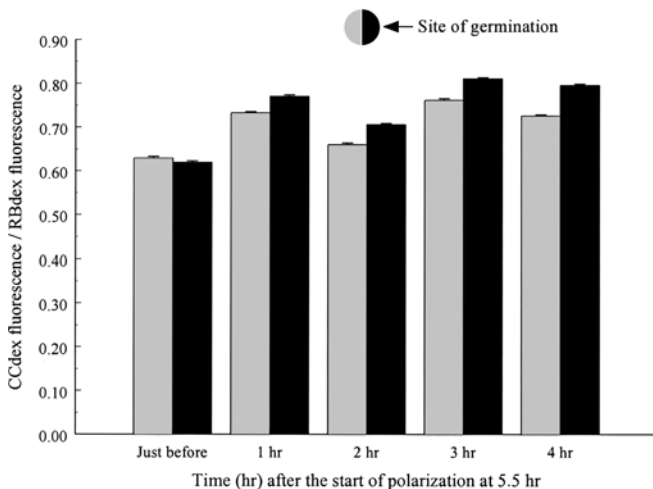


Fig. 4 Bar chart analysis of the ratios of the average fluorescent pixel values of 5 Calcium Crimson dextran images over the corresponding average fluorescent pixel values of 5 Rhodamine B dextran images obtained from 10 different *S. compressa* zygotes grown in the dark. The ratios were divided into two equal sections, with one section containing the future germination site at its center. Means and SE were calculated for both sections at each time point of imaging. All zygotes germinated on schedule with non-injected controls. On average, less CCdex was injected into dark-grown zygotes than RBdex; therefore, the overall CCdex fluorescence obtained was lower than the overall RBdex fluorescence and the ratio of CCdex fluorescence over RBdex fluorescence was less than 1.0

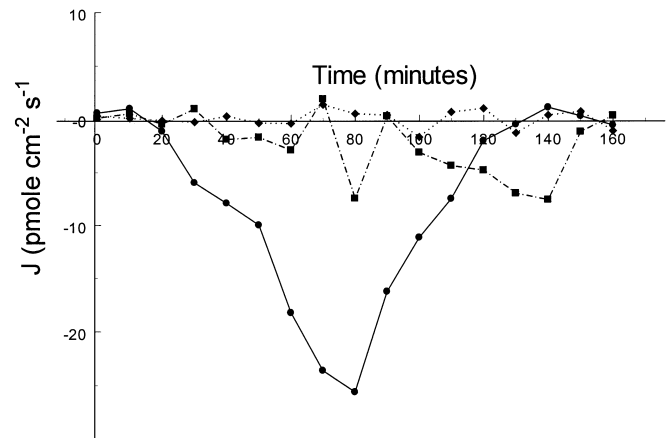


Fig. 5 Localized Ca^{2+} fluxes in polarizing zygotes. Two examples of Seris-detected Ca^{2+} fluxes at the shaded pole (future germination site) of *S. compressa* zygotes are shown (circles and squares). In addition, one example of measurement at the illuminated pole (future thallus pole) is shown (diamonds)

Student's two-tailed *t*-test), the formation of a small cytoplasmic Ca^{2+} gradient was detectable after 1 h of observation (5% greater on the hemisphere centered on the future germination site, $P < 0.005$), gradually increasing in magnitude over the next few hours and reaching a maximum of 10% greater in the germinating hemisphere by 4 h ($P < 0.001$). This difference declined as the time of germination approached.

In order to better understand the mechanism of formation of Ca^{2+} gradients during polar axis formation, we used the self-referencing ion-specific (Seris) probe to measure local Ca^{2+} fluxes through the membranes of polarizing cells. Because of the difficulty of mapping the currents completely around the cells with good temporal resolution, we mainly focused on the process of photopolarization, in which the site of germination could be known with certainty well before germination. Because the sensitivity of the Seris method declines with the average concentration of the ion being sensed, it was necessary to reduce the Ca^{2+} concentration in the seawater bathing the cells to 10% of its normal concentration. Under those conditions, the zygotes photopolarize, germinate and extend their rhizoids completely normally (Robinson 1996).

We find that a detectable influx of Ca^{2+} occurs in a restricted region of the zygotes in response to unilateral blue light. Figure 5 shows two examples of the measurement of Ca^{2+} fluxes at the center of the shaded hemisphere. Detectable influx of Ca^{2+} did not begin until at least 25 min after the polarizing light was turned on. This net influx continued for the following 1–2 h and subsided before germination. The maximum flux typically occurred near the middle of this period, and in the five complete records, the maximum influx was $17.5 \pm 3.9 \text{ pmol cm}^{-2} \text{ s}^{-1}$ (the uncertainty reported here and elsewhere, unless otherwise noted, is the standard error of the mean). The locus of the inward Ca^{2+} flux was highly localized. No detectable influx was measured

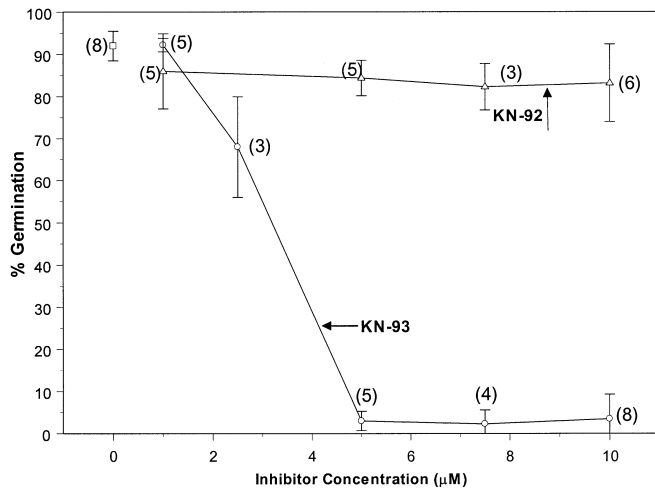


Fig. 6 The germination response of *S. compressa* zygotes to KN-92 and KN-93. The drugs were added at 5.5 h after fertilization, at the same time that a 2-h exposure to unilateral white light was begun. At 7.5 h after fertilization, the cells were put in the dark and were scored for germination at 24–26 h after fertilization. Error bars indicate the standard deviation and the numbers in parentheses are the number of independent experiments. At least two shipments of *S. compressa* were used for each reported point

if the electrode was moved by 20° in the horizontal plane from the site of maximum influx ($n = 3$).

Also shown in Fig. 5 is one of three records where the Ca^{2+} flux was measured at the center of the illuminated hemisphere. In none of these cases was significant influx or efflux detected during the entire polarization process.

We have used two approaches to evaluate the possible target of activated calmodulin in this system. We find that an inhibitor of CaM kinase II, KN-93, reduces germination of *Silvetia* zygotes to less than 5% at a concentration of 5 μM or greater when present from 6 h after fertilization until the cells were scored at 24 h after fertilization while the less effective analog of KN-93, KN-92, had very little effect on germination over the same range of concentrations. The inhibition of germination as a function of inhibitor concentration is shown in Fig. 6 and examples are shown in Fig. 7. When KN-93 (5 μM) was present only during a 90-min exposure to unilateral light starting at 6 h after fertilization, subsequent germination was reduced to $70 \pm 6\%$ of controls (which germinated $>92\%$ in all cases). The degree of photopolarization (see Materials and methods) of the treated cells that germinated was not significantly different ($P > 0.05$, Student's *t*-test) from controls, $66 \pm 7\%$ versus $73 \pm 6\%$ ($n = 6$ experiments).

The period of sensitivity to KN-93 was explored by adding the inhibitor at various times with respect to the polarizing light treatment. It was found that if the inhibitor was added at a final concentration of 10 μM after the 2-h exposure to polarizing light, the zygotes subsequently germinated to some extent (Figs. 7, 8), although those cells never divided. It is important to note that the cells germinated even though the inhibitor

was added well before untreated control cells began to germinate and remained present throughout the remainder of the experiment. This difference was especially clear when the light treatment was begun at 4 h after fertilization rather than at 5.5 h after fertilization. In that case, 40% of the cells germinated when the inhibitor was added more than 2 h before control cells began to germinate and 85% of the cells germinated if the inhibitor was added an hour before any control cells had germinated (Fig. 8). In no case did the zygotes undergo cell division when exposed continuously to KN-93, regardless of when it was added in the 4- to 10-h period after fertilization.

A second approach to the study of CaM kinase II was to inject a peptide that interferes with the interaction of substrates with CaM kinase II. The CaM kinase II inhibitory peptide (AIP) is derived from the CaM kinase II substrate, autocamtide-2, and it is a highly specific inhibitor of CaM kinase II, but does not affect protein kinase C or CaM kinase IV (Ishida and Fujisawa 1995; Ishida et al. 1995). As a control against non-specific damage due to the injection procedure, we injected a myosin light-chain kinase (MLCK) inhibitory peptide of nearly identical molecular weight. Final cytosolic concentrations of less than 44 μM of AIP had no effect on germination, but at concentrations of 49–96 μM , the peptide inhibited germination of 85% of the injected zygotes (Table 1). However, injection of up to 116 μM of MLCK inhibitor had only a small effect on germination (Table 1).

Discussion

The results presented here show that cytosolic Ca^{2+} gradients underlie the expression of polarity in fucoid zygotes under circumstances in which the dominant polarity signal, blue light, is absent. In no case was the development of a rhizoid seen to occur without a prior Ca^{2+} gradient that consisted of elevated Ca^{2+} at the germination site. This argues strongly that all of the various directional signals that can polarize fucoid zygotes act, finally, by creating a Ca^{2+} gradient. These results are consistent with the previous finding that the imposition of a Ca^{2+} gradient in the dark is sufficient to polarize a population of cells quite effectively (Robinson and Cone 1980). The development of polarity is a process involving a number of steps, and in the case of light-induced polarity, the formation of a Ca^{2+} gradient is clearly not the first step, but one that follows an increase in cortical actin filaments (Pu et al. 2000).

The Seris method reveals that a significant influx of Ca^{2+} occurs in response to light and this influx is centered on the site that will later germinate. The detectable influx is localized to a solid angle of less than 20° around the point of maximum influx. The earliest time that net influx could be detected after the start of illumination was about 25 min and the flux reached a peak after

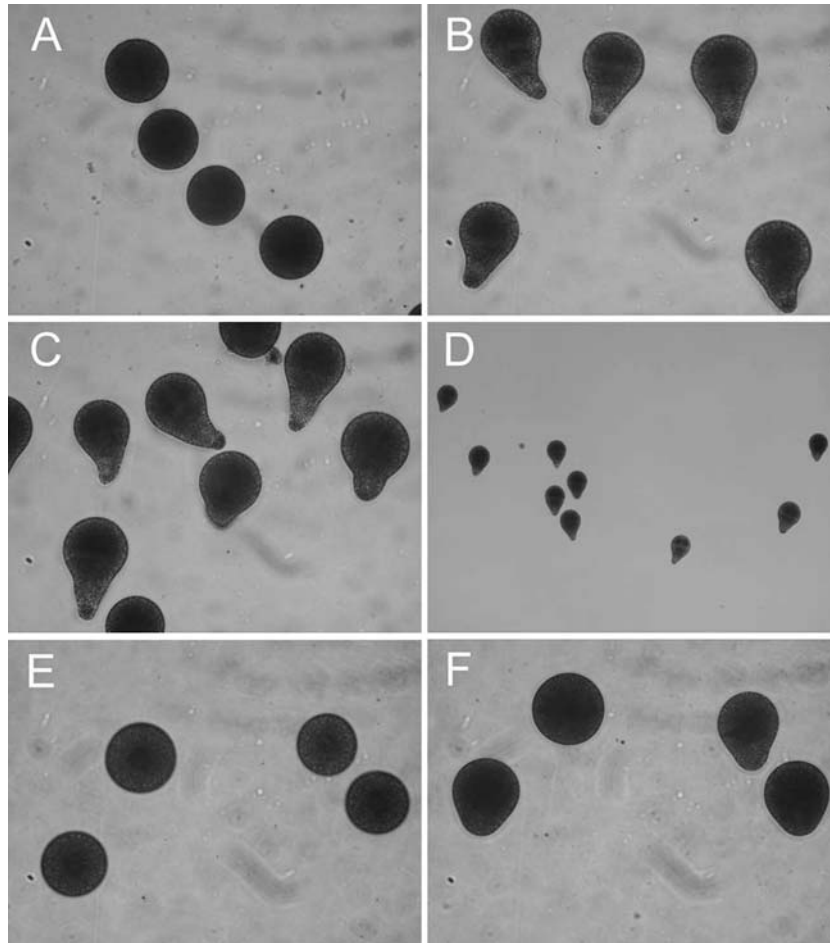


Fig. 7A–F Representative photographs of *S. compressa* zygotes' responses to KN-92 and KN-93. The average diameter of the cells is about 92 μm . **A** Cells treated with 10 μM KN-93 at 5.5 h after fertilization, at the beginning of a 2-h light treatment, and photographed at 24 h after fertilization. The KN-93 was present continuously after it was added. **B** Untreated control cells from the same experiment as shown in **A**. The high degree of germination, cell division and response to light is typical. **C** Cells treated with 5 μM KN-93 during a 2-h light treatment beginning at 5.5 h after fertilization. The KN-93 was washed off at the end of the light treatment and development allowed to proceed in the dark. **D** Low-magnification view of cells grown continuously in the presence of 10 μM KN-92 from 5.5 h after fertilization and photographed at 24 h after fertilization. **E, F** The same group of cells photographed at different times. **E** The cells at 8.5 h after fertilization at the time that 10 μM KN-93 was added. Note that none of the cells has detectably germinated. **F** The same cells at 25 h after fertilization. Three of the four have germinated, but none has divided

about 2 h, indicating that there must be some other responses to light prior to the establishment of a zone of net influx. It should be pointed out that net flux could be measured only in an artificial seawater in which Ca^{2+} was reduced from the normal 10 mM to 1 mM, and even then, the fluxes were near the detection limit of the method. In addition to improving the sensitivity of the Seris method, the Ca^{2+} influx in seawater with 1 mM Ca^{2+} is some 5-fold greater than in 10 mM Ca^{2+} seawater (Robinson 1977) and the cells are more sensitive

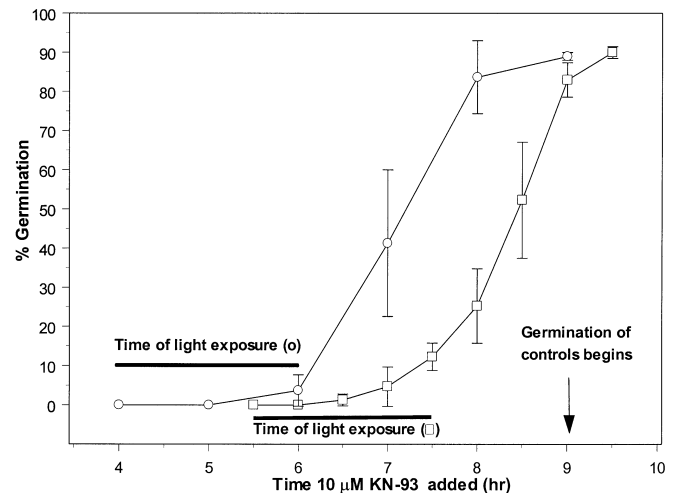


Fig. 8 The effect on germination of *S. compressa* zygotes of adding KN-93 at different times with respect to a 2-h light treatment. The curve on the *right* shows the response when the light exposure was begun at 5.5 h after fertilization and the curve on the *left* shows the response when the light treatment was begun at 4 h after fertilization. The inhibitor was continuously present after it was added. After the light treatments, the cells were allowed to develop in the dark until they were scored the next day. Controls were monitored and the earliest that any germination was detected was somewhat later than 9 h after fertilization. The *error bars* show the standard deviation. Each point is the mean of three experiments

Table 1 Germination of *Silvetia compressa* zygotes injected with CaM kinase II inhibitory peptide (CaM kinase II AIP) or myosin light-chain kinase inhibitory peptide (MLCK inhibitor)

Injection solution	Final cytosolic concentration (μM)	No. of zygotes injected and germination			
		Yes (by 12 h)		No (by 20 h)	
		<i>n</i>	%	<i>n</i>	%
CaM kinase II AIP	26–44	4	100	0	0
	49–96	2 (62 and 76 μM)	15	11	85
MLCK inhibitor	80–116	6	86	1 (109 μM)	14

to blue light as a polarization cue in reduced Ca^{2+} (Robinson 1996). No net efflux could be detected anywhere on the surface of the zygotes although tracer flux measurements previously showed net efflux on the illuminated side (Robinson and Jaffe 1975). However, if the net efflux to balance the localized net influx were distributed over a significantly larger fraction of the cells' surface area, it would fall below the limits of detection. The delay between the start of the application of the polarizing light and the detection of net influx at the shaded pole corresponds well to the similar delay in the formation of the cytosolic Ca^{2+} gradient (Pu and Robinson 1998; Pu et al. 2000). It is reasonable to conclude that the entry of Ca^{2+} from the external medium is a necessary component of the mechanism of gradient formation. The delay is also consistent with a role for newly formed actin filaments in generating localized Ca^{2+} influx. The net influx at the nascent germination site falls to undetectable levels prior to actual germination. This is consistent with measurements of cytosolic Ca^{2+} gradients and localized $^{45}\text{Ca}^{2+}$ flux entry in light-polarized cells (Robinson and Jaffe 1975; Pu and Robinson 1998), both of which also largely disappear by the time of overt germination.

CaM kinase II is a well-characterized, ubiquitous member of the multifunctional CaM kinase family that includes CaM kinases I, II, and IV (Hook and Means 2001). The inhibitor, KN-93, inhibits all of these kinases to some extent. At 5 μM or greater, the continuous presence of KN-93 almost completely inhibited germination of *Silvetia* zygotes. The inactive analog of KN-93, KN-92, had little effect on germination at any concentration. When present only during a 90-min light treatment, 5 μM KN-93 had no effect on the response of germinating cells to polarization by the light. AIP is considerably more specific for CaM kinase II, and when microinjected into cells at sufficient concentration, strongly inhibited germination. Similar injected concentrations of an MLCK inhibitory peptide had little effect on germination. Unfortunately, it is not possible to reverse the inhibition of the injected compounds, so their effects on photopolarization could not be tested.

The present experiments and our previously published results support the conclusion that calmodulin and CaM kinase II are necessary as downstream effectors of the Ca^{2+} gradient. When inhibitors of the two molecules are present from the beginning of a polarizing light pulse until 24 h after fertilization, no germination occurs. If the inhibitors are present only during a

polarizing light pulse of 30–120 min, the zygotes recover and most germinate. Zygotes treated with an inhibitor to calmodulin, ophiobolin A, during a polarizing light pulse actually show a significantly greater degree of polarization than untreated controls (Robinson 1996). Thus, neither activated calmodulin nor CaM kinase II appears to be necessary for the initial detection of the polarizing light signal. This is perhaps not surprising as the development of elevated Ca^{2+} on the future rhizoidal hemisphere occurs gradually over a 2-h period in response to unilateral light and presumably the role of calmodulin and its downstream partners are to read and interpret that gradient. What was surprising was the finding that germination could occur in the presence of KN-93 if its addition was delayed until 1–2 h before germination. This was especially evident in the case when the polarizing light pulse was given at 4–6 h after fertilization. Under those circumstances, 85% of zygotes treated with 10 μM KN-93 an hour before controls showed any sign of germination, germinated to some extent. This result suggests that CaM kinase II is not required for secretion, as it is in a number of other biological systems (Waters et al. 1998; Easom 1999), as germination requires the secretion of new membrane and cell wall materials. Likewise, KN-93 does not interfere with the ability of the zygotes to secrete the material necessary for the development of stickiness. It appears that some protein or proteins must be phosphorylated by CaM kinase II, and this event can occur in a restricted time between the forming of the zone of elevated Ca^{2+} and an hour before germination. Presumably, this phosphorylation occurs in the region of elevated Ca^{2+} . Our previous results (Pu and Robinson 1998) show that a definite Ca^{2+} gradient exists by 1 h after the beginning of a unilateral light pulse. The gradient is maximal after 2 h of light and then declines, even in the continued presence of the polarizing light. Taken together with the present results, these facts suggest that the critical time for the activity of CaM kinase II for germination is during a 1- to 2-h period beginning at 2 h after initiating polarization by unilateral light.

We also observed that cell division did not occur in the presence of 10 μM KN-93 if the inhibitor was added at any time up to 9.5 h after fertilization. Later addition of KN-93 was not tested in this regard. CaM kinase II has been shown to be necessary for progression through the cell cycle in a number of cell types (Morris et al. 1998; Patel et al. 1999; Su and Eppig 2002).

One aspect of Ca^{2+} signaling that appears not to be present in the fucoid zygotes is oscillations. Both imaging and Seris measurements indicate that the changes in cytosolic Ca^{2+} and Ca^{2+} influx are gradual, without oscillations in the frequency range of 0.1–1.0 Hz. Such oscillations are common in cells and CaM kinase II appears to be involved as a frequency-dependent response element (De Koninck and Schulman 1998; Dupont and Goldbeter 1998).

In summary, these results reinforce the notion that Ca^{2+} gradients play a central role in the emergence of asymmetry in the fucoid zygote. It will be interesting to learn if Ca^{2+} plays a similar role in the development of polarity in other biological systems. It is known that perturbations of Ca^{2+} homeostasis produce polarity defects in fission yeast (Facanha et al. 2002). Two interesting possible cases are the *Drosophila* neuroblast and the *Caenorhabditis elegans* zygote. The fly neuroblast develops from the ventral neuroectoderm where it acquires an apical–basal polarity that directs the subsequent asymmetric cell division into a larger stem cell and a smaller ganglion mother cell. The stem cell then undergoes further asymmetric cell divisions. The *C. elegans* zygote undergoes an asymmetric cell division producing the large AB blastomere and the small P₁ blastomere, and the two cells have different fates and cell cycle times. P₁ and its descendants subsequently undergo further asymmetric divisions that are necessary for the development of pattern in the embryo. The establishment and maintenance of these developmental polarities in flies and worms share a conserved complex of proteins (Doe and Bowerman 2001), including the involvement of actin microfilaments and microtubules. These cytoskeletal elements are also importantly involved in polarity establishment in the fucoid algal zygotes (Bisgrove and Kropf 1998; Kropf et al. 1999; Pu et al. 2000). The similarity of polarity formation mechanisms suggests that Ca^{2+} gradients may be a general feature in this fundamental process.

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