

Cytoplasmic calcium gradients and calmodulin in the early development of the fucoid alga *Pelvetia compressa*

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SUMMARY

The predicted existence of cytoplasmic Ca^{2+} gradients during the photopolarization of the zygotes of the brown algae, *Pelvetia* and *Fucus*, has proved to be difficult to establish, and the downstream targets of the putative gradients are not known. We have used quantitative microinjection of the long excitation wavelength Ca^{2+} indicator, Calcium Crimson, and of antibodies against calmodulin to investigate these matters in the zygotes and early embryos of *Pelvetia*.

We found that there is a window of cytoplasmic Calcium Crimson concentration that gives an adequate signal above autofluorescence yet allows normal development of the zygotes. As Calcium Crimson is not a ratiometric indicator, we injected other zygotes with a Ca^{2+} -insensitive dye, rhodamine B, and imaged the cells at the same time that Calcium Crimson-injected cells were imaged. Ratios were calculated by dividing the averaged pixel values of Calcium Crimson images by the averaged pixel values of corresponding rhodamine B images. By this method, we observed the formation of a cytoplasmic Ca^{2+} gradient within one hour of the exposure of the cells to unilateral blue light during the photosensitive period. The region of

high Ca^{2+} was localized to and predictive of the site of future rhizoid formation. We validated this somewhat indirect method by applying it to the growing rhizoid, where the existence of a tip-localized Ca^{2+} gradient is well established. The method clearly revealed the known gradient.

The injection of ungerminated zygotes with antibodies made against *Dictyostelium* calmodulin inhibited germination, and this inhibition was abolished if the calmodulin antibodies were coinjected with an excess of purified maize calmodulin. Likewise, the growth of the rhizoids was inhibited by calmodulin antibody injections. The fungus-derived calmodulin antagonist, ophiobolin A, which has previously been shown to be a potent inhibitor of germination, also inhibited rhizoidal growth. Our results provide evidence that a cytoplasmic Ca^{2+} gradient is present during photopolarization and that calmodulin acts as a mediator of Ca^{2+} gradients throughout the early developmental processes of germination and rhizoidal growth in *Pelvetia compressa*.

Key words: Calcium gradient, Calmodulin, *Pelvetia compressa*

INTRODUCTION

Fucoid algae, including species of *Pelvetia* and *Fucus*, are a model system for studying the *de novo* formation and expression of cellular polarity. Newly fertilized *Pelvetia* zygotes have no morphological or biochemical polarity, except for the point of sperm entry, but 10 to 12 hours after fertilization they develop an axis that is expressed as localized growth or germination at one end. The subsequent first mitotic division at about 18 hours after fertilization is perpendicular to the developmental axis, partitioning the zygote into two progeny cells that develop very differently: a smaller rhizoid cell containing the growth site and a larger thallus cell. The rhizoid cell becomes the adult holdfast, while the thallus cell becomes the vegetative and reproductive part, the thallus. The site of germination can be determined by a number of external gradients (reviewed by Jaffe, 1968), including unilateral blue light to which the cells are exquisitely sensitive. The light need

not be present continuously. A 90-minute exposure to unilateral light several hours before germination is sufficient to polarize a population of zygotes effectively. It has been proposed that the formation of an intracellular Ca^{2+} gradient is essential to the early development of *Pelvetia* zygotes and embryos, including the processes of polarization, germination and rhizoidal growth. Evidence supporting the hypothesis includes measurement of differential Ca^{2+} flux during photopolarization (Robinson and Jaffe, 1975), the measurement of an inward current at the nascent rhizoid (Nuccitelli, 1978) and the effects of Ca^{2+} ionophore gradients, which induced germination on the higher concentration side (Robinson and Cone, 1980). Speksnijder et al. (1989) injected a series of Ca^{2+} buffers with different K_D values for Ca^{2+} to oppose the formation of cytoplasmic Ca^{2+} gradients, and found that rhizoidal outgrowth could be blocked for up to two weeks without cell death, consistent with the requirement of a Ca^{2+} gradient preceding germination. During germination and

rhizoidal growth, an increase in Ca^{2+} level in the growing tip has been detected using Ca^{2+} -selective microelectrodes (Brownlee and Wood, 1986), ratiometric Ca^{2+} indicator Fura-2 (Brownlee and Pulsford, 1988), as well as a long-wavelength Ca^{2+} indicator, Calcium Green (Berger and Brownlee, 1993). Another essential component involved in germination and rhizoidal growth is the actin cytoskeleton (reviewed by Kropf, 1994). Cytochalasin B inhibited photopolarization of fucoid zygotes (Nelson and Jaffe, 1973; Quatrano, 1973), and cytochalasin D abolished the ionic currents entering the growing rhizoidal tip (Brawley and Robinson, 1985), suggesting that actin may be involved in initializing or stabilizing the transcellular ionic current. Recently, Hable and Kropf (1998) found that both cytochalasin D and latrunculin B were potent inhibitors of *Pelvetia* photopolarization. In addition, cyclic GMP (cGMP) has been demonstrated to play a role in photopolarization. The concentration of cGMP increased in response to unilateral blue light, and photopolarization was blocked by an inhibitor of guanylyl cyclase (Robinson and Miller, 1997). Recently, a high concentration of retinal has been extracted and purified from *Pelvetia* zygotes, suggesting that rhodopsin-like molecules might be the photoreceptors in this algal system (Robinson et al., 1998).

The resting Ca^{2+} level of fucoid zygotes has been reported to be around 300 nM (Brownlee and Wood, 1986). However, the detection of proposed cytoplasmic Ca^{2+} gradients has been surprisingly difficult. The cells are intensely autofluorescent under UV excitation, precluding the use of UV-excited ratiometric Ca^{2+} indicators, Fura-2 and Indo-1. In an effort to bypass the problem of autofluorescence, Berger and Brownlee (1993) used Calcium Green along with a pH-sensitive dye, SNARF, to monitor cytoplasmic Ca^{2+} distribution in *Fucus* zygotes at pH-insensitive wavelengths. However, most zygotes had greatly delayed germination (Berger and Brownlee, 1993), probably as a result of the buffering action of the dyes (Speksnijder et al., 1989), as well as high absorption of energy from the excitation light. In order to correlate the temporal changes of Ca^{2+} with its spatial distribution, our strategy has been to use the Ca^{2+} indicator available with the longest excitation wavelength, dextran-conjugated Calcium Crimson (CCdex), since autofluorescence of cytoplasm is much less at longer wavelengths and therefore less dye could be used. As there is no long excitation wavelength, Ca^{2+} -insensitive dye that could be coinjected and simultaneously imaged with Calcium Crimson, we were forced to use an indirect approach. Some cells were injected with CCdex and other cells were injected with dextran-conjugated rhodamine B (RBdex). The average fluorescent intensity from the CCdex-injected cells was divided by the average fluorescent intensity from the RBdex-injected cells at the same stage of development. The resultant ratio intensity should be dependent only on cytosolic Ca^{2+} levels and independent of optical asymmetries, which are slight in any case. As a test of this somewhat indirect method, we also applied it to determining the Ca^{2+} distribution in the growing rhizoid, where there is good evidence for the existence of a tip-focused Ca^{2+} gradient (Brownlee and Wood, 1986; Brownlee and Pulsford, 1988; Berger and Brownlee, 1993). This method correctly revealed the known gradient, validating this approach.

Pharmacological evidence has suggested that calmodulin

(CaM), a small, compact, highly conserved cytoplasmic protein, may be the cellular mediator of Ca^{2+} . CaM antagonists inhibited photopolarization of *Fucus serratus* zygotes, and microinjection of recombinant CaM of brown alga *Macrocystis* enhanced photopolarization (Love et al., 1997). The fungus-derived CaM inhibitor, ophiobolin A (OA), substantially enhanced photopolarization of *Pelvetia* zygotes when present during a brief light pulse, and the continued presence of as low as 300 nM of OA completely blocked germination, suggesting that CaM is involved in photopolarization, germination and growth (Robinson, 1996). CaM is present in all eukaryotic cells studied, and CaM-like proteins have been identified in prokaryotic cells (Fry et al., 1991). Ca^{2+} /CaM has a variety of downstream targets, including ion channels, receptor proteins, and enzymes such as protein kinases, calcineurins, phosphodiesterases, cyclases, and membrane Ca^{2+} -ATPases (Ehlers et al., 1996; Saimi and Kung, 1994). Ca^{2+} /CaM regulates the release of Ca^{2+} from intracellular stores essential for *Drosophila* phototransduction (Arnon et al., 1997), and CaM binding to the glutamate decarboxylase is required for the enzyme's activity and normal plant development (Baum et al., 1996). *Pelvetia* contains CaM in its gametes, zygotes and early embryos, as well as CaM-binding proteins that are developmentally regulated (Brawley and Roberts, 1989). To further investigate the role of CaM during *Pelvetia* early development, we have injected antibodies made against *Dictyostelium discoideum* CaM into zygotes shortly after fertilization, shortly after germination, and into rhizoidal cells of embryos just after first cell division. We found that the antibodies inhibited both germination and rhizoidal growth, and that the inhibition was alleviated when the antibodies were coinjected with excess purified recombinant maize CaM.

MATERIALS AND METHODS

Preparation of material

Mature *Pelvetia compressa* (formerly *P. fastigiata*) fronds were collected near Santa Cruz, California and shipped to us via Federal Express on ice. Fronds were dried with absorbent paper and stored in monolayers in loosely-capped containers at 4°C for up to two weeks before use. Gametes were obtained by the method of Jaffe (1954). Briefly, the fronds were put in flat-bottom glass vials in filtered natural seawater (NSW, Marine Biological Laboratory, Woods Hole, MA) and placed near a unilateral white light source of 40 to 90 $\mu\text{mole photons} \cdot \text{m}^{-2} \cdot \text{second}^{-1}$ overnight at $16 \pm 1^\circ\text{C}$. The next day, the fronds were rinsed thoroughly with NSW and transferred to a beaker in the dark for 15 minutes. Fertilization was arbitrarily taken as 30 minutes after the fronds were first placed in the dark. The zygotes were then filtered through a 125 μm Nitex nylon filter, and either put in the dark or back in the original light source until use. Artificial seawater (ASW) was prepared as previously described (Robinson, 1996), with the addition of 2.5 mM NaHCO_3 .

Microinjection

Microinjection pipettes were made from no-filament glass capillaries (Drummond Scientific Co., Broomall, PA) pulled on a vertical pipette puller (Sutter Instrument Co., model P-30) to obtain a tip size of approximately 0.5 μm . To minimize cytoplasmic leakage, silanization of micropipettes was done the day before by drying them at 160–220°C for at least one hour or overnight, and baking with *N,N*-dimethyl-trimethylsilylamine (Fluka Chemical Corp., Milwaukee, WI) at the same temperature for 15–30 minutes.

To facilitate injection, holding chambers were constructed according to Kiehart (1982), utilizing a piece of Scotch double-sided tape as a spacer between two coverslips to create a chamber for zygote loading. Each chamber had 20 to 30 zygotes placed about 100 to 200 μm apart. Zygotes in such chambers develop normally.

We utilized a high pressure microinjection system to allow quantification of injection volume (Kiehart, 1982; Miller et al., 1994). The slightly modified system consisted of a microinstrument holder, a horizontal micropositioner, a micrometer syringe and tubing filled with fluorinert (FC-40, Sigma) oil. Injection pipettes were backfilled with mercury, tip-loaded with the injection solution and dimethylpolysiloxane (Sigma) oil cap from a loading capillary. Occasionally pipette tips were gently broken off to a diameter of about 1 μm to facilitate tip-loading. Injection volumes were calibrated regularly by using pipettes from the same batch to inject oil columns of various lengths into seawater, measuring the volume of the oil droplet, and constructing a standard curve. All injections were done on an inverted Nikon microscope under a $\times 20$ objective according to the procedure of Speksnijder et al. (1989). Injection of young zygotes was done in NSW within 2 hours after fertilization. The presence or absence of germination was scored for from 12 to 20 hours after fertilization. For rhizoid injection, zygotes were loaded into the holding chamber the night before, illuminated from the opposite side of the tape, so that germination would occur toward the lumen of the chamber. Rhizoid injection was done in ASW made hypertonic with 450 to 550 mM mannitol between 12-15 hours after fertilization. After injection, the chamber was transferred to several dishes containing ASW of declining hypertonicity, and finally back to NSW near the same light source. The longest length of each injected zygote was recorded every hour onwards until first cell division had occurred.

The volume of zygotes was estimated in order to calculate the amount of injected solution (oil cap excluded) relative to the zygote's volume (%V). Zygotes prior to germination were spherical, and their diameters (d) were measured shortly after injection. Volumes were calculated by using the formula $V = 4\pi/3(d/2)^3$. Zygotes between germination and first cell division had blunt rhizoids. Each zygote was assumed to consist of a half sphere and a half oblate spheroid. The oblate spheroid included the germinating region, its minor axis being the diameter of the zygote (d). Volume was calculated using the formula $V = 2\pi/3(d/2)^2 \cdot l$, where l was the longest length of the zygote shortly after injection. All measurements were made under a $\times 20$ or $\times 40$ objective lens with a calibrated image splitting eyepiece (Vickers-A.E.I., loaned to us by Dr Lionel Jaffe, Marine Biological Laboratory) using the double width method. Briefly, the image splitting eyepiece split a single image into overlapping twin images, one red, the other green. The measurement knob was adjusted so that the two images would separate and juxtapose, until their closest edges were just barely touching. This could be easily visualized since the touching edges appeared black. Then the knob was adjusted again so that the position of the two images was reversed. Repetitive measurements indicate that the image splitting eyepiece measured with a precision of less than 1 μm .

Purified recombinant maize CaM stock solution (1.0 $\mu\text{g}/\mu\text{l}$) was provided by Dr Daniel Roberts at University of Tennessee, Knoxville, TN (Roberts et al., 1992). Mixture of three monoclonal antibodies against *Dictyostelium* CaM (anti-CaM) in mouse ascites fluid was from Sigma Biosciences (catalogue #C7055; Bazari and Clarke, 1982; Hulen et al., 1991). To minimize any toxic effect of sodium azide contained in it, the antibody solution was processed by repetitive dilution in 200 mM KCl, 5 mM Hepes (KCl/Hepes, pH 7.0) and centrifugation in a microcentrifuge filter (MWCO 10,000, ultrafree-MC filter unit, Sigma). The final concentration of sodium azide in anti-*Dictyostelium* CaM was less than 0.002%, and the final effective concentration of the antibodies was 1.55 mg/ml, or 10 μM . A mixture of equal volumes of maize CaM and anti-*Dictyostelium* CaM (CaM/anti-CaM) was also prepared. Dilution buffer for CaM and anti-CaM antibodies was either phosphate buffered saline (PBS, pH 7.1)

or KCl/Hepes. All injection solutions, except for KCl/Hepes, were stored at -20°C in small aliquots until use.

Ophiobolin A treatment

Ophiobolin A (OA) was made as a 10 mM stock solution in dimethyl sulfoxide (DMSO). For OA treatments, zygotes were obtained as above and dispersed in NSW at 1 to 2 hours after fertilization at low density into 35 mm plastic Petri dishes and placed in the unilateral white light source. At 12 to 12.5 hours or 18 to 19.5 hours after fertilization, each dish was rinsed three times and replaced with, respectively, ASW, ASW with 0.01% DMSO (v/v), ASW with 100 nM OA, ASW with 300 nM OA, and ASW with 1.0 μM OA. For second light treatment, all dishes were then turned by 90° relative to the original light source after the bathing media change.

Cytoplasmic protein extraction and immunoblotting

Cytoplasmic protein extraction was performed on 2-3 hour old zygotes according to the methods described by Brawley and Roberts (1989). Extracts were centrifuged at 12,000 g for 10 minutes, and the supernatants were collected and stored at -20°C . SDS-PAGE was carried out in 0.75 or 1.0 mm thick, 12.5% polyacrylamide gels using the Laemmli discontinuous buffer system and a Bio-Rad mini-gel apparatus. Immunoblotting was done on polyvinylidene difluoride (PVDF) membranes (0.2 μm pore size, Bio-Rad cat. no. 162-0185). For immunoblotting, gels were soaked in 25 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer, pH 7.0 (KP buffer) for 15 minutes at room temperature after SDS-PAGE. PVDF membranes were pre-wetted in 100% methanol and washed in KP buffer for 15 minutes at room temperature. Proteins were transferred overnight in KP buffer at 20 V at 4°C using a Bio-Rad mini trans-blot apparatus. After transfer, PVDF protein blots were fixed in KP buffer containing 0.2% (v/v) glutaraldehyde for 45 minutes at room temperature. Blots were then rinsed in KP buffer (3×15 minutes/rinse at room temperature), blocked with 10% (w/v) nonfat dry milk (Carnation) in PBS for 1 hour at 37°C . After blocking, blots were rinsed in PBS containing 0.05% (v/v) Tween-20 (PBST, 3×15 minutes/rinse) at 37°C . Incubation with primary mouse anti-*Dictyostelium* CaM antibodies (1:50 in PBS) was carried out for 1.5 hours at 37°C . Blots were rinsed as above in PBST, and incubated with secondary antibody (horseradish peroxidase-conjugated anti-mouse IgG, Sigma) diluted 1:500 in PBST for 1 hour at 37°C . Blots were rinsed again in PBST as above. Protein bands were visualized by incubating for 10-15 minutes with the enzyme substrate prepared by mixing 0.2 ml of 20 mg AEC (3-amino-9-ethylcarbazole, Sigma) in 2.5 ml DMF (dimethylformamide, Sigma), 3.8 ml 0.05 M acetate buffer (pH 5.0), and 0.02 ml 3% hydrogen peroxide.

Fluorescent imaging of zygotes and rhizoidal cells

Calcium Crimson dextran (10 kDa) (CCdex) and rhodamine B dextran (10 kDa) (RBdex) were from Molecular Probes, Inc. (Eugene, OR). Both were dissolved in 200 mM KCl, 5 mM Hepes, pH 7.0, at a final concentration of 2.5 mM. Only those rhizoidal cells that showed active tip growth and zygotes that germinated on schedule during an imaging experiment were chosen to be analyzed. Furthermore, among the imaged zygotes that germinated on schedule, only those that did not exhibit obvious overall asymmetry in brightness prior to blue polarizing light exposure were included in the final analysis of the ratio of the average CCdex fluorescent intensity over the average RBdex fluorescent intensity. For rhizoidal cell imaging, zygotes were cultured in unilateral white light as described above until first cell division had occurred. Rhizoidal cells were injected with 1.0 to 2.3 pl of either CCdex or RBdex. Rhizoidal cell imaging was started at approximately 20 hours after fertilization. To minimize the interference of the excitation light with normal growth, individual rhizoidal cells were imaged once every hour until 24 hours after fertilization. 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 shortly after fertilization. Polarizing blue light ($3\text{-}10 \mu\text{mole photons} \cdot \text{m}^{-2} \cdot \text{second}^{-1}$) was turned on at 5.5 hours 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 (numerical aperture 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 512×512 , 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 reached the maximum value of 255. Cells were maintained at $16 \pm 1^\circ\text{C}$ 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. To combine rhizoidal cell images, images of the apical $60 \mu\text{m}$ of the rhizoidal tip were rotated when necessary inside 'NIH Image' software program, so that all rhizoids showed horizontal extension in the final images. The three CCdex and three RBdex images of the rhizoids were aligned at the tip and each divided by 3 and the resultant images added up to yield one single combined image for CCdex and another one for RBdex. The ratio image was obtained by dividing the two single images and multiplying by an appropriate scaling factor, so that the entire spectrum of 256 pixel values was utilized. For zygotes, the cortical 5% 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 starting point of each 'linescan' was always chosen at the point on the cell on which the polarizing light was imposed. The site of germination was within 5 degrees of the axis of the light. The 'linescan' function scanned along the selected area just beneath the circumference of each cell, obtaining an ordered set of numbers each corresponding to

the average pixel value of a small region measuring approximately 17×1 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 average pixel values of five RBdex zygotes. The ratios were then divided into two equal sections, one corresponding to the illuminated hemisphere, the other corresponding to the shaded hemisphere. Means and standard errors of the mean were calculated for both sections at each time point of imaging. To construct polar line plots, average pixel values of 5 CCdex images were divided by the corresponding average pixel values of 5 RBdex images at each time point of the imaging process. The ratios thus obtained were further averaged over 10 degree intervals to obtain 36 data points for each time point.

RESULTS

Fluorescent imaging of zygotes and rhizoidal cells

We first established the amount of Calcium Crimson dextran (CCdex) that could be injected into *Pelvetia* cells without perturbing development. We found that zygotes containing a cytoplasmic concentration of CCdex between 13 and $18 \mu\text{M}$ both germinated at the same time as uninjected zygotes and also generated adequate signal for fluorescent imaging by confocal microscopy. Autofluorescence of uninjected *Pelvetia* zygotes that were imaged under identical conditions was 20- to 40-fold lower than the signal from CCdex-injected cells. Cells that were imaged once every hour almost always

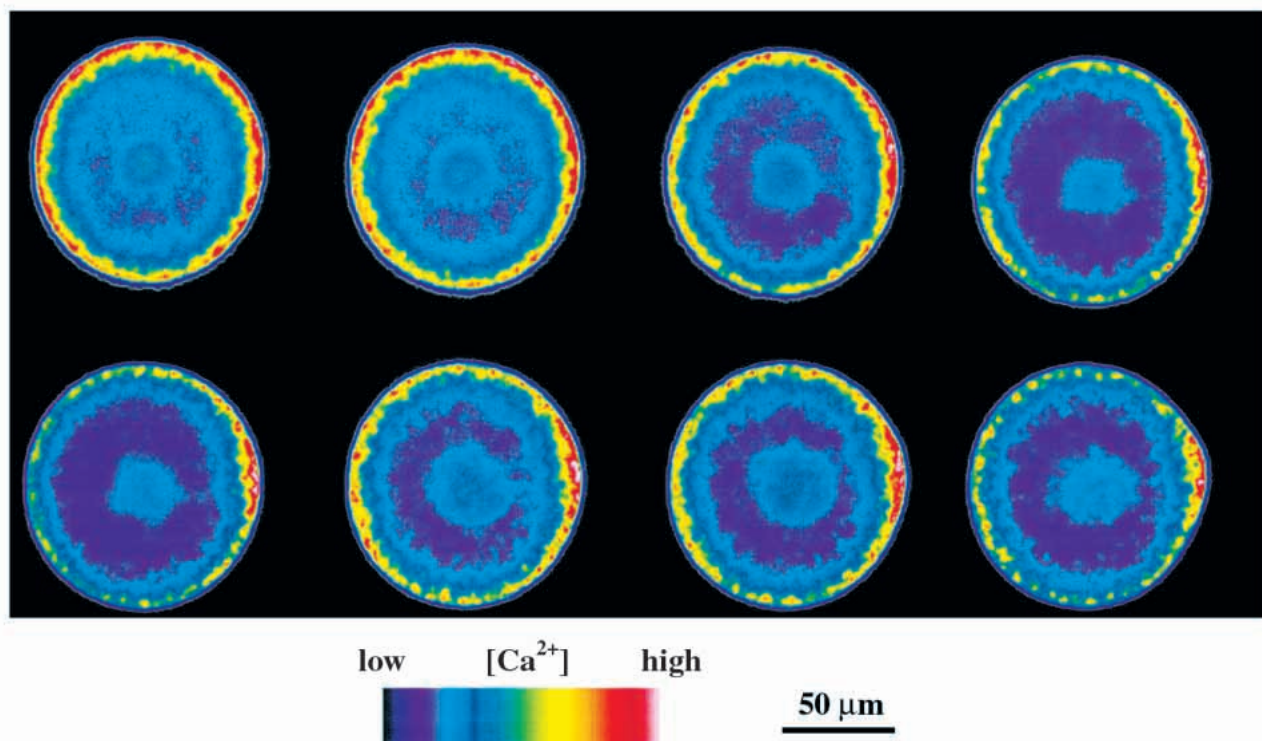


Fig. 1. Pseudocolor images of a zygote injected with Calcium Crimson dextran. Time 0 was defined as when the blue polarizing light was turned on, at 5.5 hours after fertilization. From left to right, the images were obtained at 0, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours (at which time the zygote had germinated), and 7 hours. The radial asymmetry in the signal is due to the opacity of the cells (the same for Fig. 2).

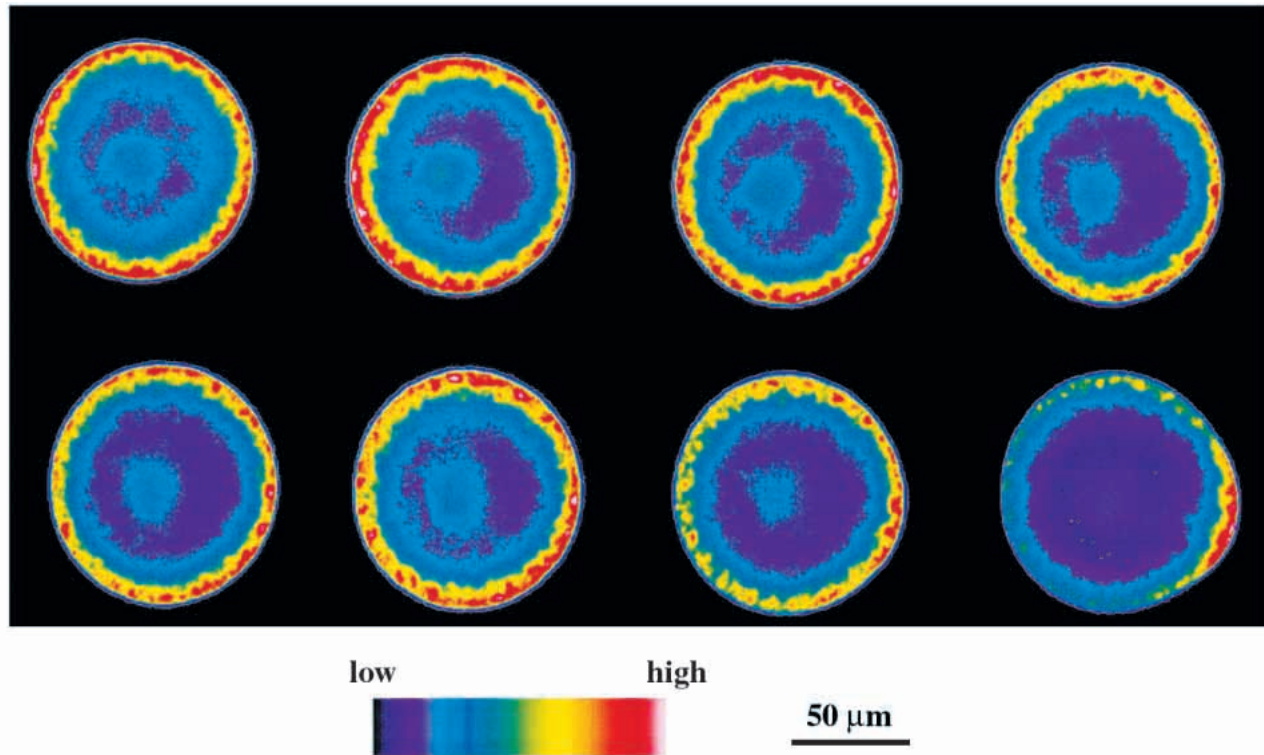


Fig. 2. Pseudocolor images of a zygote injected with rhodamine B dextran. Time 0 was defined as when the blue polarizing light was turned on, at 5.5 hours after fertilization. From left to right, the images were obtained at 0, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours (at which time the zygote had germinated), and 7 hours.

germinated normally. With increased frequency of imaging, the rate of normal germination decreased. As a control for any asymmetries in cytoplasmic dye distribution and aberrations in light paths that would affect the imaging output, we injected other zygotes with a Ca^{2+} -insensitive dye, rhodamine B dextran (RBdex), and imaged them in the same way as we did the ones injected with Calcium Crimson. Appropriate cytoplasmic concentrations for RBdex were found to be between 10 and 21 μM . We then ratioed the average CCdex intensity over the average RBdex intensity to detect any cytoplasmic Ca^{2+} changes during photopolarization and germination.

Figs 1 and 2 are pseudocolor images of a zygote injected with CCdex (Fig. 1) and another zygote injected with RBdex (Fig. 2) and imaged during photopolarization and germination. The opacity of the cells prevented the collection of significant signal from the interior of cells, but adequate signal was obtained from the cortical 10 μm or so. Prior to the turning on of the blue polarizing light, fluorescent signals from both cells were uniformly distributed in the cortical cytoplasm. Within one hour after the polarizing light was turned on, fluorescent intensity from the zygote injected with CCdex was seen to be greater on the side facing away from the light than on the side facing the light. This difference in CCdex signal output from the side of the zygote facing the light and the opposing side became more evident with time and the onset of germination. In the zygote injected with RBdex, fluorescent signals also exhibited different patterns on the illuminated side and on the shaded side that progressed with time after the polarizing light was turned on. More and more fluorescence was seen at the side of the zygote facing away from the light, and by the time

of germination, RBdex fluorescence was most prominently seen at the germination site.

The patterns of both CCdex fluorescence and RBdex fluorescence during the processes of photopolarization and germination are similar in that both became more uneven during photopolarization and eventually became brightest at the future germination site. We have obtained images of five zygotes injected with CCdex and another five injected with RBdex, all of which germinated at the same time as uninjected zygotes. The blue light completely polarized the zygotes, and their axes of germination were within 5 degrees of the axis of the light. Since aligning and combining all 10 zygotes were difficult, we were not able to obtain an averaged image for CCdex and another one for RBdex for indirect ratioing as described below for rhizoidal cells. Instead, we measured the pixel values around the cortical 5% (approximately 4.5 to 5 μm thick) of each individual fluorescent image obtained from all of the 10 zygotes. From those measurements, we were then able to calculate the average pixel values around the cortical cytoplasm for the five CCdex zygotes and also for the five RBdex zygotes. Fig. 3 is a polar line plot of the ratio obtained by dividing the averaged signal of five CCdex zygotes by the averaged signal of five RBdex zygotes during photopolarization and germination. Each data point represents the average ratio over 10 degree intervals, whereas the outer solid circle represents a ratio of 1. Standard errors of the mean were always less than 0.02. It can be seen that prior to the turning on of the blue polarizing light, the ratio around the zygote exhibited no apparent asymmetry in the hemisphere to be illuminated and in the hemisphere to be shaded from the

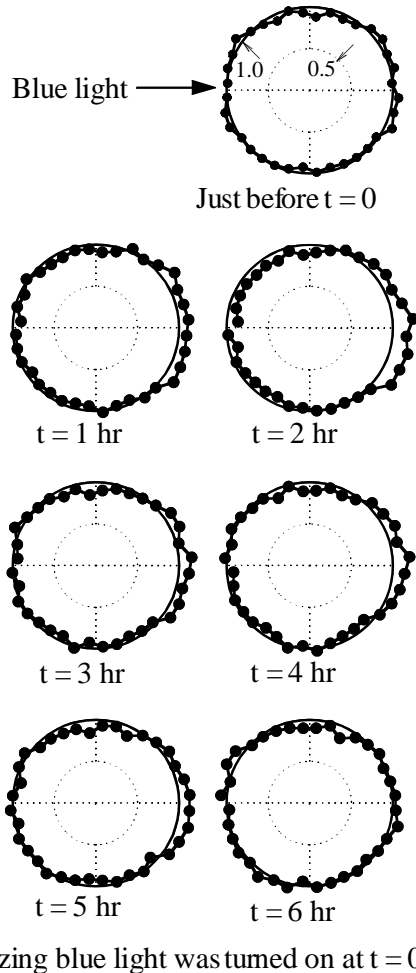


Fig. 3. Polar line plots of the ratio of corresponding pixel values of the average of 5 Calcium Crimson dextran images over the average of 5 rhodamine B dextran images obtained from 10 different zygotes. Time 0 was defined as when the blue polarizing light was turned on, at 5.5 hours after fertilization.

light. A higher ratio at the shaded side had formed after one hour of illumination, as seen by the skewed appearance of the plot on the shaded side, and this asymmetry was greatest after two hours of light exposure and correlated well with the future site of germination. With continued photopolarization and germination, this asymmetry declined. A more quantitative analysis of the same processes on the illuminated hemisphere and the shaded hemisphere at seven different time points is shown in Fig. 4. Prior to the polarizing light exposure, the average ratio on the side to be illuminated and that on the side to be shaded were the same ($P > 0.05$, Student's two-tailed t -test). Within one hour after the polarizing light was turned on, the average ratio on the shaded side had become 10% higher than that on the lighted side ($P < 0.0001$). After two hours of photopolarization, this ratio difference increased to being 17% higher on the shaded side ($P < 0.0001$). With further continued illumination, this difference in average ratio on the two hemispheres actually declined until germination, which occurred after 5 to 6 hours of illumination. With the onset of germination, the asymmetry in the average ratio of the two

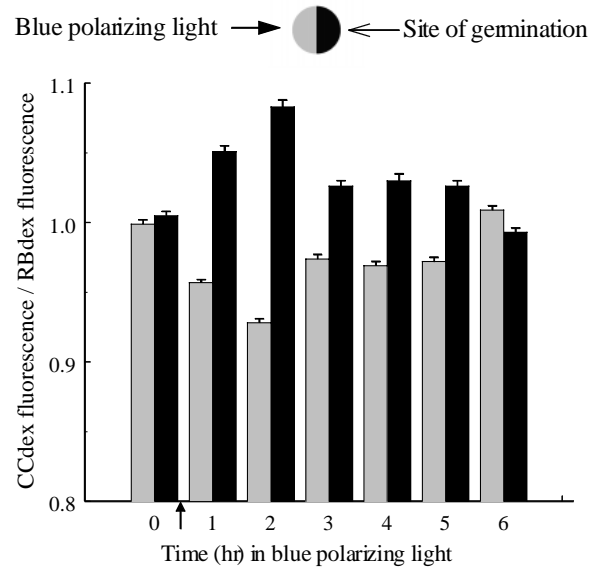


Fig. 4. Bar chart analysis of the ratios of corresponding pixel values of the average of 5 Calcium Crimson dextran images over the average of 5 rhodamine B dextran images obtained from 10 different zygotes. The ratios were divided into two equal sections, one containing the illuminated hemisphere, the other containing the shaded hemisphere. Means and s.e.m. were calculated for both sections at each time point of imaging. All zygotes had germinated within 6 hours after the blue polarizing light was turned on (arrow).

fluorescent signals became less substantial. Our results indicate that a cytoplasmic Ca^{2+} gradient formed in response to the polarizing light within one hour. This Ca^{2+} gradient was steepest after two hours of light exposure and it declined afterwards. Given that the resting cytoplasmic Ca^{2+} concentration is 300 nM (Brownlee and Wood, 1986), the steepest Ca^{2+} gradient seen after two hours of light exposure corresponds to approximately a twofold increase (Haugland, 1996) in cytoplasmic free Ca^{2+} concentration at the shaded hemisphere of the zygote which contained the future germination site.

To validate this indirect ratioing method, we injected some rhizoidal cells with CCdex and other rhizoidal cells with RBdex after first cell division had occurred. As shown in Fig. 5, we were able to combine and average the images obtained from the apical 17 μm of three rhizoidal cells injected with CCdex and the images obtained from three other rhizoidal cells injected with RBdex, respectively. All cells showed active rhizoidal growth throughout the duration of the imaging experiments. By ratioing the averaged CCdex image over the averaged RBdex image, a zone of high Ca^{2+} localized at the apical 3.7 to 7.2 μm of the rhizoid was detected which persisted along with the rhizoidal tip growth. This result is in agreement with earlier experiments using Fura-2 and Ca^{2+} microelectrodes which detected a tip-focused cytoplasmic Ca^{2+} gradient associated with fucoïd rhizoidal growth.

Immunoblotting of CaM in *Pelvetia* cell extracts

CaM is highly conserved across eukaryotic species. Invertebrate and vertebrate CaMs have high sequence homology, and despite their significant sequence divergence,

vertebrate CaM can functionally replace yeast CaM (Davis and Thorner, 1989). Brawley and Roberts (1989) have identified CaM from *Pelvetia compressa* gametes, zygotes and embryos, and we confirmed the presence of CaM in cytoplasmic protein extracts of *Pelvetia* by SDS-PAGE utilizing its differential migration ability in the presence and absence of Ca^{2+} (Burgess et al., 1980) (data not shown). It has been reported that antibodies made against bovine testes CaM recognized purified *Pelvetia* CaM (Brawley and Roberts, 1989). We immunoblotted *Pelvetia* protein extracts with the cocktail of three monoclonal anti-*Dictyostelium* CaM antibodies. As shown in lane 2, Fig. 6 (arrowhead), the antibodies crossreacted with a single band of apparent molecular mass of between 17 and 20 kDa in *Pelvetia* cytoplasmic protein extract. The band also had the same mobility as the exogenous maize CaM that was added to the same extract preparation (Fig. 6, lane 3). Therefore, the anti-*Dictyostelium* CaM antibodies recognized the putative *Pelvetia* CaM and not any other cytoplasmic proteins. It would be expected that once microinjected into *Pelvetia* zygotes or embryos, the anti-*Dictyostelium* CaM antibodies would specifically bind to the endogenous *Pelvetia* CaM and perhaps inhibit its activity.

Injection prior to germination

When newly fertilized zygotes were injected with control solutions or simply poked on the plasma membrane and grown in dark until germination, the site of germination was biased toward the penetration site compared to unpenetrated controls. The penetrated zygotes developed indistinguishably from the controls. It has been reported that injection volumes of up to 16% that of zygotes did not affect normal growth (Speksnijder et al., 1989). To minimize any damage incurred by the microinjection process itself, injection volumes were kept below 10% that of zygotes.

The cytosolic volume of *Pelvetia* has been estimated to be approximately 45% of the total volume of a single zygote (Allen et al., 1972). This value was used to calculate the final cytosolic concentrations of injected solutions. As shown in Table 1, microinjections of KCl/Hepes or maize CaM alone did not affect normal germination of the injected zygotes (37/38, or 97% normal germination). This gave us confidence that

should we see any inhibitory effect after microinjection, it was not due to the injection process itself or the presence of as much as 5 μM maize CaM in the cytosol. Cytosolic concentrations of less than 0.33 to 0.39 μM anti-*Dictyostelium* CaM antibodies had little effect upon germination (80% germination), although more concentrated antibody solution (10 μM versus 5 μM stock) seemed to be inhibitory at a lower final cytosolic concentration. However, when the cytosolic concentrations of the antibodies were above 0.33 μM to 0.43 μM , up to 0.93 μM , germination was blocked in 90-100% of all injected zygotes. To further investigate the specificity of the antibodies, other zygotes were injected with the same concentration of the antibodies along with a severalfold higher concentration of maize CaM. When the final cytosolic concentration of the antibodies was above 0.43 μM , germination rate of the coinjected zygotes was 73%, while that of the zygotes injected with antibodies alone was 10%. Since the inhibitory effect of the anti-CaM antibodies was significantly alleviated by excess maize CaM, our results suggest that CaM plays a vital role in mediating the process of germination.

Among those zygotes whose germination was inhibited by anti-CaM or CaM/anti-CaM, some were followed for longer periods of time. As shown by the pictures in Fig. 7, it was observed that unless germination occurred sufficiently early (before or on day 2), division generally preceded germination. On the other hand, once germination occurred, cell divisions always followed. Four zygotes injected with CaM/anti-CaM divided by day 3 or day 4, but no germination was observed for the same period of time. The embryos remained round yet viable (as shown by their ability to plasmolyze in hypertonic seawater) and their diameter increased over time. Among the 16 ungerminated zygotes injected with 10 μM anti-CaM, only the one injected with the smallest amount of the antibodies germinated and divided by the end of day 2; the other 15 zygotes did not germinate for 3 to 6 days. Among the zygotes injected with 5 μM anti-CaM, 2 germinated by day 2 and subsequently underwent multiple cell divisions, 1 divided by day 7 but did not germinate, 6 had one or more division plane formation prior to germination several days later, 2 lysed by day 5 without any division or germination.

Table 1. Injection of young zygotes with KCl/Hepes, maize CaM and anti-*Dictyostelium* CaM antibodies

Injection solution	Injection volume (% cell vol.)	Final cytosolic conc. (μM)	Number of zygotes injected	Germination			
				Yes		No	
				<i>n</i>	%	<i>n</i>	%
KCl/Hepes	2.0-4.5	—	24	23	96	1	4
CaM (29 μM)	1.5-5.9	0.97-3.80	7	7	100	0	0
CaM (58 μM)	2.0-4.0	2.58-5.16	7	7	100	0	0
Anti-CaM (10 μM)	0.2-1.4	0.04-0.31	4	4	100	0	0
	1.5-4.2	0.33-0.93	16	0	0	16	100
Anti-CaM (5 μM)	1.5-3.5	0.17-0.39	16	12	75	4	25
	3.9-6.4	0.43-0.71	20	2	10	18	90
CaM/anti-CaM	1.7-3.0	1.10/0.19-1.93 / 0.33	16	15	94	1	6
(29 μM /5 μM)	3.9-6.4	2.51/0.43-4.12/0.71	22	16	73	6	27

Germination was monitored starting at 12 hours after fertilization. Zygotes that did not germinate by 20 hours after fertilization were assigned the term 'no germination'.

OA inhibition of rhizoidal growth

In view of the potent effects of OA on germination and the confirmation of the involvement of CaM in germination by our antibody injections, we have investigated the effects of both OA and injected antibodies on rhizoidal growth. Fucoid rhizoids have the intrinsic property of growing away from a unidirectional light source. We utilized this property of the rhizoids to test the effects of OA on rhizoidal growth on a large scale by bathing germinated zygotes and embryos just after first cell division in OA while exposing them to a second polarizing light that was at a right angle to the first polarizing light (Fig. 8). Although the four different batches of material used for both experiments showed varying degrees of sensitivity to OA, the rhizoids of untreated cells were always negatively phototropic; that is, they redirect tip growth away from the second light source. In all experiments, the inclusion of 100 nM OA in the bathing ASW visibly inhibited rhizoidal growth compared to the controls. The rhizoidal growth was further inhibited or blocked by 300 nM OA. In most cases, when such low concentrations of OA were washed out and replaced with normal ASW, the zygotes or embryos were able to recover in 1 or 2 days. When present at 1.0 μ M, OA completely blocked any rhizoidal growth in all cases, and cells started to lyse within 12 hours to a few days, and this inhibitory effect was not reversed by removing OA from the bathing

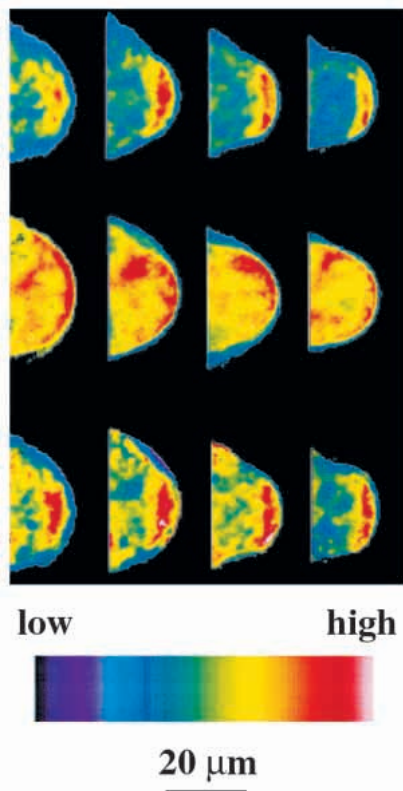


Fig. 5. Pseudocolor images of rhizoidal cells after first cell division. Top: Calcium Crimson dextran images averaged from 3 different cells; middle: rhodamine B dextran images averaged from 3 different cells; bottom: corresponding ratioed images obtained from top and middle rows of images. From left to right, images were obtained at 21, 22, 23 and 24 hours after fertilization, during which time all rhizoids showed normal rates of growth.

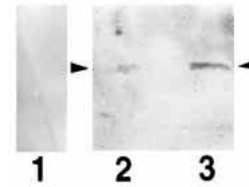


Fig. 6. Immunoblotting of *Pelvetia* protein extract with anti-*Dictyostelium* CaM antibody cocktail. Lane 1: negative control (*Pelvetia* protein extract, no primary antibody); lane 2: *Pelvetia* protein extract; lane 3: *Pelvetia* protein extract containing added exogenous maize CaM. The antibodies crossreacted with a single band of between 17 and 20 kDa.

ASW. Although cells bathed in 1.0 μ M OA were not able to initiate rhizoidal growth, cell division planes were nevertheless observed in some cases, although sometimes at much delayed time and with abnormal division patterns. Embryos bathed in 300 nM OA did not exhibit any obvious growth until at least 6 hours after exposure to the redirected light, during which time they had undergone one or more cell divisions. Cells bathed in ASW containing 0.01% (v/v) DMSO developed indistinguishably from control cells bathed in ASW. Our results of OA experiments on both germinated zygotes and early embryos again suggest that CaM is essential for rhizoidal

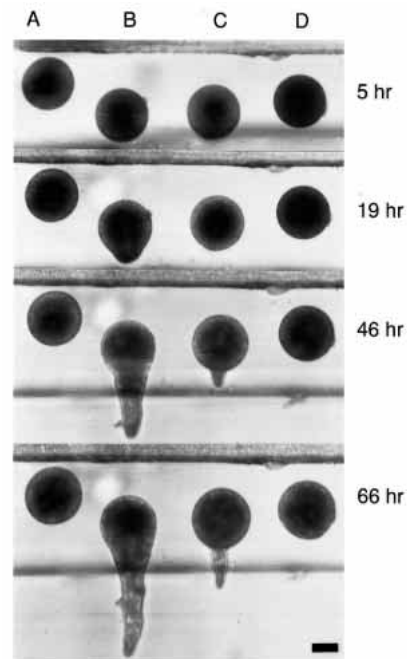


Fig. 7. Germination and growth of zygotes injected with anti-CaM or CaM/anti-CaM shortly after fertilization. Zygotes A, C and D were injected with 5 μ M anti-CaM (4.1–6.1% V zygote); zygote B was coinjected with 5 μ M/29 μ M anti-CaM/CaM (4.3% V zygote). B germinated and had first cell division by 19 hours, as did uninjected controls. The antibody-injected zygotes neither germinated nor divided at 19 hours, but they all had at least one cell division plane by 46 hours, and C had germinated by this time and continued rhizoidal growth. A and D did not have an outgrowth until after 7 days, when they had had multiple cell divisions. Bar, 50 μ m.

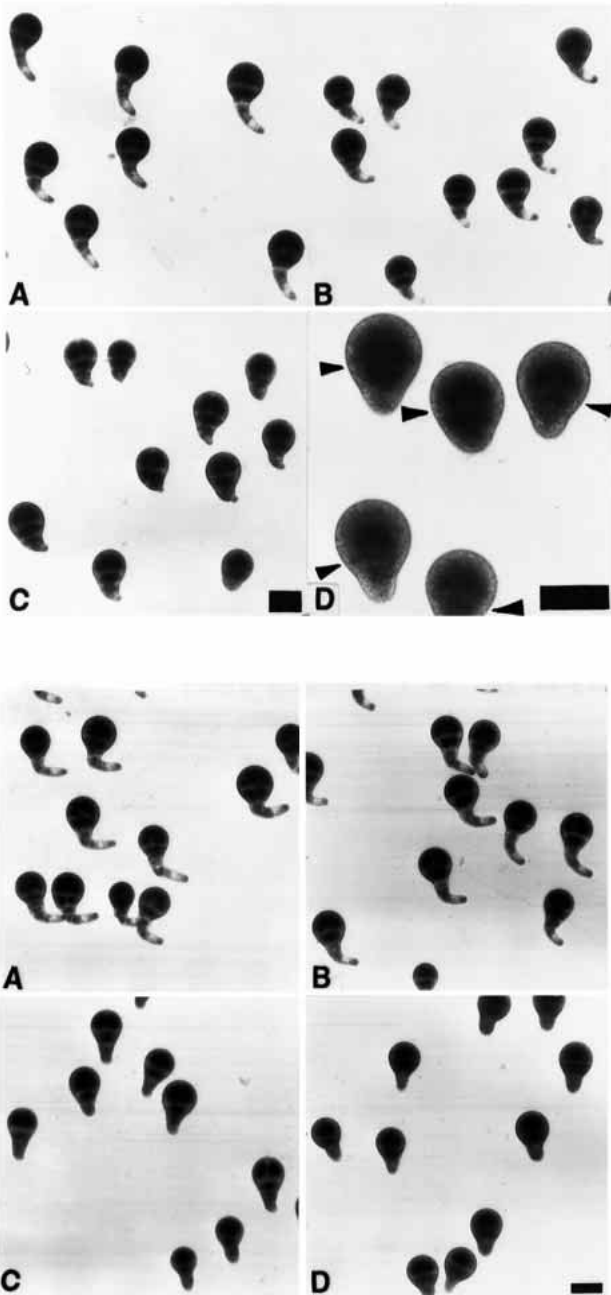


Fig. 8. Effects of Ophiobolin A on rhizoidal growth of germinated zygotes (top panel) and embryos after first cell division (bottom panel). In all cases, zygotes were cultured in filtered NSW in the first polarizing white light (from top of page) until 12 hours (top) or 18 hours (bottom) after fertilization. NSW was then replaced with normal ASW or ASW containing OA and the dishes were placed in the second polarizing light (from left of page). Pictures were taken 24 hours later. (A) Control cells in DMSO-containing ASW (top) or ASW (bottom); (B) cells cultured in 100 nM OA; (C) cells cultured in 300 nM OA; (D) cells cultured in 1.0 μM OA. The presence of 100 nM (B) visibly inhibited the negative phototropic rhizoidal growth, while 1.0 μM OA (D) completely blocked this growth. The effect of 300 nM OA (C) was intermediate. However, one or more cell divisions proceeded in all cells treated with 100 or 300 nM OA, and in most of the cells treated with 1.0 μM OA (arrowheads), suggesting that cell division is less sensitive to CaM inhibition than rhizoidal growth. Bars, 100 μm .

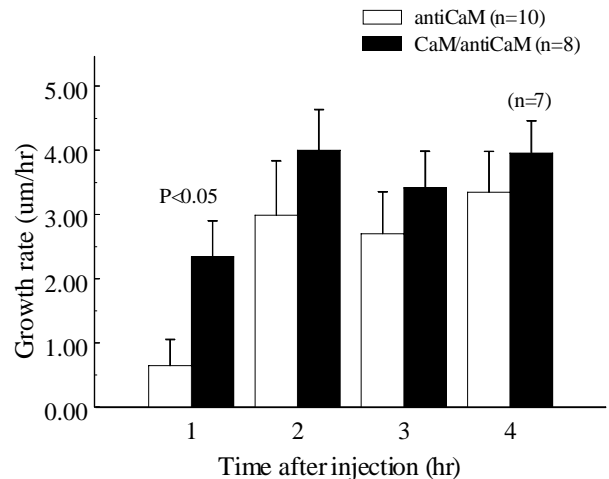


Fig. 9. Growth measurements of zygotes injected at 15 hours after fertilization with 3.2–6.2% v/v zygote of 5 μM anti-CaM antibodies or 5 μM /29 μM anti-CaM/maize CaM. Injections were done in ASW containing 450–550 mM mannitol to minimize cytoplasmic leakage. Zygotes were grown in the same continuous unilateral white light before and after injection. Triplicate measurements were made using an eyepiece image splitting device.

growth and precise cell division planes, but the formation of cell division planes itself is less sensitive to CaM antagonists.

Injection of germinated zygotes and rhizoidal cells after first cell division

Fig. 9 summarizes the results obtained with antibody injections of 15-hour-old zygotes. Zygotes injected with small volumes (1.7–2.9% those of the zygotes') of 5 μM anti-CaM showed no significant difference in rhizoidal growth rate than other zygotes coinjected with the same antibody along with excess maize CaM (29 μM) ($n=7$ for anti-CaM, $n=6$ for CaM/anti-CaM). With larger volumes of either solution (Fig. 9), zygotes injected with the antibodies alone consistently grew at a slower rate than those coinjected with excess maize CaM along with the antibody. It seems that although at the concentrations and the amounts tested, the antibody did not stop growth, it nonetheless slowed the growth rate, especially shortly after the injection was done and before the onset of first cell division. In either group of injected zygotes, first cell division usually occurred at 18–20 hours after fertilization, at the same time as those uninjected zygotes that were grown in the same chamber. Sometimes the division plane was not readily discernible at the same focal plane as the circumference of the zygote at 18–20 hours, but it was clear before 24 hours after fertilization. Measurements of growth rate also indicate that the two groups of embryos continued rhizoidal growth at indistinguishable rates after first cell division, which could have partitioned much of the antibody away from the expanding rhizoid, thus diluting the antibody concentration. In addition, more CaM proteins could have been synthesized by this time, further compensating for any inhibition that the antibodies might have imposed upon rhizoidal growth.

The growth of rhizoidal cells after first cell division was also significantly inhibited by an injection amount of 10–20% cell volume of 5 μM anti-CaM when compared with those rhizoidal cells injected with comparable amounts of CaM/anti-CaM (5

$\mu\text{M}/29 \mu\text{M}$) for up to four hours until next cell division had occurred (data not shown). Those results further suggest that CaM is important for rhizoidal growth.

DISCUSSION

We investigated the existence of putative cytoplasmic Ca^{2+} gradients during photopolarization in *Pelvetia compressa* zygotes and whether CaM is involved in germination and rhizoidal growth by acting as the cellular receptor of Ca^{2+} . By an indirect ratiometric method utilizing the ratio of averaged CCdex fluorescent images over averaged RBdex fluorescent images separately obtained from several zygotes, we detected a cytoplasmic Ca^{2+} gradient, with elevated Ca^{2+} on the hemisphere where germination would occur. The magnitude of the gradient increased during the first two hours of exposure to unilateral blue light, and then declined as the time of germination approached. We assume that the increasing RBdex fluorescence at the developing rhizoidal pole is due to organelle rearrangements resulting in the formation of a clear zone there that has been noted by others (Nuccitelli, 1978). It is important to note that there is little or no asymmetry in the RBdex signal during the first two hours of light polarization, but that a substantial CCdex fluorescence asymmetry appears during that time.

To validate this indirect ratiometric Ca^{2+} imaging method, we imaged CCdex and RBdex fluorescence from the tip of growing rhizoidal cells after first cell division had occurred. The rhizoidal cells are not heavily pigmented, especially at the apical tip region and others have reported Ca^{2+} gradients at the tip. Because of this homogeneity of the rhizoidal tip, we were able to ratio the averaged images of CCdex over the averaged images of RBdex and obtain an artificial ratiometric image. This method showed clearly the formation of a tip-localized high Ca^{2+} zone within about 3.7 to 7.2 μm of the tip and it persisted along with tip growth. This result was in good agreement with earlier reports on Ca^{2+} gradients in growing fucoid algae rhizoidal tip using other methods.

The cytoplasmic Ca^{2+} that we observed here are in surprisingly good agreement with the Ca^{2+} influx gradients reported more than twenty years ago (Robinson and Jaffe, 1975). Using a nickel screen method that allowed the measurement of $^{45}\text{Ca}^{2+}$ influx on the illuminated and shaded sides of zygotes separately, it was found that there was a large asymmetry in influx by two hours after illumination was begun. This influx asymmetry declined as the time of germination approached, despite continued illumination. Unfortunately, the first tracer flux measurement was not begun until two hours after the start of the polarizing light treatment. Nevertheless, the two methods agree that the major asymmetry in Ca^{2+} occurs early in the photopolarization response and then declines despite continued unilateral illumination. It may be that as polarization proceeds, the region of elevated Ca^{2+} influx and resultant elevated cytosolic Ca^{2+} becomes increasingly restricted and thus less readily detectable by our relatively low resolution measurements. In any case, a tip-focused Ca^{2+} gradient was detected later in the growing rhizoid.

Our experience with microinjection indicates that zygotes have a tendency to germinate from the penetration site when grown in the dark, and sometimes even in the presence of

continuous polarizing light that was directed to induce germination opposite the penetration site. This is in contrast to the report of Love et al. (1997), in which it was stated that *Fucus* zygotes tended to germinate away from the injection site in dark. Since fucoid zygotes are intrinsically sensitive to a number of external gradients, including those of pH, temperature, flow and the presence of a nearby thallus, this discrepancy could be due to other polarizing factors that were not taken into account by Love et al. (1997). Considering the fact that the zygotes had the potential to germinate in all three dimensions, the results indicate that external Ca^{2+} entry from seawater through the injection or poking wound may play an important role in germination site selection. Love et al. (1997) exposed *Fucus serratus* zygotes to unilateral light in various inhibitors of plasma membrane Ca^{2+} channels and intracellular Ca^{2+} release channels, and found that the inhibitors significantly reduced photopolarization.

We set forth to find if CaM is the downstream target of Ca^{2+} that mediates its effects on germination and growth. CaM is the major intracellular Ca^{2+} receptor. It regulates a variety of cellular events upon activation by Ca^{2+} binding. Through the activation of myosin and axonemal dynein ATPases (Klee and Vanaman, 1982), CaM plays an important role in regulating cytoskeletal function. Cyclic GMP has been found to be involved in *Pelvetia* photopolarization process (Robinson and Miller, 1997), and CaM was discovered as the stimulator of bovine brain cyclic nucleotide phosphodiesterase, which is important for cyclic nucleotide metabolism. A fungus-derived CaM antagonist, ophiobolin A, inhibited germination and rhizoidal growth when present in seawater at concentrations as low as 100 nM. We utilized microinjections of recombinant maize CaM, an anti-*Dictyostelium* CaM antibody cocktail, and a mixture of both CaM and the anti-CaM antibody to investigate whether CaM is involved in germination and rhizoidal growth of *Pelvetia* zygotes and early embryos. By microinjecting anti-*Dictyostelium* CaM antibodies, we found that a cytoplasmic concentration of anti-*Dictyostelium* CaM antibodies greater than 0.43 μM blocked germination and at higher concentrations delayed rhizoidal growth prior to and after first cell division, while coinjection of the antibodies with excess recombinant maize CaM almost completely reversed the inhibitory effect of the antibodies. On the other hand, zygotic and rhizoidal cell divisions can occur in the presence of an inhibitory concentration of the antibody, although the divisions were usually delayed and exhibited abnormal patterns. The above observations are consistent with those of Speksnijder et al. (1989), in which they found that appropriate Ca^{2+} buffer injections were able to block germination of *Pelvetia* zygotes, and that some ungerminated zygotes were able to divide nonetheless. They also found that embryos that formed substantial rhizoidal outgrowth never failed to divide. Berger and Brownlee (1993) also observed that in some *Fucus* zygotes injected with Calcium Green and SNARF, cell division preceded germination. Our results indicate that CaM is essential for germination in *Pelvetia* zygotes, but it is not required at the same level for cell divisions to occur. It is possible that small amounts of CaM present in the zygotes unbound by the antibodies would still permit cell divisions to occur. The partitioning of cytoplasm after cell division could locally increase the concentration of CaM to above the threshold level for germination. If the increased CaM happens

to localize at the presumptive germination site, the daughter cell would be able to germinate. On the other hand, it has been reported that protein synthesis and morphogenesis are relatively independent of each other during *Fucus* embryogenesis (Kropf et al., 1989). Among those zygotes whose germination had been blocked, over the period of several days, enough CaM protein could eventually be synthesized to allow germination to proceed, and subsequently cell divisions could occur. Our observations of germination, rhizoidal growth and cell divisions in *Pelvetia* zygotes and early embryos indicate that CaM is essential for germination and required for rhizoidal growth, but cell division is less sensitive to CaM inhibitors. It appears that cell division occurs when there is sufficient rhizoidal outgrowth, and that cell division does not require germination. We conclude that CaM is involved in germination and rhizoidal tip growth in *Pelvetia*, and to a lesser degree, if at all, in cell division.

Our results indicate that cytoplasmic Ca²⁺ gradients mediate photopolarization and germination, and that localized intracellular Ca²⁺ changes in developing *Pelvetia* zygotes may affect CaM activity, which in turn participates in germination and rhizoidal growth, and possibly polarization as well. Love et al. (1997) found that photopolarization of *Fucus serratus* zygotes was enhanced by microinjection of recombinant CaM from another brown algal species, indicating that CaM mediates the photopolarization response in this system. The results encourage us to continue our efforts to image Ca²⁺ distribution during polarization under other conditions, such as induction by external vectors other than light, and in Ca²⁺-free seawater.

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