Planta (2011) 233:911–920
DOI 10.1007/s00425-010-1343-2

ORIGINAL ARTICLE

Changes in gravity rapidly alter the magnitude and direction of a cellular calcium current

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Received: 24 October 2010 / Accepted: 21 December 2010 / Published online: 15 January 2011

Abstract In single-celled spores of the fern *Ceratopteris richardii,* gravity directs polarity of development and induces a directional, trans-cellular calcium (Ca^{2+}) current. To clarify how gravity polarizes this electrophysiological process, we measured the kinetics of the cellular response to changes in the gravity vector, which we initially estimated using the self-referencing calcium microsensor. In order to generate more precise and detailed data, we developed a silicon microfabricated sensor array which facilitated a lab-on-a-chip approach to simultaneously measure calcium currents from multiple cells in real time. These experiments revealed that the direction of the gravity-dependent polar calcium current is reversed in less than 25 s when the cells are inverted, and that changes in the magnitude of the calcium current parallel rapidly changing *g*-forces during parabolic flight on the NASA C-9 aircraft. The data also revealed a hysteresis in the response of cells in the transition from 2*g* to micro-*g* in comparison to cells in the micro-*g* to 2-*g* transition, a result consistent with a role for mechanosensitive ion channels in the gravity response. The calcium current is suppressed by either nifedipine (calcium-channel blocker) or eosin yellow (plasma membrane calcium pump inhibitor). Nifedipine disrupts gravity-directed cell polarity, but not spore germination. These results indicate that gravity perception in single plant cells may be mediated by mechanosensitive calcium channels, an idea consistent with some previously proposed models of plant gravity perception.

Keywords Gravity · Calcium channel · Calcium pump · Polarity

Electronic supplementary material The online version of this article (doi:10.1007/s00425-010-1343-2) contains supplementary material, which is available to authorized users.

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Introduction

Experiments in space (Roux et al. 2003) and on earth (Edwards and Roux 1998) demonstrate that gravity directs the polarity of development in single-celled spores of the fern *Ceratopteris richardii*. The earliest measured gravity-directed event in these cells is a vertically polarized, trans-cellular calcium current that peaks between 8 and 12 h after the induction of germination by light (Chatterjee et al. 2000). This current is coincident with the period of axis fixation by gravity (Edwards and Roux 1998) and appears to be critical for subsequent gravity-directed polarized

events, including nuclear migration, the first cell division, and rhizoid emergence, because the ability of gravity to direct these events, collectively known as gravimorphogenesis, is suppressed by a calcium-channel blocker that also blocks the calcium current (Chatterjee et al. 2000).

The first experiments to measure a gravity-directed calcium flux in *C. richardii* spores used a self-referencing ion-selective electrode system that sequentially measured the directional flux of calcium at various points around the spore (Chatterjee et al. 2000). This study also included experiments that demonstrated the current reorients after the spore is rotated, but did not reveal how quickly the gravitational stimulus changed the direction of the current. This limited an evaluation of the underlying mechanisms and made it difficult to clarify whether gravity was directly or indirectly altering the activity of the calcium pumps and channels that drove the current. That is, the earlier data were insufficient to differentiate between competing hypotheses to explain the mechanism for generating the directional calcium current. Does gravity direct calcium pumps and channels to redistribute by diffusion through the plasma membrane to the poles of the cell, or does it activate mechanosensitive ion channels at the bottom pole of the cell?

To answer the question of how fast the current reorients when the cells are turned 180°, we took two approaches. First we upgraded the self-referencing microsensor system with a computer-controlled stepper motor to rotate individual spores (Supplemental Figure 1). Secondly, to generate even more precise and detailed data, we developed a silicon microfabricated sensor array that allowed us to simultaneously measure calcium currents from multiple cells in real time. The design, fabrication, and testing of this cell electrophysiology lab-on-a-chip device, or CEL-C, and the interface instrumentation and software for measuring differential calcium concentrations have been previously described (Rokkam et al. 2007; ul Haque et al. 2007). We used the CEL-C to record rapid changes in the current both when the cells were rotated and when they were subjected to a wide range of different *g*-forces during parabolic flight.

The NASA "Reduced Gravity Research Program" at Johnson Space Center operates flights of a C-9B aircraft through a series of parabolas referred to as "zero-*g* maneuvers." This program is designed to allow researchers to conduct experiments in a true microgravity environment. During flight through multiple parabolas, the climb and dive of each parabola produces hypergravity conditions of approximately 2*g*, and when the aircraft has reached the peak of each parabola, it experiences a period of 15–25 s of true microgravity (0.002*g*). Here, we report the results of these *g*-changes on the trans-cell calcium current and of pharmaceutical experiments that assess the relative contributions of calcium channels and pumps to the ion current and to the gravity-directed cell polarization.

Materials and methods

Plant materials

Spores from the fern *Ceratopteris richardii* were surface-sterilized by soaking for 90 s in 0.875% sodium hypochlorite, and rinsed with sterile water. In order to enhance the synchronization of spore germination, the spores were resuspended in deionized water and soaked for 4–6 days in complete darkness at 29°C (Edwards and Roux 1998). Petri dishes contained solid growth media made from 1% agarose in half-strength Murashige and Skoog Basal Salt Mixture pH 6.2 (Caisson Labs, Rexburg, ID).

Calcium measurements with the CEL-C device

The CEL-C device (ul Haque et al. 2007) consists of 16 wells (150 μm^2) on a silicon substrate with Ag/AgCl electrodes (10 μm) leading into each well at each of the four poles of the cell (top, bottom, and the two sides (Supplemental Figure 2). An SU-8 polymer layer is used as the structural and insulating layer and a solid-state calcium ion selective membrane is used to impart ion selectivity to the Ag/AgCl electrodes. The calcium electrodes are each tested against a reference electrode by calibration in standard $CaCl_2$ solutions. We interrogate the cellular events by operating these sensors without a reference, as potentiometric pairs (Rokkam et al. 2007), using a technique known as dual electrode differential coupling (DEDC). The electrode pairs still behave in accordance with the Nernst equation, where a 28 mV change in differential output corresponds with a tenfold difference in calcium concentration between the two paired electrodes. While it is possible to use the calibration data, and intermittent reference data to convert the DEDC differential into concentration values, the data here are presented in terms of relative calcium concentration differentials that result from changes in calcium flux into, and out of the cell at different positions. Note that the DEDC conversion is based on the Nernstian pre-calibration of the sensors operated against a Ag/AgCl reference, and background reference concentrations recorded intermittently by changing from DEDC mode using the switching matrix controlled by the data acquisition (DAQ) hardware/software system. The advantage of DEDC is the system is auto-zeroing, without the need for extensive electronics and DAQ functions, which dramatically improves signal to noise when the signals are being amplified by filtering out common transients associated with the single amplifier circuit. While we do know

concentrations, the direct DEDC conversion is more important as we are precisely measuring the concentration differential which is a direct measure of the trans-cellular current. For full details of the approach please refer to ul Haque et al. (2007) and Rokkam et al. (2007).

Individual spores were pipetted and placed into individual pores using pulled glass micro-capillaries and a dissecting microscope. Once in place on the chip the fluidic chamber over the CEL-C microchip was filled with 1% agarose in half-strength Murashige and Skoog basal salt mixture pH 6.2 (Caisson Labs, Rexburg, ID). The agarose was a low melting point grade and was cooled to at least 37°C before being pipetted carefully into the fluidic chamber. This CEL-C system was used to investigate the behavior of spores under a variety of gravity-stimulating conditions. The calcium flux of individual spores was measured during and after 180° vertical rotations (Fig. 2). The effects of micro-and hyper-gravity were also examined in real time during parabolic flights on the NASA-operated C-9B aircraft (Figs. 3, 4). Additionally the system was used to monitor the effects of pharmaceutical agents that alter calcium pump and channel activity on spores (Fig. 6). For both in-flight and on-ground studies null-sensor controls were performed that included empty pore chambers, and dead (boiled) spores.

Drug preparation and use

DMSO was chosen as the solute for the majority of experiments for consistency with other drug treatments. Stock solutions were used full strength or diluted with 100% solvent to make "1,000×" working solutions. Solvent was added to control medium at a matching concentration of 0.1%. Treatment media was added and removed at the indicated times post light exposure. Removal of the drug was aided by a rinse step consisting of soaking the spores in 10 mL of drug-free medium for at least 60 min. This was followed by an exchange for fresh medium for the remainder of the 3 days of growth.

The free acid form of EY, 2,4,5,7-tetrabromofluorescein (H_2EY; Sigma-Aldrich, Inc.) was used to make 0.2 M stock solution in 100% DMSO for direct or diluted use as above. The solubility of the free acid form limited final concentrations of EY in the 1/2 MS medium to 2 μM or less. The disodium salt form of EY (2,4,5,7-tetrabromofluorescein disodium salt, Na_2EY) is water soluble and was used at final concentration up to 100 μM. Stocks were prepared as above but using sterile distilled water as the solvent. Untreated controls for Na_2EY used either unmodified 1/2 MS or, in experiments involving DMSO-soluble drugs and controls, the number of necessary samples was limited by adding 0.1% DMSO to the Na_2EY treatments.

Scoring and statistics

Directionality of rhizoid growth was scored visually under a microscope at 40× total magnification. Rhizoids were categorized according to the major growth direction in relation to a horizontal midline. For consistency with calculations from Edwards and Roux (1994) "% down" was calculated based on the number of "down" rhizoids versus "midline" and "up", which make up the population of spores considered to be non-gravity responsive.

Percent rhizoid emergence and downward rhizoid growth were calculated for each population. Populations of the same condition were grouped and averaged. A two tailed, two-sample unequal variance Student's t test was performed to determine statistical difference between control and treatment averages, using a cut off for significance of $P < 0.05$.

Results and discussion

Initially, we confirmed that gravity directed the polarity of the trans-cell calcium current using an improved version of the self-referencing ion-selective (SRIS) microelectrode technique previously used (Chatterjee et al. 2000). We corroborated the previous findings that calcium efflux from the top of cells ranged between 250 and 3,000 fmol cm^{-2} s^{-1}, always 3–4 times greater than the efflux measured from the side of the spore (Fig. 1), which ranged from 0 to 200 fmol cm^{-2} s^{-1}. These experiments also showed that there in an influx of calcium at the bottom of the cell, which quickly was re-established upon rotation of the cell by 180°. The new platform for these SRIS measurements allowed us to move the calcium microsensor around the external surface of the spore while the spore was held in position by a micropipette that was mounted on a computer-controlled microstepper motor to facilitate controlled rotation of the cell. With this setup we could measure the kinetics of the calcium current reorientation after cell rotation more rapidly than in the earlier study. Because the microsensor had to be moved away from the spore during rotation, there was still some time-lag between rotation of the spore and current measurement. Our fastest measurement was able to resume recording after rotation in approximately 44 s (data not shown). In all experiments, the directionality of the calcium current was reoriented, and it resumed to at least 69% its pre-rotation value, within the time recording was resumed. These single-microsensor experiments provided a valuable independent data set to compare against the subsequent results obtained with the CEL-C device; however, the results obtained were limited in that we could not continuously measure the calcium flux while the cell was being rotated,

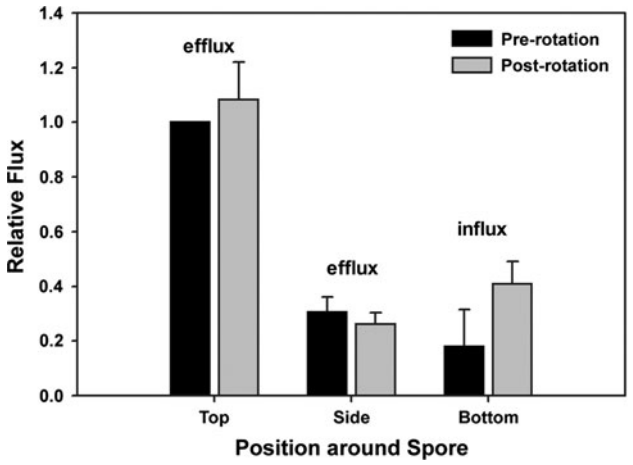

Fig. 1 Relative Ca^{2+} flux before and after spore rotation. For each rotation recorded, measurements taken at each position before and after rotation were averaged and then normalized to the Ca^{2+} flux recorded at the top of the spore prior to rotation. The top efflux was significantly greater than the measured efflux from the side or influx at the bottom (Student's t test, $P < 0.05$). There were no significant differences within a position before or after rotation (Paired t test, $P > 0.05$). For top measurements, $n = 28$; for side, $n \geq 16$; for bottom, $n = 3$. *Error bars* represent standard error

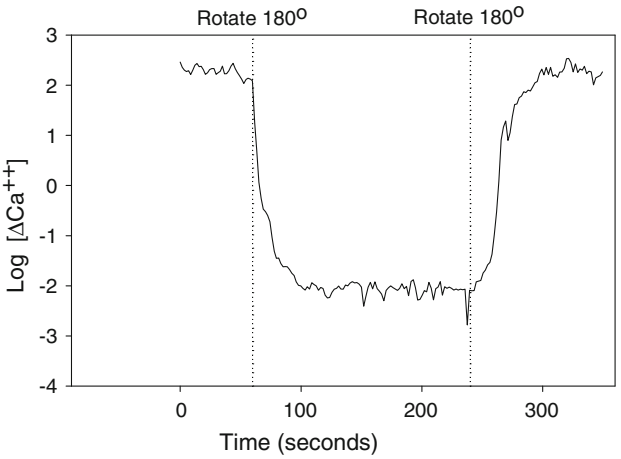

Fig. 2 Calcium current reorientation during rotation of spores as detected by CEL-C calcium sensor device. The top/bottom differential electrode signal was monitored continuously during these rotation experiments using dual electrode differential coupling. The rotation of the shielded metal housing that contain the spore loaded CEL-C chip was facilitated by a computer-controlled stepper motor that rotated the chip with the cells by 180° in 5 s. Data were collected every 0.5 s, and the chip was loaded with 14 spores, of which 12 responded. The average response time for current reorientation was 24.1 ± 3.2 s. This plot is a representative reading from one spore. The experiment was replicated 5 times with similar results

nor could we simultaneously monitor calcium flux at multiple locations around the cell.

In order to answer the question of how rapidly the calcium current is redirected after cell inversion we engineered a MEMS-based system to integrate single cells with microsensors, employing microfabrication techniques commonly used in the microelectronics industry. The resulting CEL-C system was fabricated on a silicon substrate with an electronics interface and supporting data acquisition system and software (Rokkam et al. 2007; ul Haque et al. 2007). Hydrated and developmentally synchronized spores were placed into individual microfluidic chambers of the CEL-C device (Supplemental Figure 2) that act as cell culture chambers. The cells were immobilized using 1% agar media to prevent their movement during g-force changes.

Data analysis revealed that the calcium differential signals measured by the CEL-C were statistically significant but relatively small. Although the CEL-C was capable of recording the calcium concentration at the top and bottom simultaneously, operating the chip in a dual electrode differential coupling (DEDC) configuration was necessary to measure the low-level signals coming from a cell (Rokkam et al. 2007). In this mode, the electrodes are electrically coupled to use each other as a reference. The electrodes do not directly measure bulk concentrations, but instead measure relative differences in concentration between themselves. The advantage is the elimination of reference noise and background offsets, allowing direct signal amplification to measure the subtle signal changes induced by gravity in the cells. This approach was designed and validated by comparison to previous work with self-referencing calcium microelectrodes that first showed differences in calcium efflux (top) and influx (bottom), suggesting a trans-cellular calcium current is active in directing cellular polarity (Chatterjee et al. 2000).

The top/bottom differential electrode signal was monitored continuously during the rotation experiments (Fig. 2) using the DEDC configuration noted above. We recorded continuously during a 5-s rotation of the spore and found that the average response time for reorientation of the calcium differential started in less than 5 s and was complete in 24.1 ± 3.2 s (Fig. 2).

We also used the CEL-C system to observe the effect of microgravity on the magnitude of the calcium current. Spores were flown onboard the NASA-operated C-9 aircraft during parabolic flights. In these experiments, the calcium current was monitored in alternating hyper-g (1.8g) and reduced-gravity (0.002g) conditions (Fig. 3). Cells were flown on three separate flights. Each flight consisted of the aircraft flying through four sets of approximately ten parabolas. On each flight 8–10 cellular recordings were sufficiently free of background and transient noise artifacts to observe the top–bottom calcium differential. The cells responded to hyper-g by increasing the magnitude of the top–bottom calcium differential, whereas the transition of flight into micro-g caused the signal to decrease (Figs. 3b, 4). Evidence that the cells did

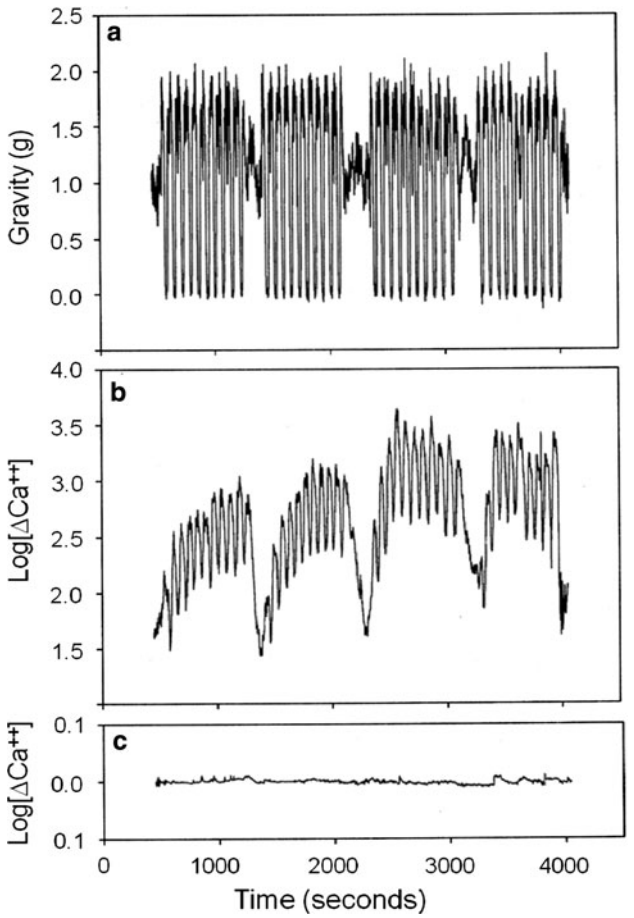

Fig. 3 Representative data depicting the measured changes in top–bottom trans-cellular calcium flux related to gravitational force during a parabolic flight experiment on NASA's C-9 aircraft. **a** The aircraft flies through four sets of ten parabolas, during which the *g*-force within the plane is modulated between micro-*g* and approximately 1.8*g*, as recorded by an accelerometer. Between each set of parabolas the spores experienced a period of 1-*g* flight. **b** Each cell responded to hypergravity by increasing the calcium differential between top and bottom, whereas microgravity caused the signal to decrease. For these experiments 2–4 cell chambers were left empty, without a cell, as a control for signal artifacts. On each of three flights we obtained 8–10 cellular recordings that were sufficiently free of noise, and transient noise artifacts. **c** Voltage reading from a null fluidic sensor chamber that lacked a cell did not have any measurable signal that correlated with the gravitational force. Data were collected at a rate of 1 data point per second

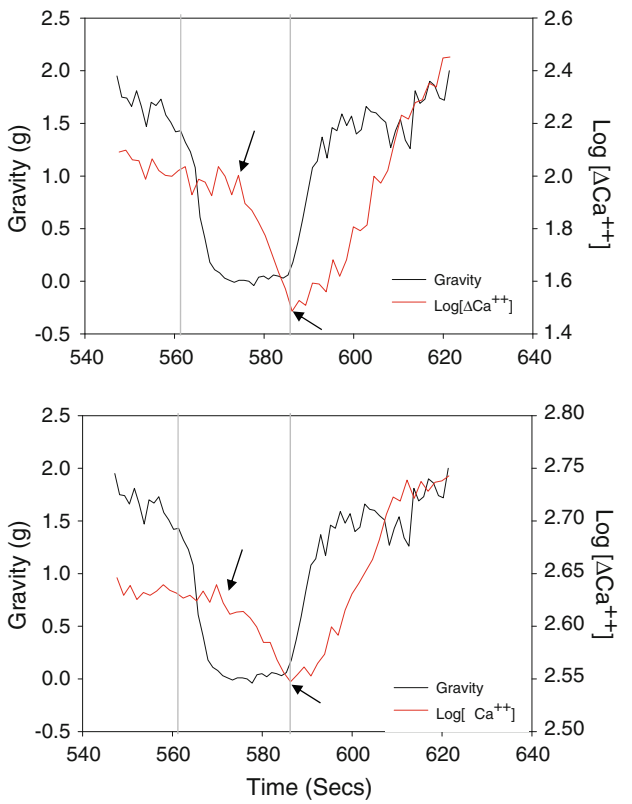

Fig. 4 Representative plots of two different cells (*top* and *bottom plots*) associated with changes in *g*-force. *Dashed lines* indicate the time of the change in *g*-force as recorded by an accelerometer. Here we are focusing on the detailed signals during the transitions in gravitational force from hyper-*g* to micro-*g*, and back to hyper-*g*. *Arrows* point to the time point at which the calcium current was altered. Note that the hyper-*g* to micro-g phase has approximately a 10- to 15-s lag before the cell starts to respond. The micro-*g* to hyper-*g* change occurs within 2 s. Similar results were observed in all responding cells in all of the flight experiments. Data were collected at a rate of 1 data point per second

not move downward during hypergravity stimuli was that if they did, this should have moved the cells closer to the bottom electrodes and further from the top ones, thus lowering the ratio of top-to-bottom calcium measurements, but instead hypergravity actually increased this ratio.

The decrease in the calcium differential in micro-*g* was reliable and significant compared to hyper-*g* in all parabolas, but the magnitude of the signal in micro-*g* did not decrease to the magnitude observed in 1-*g* condition. This may have been due to the limited length of time spent in micro-*g* condition and/or a significant hysteresis in the response. During the transitions in gravitational force from hyper-*g* to micro-*g*, we observed a 10–15 s lag before the cell started to respond (Fig. 4); in the micro-*g* to hyper-*g* change, however, the change in calcium current occurred within 2 s.

One explanation of the hysteresis phenomenon could be the *g*-force threshold necessary for channel activation. The force necessary to activate these channels may be in the 0.01-*g* range rather than 1*g*. In this case, the spores would actually spend more time in *g*-force conditions that activate ion channel opening (above 0.01*g*) than in the micro-*g* condition that causes relaxation or closing of the channels.

The hysteresis could also have been due to differences in closing and opening rates in mechanosensitive channels that have been previously reported in other cells. Mechanosensitive channels of *Xenopus* oocytes can be activated

to full capacity an order of magnitude more rapidly than they can be inactivated (McBride and Hamill 1993). While the patch clamp studies reveal response times faster than 100 ms (Mcbride and Hamill 1993), our recordings suggest that the concerted activities of thousands of channels at the whole cell level takes seconds for full trans-cellular current changes. Similarly, hysteresis in mechanosensitive ion channels in mammalian smooth and cardiac muscle is well documented (Lin et al. 2007). Another example is a mechanosensitive ion channel from the chloroplast of *Chlamydomonas* (Nakayama et al. 2007), which opens readily when mechanical stimulation begins but will not close until the mechanical stimulus is completely removed. Our results do not require that the mechanosensitive channel be on the plasma membrane. In addition to the *Chlamydomonas* example, plant mechanosensitive channels have been identified in Arabidopsis plastid locales (Haswell and Meyerowitz 2006), and mechanically induced transport changes in these organelles could induce the ion uptake changes across the plasma membrane we observe.

Mechanosensitive channels could also be involved in the change in the calcium current observed during parabolic flight when the spores underwent transition from micro-*g* to hyper-*g* (Figs. 3, 4). In the few cases where it has been identified, ion transport across a membrane in response to mechanical stimulation occurs through channel activity, driven by a chemiosmotic gradient (Syntichaki and Tavernarakis 2004). In keeping with this, the alteration in calcium current that occurred in parabolic flight (Fig. 3) and upon rotation of spores (Fig. 2) may be due to regulation of mechanosensitive or stretch-activated (SA) calcium channels. Of course, the increase in calcium current when the spores transited into hyper-*g* would very likely have involved changes in efflux (calcium pump activity) as well as changes in calcium-channel activity. This gravity-induced regulation of trans-cellular calcium flux occurred so quickly it must be very closely associated with the primary gravity perception mechanism in *C. richardii*.

Finding mechanosensitive ion channels (MSCs) in *Ceratopteris* spores would not be surprising, since MSCs have been detected in all biological systems tested, including many plant cells (Ding and Pickard 1993). They have been detected and analyzed by diverse methods, including patch-and pressure-clamp (Leonetti et al. 2004; Pickard and Fujiki 2005; Robinson and Jaffe 1975), and pharmacological approaches employing drugs that specifically inhibit SA channels (Chen et al. 1996; Dutta and Robinson 2004). Many members of the bacterial gene family Mechanosensitve channel Large (MscL) have been cloned and extensively characterized (Sachs 2002). Recently, a mechanosensitive SA calcium channel was cloned from Arabidopsis (Nakagawa et al. 2007). The Arabidopsis gene mid1-complementing activity 1 (MCA1), rescues the mutant phenotype when transformed into *mid1*yeast. There is another possible orthologue of this gene in Arabidopsis, as well as a possible orthologue in rice, but the authors found no gene sequences with significant similarity to MCA1 in any other plants and suggested that this may be a gene family unique to flowering plants.

Given the typically low cytoplasmic concentration of free calcium in plant cells relative to their extracellular environment, we expect that the calcium influx in the fern spore is mediated by calcium channels (Chatterjee et al. 2000), and the efflux is mediated by calcium pumps. Finding that the *C. richardii* calcium current reorients in response to rotation within 25 s (Fig. 2) suggests a gravity-induced localized change in the activity of the ion channels and/or pumps is involved in this response. We propose that the calcium transport components mediating this current are uniformly distributed in the spore plasma membrane and are locally activated to generate the polar calcium current (Fig. 5a) measured by both the CEL-C and the self-referencing ion-selective electrode systems.

An alternative hypothesis (Fig. 5b) would require that the channel and pump transporters have localized positions which are redistributed in the cell membrane upon reorientation. This localized redistribution could happen by polarized turnover and secretion of channels and pumps or by their polarized movement in the plane of the fluid membrane. *C. richardii* spores typically have a circumference of 300 μm or more. To travel from one pole to the opposite, the transporters would have to move at a velocity of approximately 6.0 μm s^{-1} in order to reorient the calcium transport in 25 s or less. Recently, the diffusion constant for the free movement of a transporter in a membrane was estimated to be 0.6 μm^2 s^{-1} (Adkins et al. 2007) and the typical diffusion coefficients for the directed movement of proteins in membranes are even smaller (Carlsson et al. 2002; Kucik et al. 1989; Pelham and Chang 2001; Waddle et al. 1996). Thus, it would be highly unusual for a transporter protein to move at a velocity sufficient to travel half the perimeter of a cell the size of a *C. richardii* spore in less than 25 s.

In order to dissect the connection between the gravity-directed calcium current and spore polarity alignment, pharmacological agents were chosen that could selectively inhibit plasma membrane calcium channels or plasma membrane Ca^{2+}-ATPase activities (Fig. 6). In previous experiments we used nifedipine, a calcium-channel blocker, and demonstrated that this drug inhibited both gravity-dependent cell polarity, and calcium flux in the spores (Chatterjee et al. 2000). In this study we replicated the previous work with nifedipine using the CEL-C system to assay the effects of the drug on the calcium current.

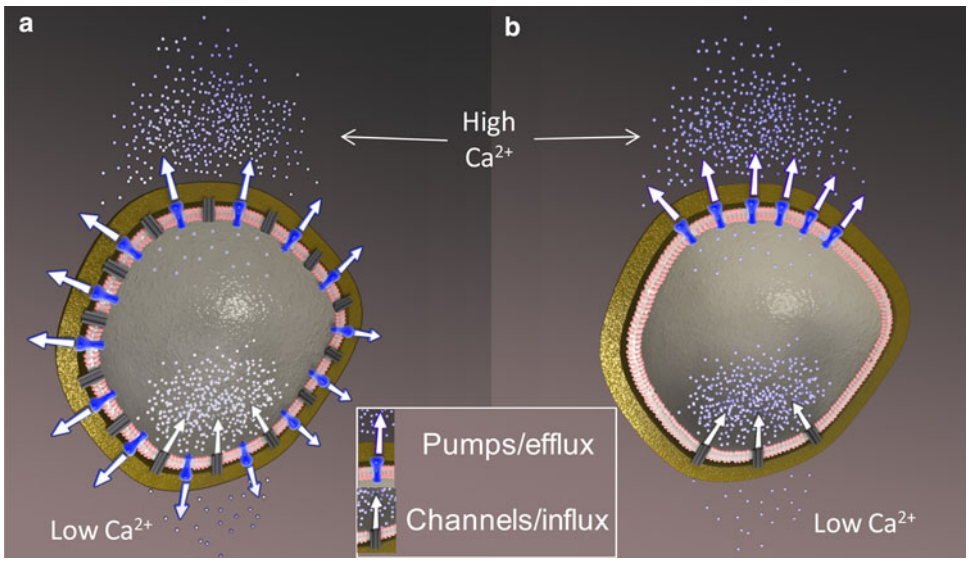

Fig. 5 Two alternative models of the role of calcium pumps and channels in the transcellular current. In the localized activation hypothesis (**a**), membrane calcium pumps and mechanosensitive calcium ion channels are all evenly distributed in the membrane of the cell, and activation of the ion channels at the bottom of the cell allows calcium to move into the cell. This influx would supersede the efflux driven by calcium pumps in this region only. The membrane redistribution hypothesis (**b**) requires gravity-driven movement of channels and pumps to the bottom and top of the cell, respectively. In this model the calcium current might promote cell polarity but may not itself be the primary mechanism of gravity sensing

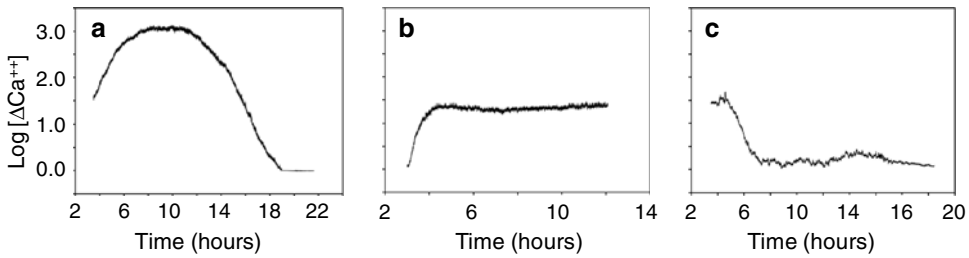

Fig. 6 Representative data depicting the localized top and bottom calcium concentration differential associated with cell polarity development in untreated or drug-treated *C. richardii* spores during germination. The top–bottom electrode pair was measured continuously in DEDC mode for the indicated time. The differential signal was recorded once every 1.75 s. It took approximately 3 h to load and configure the device after illumination. **a** This curve is representative of the pattern exhibited by untreated spores held in fixed orientation. The peak calcium differential was between 2 and 3 mM and occurred between hour 8 and 12 of the germination process. This experiment was replicated 7 times with similar results. **b** Representative effects of the calcium-channel blocker nifedipine on the localized top/bottom calcium concentration differential. Nifedipine was added to the spores at 4 h. Similar results were achieved in 2 additional replicates. **c** Representative effects of the calcium pump blocker eosin Y on localized top/bottom calcium concentration differential. Eosin Y was added to the spores at 5 h. Similar results were obtained in 2 additional replicate experiments

Nifedipine treatment resulted in an attenuated differential current that reached a plateau at a much lower ratio than observed in untreated spores (Fig. 6). In the previous electrode studies, 100 μM nifedipine dramatically reduced (~40-fold) the efflux of calcium from the top of the spore as well as the side (~15-fold less) (Chatterjee et al. 2000). As measured by the CEL-C system, nifedipine reduced the trans-cellular calcium current by 97%. The residual 3% of the trans-cellular calcium current measured may reflect remaining channel, and associated pump, activity.

In untreated spores (Fig. 6a), the extracellular calcium concentration at the top of the cell is approximately 1,000-fold higher ($\Delta[Ca^{+}] = 10^{3}$, approximately 1 mM at the top and 1 μM at the bottom) than the bottom, whereas in the nifedipine-treated spores the differential was only 25-fold higher ($\Delta[Ca^{+}] = 10^{1.4}$). This 97% reduction in the trans-cellular current is comparable to that measured by SRIS method (Chatterjee et al. 2000). The nifedipine treatment also randomized the direction of rhizoid emergence; i.e., it blocked the ability of the cells to establish a downward polarity in response to gravity. The fact that nifedipine can impact axis alignment without completely removing the calcium flux implies that there is a threshold level of stimulus required to induce gravity-directed polarity.

Neither nifedipine nor the pump inhibitor used in these experiments had an inherent impact on CEL-C calcium electrode readings or functionality (Fig. 6).

Calcium extrusion rates are clearly linked to Ca^{2+}-ATPase activity (Luoni et al. 2000; Rasi-Caldogno et al. 1987), and these rates play a key role in adjusting intracellular calcium concentrations (Kordyum 2003). A lower influx of calcium into cells would be expected to induce a lower efflux, a pattern seen here in the 97% decrease in the overall differential induced by nifedipine.

We used EY to assess the role of plasmalemma calcium pumps in the gravity response. EY is a fluorescein derivative, a class of compounds known to bind to Ca^{2+}-ATPases in an inhibitory manner. De Michelis et al. (1993) demonstrated a 10,000-fold higher specificity of EY for plasma membrane Ca^{2+}-ATPases over H^+-ATPases. A 24 h treatment of spores with EY (2 μM H_2EY) suppressed the trans-cellular calcium current (Fig. 6c), but had no impact on spore gravity perception and response. Exposure to the chemical caused a nearly complete abolishment of the calcium differential between the two recording electrodes. In these treatments, when EY was removed after 24 h, the treatment did not induce statistically significant alterations in spore polarity at any tested concentration, although germination was delayed. Longer treatment with this compound caused significantly reduced rhizoid emergence, so was not used. If endomembrane Ca-ATPases, for which EY has lower affinity (De Michelis et al. 1993), are still functional, spores treated with EY may still have the ability to remove calcium from the cytosol.

Although EY did not alter the ability of gravity to direct cell polarity, nifedipine did. This finding demonstrated that the general disruption of the calcium current does not impact spore polar development, while specific inhibition of calcium entry into cells, as demonstrated by the treatment with nifedipine (Fig. 6b), can hinder the gravity-directed polar development.

As yet, the identity of the channels and pumps involved in gravity-regulated polarity of *C. richardii* spores are unknown. However, a translated query BLAST search (blastx) against *C. richardii* EST sequences identified one positive match for a possible Ca^{2+}-ATPase, which we refer to as CrACA1 (AY333123). The full-length gene sequence, obtained from a chromosome walking technique, matches sequences for plasma membrane Ca^{2+}-ATPases in *Oryza sativa*, *Arabidopsis thaliana*, *Medicago truncatula*, and *Glycine max*. Quantitative real-time RT-PCR assay indicates that CrACA1 expression is maintained in a time frame that correlates with the profile of the trans-cellular calcium current (Supplemental Fig. 3). Further tests are needed to determine if this pump is the principal Ca^{2+}-ATPase driving the efflux of calcium from the top of graviresponding spore cells.

The data presented do not predict where the gravity sensor is. In plants, nifedipine is a channel blocker of both internal membranes (Vallée et al. 1997) and the plasma membrane (Demidchik and Tester 2002). In principle, a gravity sensor anywhere in the cell whose activation resulted in shutting down calcium channels throughout the cell could explain the results shown. Nonetheless, the model we propose (Fig. 5a) gives the specific example of a stretch-activated channel in the plasma membrane, because those localized at the plasma membrane are better characterized in the literature, and because some stretch-activated plasma membrane channels are blocked by nifedipine (Ryder and Duncan 2001).

We postulate that the gravity-directed calcium current reported here is regulated primarily by the activation of calcium channels at the bottom of the spore. How a change in the *g*-force vector would activate these channels is not clear, but one speculative model would propose, in accord with the tensegrity model of Ingber (1999) and the recent results of Yang et al. (2008) that gravity can deform the cytoskeleton sufficiently to alter the hinge motion of proteins, including, we propose, mechanosensitive calcium channels. These calcium channels would be located on the plasma membrane or in organelles all around the spore periphery, but only active on the lower half of the spore with respect to the gravitational field. Such a mechanosensitive channel would have a threshold force necessary for activation. Assuming the cytoskeleton-linked mass of the cytoplasm and organelles would shift downward under the force of gravity, this compression force would be highest at the bottom-most point of the spore, where these channels would have a higher probability of activation. The compression force impacting mechanosensitive channels would decrease with distance from the bottom of the cell, and thus fewer channels would be activated.

In principle, the mechanosensitive channels could be in organelles near the spore bottom, and the release of calcium from these organelles could then activate plasma membrane-localized channels to allow entry of calcium from the ECM. More simply, these channels could be on the plasma membrane, as depicted in the model. Because channels have on the order 10^3 times the activity of pumps, the entry of calcium near the bottom of the spore could counter the effect of any calcium pumps that were active. The export of calcium from all surfaces of the cell membrane, with only localized influx at the bottom would create a standing cytoplasmic [Ca^{2+}] gradient, with the elevated calcium concentrations in the basal region of the spore (Fig. 5). This localized cytoplasmic [Ca^{2+}] gradient may be the initial gravity perception event that is transduced into the polarization events of nuclear migration, polar cell division, and direction of primary rhizoid growth.

The role of calcium in signaling, polarity, and mechanosensing in plants is well established (see reviews Braam 2005; Hepler 2005; Hetherington and Brownlee 2004; Sanders et al. 1999). In algae, *Fucus* and *Pelvetia* zygotes are two well studied systems where polar calcium currents are known to precede cell polarity in single cells (Leonetti et al. 2004; Robinson and Jaffe 1975). The recent review by Messerli and Robinson (2007) discusses studies that have characterized the role of SA channels in the tip growth of four different organisms. Tip growth in pollen tubes is dependent on localized calcium influx to establish polarity (Holdaway Clarke et al. 1997), and SA calcium and potassium channels localized in the plasma membrane have been well characterized in protoplasts and pollen grains of *Lilium longiflorum* (Dutta and Robinson 2004).

Although the *Ceratopteris* cellular events documented here happen many hours before the spore rhizoid will commence its tip growth, the underlying cellular mechanisms that control this growth may be interconnected with the establishment of the spore's polarity. There are notable similarities between the polarized growth of fern rhizoids and the tip growth of pollen tubes (Bushart and Roux 2007), and the involvement of SA calcium channels in establishing polarity may prove to be an evolutionarily conserved feature, as it appears to be in the process of tip growing. Only recently have any specific molecular markers been identified in the development of cell polarity that is guided by bio-electric mechanisms; e.g., in ion current-driven wound healing in animal cells (Zhao et al. 2006).

Our data are consistent with the model that mechanical regulation of a calcium transport channel is important for initiating and controlling the gravity-directed calcium current that directs polar development in a single plant cell. Further tests of the model proposed here will include identifying the gravity-regulated channels and the pumps that drive the current and assaying what, if any, post-translational changes are induced in these transporters in response to altered gravity. Investigation of other components that may modify internal calcium concentrations, such as endomembrane Ca^{2+}-ATPases, should also allow us to further refine our model. Because calcium signaling is a universal regulatory feature of cell polarization in both plants and animals (Pickard and Fujiki 2005; Rasi-Caldogno et al. 1987) the CEL-C device, which is adaptable to many cell types, could be used on future C-9 flights to test other cells which respond dramatically to the micro-*g* environment (Kordyum 2003; Luoni et al. 2000). Such tests would reveal whether the transport phenomena reported here are evolutionarily conserved features of cellular responses to gravity or is unique to only some cell types.

Acknowledgments We thank Guy Thompson and Greg Clark for discussion and suggestions, Alan Shipley at Applicable Electronics for assistance with ion-selective electrode system setup, Harvey Fishman at U. Texas Medical Branch at Galveston for equipment used in microelectrode experiments and Bill McLamb for help in the parabolic flight experiments. This work was supported by NASA grants to S.J.R. (NAG10-295), S.C.S. (NGT5-50371), and D.M.P.

References

Adkins EM, Samuvel DJ, Fog JU, Eriksen J, Jayanthi LD, Vaegter CB, Ramamoorthy S, Gether U (2007) Membrane mobility and microdomain association of the dopamine transporter studied with fluorescence correlation spectroscopy and fluorescence recovery after photobleaching. Biochemistry 46:10484–10497

Braam J (2005) In touch: plant responses to mechanical stimuli. New Phytol 165:373–389

Bushart TJ, Roux SJ (2007) Conserved features of germination and polarized cell growth: a few insights from a pollen-fern spore comparison. Ann Bot 99:9–17

Carlsson AE, Shah AD, Elking D, Karpova TS, Cooper JA (2002) Quantitative analysis of actin patch movement in yeast. Biophys J 82:2333–2343

Chatterjee A, Porterfield DM, Smith PS, Roux SJ (2000) Gravity-directed calcium current in germinating spores of *Ceratopteris richardii*. Planta 210:607–610

Chen Y, Simasko SM, Niggel J, Sigurdson WJ, Sachs F (1996) Ca^{2+} uptake in GH3 cells during hypotonic swelling: the sensory role of stretch-activated ion channels. Am J Physiol 270:C1790–C1798

De Michelis MI, Carnelli A, Rasi-Caldogno F (1993) The Ca^{2+} pump of the plasma-membrane of *Arabidopsis thaliana*—characteristics and sensitivity to fluorescein derivatives. Bot Acta 106:20–25

Demidchik V, Tester M (2002) Sodium fluxes through nonselective cation channels in the plasma membrane of protoplasts from Arabidopsis roots. Plant Physiol 128:379–387

Ding JP, Pickard BG (1993) Mechanosensory calcium-selective cation channels in epidermal cells. Plant J 3:83–110

Dutta R, Robinson KR (2004) Identification and characterization of stretch-activated ion channels in pollen protoplasts. Plant Physiol 135:1398–1406

Edwards ES, Roux SJ (1994) Limited period of graviresponsiveness in germinating spores of *Ceratopteris richardii*. Planta 195: 150–152

Edwards ES, Roux SJ (1998) Influence of gravity and light on the developmental polarity of *Ceratopteris richardii* fern spores. Planta 205:553–560

Haswell ES, Meyerowitz EM (2006) MscS-like proteins control plastid size and shape in *Arabidopsis thaliana*. Curr Biol 16:1–11

Hepler PK (2005) Calcium: a central regulator of plant growth and development. Plant Cell 17:2142–2155

Hetherington AM, Brownlee C (2004) The generation of Ca^{2+} signals in plants. Ann Rev Plant Biol 55:401–427

Holdaway Clarke TL, Feijo JA, Hackett GR, Kunkel JG, Hepler PK (1997) Pollen tube growth and the intracellular cytosolic calcium gradient oscillate in phase while extracellular calcium influx is delayed. Plant Cell 9:1999–2010

Ingber D (1999) How cells (might) sense microgravity. FASEB J 13:S3–S15

Kordyum EL (2003) Calcium signaling in plant cells in altered gravity. Adv Space Res 32:1621–1630

Kucik DF, Elson EL, Sheetz MP (1989) Forward transport of glycoproteins on leading lamellipodia in locomoting cells. Nature 340:315–317

Leonetti M, Dubois-Violette E, Homble F (2004) Pattern formation of stationary transcellular ionic currents in *Fucus*. Proc Natl Acad Sci 101:10243–10248

Lin W, Laitko U, Juranka PF, Morris CE (2007) Dual stretch responses of mHCN2 pacemaker channels: accelerated activation, accelerated deactivation. Biophys J 92:1559–1572

Luoni L, Bonza MC, Michelis MID (2000) H^+/Ca^{2+} exchange driven by the plasma membrane Ca^{2+}-ATPase of *Arabidopsis thaliana* reconstituted in proteoliposomes after calmodulin-affinity purification. FEBS Lett 482:225–230

McBride DW, Hamill OP (1993) Pressure-clamp technique for measurement of the relaxation kinetics of mechanosensitive channels. Trends Neurosci 16:341–345

Messerli MA, Robinson KR (2007) MS channels in tip-growing systems. Curr Top Membr 58:393–412

Nakagawa Y, Katagiri T, Shinozaki K, Qi Z, Tatsumi H, Furuichi T, Kishigami A, Sokabe M, Kojima I, Sato S, Kato T, Tabata S, Iida K, Terashima A, Nakano M, Ikeda M, Yamanaka T, Iida H (2007) Arabidopsis plasma membrane protein crucial for Ca^{2+} influx and touch sensing in roots. Proc Natl Acad Sci 104: 3639–3644

Nakayama Y, Fujiu K, Sokabe M, Yoshimura K (2007) Molecular and electrophysiological characterization of a mechanosensitive channel expressed in the chloroplasts of *Chlamydomonas*. Proc Natl Acad Sci 104:5883–5888

Pelham RJ, Chang F (2001) Role of actin polymerization and actin cables in actin-patch movement in *Schizosaccharomyces pombe*. Nat Cell Biol 3:235–244

Pickard BG, Fujiki M (2005) Ca^{2+} pulsation in BY-2 cells and evidence for control of mechanosensory Ca^{2+}-selective channels by the plasmalemmal reticulum. Funct Plant Biol 32:863–879

Rasi-Caldogno F, Pugliarello MC, De Michelis MI (1987) The Ca^{2+}-transport ATPase of plant plasma membrane catalyzes a nH^+/Ca^{2+} exchange. Plant Physiol 83:994–1000

Robinson KR, Jaffe LF (1975) Polarizing fucoid eggs drive a calcium current through themselves. Science 187:70–72

Rokkam M, Chatni MR, Haque Au, De Carlo AR, Robinson BF, Irazoqui PP, Porterfield DM (2007) High-density data acquisition system and signal preprocessor for interfacing with microelectromechanical system-based biosensor arrays. Rev Sci Instrum 78:044303–044309

Roux SJ, Chatterjee A, Hillier S, Cannon T (2003) Early development of fern gametophytes in microgravity. Adv Space Res 31:215–220

Ryder KD, Duncan RL (2001) Parathyroid hormone enhances fluid shear-induced $[Ca^{2+}](i)$ signaling in osteoblastic cells through activation of mechanosensitive and voltage- sensitive Ca^{2+} channels. J Bone Miner Res 16:240–248

Sachs F (2002) Retaining your identity under stress. Nat Struct Mol Biol 9:636–637

Sanders D, Brownlee C, Harper JF (1999) Communicating with Calcium. Plant Cell 11:691–706

Syntichaki P, Tavernarakis N (2004) Genetic models of mechanotransduction: the nematode *Caenorhabditis elegans*. Physiol Rev 84:1097–1153

ul Haque A, Rokkam M, De Carlo AR, Wereley ST, Roux SJ, Irazoqui PP, Porterfield DM (2007) A MEMS fabricated cell electrophysiology biochip for in silico calcium measurements. Sens Actuators B: Chem 123:391–399

Vallée N, Brière C, Petitprez M, Barthou H, Souvré A, Alibert G (1997) Studies on ion channel antagonist-binding sites in sunflower protoplasts. FEBS Lett 411:115–118

Waddle JA, Karpova TS, Waterston RH, Cooper JA (1996) Movement of cortical actin patches in yeast. J Cell Biol 132:861–870

Yang C, Wei D, Zhuang FY (2008) The force induced by organelles' weight in the microfilament is in the range of 0.1–1 pN. Acta Astronaut 63:923–928

Zhao M, Song B, Pu J, Wada T, Reid B, Tai G, Wang F, Guo A, Walczysko P, Gu Y, Sasaki T, Suzuki A, Forrester JV, Bourne HR, Devreotes PN, McCaig CD, Penninger JM (2006) Electrical signals control wound healing through phosphatidylinositol-3-OH kinase-[gamma] and PTEN. Nature 442:457–460