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The Cytosolic Ca^{2+} Concentration Gradient of *Sinapis alba* Root Hairs as Revealed by Ca^{2+}-Selective Microelectrode Tests and Fura-Dextran Ratio Imaging[1]

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Using Ca^{2+}-selective microelectrodes and fura 2-dextran ratio imaging, the cytosolic free $[Ca^{2+}]$ was measured in *Sinapis alba* root hair cells. Both methods yielded comparable results, i.e. values between 158 to 251 nM for the basal $[Ca^{2+}]$ of the cells and an elevated $[Ca^{2+}]$ of 446 to 707 nM in the tip region. The zone of elevated $[Ca^{2+}]$ reaches 40 to 60 μm into the cell and is congruent with the region of inwardly directed Ca^{2+} net currents measured with an external Ca^{2+}-selective vibrating electrode. The channel-blocker La^{3+} eliminates these currents, stops growth, and almost completely eliminates the cytosolic $[Ca^{2+}]$ gradient without affecting the basal level of the ion. Growth is also inhibited by pressure-injected dibromo-1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, which causes a decrease in the $[Ca^{2+}]$ in the tip in a concentration-dependent manner. Indole-3-acetic acid, used as a model stimulus, decreases cytosolic free $[Ca^{2+}]$ by 0.2 to 0.3 pCa units in the tip, but only by about 0.1 pCa unit in the shank. Nongrowing root hairs may or may not display a $[Ca^{2+}]$ gradient, but still reversibly respond to external stimuli such as La^{3+}, Ca^{2+}, or indole-3-acetic acid with changes in cytosolic free $[Ca^{2+}]$. During short time periods, dicyclohexylcarbodiimide inhibition of the plasma membrane H^+-ATPase, which stops growth, does not abolish the $[Ca^{2+}]$ gradient, nor does it change significantly the basal $[Ca^{2+}]$ level. We conclude that the cytosolic $[Ca^{2+}]$ gradient and an elevated $[Ca^{2+}]$ in the tip, as in other tip-growing cells, is essential for tip growth in root hairs; however, its presence does not indicate growth under all circumstances. We argue that with respect to Ca^{2+}, tip growth regulation and responses to external signals may not interfere with each other. Finally, we suggest that the combination of the methods applied adds considerably to our understanding of the role of cytosolic free $[Ca^{2+}]$ in signal transduction and cellular growth.

Tip-growing cells develop gradients in cytosolic free $[Ca^{2+}]$. Using Ca^{2+} dyes or Ca^{2+}-selective microelectrodes, this has been demonstrated for pollen tubes (Jaffe et al., 1975; Obermeyer and Weissenseel, 1991; Rathore et al., 1991; Miller et al., 1992; Pierson et al., 1994, 1996), fungal hyphae (Jackson and Heath, 1993), rhizoid cells of algae (Brownlee and Wood, 1986; Taylor et al., 1996), and root hair cells (Clarkson et al., 1988; Tretyn et al., 1991; Herrmann and Felle, 1995). Since the $[Ca^{2+}]$ gradient is present only in growing cells, the generalization has emerged that the gradient is a prerequisite for tip growth, in which it could participate in controlling the structure and activity of the cytoskeleton and in the docking and fusion of vesicles necessary for cell extension. Despite this consensus, we were interested in determining whether tip growth of root hairs follows the pattern of other tip-growing cells. Recently, it was demonstrated that *Sinapis alba* root hairs respond to changes in external $[Ca^{2+}]$ with transient growth changes rather than with altered steady-state growth (Herrmann and Felle, 1995). With respect to cytoplasmic pH we did not find the substantial gradient that was reported for rhizoid cells of *Pelvetia* (Gibbon and Kropf, 1994), but observed that a tightly regulated cytoplasmic pH is apparently rather critical for tip growth. Since the cytosolic free $[Ca^{2+}]$ and pH may be interrelated to some extent (Felle, 1988), the absence of a pH gradient within a $[Ca^{2+}]$ gradient suggests a high degree of regulation, which may play an important role in intracellular signal transduction. To answer these questions we measured the cytosolic free $[Ca^{2+}]$ in root hairs of *S. alba* and tested different agents for their ability to change the $[Ca^{2+}]$ gradient and, when applicable, the cytoplasmic pH at the tip and approximately 100 μm behind it.

To measure the cytosolic $[Ca^{2+}]$ gradient accurately in root hairs of *S. alba* both quantitatively and spatially, we applied two different techniques: Ca^{2+}-selective microelectrodes to obtain continuous responses, and ratio imaging using fura 2-dextran to determine the spatial resolution of the $[Ca^{2+}]$ within the root hairs. By testing the effect of several different growth-perturbing and Ca-specific agents on the root hairs, we have attempted to gain further information about the maintenance of the $[Ca^{2+}]$ gradient and its relevance for tip growth.

In this paper we demonstrate that growing root hairs indeed possess a cytosolic free $[Ca^{2+}]$ gradient, and that the rate of root hair elongation is correlated with the free cytosolic $[Ca^{2+}]$ in the tip. However, nongrowing root hair cells, which can respond in a physiologically relevant man-

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Abbreviations: BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; DCCD, dicyclohexylcarbodiimide.

ner to external stimuli, may still possess a $[Ca^{2+}]$ gradient. These observations add further complexity to the role of cytosolic $[Ca^{2+}]$ gradients in tip growth, showing that growth may require a gradient, but that the gradient by itself does not dictate growth.

MATERIALS AND METHODS

General Conditions

Seedlings of *Sinapis alba* were grown for 48 h at 23°C in a moist chamber. Since the root hairs usually collapse upon transfer into the medium, the roots were incubated in the basic test solution for approximately 3 h. New root hairs began to emerge after about 1 h and grew at a maximum rate of 1 to 2 $\mu m\ min^{-1}$, reaching a length of 150 to 250 μm within 3 to 4 h, at which time they were used for tests. Root hairs of that size are already highly vacuolated, with tips of rapidly changing cytoplasmic content. From our experience, this is a sign of healthy, growing *S. alba* root hairs. Excised roots or whole plantlets were mounted in a Plexiglas cuvette that was constantly perfused with the test medium. Unless stated otherwise, this comprised 0.1 mM KCl, 0.1 mM NaCl, and 0.1 mM $CaCl_2$. By mixing 5 mM Mes and 5 mM Tris the pH was adjusted to 7.2 or to the values given in the figure legends. DCCD was added from an ethanolic solution; the ethanolic concentration never exceeded 0.1%. IAA was added from an aqueous stock solution that was prepared weekly and maintained in the dark at 4°C.

Electrophysiology and Ion-Selective Microelectrodes

The electrical setup for the impalement of the root hairs, and both the fabrication and basic application of the ion-selective microelectrodes, have been described in detail previously (Felle and Bertl, 1986; Felle, 1989). The test chamber was open on both sides for the horizontal approach of two or more separate electrodes, which was necessary for the simultaneous measurements of cytosolic pH, free $[Ca^{2+}]$, and membrane potential. Since ion-selective microelectrodes pick up a mixed signal that consists of both the membrane potential difference and the free ion concentration, a separate microelectrode that measures the membrane potential had to be inserted into the cytoplasm of the same cell. These electrodes were connected with a high-impedance differential amplifier (FD 223 or Duo 773, World Precision Instruments, Sarasota, FL), which simultaneously measured and then subtracted both signals to obtain the net signal of the ion under investigation. Due to the different response times of the two electrodes (the ion-selective being slower), this procedure may produce artifactual shifts of the difference trace in instances of rapid changes in membrane potential. Since the ion-selective electrodes may alter their physicochemical properties during and after their first impalement, data were selected from electrodes that displayed satisfactory calibration after the respective tests.

Injections

Pressure injections were carried out using a home-built device. A micrometer syringe (Gilmont Instruments, Great Neck, NY) was tightly interconnected with the micropipette holder through Teflon tubing. This holder was mounted on a manipulator (Leitz, Wetzlar, Germany). Dry-beveled micropipettes were back-filled with the injectate. Degassed mineral oil, which filled the entire pressure device, was placed directly behind the injectate. For beveling, a 0.3-μm abrasive film (A. Thomas Co., Philadelphia, PA) was fixed on the rotating plate of a commercial beveler (World Precision Instruments). Since dry beveling cannot be controlled directly by monitoring the electrical behavior of the pipette, subsequent micropipette production was standardized by optimizing the angle of the pipette to the plate and the time of beveling. During the entire injection procedure the membrane potential was continuously monitored with a separate electrode inserted into the same cell. For controlled injections, the shift of the interface between injectate and oil was monitored and measured microscopically. The injected volume was then calculated from the respective pipette dimensions. The injected amounts were between 0.05 and 0.3 pL.

Upon injection of the dye, the cytoplasmic streaming will stop in root hairs shorter than about 100 μm. Provided that the cell injury through the injecting pipette did not cause leakage and that the dye was not injected too rapidly, root hairs of 150 μm or longer remained with their usual growth pattern after an initial deceleration of cytoplasmic streaming and tip growth. Cells that did not resume tip growth within minutes were not investigated further.

Growing root hairs of at least 200 μm in length were loaded with fura 2-dextran (20 mg mL^{-1} in 100 mM KCl; Molecular Probes, Eugene, OR) by pressure injection, as described by Miller et al. (1992).

Fluorescence ratio-imaging microscopy (Grynkiewicz et al., 1985; Bolsover and Silver, 1991; Callaham and Hepler, 1991; Bolsover et al., 1993) was applied on a modified microscope (Diaphot, Nikon, Tokyo, Japan) equipped with a 40 × 1.3 numerical aperture fluorescence objective (Nikon).

Excitation was stimulated by light from a mercury arc lamp (HBO 100W/2, Osram, Berlin, Germany) operated at constant current on a purpose-built power supply. The excitation light was filtered by a 340-nm and a 360-nm interference filter combination (10 nm FWHM, Corion Corp., Holliston, MA). The filters were mounted on a slider, and thus were sequentially introduced in the excitation path with an approximately 50-ms delay between them. To provide a balance between the transmitted light beams, a 0.15-optical density (70.79% transmittance) neutral filter was paired with the 360-nm interference filter. The fluorescence emission cassette in the microscope contained a 380-nm dichroic reflector and a 450-nm long-pass glass emission filter (Omega Optical, Brattleboro, VT). The emission was imaged separately at the two excitatory wavelengths by a thermoelectrically cooled (−45°C), charge-coupled device camera (Photometrics, Tucson, AZ; type AT200 with a Thomson TH7883A CCD chip, 500-kHz

data transfer rate) installed in an 80486DX-based PC compatible computer. Ratio images were computed after subtraction of background images taken immediately adjacent to the cell. The final images were displayed in 256 colors with the condition that when 360 nm (cell) minus 360 nm (background) was less than a preselected threshold value, then the ratio was set to zero. The threshold value was chosen as the minimum pixel intensity value with the cytoplasm of the Ca^{2+}-independent image. When applied to the whole image, regions outside the cell will thus appear black. The absolute intracellular Ca^{2+} concentration was determined according to methods published previously (Grynkiewicz et al., 1985; Miller et al., 1992; Pierson et al., 1994). A calibration curve was established in which the ratio numbers were correlated with standardized Ca^{2+} concentrations obtained by imaging fura 2-dextran (40 μg mL^{-1}) in 2.5 mM Hepes, pH 7.0, 100 mM KCl, 60% (w/w) Suc, and 2.5 mM BAPTA buffer, with the latter being either Ca^{2+}-free, Ca^{2+}-bound, or an equal mixture of both. The same exposure time was used for the calibrations that was used for the cell images. Numerical data were collected digitally from a charged coupled device camera.

Vibrating-Probe Experiments

All measurements were carried out at the National Vibrating Probe Facility of the Marine Biological Laboratories (Woods Hole, MA). The construction and use of the Ca^{2+}-selective electrode has been described by Kühtreiber and Jaffe (1990). Medium-adapted (3 h) seedlings of *S. alba* were fixed with candle wax to the bottom of a commercial plastic Petri dish. The probe was vibrated with a frequency of 0.3 Hz at the desired angle to a distance of 2 to 5 μm from the respective root hair surface, and with an excursion (moving the electrode forward and backward) of 10 μm. Reference was obtained by moving the electrode approximately 500 μm from the nearest root hair.

RESULTS

Cytosolic $[Ca^{2+}]$ Gradient of the Growing Root Hair

It has been shown previously that root hairs of *S. alba* grow 1 to 2 μm min^{-1} at room temperature (Herrmann and Felle, 1995). Our measurements with Ca^{2+}-selective microelectrodes showed that these cells develop a zone of elevated $[Ca^{2+}]$ of 446 to 707 nM (mean 602 ± 135 nM; $n = 58$) at the tip, compared with a basal level of 158 to 251 nM (mean 188 ± 52 nM; $n = 12$) in the shank of the hair, about 100 μm behind the tip. The basal free concentration agrees closely with that commonly found in the cytoplasm of plant cells (Hepler and Wayne, 1985; Felle, 1989; Bush, 1993).

Using pressure-injected fura 2-dextran, ratio imaging confirmed these values, revealing a relatively shallow $[Ca^{2+}]$ gradient that reached 40 to 60 μm from the tip into the cell (Fig. 1a). Figure 1, a through d, shows the $[Ca^{2+}]$ gradient representative of an undisturbed, growing *S. alba* root hair cell, with cytoplasmic streaming indicated by the changing position of the vacuole. The cells react to a 100-fold increase in external $[Ca^{2+}]$ from 0.1 to 10 mM with a clear increase in cytosolic $[Ca^{2+}]$ (Fig. 1e), and to the reduction of external $[Ca^{2+}]$ to 0.01 mM with an immediate decrease in cytosolic free $[Ca^{2+}]$ (Fig. 1, f and g). These findings confirm earlier measurements obtained with Ca^{2+}-selective microelectrodes (Felle et al., 1992; Herrmann and Felle, 1995), in which it was shown that sudden changes in external $[Ca^{2+}]$ cause only transient shifts in both growth and cytosolic free $[Ca^{2+}]$ (i.e. an increase in external $[Ca^{2+}]$ would increase cytosolic $[Ca^{2+}]$ and stimulate growth, whereas a sudden decrease of external $[Ca^{2+}]$ would have the opposite effect).

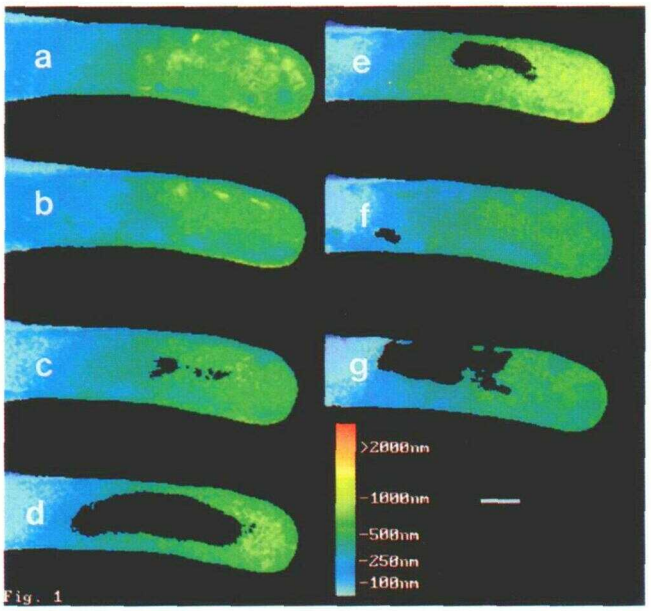

Figure 1. Cytosolic gradient of free $[Ca^{2+}]$ in a growing (1.2 μm/min) *S. alba* root hair as detected through ratio imaging. The first four images (a–d) are taken at 2-min intervals without changing conditions. After taking the fourth image the external $[Ca^{2+}]$ was increased 100-fold from 0.1 to 10 mM, and an image was taken 3 min later (e). Images were also taken at 3-min intervals following the reduction of the external $[Ca^{2+}]$ to 0.01 mM (f and g). The color scale refers directly to cytosolic free $[Ca^{2+}]$ as determined by in vitro calibration. Tips were aligned. Bar = 10 μm. Six similar cells were examined.

The Auxin Effect

Auxin (IAA) rapidly depolarizes the root hairs of *S. alba* and reduces the tip growth by about 70%. As demonstrated in Figure 2A, both responses are transient; i.e. after about 45 min membrane potential and tip growth are almost back to normal. The observation that the response to auxin at the tip is not different from the response in the shank of the same root hair is not really surprising because cortex cells react in a similar way to auxin (data not shown).

Using Ca^{2+}-selective microelectrodes it has been demonstrated that IAA at submicromolar concentrations decreases the cytosolic free $[Ca^{2+}]$ in *S. alba* root hairs (Tretyn et al., 1991). As shown in Figure 2B, this IAA-induced drop in cytosolic free $[Ca^{2+}]$ occurs along the length of the entire

root hair, but more so in the tip than in the shank. As tip growth slowly recovers, within about 45 min the cytosolic $[Ca^{2+}]$ in both the tip and the shank spontaneously (i.e. in the presence of IAA) returns to about the levels measured prior to the IAA treatment.

Cytosolic pH is different. pH values between 7.17 and 7.52 (mean 7.35 ± 0.07; $n = 21$) in the shank and between 7.06 and 7.38 (mean 7.24 ± 0.04; $n = 45$) in the tip suggest at most a rather shallow pH gradient. As Figure 2C shows, the pH responses to auxin at the tip and in the shank are statistically almost equal in pH units.

Ratio images taken from the tip region 2 min after the addition of 10^{-7} M IAA (Fig. 3) confirm the decrease in cytosolic $[Ca^{2+}]$ from 6.12 to 6.45 pCa (i.e. 758–355 nM) measured with the Ca^{2+}-selective microelectrode (Fig. 2B). The $[Ca^{2+}]$ decrease about 100 μm behind the tip is lower in magnitude, which is also in full agreement with the kinetics obtained with Ca^{2+}-selective microelectrodes. Measurements of the inwardly directed net Ca^{2+} currents (Fig. 3), carried out with an externally vibrating, ion-specific microelectrode (Kühtreiber et al., 1990), show that these currents are confined to the tip and appear congruent with the expansion of the internal gradient of cytosolic free $[Ca^{2+}]$. IAA reduces these currents (Fig. 3) and slows tip growth by roughly 70% (Fig. 2A; Table I).

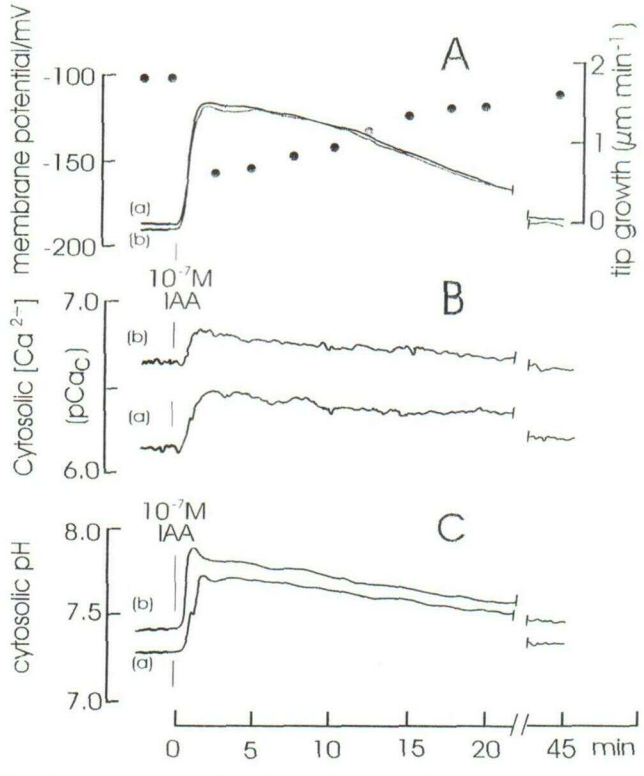

Figure 2. The effect of 10^{-7} M IAA on membrane potential and tip growth (A), cytosolic free $[Ca^{2+}]$ (B), and cytosolic pH (C) in *S. alba* root hairs measured with ion-selective microelectrodes about 5 μm behind the tip (trace a) and about 100 μm behind the tip (trace b). Each of these traces is representative of single kinetics of four to six equivalent measurements.

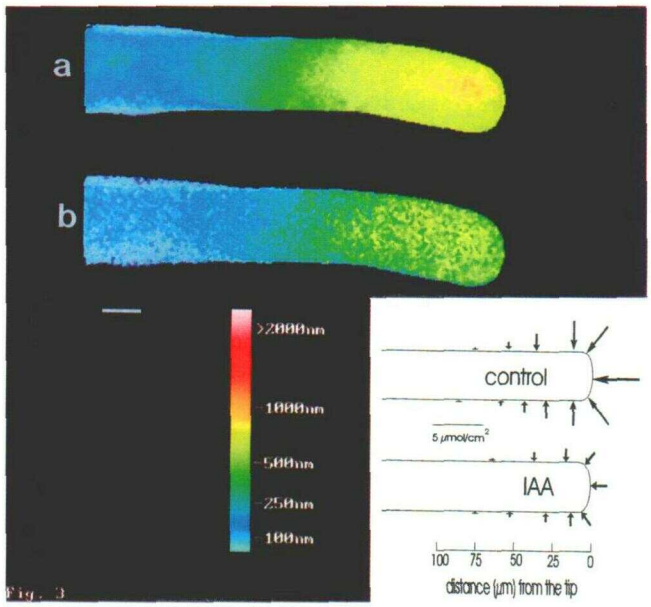

Figure 3. Cytosolic free $[Ca^{2+}]$ gradient of a growing *S. alba* root hair (compare Table I; $n = 8$). Ratio images were taken before (a) and 3 min after the addition of 10^{-7} M IAA (b). As in Figure 1, tips were aligned; the color scale refers directly to the cytosolic free $[Ca^{2+}]$, as determined by in vitro calibration. Bar = 10 μm. Inset: Arrows, inwardly directed Ca^{2+} net currents as measured with the Ca^{2+}-selective vibrating probe (Kühtreiber et al., 1990) before (control) and after the addition of 10^{-7} M IAA (IAA). Note that the arrows only reach about as far as the $[Ca^{2+}]$ gradient. External pH was 6.1.

The Cytosolic $[Ca^{2+}]$ in the Nongrowing Root Hair

Nongrowing root hairs may or may not display a $[Ca^{2+}]$ gradient, depending on how the growth was inhibited (see below). Those hairs lacking a gradient and exhibiting a uniform Ca^{2+} concentration throughout the entire cell still perform slow cytoplasmic streaming, as was observed during the measurements, but that was also indicated by the changing position of the vacuoles (Fig. 4). The cells also respond to different treatments as follows: (a) when the external $[Ca^{2+}]$ was increased from 0.1 to 10 mM, the cytosolic $[Ca^{2+}]$ increased uniformly within the root hair (Fig. 4, b–d), whereas in growing cells the increase was confined to the apical region of the hair (Fig. 1e); (b) the cells responded to IAA in the usual manner, i.e. with a transient decrease in cytosolic $[Ca^{2+}]$ (Fig. 4, e–h); and (c) the cells responded to La^{3+} with a further decrease in $[Ca^{2+}]$ (Fig. 4, i–l) and only a slight recovery (Fig. 4m).

Root hair tip growth can be stopped in different ways: by blocking the net Ca^{2+} currents at the tip (e.g. with La^{3+}), by manipulating cytosolic $[Ca^{2+}]$ (e.g. with BAPTA-type buffers or with Ca^{2+}-ionophores), by manipulating cytosolic pH, or by inhibiting the plasma membrane proton ATPase (Herrmann and Felle, 1995). La^{3+}, for instance, by blocking Ca^{2+}-conducting channels at the plasma membrane, eliminates the inwardly directed net Ca^{+} currents at the tip (Table I). Concomitantly, the cytosolic free $[Ca^{2+}]$ drops by about 0.3 pCa units, mainly at the tip, thus virtually eliminating the gradient and creating a constant level of cytosolic free $[Ca^{2+}]$ throughout the root hair (Fig. 5). On the

Table I. *Ca^{2+} net currents into S. alba root hair cells and rates of tip growth*

Tip growth was monitored through microscopic observation, and net Ca^{2+} currents were measured with the vibrating-probe technique (Kühtreiber et al., 1990; Schiefelbein et al., 1992). The electrode was vibrating at 0.3 Hz, 5 μm behind the tip, 2 μm from the cell surface, at an angle of 90° with respect to the cell surface. Data reflect measurements 5 min after adding the respective agents. External pH was 6.1.

Treatment	Ca^{2+} Net Current	Tip Growth	Nos. of Cells Examined
	pmol cm^{-2} s^{-1}	*μm min^{-1}*	
Control	1–4	1–2	13
La^{3+} (30 μM)	0	0	6
IAA (0.1 μM)	0.5–1	0.3–0.4	8

other hand, DCCD, which inhibits growth for energetic reasons during at least the first 25 min, does not appreciably change cytosolic free $[Ca^{2+}]$ either in the tip (Fig. 5) or along the shank of the root hair (data not shown).

It has been demonstrated that BAPTA-type buffers effectively eliminate the cytosolic $[Ca^{2+}]$ gradient in pollen tubes (Pierson et al., 1994). In *S. alba* root hairs we found a similar result, a decrease in elevated $[Ca^{2+}]$ in the tip zone to approximately basal levels (Fig. 6), but only a minimal change in the shank (data not shown). The data from Figure 6 indicate that in those instances in which the $[Ca^{2+}]$ had not decreased considerably, growth was affected only minimally or not at all. However, when $[Ca^{2+}]$ had decreased transiently to basal levels, growth occasionally resumed (four of seven tests) and was accompanied by a slow but more or less complete regeneration of the tip Ca^{2+} gradient. In experiments in which the tip $[Ca^{2+}]$ remained low, growth never resumed.

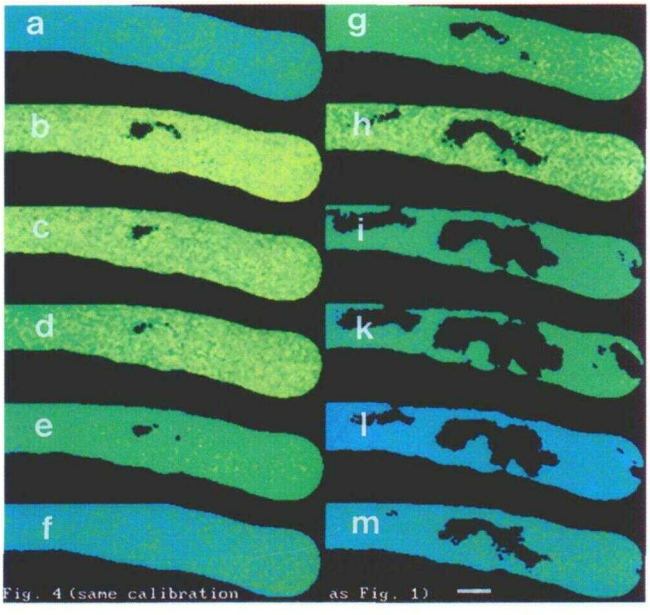

Figure 4. Ratio images showing the cytosolic free $[Ca^{2+}]$ of a nongrowing *S. alba* root hair (n = 6) taken at 0.1 mM external Ca^{2+} (a), after increasing external $[Ca^{2+}]$ to 10 mM (b–d; 3-min intervals each), after adding 10^{-7} M IAA (e–h; 3-min intervals each), and after adding 30 μM $LaCl_3$ (i–m; 3-min intervals each). As in Figure 1, the color scale refers directly to the cytosolic free $[Ca^{2+}]$, as determined by in vitro calibration. Bar = 10 μm. External pH was 6.1.

DISCUSSION

Ca^{2+}-Selective Microelectrodes and Fura 2-Dextran Ratio Imaging Produce Comparable Results

We demonstrate that both ratio imaging and Ca^{2+}-selective microelectrodes yield results that are comparable (Figs. 1–3) and complementary. Although ratio imaging essentially confirms the absolute $[Ca^{2+}]$ values, as reported previously using microelectrodes (Felle et al., 1992), it also provides the spatial resolution of the cytosolic $[Ca^{2+}]$ gradient. Moreover, these values agree well with the region of the inwardly directed Ca^{2+} net currents as measured by the vibrating-probe technique (Fig. 3, inset). The advantage of the microelectrodes is that they uninterruptedly give information about $[Ca^{2+}]$ changes during the measurement, and also, through continuous monitoring of the membrane potential, provide information about the physiological state of the cell at any time during the experiment. The ratio images further prove that the precise location of the Ca^{2+} microelectrode in the tip region of the root hairs is not critical.

The $[Ca^{2+}]$ Gradient and Tip Growth in *S. alba* Root Hairs

The root hair cells of *S. alba* show an elevated tip $[Ca^{2+}]$, similar to other tip-growing cells such as pollen tubes (Obermeyer and Weissenseel, 1991; Rathore et al., 1991; Pierson et al., 1994, 1996; Malhó et al., 1995), rhizoid cells (Brownlee and Wood, 1986; Brownlee and Pulsford, 1988; Taylor et al., 1996), fungal hyphae (Jackson and Heath,

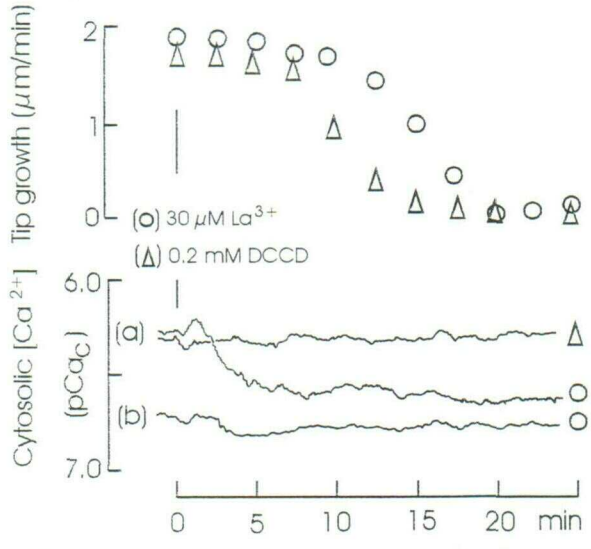

Figure 5. Effect of micromolar concentrations of La^{3+} (□) and DCCD (Δ) on tip growth (measured by microscopic observation), and on cytosolic free $[Ca^{2+}]$, measured both 5 μm (a) and about 100 μm (b) behind the tip of *S. alba* root hairs with Ca^{2+}-selective microelectrodes. Each of these traces is representative of single kinetics of five to seven equivalent measurements.

1993; Levina et al., 1995), and root hairs of other species (H. Felle and P.K. Hepler, unpublished data). There are differences between various tip-growing cells in the magnitude and extent of the $[Ca^{2+}]$ gradient. For instance, the gradient in *S. alba* root hairs is less high and diminishes more gradually compared with pollen tubes in lily (Pierson et al., 1994, 1996) or rhizoids of *Fucus serratus* (Taylor et al., 1996). When indexed according to the cell dimensions, the length of the *S. alba* $[Ca^{2+}]$ gradient is two to three times the diameter of the cell. However, steeper gradients of about one cell diameter, as observed in pollen tubes, are found in root hairs of other species (*Medicago sativa*; H. Felle and P.K. Hepler, unpublished data). Presumably, the significance of the gradient steepness relates to the degree to which a reaction, dependent on the elevated ion concentration, can be spatially defined; therefore, a steep $[Ca^{2+}]$ gradient will spatially confine the Ca^{2+}-dependent processes.

At first sight the data presented here seem to support the general idea that a cytosolic $[Ca^{2+}]$ gradient is a basic requirement for tip growth in *S. alba* root hairs, as it is for other tip-growing cells. As such, treatments that abolish or diminish this gradient should lead to the cessation or at least a severe disturbance of polar growth. This is true for La^{3+}, which strongly diminishes the $[Ca^{2+}]$ gradient from about 0.4 to 0.1 pCa units and completely inhibits growth (Fig. 5; Table I). It also seems to be true for experimental conditions in which dibromo-BAPTA (Fig. 6) and IAA (Fig. 2; Table I) first decrease cytosolic $[Ca^{2+}]$ and inhibit growth (which then revert to normal as the cytosolic $[Ca^{2+}]$ spontaneously increases). However, a closer look reveals that it is the $[Ca^{2+}]$ in the tip and not necessarily the gradient that is primarily changed. For instance, during the IAA treatment the cytosolic $[Ca^{2+}]$ is reduced along the entire cell, diminishing the $[Ca^{2+}]$ only gradually (Figs. 2 and 3); still, the tip growth transiently slows down. These observations are supported by earlier findings that tip growth in *S. alba* root hairs follows changes in external $[Ca^{2+}]$, i.e. a transient increase in cytosolic $[Ca^{2+}]$ entails a likewise transient acceleration of tip growth (Herrmann and Felle, 1995). Only recently, Malhó and Trewavas (1996) demonstrated that pollen tubes of *Agapanthus umbellatus* bent toward higher $[Ca^{2+}]$ and away from decreasing $[Ca^{2+}]$, effects that could be mimicked with localized external gradients of A23187 and $GdCl_3$. It was shown that the tubes bent toward the highest concentration of A23187 and away from $GdCl_3$. Since the A23187 at least temporarily will increase the cytosolic $[Ca^{2+}]$ mainly in the tip, whereas $GdCl_3$ decreases it, these observations emphasize the importance of elevated $[Ca^{2+}]$ for tip growth.

It is interesting that not all conditions that lead to growth inhibition dissipated the cytosolic $[Ca^{2+}]$ gradient. Whereas La^{3+} eliminated the inwardly directed Ca^{2+} net currents at the tip (Table I) and almost completely leveled the cytosolic $[Ca^{2+}]$ gradient, presumably by blocking Ca^{2+}-conducting channels, inhibition of the plasma membrane proton pump through DCCD had no such immediate effect, even though growth was completely stopped (Fig. 5). These observations indicate that although an elevated

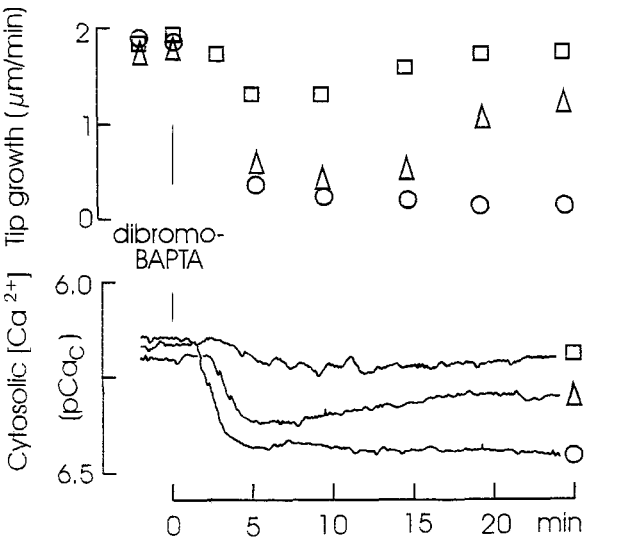

Figure 6. Effect of dibromo-BAPTA pressure-injected at different concentrations into the cell shank (injectates: □, 0.5 mM; △, 3 mM; and ○, 10 mM basic concentration) on tip growth and cytosolic free $[Ca^{2+}]$, as measured with Ca^{2+}-selective microelectrodes in the tip region of *S. alba* root hairs. Each of these traces is representative of single kinetics of four equivalent measurements.

apical $[Ca^{2+}]$ may be an important condition for tip growth, inversely, the presence of a $[Ca^{2+}]$ gradient does not necessarily mean that the root hair is growing. We would concede, however, that under conditions in which growth has been stopped by DCCD for a longer time period, the $[Ca^{2+}]$ gradient would be expected to slowly decline, since deactivation of the primary energy source will finally result in a breakdown of plasma membrane transport processes. Also, the absence of a $[Ca^{2+}]$ gradient does not seem to impair Ca^{2+} regulation considerably. As shown in Figure 4, the responses to external Ca^{2+} and to IAA are clearly transient, and even in the presence of La^{3+} a slight recovery is apparent. However, comparing Figures 1 and 4, it appears that nongrowing cells respond more strongly to changes in external $[Ca^{2+}]$.

A Role of Cytosolic pH for Tip Growth?

Given the distinct $[Ca^{2+}]$ gradient and the likely interaction between Ca^{2+} and H^+, the question arises whether these cells also contain pH gradients. Levina et al. (1995) have raised the issue of a pH gradient in tip-growing cells, suggesting that the apparent low $[Ca^{2+}]$ in *Neurospora crassa* may be a massive underestimation, since the pH in the tip may be more acidic by 1 to 1.5 pH units relative to subapical regions. Fluorescence ion imaging has revealed a gradient of about 0.3 to 0.4 pH unit in tip-growing rhizoid cells of *Pelvetia*, which was supported by measurements using pH-sensitive microelectrodes (Gibbon and Kropf, 1994). However, a careful investigation of *S. alba* root hairs using pH-sensitive microelectrodes (Felle, 1987; Herrmann and Felle, 1995) failed to detect a significant cytosolic elevation of the free $[H^+]$ in the tip, or a substantial spatial

difference of the IAA-induced alkalinization (Fig. 2C). In general, the reports on cytosolic pH gradients of tip-growing cells using different dyes differ considerably (Feijo et al., 1995), leaving open the question of their requirement for tip growth. Since there is evidence of H^+/Ca^{2+} interactions within the cytosol of animal cells (Busa and Nuccitelli, 1984) as well as in plant cells (Felle, 1988), the shallow slope of the [Ca^{2+}] gradient would provide evidence against a steep pH gradient in these root hairs. Many questions concerning pH gradients and the role of protons possibly interacting with Ca^{2+} in tip-growing cells remain open and deserve attention in future work.

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