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Calcium Channel Activity during Pollen Tube Growth and Reorientation

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We have shown previously that the inhibition of pollen tube growth and its subsequent reorientation in *Agapanthus umbellatus* are preceded by an increase in cytosolic free calcium ($[Ca^{2+}]_c$), suggesting a role for Ca^{2+} in signaling these processes. In this study, a novel procedure was used to measure Ca^{2+} channel activity in living pollen tubes subjected to various growth reorienting treatments (electrical fields and ionophoretic microinjection). The method involves adding extracellular Mn^{2+} to quench the fluorescence of intracellular Indo-1 at its Ca^{2+}-insensitive wavelength (isosbestic point). The spatial and temporal kinetics of Ca^{2+} channel activity correlated well with measurements of $[Ca^{2+}]_c$ dynamics obtained by fluorescence ratio imaging of Indo-1. Tip-focused gradients in Ca^{2+} channel activity and $[Ca^{2+}]_c$ were observed and quantified in growing pollen tubes and in swollen pollen tubes before reoriented growth. In nongrowing pollen tubes, Ca^{2+} channel activity was very low and $[Ca^{2+}]_c$ gradients were absent. Measurements of membrane potential indicated that the growth reorienting treatments induced a depolarization of the plasma membrane, suggesting that voltage-gated Ca^{2+} channels might be activated.

INTRODUCTION

The pollen tube exhibits extraordinarily pronounced polarized growth; its primary function is to deliver male gametes to the egg to effect fertilization. Pollen tubes can grow to great lengths (for example, >50 cm in maize) and often have extremely rapid growth rates (>1 μm per sec). During their long and often tortuous journey from the stigma to the egg, pollen tubes undergo numerous reorientations in growth direction. How these changes in direction are specified is not understood, but mechanical, chemical, and electrical guidance signals have all been suggested as contributors (Heslop-Harrison, 1987; Hülskamp et al., 1995).

Pollen tube elongation results from growth confined to the tip of the tube; this involves localized secretion and cell wall synthesis (Heslop-Harrison, 1987; Steer and Steer, 1989; Battey and Blackbourn, 1993). It is now well established that there is a tip-high gradient in cytosolic free calcium ($[Ca^{2+}]_c$) in growing but not in nongrowing pollen tubes. Evidence for the existence of a Ca^{2+} gradient was first obtained by Jaffe et al. (1975), who provided evidence that Ca^{2+} influx is localized at the pollen tube apex. The presence of the gradient was later confirmed by direct measurements of $[Ca^{2+}]_c$ in living pollen tubes using fluorescence ratio imaging of Ca^{2+}-sensitive dyes (Obermeyer and Weisenseel, 1991; Rathore et al., 1991; Miller et al., 1992; Malhó et al., 1994; Pierson et al., 1994).

How a gradient of $[Ca^{2+}]_c$ might regulate polarized pollen tube growth is not known. It seems likely that this gradient could be important in regulating the F-actin network, which has a multifunctional role in coordinating different aspects of tip growth (Jackson and Heath, 1993). The $[Ca^{2+}]_c$ gradient probably serves other functions as well, including the regulation of localized vesicle-mediated secretion at the pollen tube tip via Ca^{2+}-dependent, phospholipid-binding annexins (Blackbourn et al., 1992; Battey and Blackbourn, 1993). The mechanisms that maintain the gradient of $[Ca^{2+}]_c$ are also not understood, but recent results indicate that localization of Ca^{2+} channel activity in the growing tip could be an important factor (Pierson et al., 1994).

We have recently reported that pollen tube growth can be reoriented by ionophoretic injection, photolysis of caged Ca^{2+}, or an electrical field (Malhó et al., 1994). The following sequence of events was noted within 10 min of ionophoresis or photorelease of caged Ca^{2+}: (1) an immediate increase in $[Ca^{2+}]_c$, (2) an inhibition of growth, (3) return of $[Ca^{2+}]_c$ to normal cytosolic levels, (4) tip swelling, and (5) a recovery of pollen tube growth, usually in a different orientation. Similar results have also been observed after applying electrical fields, except obvious tip swelling was not apparent and growth reorientation was not abrupt. With electrical fields, we have also found that adding a very low concentration of the Ca^{2+} channel blocker La^{3+} to the growth medium prevents the

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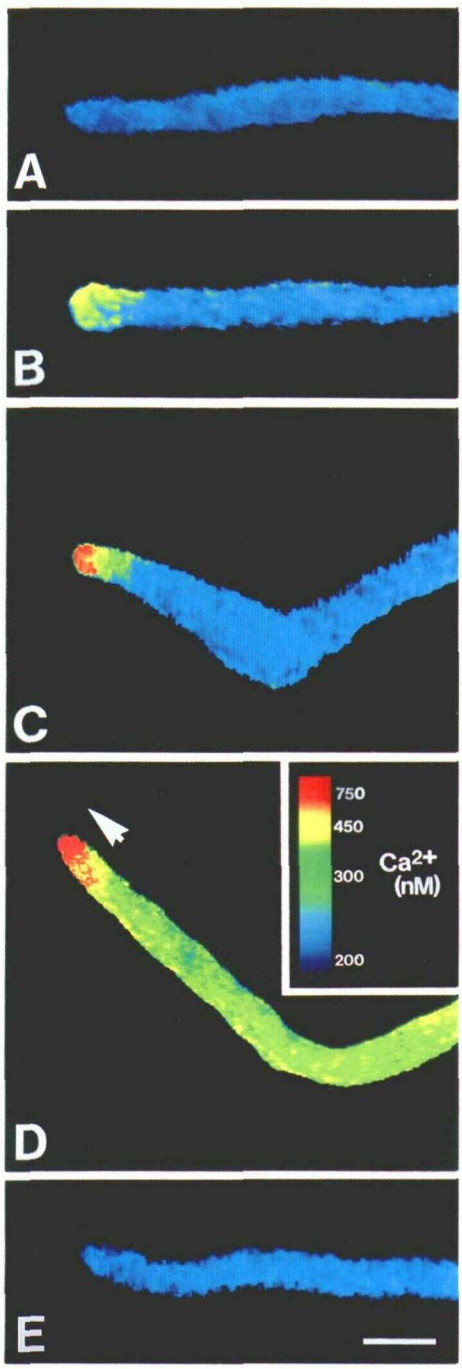

Figure 1. Fluorescence Ratio Images of $[Ca^{2+}]_c$ in Pollen Tubes during and after Different Growth Reorienting Treatments.

(A) Nongrowing pollen tube 3 min after ionophoresis. Note the absence of a $[Ca^{2+}]_c$ gradient.

(B) Swollen pollen tube 6 min after ionophoresis.

(C) Pollen tube outgrowth (8 min after ionophoresis) from a swelling that is in an orientation different from the original growth axis. The tip-focused $[Ca^{2+}]_c$ gradient and the growth reorientation are abrupt (cf. **[D]** and Figure 2D).

(D) Pollen tube growth reoriented by a continuous electrical field of 5 V cm^{-1} that was maintained during ratio imaging. The reorientated pollen tube is growing directly toward the cathode. The resting level of $[Ca^{2+}]_c$ has been raised by the electrical treatment, but the tip-focused gradient in $[Ca^{2+}]_c$ is still present in this growing pollen tube. Also, the growth curvature is smoother than after ionophoresis (cf. **[C]** and Figure 2C). The arrow indicates the direction of the cathode.

(E) Nongrowing pollen tube 2 min after treatment with 100 μM La^{3+}. Bar = 10 μm.

reduction in growth rate, $[Ca^{2+}]_c$ increase, and subsequent growth reorientation. These data thus suggest Ca^{2+} channel involvement in the events leading to growth reorientation.

These observations pose important questions concerning Ca^{2+} channel activity during the growth and reorientation of pollen tubes. A simple procedure has been devised to probe Ca^{2+} channel activity in living animal cells (Hallam et al., 1988). The basis of this method is that, first, Mn^{2+} quenches the fluorescence of ratiometric Ca^{2+} dyes, such as Indo-1 (Grynkiewicz et al., 1985; Owen, 1993), and second, Mn^{2+} can act as a surrogate for Ca^{2+} and thus commonly enters cells through open Ca^{2+} channels (Jacob, 1990). By adding a noninhibitory concentration of Mn^{2+} to a cell loaded with Indo-1, it is possible to image, or measure photometrically, the quenching of the dye at its Ca^{2+}-insensitive wavelength (that is, isosbestic point; Haugland, 1993). This provides an indirect method for localizing and quantifying Ca^{2+} channel activity. In this study, we used this procedure and showed a close spatial and temporal correlation between Ca^{2+} channel activity and $[Ca^{2+}]_c$ levels in living pollen tubes during growth and reorientation.

RESULTS

Ca^{2+} Gradients in Pollen Tubes Undergoing Reorientation

To determine whether there is a close spatial and temporal correlation between $[Ca^{2+}]_c$ levels and Ca^{2+} channel activity in pollen tubes, it was first necessary to image and quantify $[Ca^{2+}]_c$ at different stages during and after growth reorienting treatments. This was done by fluorescence ratio imaging of Indo-1–loaded pollen tubes that were subjected to ionophoretic microinjection and allowed to recover (Figure 1). Light micrographs of different pollen tubes at equivalent stages of growth and reorientation following ionophoresis are shown in Figure 2.

In Figures 1A to 1C and 2A to 2C, three successive stages of recovery of the pollen tube leading to reorientation are shown. Immediately following ionophoresis, there is an increase in $[Ca^{2+}]_c$, which returns to resting levels after about 3 min (Malhó et al., 1994). At this time, the pollen tubes show no $[Ca^{2+}]_c$ gradient and no growth (Figures 1A and 2A).

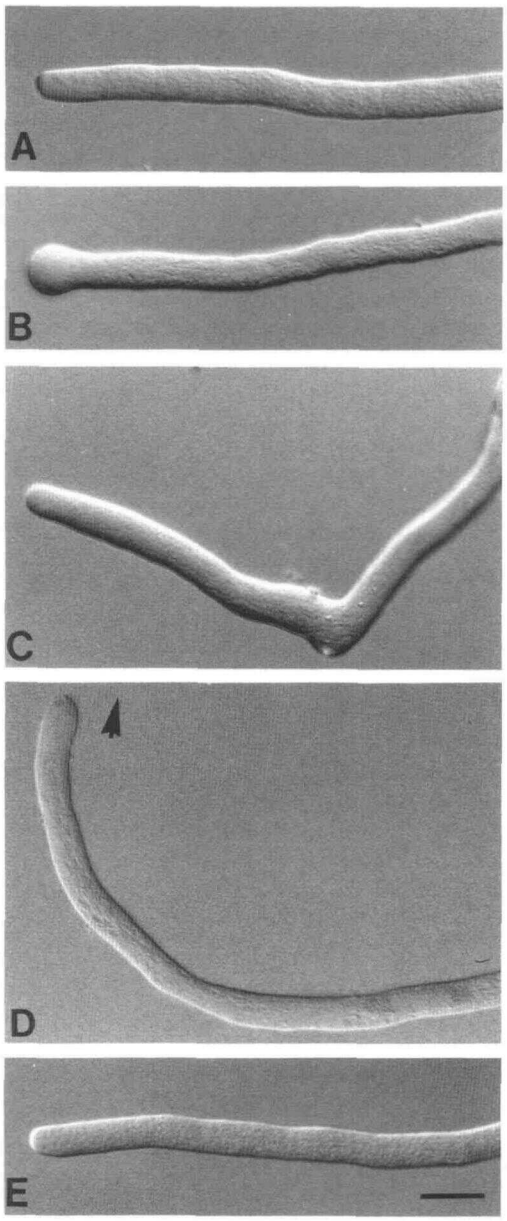

Figure 2. Differential Interference Contrast Microscopy of Pollen Tubes during and after Similar Treatments to the Pollen Tubes as Shown in Figure 1.

(A) Nongrowing pollen tube 3 min after ionophoresis.
(B) Swollen pollen tube 6 min after ionophoresis.
(C) Pollen tube growth reestablished (8 min after ionophoresis) in an orientation different from that before growth cessation. The growth reorientation is abrupt (cf. Figure 1D and **[D]**).
(D) Pollen tube growth reoriented by a continuous electrical field of 5 V cm^{-1} that was maintained while imaging. The reorientated pollen tube is growing directly toward the cathode. The growth reorientation is gradual (cf. Figure 1C and **[C]**). The arrow indicates the direction of the cathode.
(E) Nongrowing pollen tube 2 min after treatment with 100 μM La^{3+}. Bar = 10 μm.

Approximately 4 to 8 min after ionophoresis, the pollen tube tip swells, and this coincides with the reestablishment of a new $[Ca^{2+}]_c$ gradient with a free Ca^{2+} concentration of ~450 nM (Figures 1B and 2B). After ~8 min, pollen tube growth recovers, typically in an abrupt and random, new orientation (Malhó et al., 1994), and the coincident $[Ca^{2+}]_c$ gradient has a maximal tip-high free Ca^{2+} concentration of ~750 nM or higher (Figures 1C and 2C). There was an indication in some of our ratio images of swollen pollen tubes (for example, Figure 1B) that the $[Ca^{2+}]_c$ concentration was not uniform within the swelling but was highest in the region where the new tube tip subsequently emerged. However, clear evidence of this phenomenon will require more detailed and rigorous analysis.

Growing pollen tubes that were reoriented by an applied electrical field of 5 V cm^{-1} are shown in Figures 1D and 2D. The gradient of $[Ca^{2+}]_c$ is still clearly detectable in the tip of the pollen tube (Figure 1D), even though there had been a general elevation of the $[Ca^{2+}]_c$ level behind this region. In contrast with ionophoresis, growth of pollen tubes does not stop when they are treated with an electrical field of 5 V cm^{-1} (Malhó et al., 1994). Thus, there is a correlation between pollen tube growth and the presence of the tip-focused $[Ca^{2+}]_c$ gradient, even when the resting level of $[Ca^{2+}]_c$ is increased during the application of an electrical field.

Figure 1E shows a ratio image of $[Ca^{2+}]_c$ in a pollen tube that had been incubated for 2 min in 100 μM of the Ca^{2+} channel blocker La^{3+}. Growth ceased and the $[Ca^{2+}]_c$ gradient disappeared concomitantly after treatment. This observation suggests that the tip-focused $[Ca^{2+}]_c$ gradient is dependent on active Ca^{2+} channels located in this region of pollen tubes.

Indo-1 Quenching by Mn^{2+} To Assess Ca^{2+} Channel Activity

Figures 3 and 4 demonstrate that measurement of Indo-1 at its Ca^{2+}-insensitive wavelength (isosbestic point) may be used to monitor Mn^{2+} uptake into a cell and thus provide a measure of Ca^{2+} channel activity (Jacob, 1990). When analyzed in vitro using the filter system (450 nm, 10-nm half-bandwidth) employed for in vivo studies, little change in Indo-1 fluorescence was noted at the isosbestic point compared with that observed at 405 and 480 nm when Ca^{2+} was added (Figure 3A). The addition of Mn^{2+} to Indo-1, however, resulted in marked quenching of fluorescence at all three wavelengths (Figure 3B).

Quenching of Indo-1 fluorescence at its Ca^{2+}-insensitive wavelength was also observed within pollen tubes when Mn^{2+} was added. This showed the potential of this fluorescence loss as a reporter for localizing Ca^{2+} channel activity in vivo. Figure 4 illustrates the quenching induced by 200 μM Mn^{2+} in selected frames taken from a video recording of a growing pollen tube over a 15-sec period. Immediately following this treatment, there was a very slight reduction in pollen tube growth rate, which fully recovered within 6 min (Figure 5).

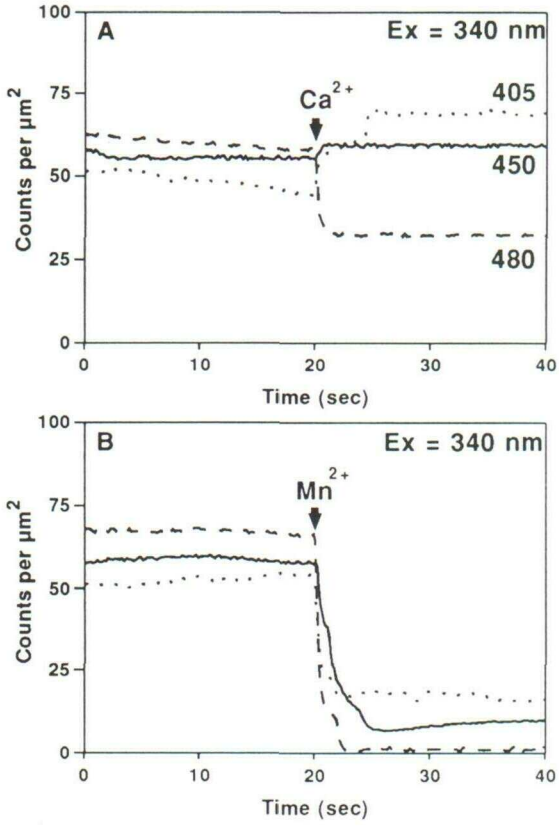

Figure 3. Photometry Traces of Indo-1 Fluorescence at 405, 450 (Ca^{2+}-Insensitive, Isosbestic Point), and 480 nm.

(A) Effect of 1 mM Ca^{2+} added at 20 sec. There is negligible change in fluorescence at 450 nm.
(B) Effect of 200 µM Mn^{2+} added at 20 sec. There is a significant quenching of fluorescence at 450 nm.
Ex, excitation.

Spatial Localization of Ca^{2+} Channel Activity in Pollen Tubes

On close examination, quenching by extracellular Mn^{2+} of Indo-1 fluorescence in the growing pollen tube shown in Figure 4 seemed to be more rapid in the tip region than behind it. This is what we would expect if there were more active Ca^{2+} channels in the pollen tube tip. To analyze this localization of Ca^{2+} channel activity more precisely, we quantified the fluorescence loss in pollen tubes from individual frames captured on videotape at different time intervals following the addition of Mn^{2+}.

Loaded pollen tubes at various stages following ionophoresis or electrical field or inhibitor treatment were incubated for 30 sec in 200 µM Mn^{2+}. Fluorescence images were captured at time zero before treatment and 30 sec after the addition of Mn^{2+}. The fluorescence intensity was measured along the midline of the first 50 µm of each pollen tube at these two time points. To reduce the inherent noise of the pixel intensities along these transects, each successive group of 10 pixels was averaged. The measurements obtained after Mn^{2+} treatment were subtracted from those obtained before treatment. This provided a measure of fluorescence loss down the length of the pollen tube during the intervening 30 sec (Figure 6).

In nongrowing pollen tubes following ionophoresis (Figure 6A), the fluorescence quenching was <20% and more or less uniform along the length of a pollen tube, implying a low uptake of Mn^{2+}. This indicates that in the nongrowing state, there are relatively few open divalent cation channels through which Mn^{2+} can enter the pollen tube and is consistent with the absence of a Ca^{2+} gradient (Figure 1A). However, during the swelling process prior to the recovery of growth, 80% fluorescence loss was evident in the pollen tube tip (Figure 6B). The extent of quenching in the tip region was similar to that of pollen tubes that had grown out from the swelling, although in this case the loss of fluorescence further back from the tip was greater than in pollen tubes that were just swollen (Figure 6C). The greatest quenching (95% fluorescence loss) was observed in pollen tubes subjected to an external electrical field of 5 V cm^{-1} (Figure 6D). The addition of 100 µM La^{3+} to the medium resulted in almost total inhibition of Mn^{2+} entry, with only 10% fluorescence loss (Figure 6E). Comparison with the control in Figure 6F, in which no Mn^{2+} was added, demonstrates that 5% of the fluorescence loss, probably in all of the treatments (Figures 6A to 6E), was due to a combination of dye leakage from the cytosol and dye quenching by the

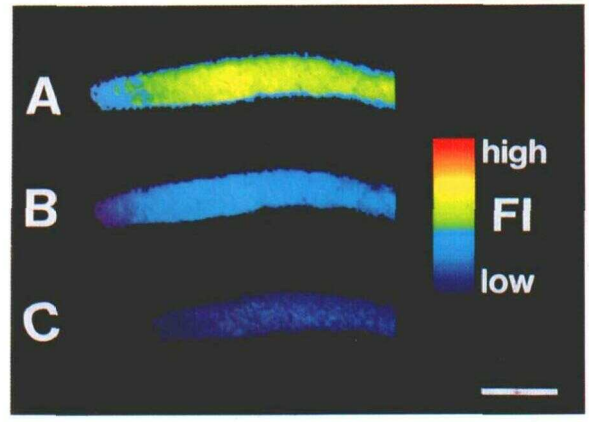

Figure 4. Time Course of Quenching by 200 µM Mn^{2+} of Indo-1 Fluorescence at Its Ca^{2+}-Insensitive Wavelength (450 nm) within the Apical Region of a Pollen Tube.

The three images are selected frames from the videotape.
(A) Before treatment. The fluorescence intensity, imaged at a single wavelength, is lower in the apical region because less dye is present in the cytosol surrounding the high concentration of vesicles in the tips of growing pollen tubes.
(B) Five seconds after the addition of Mn^{2+}.
(C) Fifteen seconds after treatment with Mn^{2+}.
FI, fluorescence intensity. Bar = 10 µm.

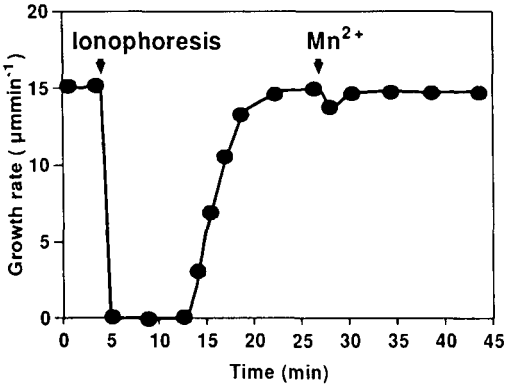

Figure 5. Growth Rate of a Typical Pollen Tube after Treatment with Mn^{2+}.

The pollen tube was ionophoresed with Indo-1 and allowed to recover and was then treated with 200 µM Mn^{2+}. Note the Mn^{2+} only results in a very slight transient reduction in growth rate.

irradiating UV light source. Other organic channel blockers, such as verapamil or nifedipine, were not used in place of La^{3+} because we have been unable to observe growth inhibition at acceptably low inhibitor concentrations, which are essential to minimize the problems of nonspecific side effects.

Kinetics of Ca^{2+} Channel Activity in Pollen Tube Tips

An alternative means of assessing active channel density and kinetics is indicated in Figure 7. Here, photometry was used to measure the rate of loss of Ca^{2+}-insensitive, Indo-1 fluorescence in the apices of pollen tubes when Mn^{2+} was added (photometry provides greater temporal resolution and sensitivity than can be achieved by imaging). A circular aperture of appropriate size was used to define a 10-µm-diameter region over each pollen tube apex from which the total light detected was integrated for successive periods of 200 msec for 60 sec. These pollen tubes were analyzed at different times after ionophoresis in the following states: nongrowing (Figure 7A), swelling (Figure 7B), and growing after reorientation (Figure 7C). They were also analyzed after reorientation in growing pollen tubes during electrical field stimulation (Figure 7D). In addition, all of these treatments were repeated in the presence of 10 µM La^{3+} (Figures 7E to 7H) or 10 µM Gd^{3+} (Figures 7I to 7L) (the pollen tubes continue to grow in the presence of these inhibitors at these concentrations; Malhó et al., 1994). The initial rate of quenching relates to the initial rate of Mn^{2+} entry into a pollen tube and thus should reflect the number of active, open channels. These initial rates of quenching are shown in Table 1.

The data of Figure 7 and Table 1 are in broad agreement with the results shown in Figure 6. The quenching rate is very slow in nongrowing pollen tubes following ionophoresis but 60-fold higher in the electrically stimulated pollen tubes. Both the swelling pollen tubes and the growing pollen tubes reorientated by ionophoresis exhibited approximately similar quenching rates. Inclusion of La^{3+} or Gd^{3+} in the growth medium reduced quenching rates substantially in all treatments. This inhibition was most pronounced in the electrically stimulated tubes (over fourfold reduction) than in other treatments not subjected to electrical fields, where it was approximately twofold.

Membrane Potential Changes in Response to Ionophoresis and Electrical Fields

Data from Figures 1, 4, 6, and 7 and Table 1 suggest that changes occur in Ca^{2+} channel activity either after ionophoresis or during electrical field treatment, which result in growth reorientation. Because many Ca^{2+} channels are voltage gated, we tested whether the two reorienting procedures could change pollen tube membrane potential by making microelectrode measurements before and after these treatments (Figure 8).

Growing pollen tubes that were 500 to 800 µm long possess an average membrane potential of -55 ± 5 mV (n = 20; Figures 8A and 8B). Recordings were usually very stable, although there was often a slight reduction (never exceeding 5 mV) in the negative potential recorded over the 15-min duration of the experiment. This may represent either a current leakage from the site of microelectrode impalement or a slow change in membrane potential with time. After application of a 3-sec ionophoretic stimulus with a current of 0.4 nA, an average membrane depolarization of 15 ± 5 mV (n = 15) was recorded; this was followed by repolarization back to the resting level within 2 to 5 min (Figure 8A). If the membrane potential did not repolarize, the pollen tube failed to recover growth. Membrane depolarization also occurred after applying an electrical field of 5 V cm^{-1}, although with a lower magnitude (Figure 8B). Immediately following electrical stimulation, a depolarization in the membrane potential to -48 ± 3 mV (n = 15) was recorded. After switching the electrical field off, the membrane potential slowly returned, within 7 to 8 min, to its basal level. The depolarization values given here correspond to the membrane potential measurements recorded after cessation of the electrical stimuli. It is likely that during both treatments, higher depolarizations occurred but could not be detected rapidly enough using our experimental approach.

DISCUSSION

Calcium channels are considered to play prominent roles in signal perception and transduction by plant cells (Johannes et al., 1991; Schroeder and Thuleau, 1991; Bush, 1993). Results presented here provide evidence for Ca^{2+} channels playing a pivotal role in regulating pollen tube growth. This conclusion

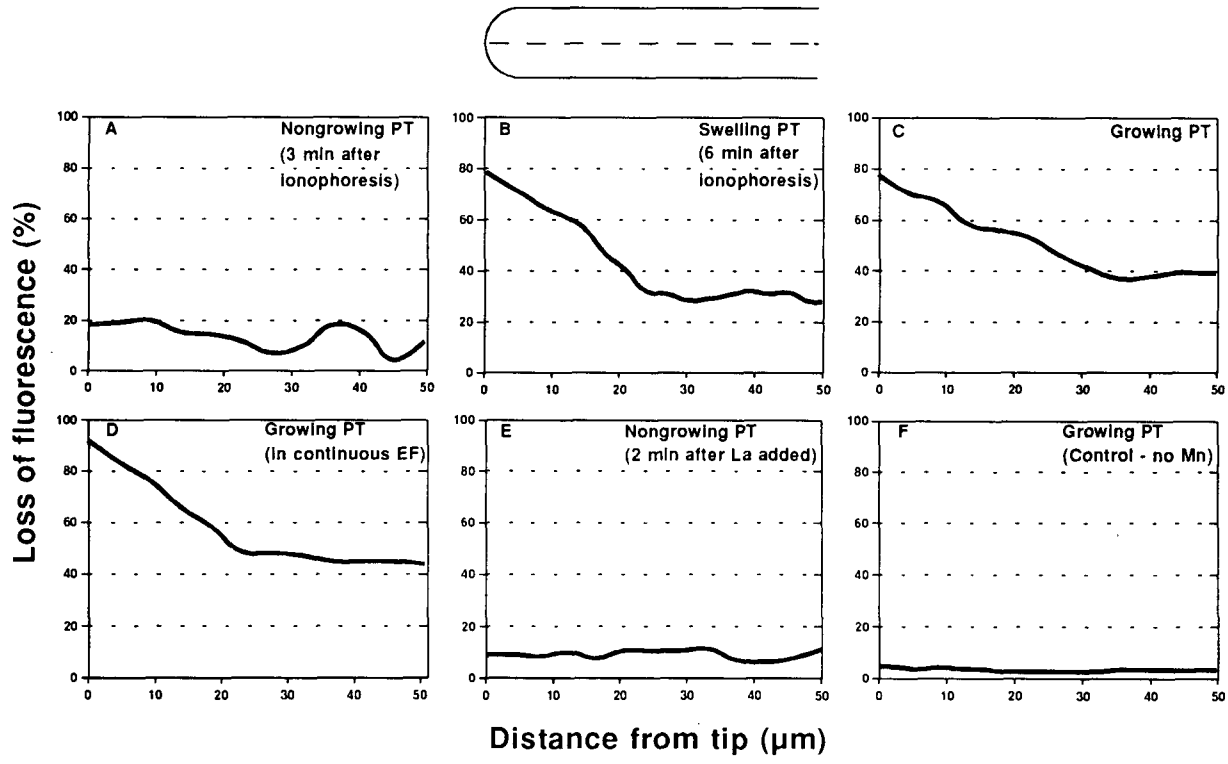

Figure 6. Spatial Localization of Ca^{2+} Channel Activity in Pollen Tubes.

Calcium channel activity was reflected by the quenching of Indo-1 fluorescence at its Ca^{2+}-insensitive wavelength in pollen tubes 30 sec after the addition of 200 μM Mn^{2+}. Measurements are the percentage of fluorescence loss during the intervening 30 sec along a midline transect down the length of the apical 50 μm of pollen tubes. (See diagram above **[A]** to **[C]**.)

(A) Nongrowing pollen tube 3 min after ionophoresis (equivalent to Figures 1A and 2A).

(B) Swelling pollen tube 6 min after ionophoresis (equivalent to Figures 1B and 2B).

(C) Reoriented pollen tube growing from a swelling following ionophoresis (equivalent to Figures 1C and 2C).

(D) Growing pollen tube reorientated in an applied electrical field of 5 V cm^{-1} (equivalent to Figures 1D and 2D).

(E) Nongrowing pollen tube 2 min after treatment with 100 μM La^{3+} (equivalent to Figures 1E and 2E).

(F) Control. Growing pollen tube with no Mn^{2+} added.

Of the treatments in which Mn^{2+} was added (**[A]** to **[E]**), only those in which the pollen tubes were swelling (**[B]**) or growing (**[C]** and **[D]**) exhibited significant dye quenching in their apical regions. EF, electrical field; PT, pollen tube.

is based on data obtained using a novel method to localize and quantify Ca^{2+} channel activity in plant cells. With this technique, a close correlation was found among the distribution of Ca^{2+} channel activity, $[Ca^{2+}]_c$ gradients, and sites of growth within pollen tubes subjected to various treatments that modify and perturb the polarity of these cells.

Role of $[Ca^{2+}]_c$ Gradients in Pollen Tube Growth and Reorientation

Two treatments (ionophoresis and applied electrical fields) were used to change the pattern of pollen tube growth and orientation. Ionophoresis induced the following sequence of cytological events: a complete cessation of tip growth; tip swelling, probably resulting from nonpolar growth; initiation of polarized growth at a more-or-less random site within the swelling; and maintenance of new polarized growth, the direction of which was not fixed. In contrast, the application of an electrical field (5 V cm^{-1}) only reduced the growth rate and resulted in a gradual change in the direction of growth, which became fixed toward the cathode until the electrical field was switched off (Malhó et al., 1994). Ionophoresis clearly resulted in a significantly more complex cytological response than did electrical field treatment, suggesting that although both responses may share features in common, they may also exhibit important differences. Various other treatments, such as heat shock, exposure to hypertonic medium, injection of 1,2-*bis*(*o*-aminophenoxy)ethane *N,N,N′,N′*-tetraacetic acid (BAPTA) buffers, or intracellular photolysis of caged Ca^{2+}, can also result in a response sequence similar to that induced by ionophoresis (van Herpen et al., 1989; Malhó et al., 1994; Pierson et al.,

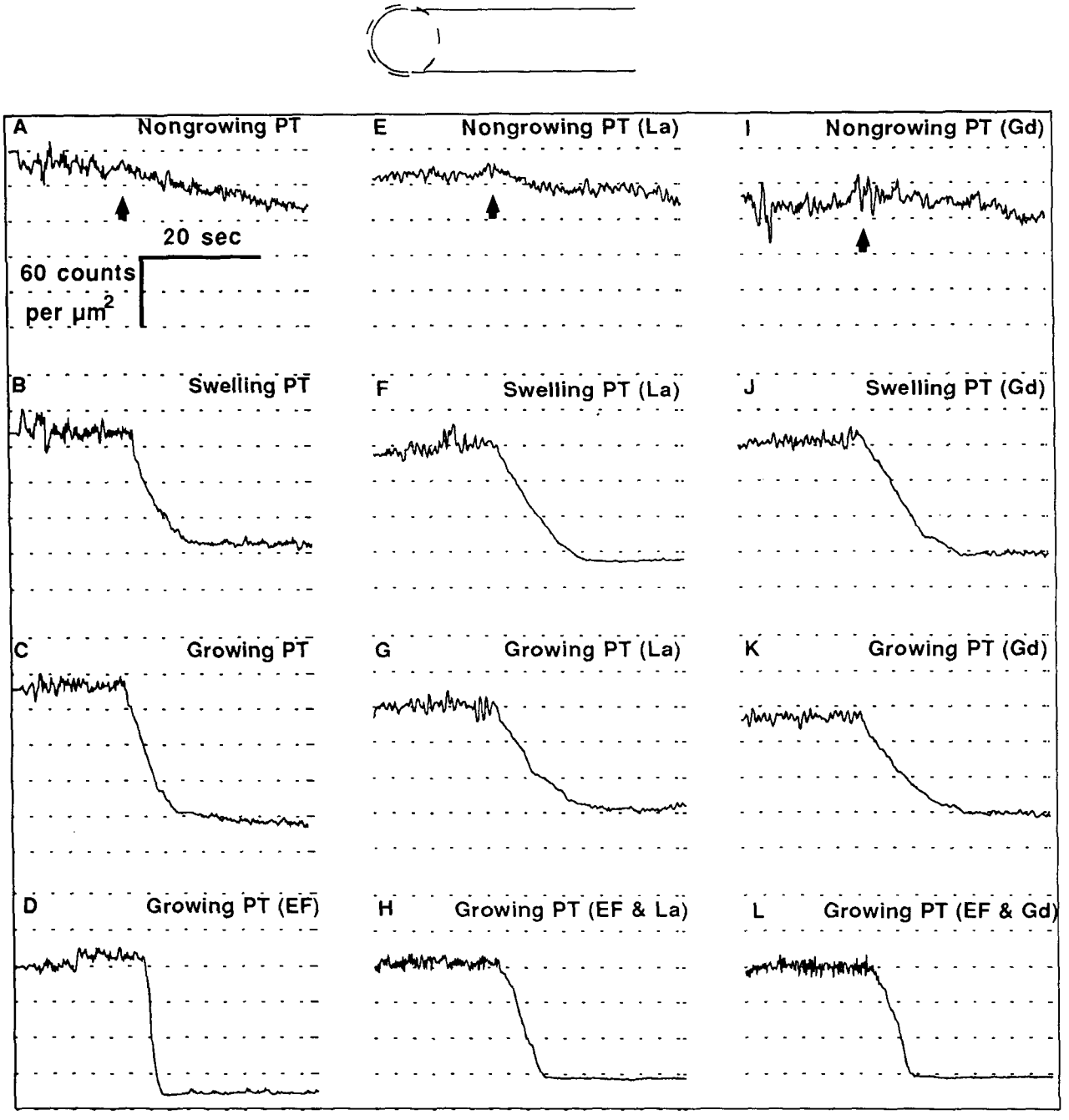

Figure 7. Kinetics of Ca^{2+} Channel Activity in the Tips of Pollen Tubes.

The photometric traces show the kinetics of Indo-1 quenching by 200 μM Mn^{2+} in the apical 10-μm region of the pollen tubes (see diagram above **[A]** to **[L]**) at different stages during recovery after ionophoresis and during electrical stimulation. PT, pollen tube; EF, electrical field.

(A) Nongrowing pollen tube (equivalent to Figures 1A and 2A). Mn^{2+} was added at the arrow or at the point when immediate fluorescence quenching was observed.
(B) Swelling pollen tube (equivalent to Figures 1B and 2B).
(C) Reoriented pollen tube growing out from a swelling (equivalent to Figures 1C and 2C).
(D) Growing pollen tube being reoriented by exposure to an electrical field of 5 V cm^{-1} (equivalent to Figures 1D and 2D).
(E) to **(H)** Same treatments as given in **(A)** to **(D)**, respectively, except that pollen tubes were continuously treated with 10 μM La^{3+}.
(I) to **(L)** Same treatments as given in **(A)** to **(D)**, respectively, except that pollen tubes were continuously treated with 10 μM Gd^{3+}.

Table 1. Initial Rates of Ca^{2+}-Insensitive Indo-1 Quenching Immediately after the Addition of 200 μM Mn^{2+} in the Apical 10-μm Region of Pollen Tubes at Different Stages during Recovery after Ionophoresis

Stage of Recovery and Treatment	Quenching Rate (% Fluorescence Loss Sec^{-1})[a]		
	No Inhibitors	+10 μM La^{3+}	+10 μM Gd^{3+}
Nongrowing after ionophoresis	0.76 ± 0.03	0.27 ± 0.04	0.32 ± 0.05
Swelling after ionophoresis	8.04 ± 0.21	5.38 ± 0.24	5.42 ± 0.22
Reorientated and growing after ionophoresis	8.75 ± 0.17	4.41 ± 0.29	4.22 ± 0.38
Reorientated and growing in electrical field (5 V cm^{-1})	45.78 ± 1.38	10.36 ± 0.31	9.94 ± 0.29

[a] The quenching rates were determined from the initial slopes of the quench curves. The treatments are the same as those given in Figure 7. The quenching rates shown represent the means (± SE) of five measurements for each treatment.

1994). The evidence presented here and elsewhere suggests that $[Ca^{2+}]_c$ gradients are essential for the maintenance and localization of pollen tube growth.

A general increase in $[Ca^{2+}]_c$ along the length of a pollen tube can be induced by ionophoresis, caged probe photoactivation, ionophore treatment, or the application of an electrical field. Each of these treatments inhibited growth, either by preventing it altogether or by reducing the growth rate (Franklin-Tong et al., 1993; Malhó et al., 1994). Data from experiments using caged Ca^{2+} and electrical fields indicated that the greater the $[Ca^{2+}]_c$ increase, the more growth was inhibited (Malhó et al., 1994). It has also been found that if growth is inhibited by injecting pollen tubes with BAPTA-type buffers, which dissipate the tip-focused $[Ca^{2+}]_c$ gradient, the growth rate is completely inhibited (Miller et al., 1992; Pierson et al., 1994). In this study, similar effects were shown by the addition of 100 μM of the Ca^{2+} channel blocker La^{3+} to pollen tubes (Figure 1E). All of these results may be explained by the effects of each treatment on the tip-focused $[Ca^{2+}]_c$ gradient. The results from this study and others (Miller et al., 1992; Malhó et al., 1994; Pierson et al., 1994) indicate that complete elimination of the $[Ca^{2+}]_c$ gradient, either by increasing the basal level of $[Ca^{2+}]_c$ or by dissipating the gradient at the tip, results in complete growth inhibition. In all cases, if the $[Ca^{2+}]_c$ gradient reappears, growth resumes. Whether a reduction in growth rate can be correlated with a reduction in the steepness of the $[Ca^{2+}]_c$ gradient needs to be addressed in future studies.

The intimate association between the presence of a tip-focused $[Ca^{2+}]_c$ gradient and growth at the pollen tube tip suggests a critical role for the gradient in maintaining growth (Obermeyer and Weisenseel, 1991; Rathore et al., 1991; Miller et al., 1992; Malhó et al., 1994; Pierson et al., 1994). In this study, we extended our previous observations and demonstrated that $[Ca^{2+}]_c$ gradients are present even in growing pollen tubes exposed to an electrical field in which the basal $[Ca^{2+}]_c$ level has been raised (Figure 1D) as well as in swelling pollen tubes prior to reorientated growth (Figure 1B). Because tip swelling probably involves nonpolarized growth in that the swelling did not disappear after the emergence of a new reorientated pollen tube, the apparently essential association between $[Ca^{2+}]_c$ gradients and growth was maintained. However, the $[Ca^{2+}]_c$ gradient became more prominent with the emergence of a new tip, and this correlated with the resumption of pronounced, polarized growth, which has been shown to result from vesicle-mediated secretion and cell wall synthesis localized at the pollen tube apex (Heslop-Harrison, 1987; Steer and Steer, 1989; Battey and Blackbourn, 1993). Whether

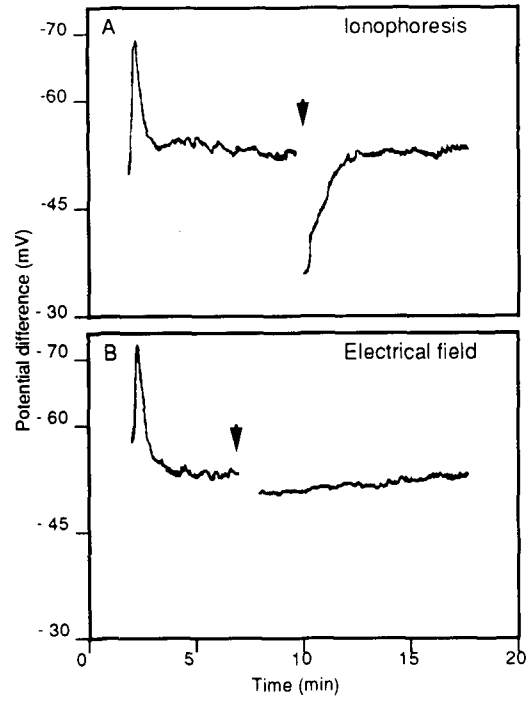

Figure 8. Membrane Potential Measurements of Pollen Tubes 500 to 800 μm Long.

In each case, the more negative potential recorded immediately after puncturing resulted from an increase in the resistance and tip potential of the impaling micropipette.

(A) Trace of membrane potential recorded in a growing pollen tube subjected to ionophoresis (arrowhead). This results in a depolarization of ~15 mV. The membrane potential recovered after ~3 min.

(B) Trace of membrane potential recorded from a growing pollen tube subjected to an electrical field (5 V cm^{-1}, arrowhead) for 1 min. After application of the electrical field, a membrane depolarization of ~4 mV was recorded, and pollen tube growth recovered after ~8 min of switching off the electrical field.

a more pronounced, asymmetric $[Ca^{2+}]_c$ gradient is generated in the swelling at the site of subsequent tip formation, thus implicating the development of a $[Ca^{2+}]_c$ gradient with the initiation of polarized growth, was not clear from our observations but is another important question to be answered.

Role of Ca^{2+} Channels in Generating $[Ca^{2+}]_c$ Gradients in Pollen Tubes

Pierson et al. (1994) have recently demonstrated in lily pollen tubes that there is a close correlation between an extracellular tip-directed influx of Ca^{2+} and a tip-focused $[Ca^{2+}]_c$ gradient; this strongly suggests that the two phenomena are coupled. Our data support their findings and provide additional evidence that the magnitude and location of Ca^{2+} channel activity in the pollen tube are critical for specifying the size and site of the $[Ca^{2+}]_c$ gradient; thus, this activity is probably instrumental in regulating and directing oriented growth.

We used quenching of intracellular Indo-1 at its Ca^{2+}-insensitive wavelength by adding external Mn^{2+} to investigate the putative functioning of Ca^{2+} channels in pollen tubes. Mn^{2+} is a divalent cation and theoretically could enter any divalent cation channels, although Ca^{2+} channels are probably the most abundant of these. The ability of Mn^{2+} to pass through Ca^{2+} channels is very widespread in animal cells, and there are only a few examples of Ca^{2+} entry pathways that are impermeable to Mn^{2+} (Jacob, 1990). Our data clearly showed that Mn^{2+} ions rapidly entered growing pollen tubes with little interruption in tube growth. Entry of Mn^{2+} was inhibited by La^{3+}, a Ca^{2+} channel blocker, and Gd^{3+}, an inhibitor of stretch-activated ion channels (Yang and Sachs, 1989). Confidence in the specificity with which the lanthanides inhibit plant cell Ca^{2+} channel function has recently been reinforced. Lanthanides induce changes in *Amaranthus* that are commensurate with inhibition of Ca^{2+} channel activity (Terry et al., 1992). Marshall et al. (1994) have shown that La^{3+} and Gd^{3+} inhibit Ca^{2+} influx in maize roots, and their data suggest that these two inhibitors may impair the activities of different Ca^{2+} channels. However, we were unable to detect any difference in the ability of these two inhibitors to inhibit Mn^{2+} influx into pollen tubes. Marshall et al. (1994) have also shown that Mn^{2+} ions can inhibit Ca^{2+} influx, but this could simply have occurred by direct competition for the same entry site.

Our data showed that the presence or absence of tip-focused $[Ca^{2+}]_c$ gradients inside the pollen tube correlates well with Mn^{2+} entry into the tube apex. When growth was temporarily halted by ionophoresis, the $[Ca^{2+}]_c$ gradient disappeared, and this was correlated with a substantive reduction in Mn^{2+} entry. Recovery of growth during swelling was accompanied by the reinstitution of a $[Ca^{2+}]_c$ gradient and marked localized Mn^{2+} entry. Electrical stimulation, which increased $[Ca^{2+}]_c$ throughout the pollen tube, also increased Mn^{2+} uptake. Thus, a close spatial and temporal correlation between putative Ca^{2+} channel activity and $[Ca^{2+}]_c$ gradients was found, and as discussed earlier, this correlation appears to be closely connected to the pattern of tube growth. Clearly, our data are consistent with the idea that Ca^{2+} channels in the plasma membrane of pollen tubes play a primary role in regulating $[Ca^{2+}]_c$ gradients.

Although putative Ca^{2+} channel activity, $[Ca^{2+}]_c$ gradients, and tube growth were focused at the pollen tube tip, the increase in $[Ca^{2+}]_c$ along the length of the pollen tube following electrical field stimulation (Figure 1D) or ionophoresis (Malhó et al., 1994) indicates that Ca^{2+} channels are present throughout the plasma membrane of the pollen tube. However, we could not distinguish between greater activity of a subgroup of Ca^{2+} channels or a larger number of Ca^{2+} channels localized in the pollen tube apex. Garrill et al. (1992, 1993) have obtained protoplasts as a linear array from different regions along the length of tip-growing hyphae of the oomycete *Saprolegnia ferax* and, by patch clamping, found the highest concentration of Ca^{2+} channels in protoplasts from hyphal tips.

In our previous study (Malhó et al., 1994), low concentrations (10 μM) of La^{3+} were found to inhibit the reorientation of pollen tubes by an electrical field of 5 V cm^{-1} without significantly reducing their growth rate. In this study, pollen tubes growing and undergoing reorientation in an electrical field of the same magnitude exhibited more than fivefold greater Mn^{2+} quenching than normally growing pollen tubes. Our data thus suggest the involvement of different Ca^{2+} channels in these processes. Thuleau et al. (1994a) have recently found that protoplasts of carrot cells possess quiescent voltage-dependent Ca^{2+} channels that could be activated by electrical pulses. The authors suggested that the quiescent channels might be rapidly recruited for Ca^{2+}-dependent signal transduction. Electrical signaling has also been suggested to be involved in the guidance mechanisms of pollen tubes (Wang et al., 1989).

Measurements of membrane potential made during ionophoresis or the application of an electrical field showed that these treatments depolarized the pollen tube plasma membrane. Voltage-gated Ca^{2+} channels might thus be activated. We were unable to observe significant inhibition of Mn^{2+} uptake with two inhibitors of voltage-gated Ca^{2+} channels (verapamil and nifedipine) at acceptably low concentrations. However, the specificity of these inhibitors for different channel types has most often been analyzed in animal cells (Hess, 1990), and thus their effects on plant cells need to be interpreted with caution (Terry et al., 1992). A further complicating factor is that stretch-activated channels are voltage-dependent (Guharay and Sachs, 1985). Direct evidence for the existence of voltage-dependent Ca^{2+} channels being activated by depolarization of the plasma membrane of higher plant cells has recently been obtained by Huang et al. (1994) and Thuleau et al. (1994a, 1994b).

The experiments performed here have not allowed us to identify precisely the nature of the Ca^{2+} channels involved in the production of tip-based gradients. Nevertheless, our results suggest that stretch-activated channels could have a role because 10 μM Gd^{3+} inhibited both Mn^{2+}-induced quenching

and pollen tube reorientation. Additional support for stretch-activated channels playing a role in pollen tube growth comes from the observation that when the osmolarity of the medium around lily pollen tubes is increased, the $[Ca^{2+}]_c$ gradient is reduced and tube growth is inhibited (Pierson et al., 1994). Patch clamp studies have identified stretch-activated channels in protoplasts from fungal hyphae (Zhou et al., 1991; Garrill et al., 1992, 1993), but it is not clear whether these channels are involved in regulating tip growth. It has been suggested that ion channels activated by stretching of the plasma membrane act as sensors of cell turgor, external mechanical stresses, voltage gradients, and chemical gradients (Ding and Pickard, 1993). In this way, Ca^{2+} channels may be involved in a reorientation mechanism in which localized channel activity, induced by mechanical (Sanders and Lord, 1989), chemical (Hülskamp et al., 1995), or electrical (Wang et al., 1989) signals, could modify ion fluxes that may regulate localized cell growth.

METHODS

Plant Material

Pollen of *Agapanthus umbellatus* was harvested and stored, and pollen tubes were grown in vitro, as described previously (Malhó et al., 1994).

Imaging and Calibration of Cytosolic Free Calcium

Growing pollen tubes were microinjected with the free acid of the Ca^{2+}-sensitive dye Indo-1 (Molecular Probes Inc., Eugene, OR). The cells were ionophoresed with Indo-1 for 15 sec with a 1 to 2 nA current and allowed to recover fully before experimental treatment. Details on the experimental procedure and criteria used to establish the success of microinjection can be found in Malhó et al. (1994).

Cytosolic free calcium ($[Ca^{2+}]_c$) was ratio imaged using Indo-1 with the low-light-level imaging system described by Read et al. (1992). Indo-1 was excited at 340 nm (10-nm half-bandwidth), and fluorescence was detected at 405 nm (10-nm half-bandwidth) and 480 nm (10-nm half-bandwidth) using a Nikon (Telford, Shropshire, UK) dry 40× fluor objective (all filters supplied by Ealing Electro-Optics, Ealing, UK). Ratio imaging and calibration of $[Ca^{2+}]_c$ were performed as described previously (Malhó et al., 1994).

Growth Reorientation and Inhibitor Treatments

Ionophoresis and external electrical fields were used as treatments to reorient pollen tube growth. Ionophoresis of 100 μM Indo-1 was performed at 1 to 2 nA for 15 sec. An external electrical field of 5 V cm^{-1} was applied to pollen tubes growing on medium on a coverslip using the system described by Malhó et al. (1992, 1994). In some cases, 100 μM of the Ca^{2+} channel blocker $LaCl_3$ (Sigma) was added to growing pollen tubes preloaded with Indo-1.

Spatial Localization and Kinetics of Dye Quenching by Mn^{2+}

To demonstrate the influence of Ca^{2+} and Mn^{2+} on dye fluorescence at different wavelengths (405, 450, and 480 nm; all provided by 10-nm half-bandwidth interference filters), a 90-μL drop of the dye was placed on a coverslip, and 10-μL drops of 1 mM $CaCl_2$ or 200 nM $MnCl_2$ were added. These concentrations and drop sizes were chosen because they have been found to induce changes in dye fluorescence within the same order of magnitude. The kinetics of dye quenching by Mn^{2+} were measured with the photometry system described by Read et al. (1992).

To analyze the spatial localization of Mn^{2+} quenching within dye-loaded pollen tubes, tubes were exposed to a solution of medium (Brewbaker and Kwack, 1963) containing 1% sucrose and 200 μM Mn^{2+}, and the quenching was recorded at 450 nm on VHS videotape using a JVC BR-S610E S-VHS video recorder (Optivision, Ltd., Ossett, West Yorkshire, UK). Individual frames from these recordings were captured, digitized, and processed using a Synapse frame store under the control of Semper 6 Plus (Synoptics Ltd., Cambridge, UK). To quantify the spatial localization of Mn^{2+} quenching in pollen tubes, the fluorescence intensity along a midline transect down the first 50 μm of each pollen tube was measured at time zero and 30 sec after the addition of Mn^{2+} using the Comos software (Bio-Rad Microscience Ltd., Hemel Hempstead, Hertfordshire, UK). To smooth the pixel values in this transect, each successive group of 10 pixels was averaged. The transect of a pollen tube 30 sec after the Mn^{2+} addition was then subtracted from that obtained previously to produce a transect that was a measure of fluorescence loss during the intervening period. The data were then plotted using Harvard Graphics version 3.0 software (Software Publishing Corporation, Bracknell, Berkshire, UK). Growth rate measurements of pollen tubes subjected to different treatments were determined using the Comos software.

To analyze the kinetics of dye quenching in pollen tube tips with greater temporal resolution and sensitivity than could be achieved by imaging, photometry was used. A circular aperture was selected to allow photometric measurements from the apical 10 μm of the tube. Pollen tubes loaded with Indo-1 were exposed to 200 μM Mn^{2+} as before, and dye quenching was recorded under different growth conditions at 450 nm. The photomultiplier signal, with a sampling time of 200 msec, was processed and analyzed using the Photon Counting System and Count version 5.2 software of Newcastle Photometric Systems (Newcastle-upon-Tyne, UK). Saved data were subsequently plotted using Harvard Graphics software.

Membrane Potential Measurements

Single microelectrodes of 0.5-μm external tip diameter were gravity-pulled from 1.5-mm–wide glass capillaries containing an inner glass filament (Clark Electromedical Instruments, Reading, UK) using a vertical Narishige 5759 DBS micropipette puller (Nikon). The micropipettes were back-filled with a 1 M KCl solution and possessed a tip resistance of 4 to 7 MΩ and tip potential of −4 to 1 mV. The micropipettes were connected to a DC preamplifier (via an Ag-AgCl wire) with an input impedance $>10^{13}$ MΩ and a leakage current of $<4.10^{13}$ A. The reference electrode (Ag-AgCl), grounded via a calibrator, served as a reference electrode in the growth medium. Membrane potentials were recorded on a chart recorder. The microelectrode was held by a Narishige NT-88 4D micromanipulator mounted on a Nikon Diaphot inverted microscope. Some membrane potential measurements were taken before and immediately after ionophoretic injection of individual

pollen tubes. Ionophoresis was performed with a second microelectrode (external tip diameter <0.3 μm) filled with a 100 μM Indo-1 or Calcium Green-1 (Molecular Probes Inc.). A second reference electrode was used between the two circuits. Impalement with the ionophoretic microelectrode was made 50 to 80 μm away from the KCl-containing microelectrode. Other membrane potential measurements were taken before and immediately after applying an external electrical field of 5 V cm^{-1} for 1 min to individual pollen tubes. In all experiments, membrane potentials were monitored for >15 min. The values obtained for membrane potentials were the potential difference between the cytoplasm and the extracellular medium. The latter was measured immediately after withdrawing the microelectrode from the cell and was chosen as the reference potential to account for changes in the tip potential of the micropipette during cell impalement (Weisenseel and Wenisch, 1980).

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