Tip-Localized Calcium Entry Fluctuates during Pollen Tube Growth

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Studies have been conducted on the dynamics of Ca²⁺ entry in pollen tubes using ratiometric ion imaging to measure the intracellular gradient and an ion selective vibrating electrode to detect the extracellular influx. A steep tip-focused gradient occurs in all species examined, including Lilium longiflorum, Nicotiana sylvestris, and Tradescantia virginiana. Analysis of Lilium pollen tubes loaded with dextran conjugated fura-2 reveals that the gradient derives from Ca²⁺ entry that is restricted to a small area of plasma membrane at the extreme apex of the tube dome. Since the apical membrane is continually swept to the flanks during tube elongation, either Ca²⁺ channels are specifically retained at the extreme apex or, as seems more likely, the Ca²⁺ channels which were active at the tip rapidly inactivate, as new ones are inserted during vesicle fusion. Ratiometric imaging further indicates that the high point of the gradient fluctuates in magnitude from 0.75 to above 3 mM, during measuring intervals of 60 sec, with the elevated points being correlated with an increased rate of tube growth. Independent analysis of the growth at 2- to 3-sec intervals reveals that the rates can fluctuate more than threefold; tubes longer than 700 µm exhibit oscillations with a period of 23 sec, while tubes shorter than 700 µm display erratic fluctuations. Inhibition of pollen tube growth caused by mild temperature shock or caffeine (1.5 to 3.0 mM) is correlated with the dissipation of the tip-focused gradient and the Ca²⁺ influx. Recovery from both treatments is denoted by a global swelling of the pollen tube tip, concomitant with a high transient entry of Ca²⁺ in the tip. The location of the highest Ca²⁺ domain within the tip region defines the point from which normal cylindrical elongation will proceed.

INTRODUCTION

Growing pollen tubes possess a localized gradient of cytosolic free Ca²⁺ at their apex (Obermeyer and Weisenseel, 1991; Rathore et al., 1991; Miller et al., 1992; Malhó et al., 1994; Pierson et al., 1994). In lily, recent work reveals that this gradient extends from above 3 µM at the extreme apex of the tube to a basal level of 0.2 µM within 20 to 30 µm from the apex (Pierson et al., 1994). Referred to as a “tip-focused” gradient, it is confined exclusively to the clear zone of the pollen tube, the region in which vesicles accumulate and fuse, secreting wall material and membrane necessary for tube elongation. An essential role for this gradient in growth is shown convincingly in those studies in which its dissipation with BAPTA-type buffers completely inhibits growth (Miller et al., 1992; Pierson et al., 1994), whereas the ability of a tube to reinitiate growth is preceded by the reappearance of elevated apical Ca²⁺ (Miller et al., 1992).

In addition to a tip-focused gradient the growing pollen tube also possesses a marked extracellular Ca²⁺ flux (Küthreiber and Jaffe, 1990), between 1.4 to 14 pmole/cm² sec in Lilium (Pierson et al., 1993; 1994), that is directed inward specifically at the tip of the tube. Growth inhibition due to treatment with BAPTA-type buffers or hypertonic media also causes the inward Ca²⁺ flux to disappear (Pierson et al., 1994); conversely, when inhibited tubes recover growth, the Ca²⁺ influx reappears. Taken together these observations establish a clear relationship between pollen tube growth and the presence of both a tip-focused intracellular gradient and a tip-directed extracellular influx of Ca²⁺ ions.
Pollen tube growth thus requires the directed entry of Ca$^{2+}$; however, the dynamics of this relationship has remained largely undeciphered (Steer and Steer, 1989; Pierson and Cresti, 1992; Feijo et al., 1995). These issues became important in our deliberations when we realized that there was a striking, natural fluctuation in the [Ca$^{2+}$] during sequential recordings on single growing pollen tubes. In the study herein, we document these fluctuations, and show that the magnitude of [Ca$^{2+}$] appears to correlate with the growth rate. We also provide new spatial information about the tip-focused gradient, showing that Ca$^{2+}$ entry is confined to a limited area at the extreme apex of the tube dome. Finally, we document both the loss and reestablishment of the gradient in pollen tubes that have been reversibly inhibited by treatment with caffeine or mild thermal shock; we show during the recovery process that there are large global increases in [Ca$^{2+}$] which accompany the swelling of the tip, but that these points of elevation become localized in anticipation of normal cylindrical tube elongation.

**MATERIALS AND METHODS**

**Pollen Culture**

Pollen of *Lilium longiflorum*, *Tradescantia virginiana*, and *Nicotiana sylvestris* were germinated in a medium containing 10% sucrose, 160 μM H$_2$BO$_3$, 15 mM 2-N-morpholinoo ethanol sulphonate acid (MES), pH 5.5, 100 μM CaCl$_2$, and up to 1 mM K$^+$ (from KC1 and KOH) (Miller et al., 1992; Joos et al., 1994). Following germination the young pollen tubes were adhered to the coverslip of a microscope slide chamber with a thin film of low-temperature gelling agarose medium (Miller et al., 1992; Pierson et al., 1994) and flooded with the culture medium. Under these conditions the tubes exhibited normal in vitro growth, but yet they were stuck to the coverslip sufficiently well such that they could be microinjected without displacement.

To observe pollen tube morphology, the cells were examined with Nomarski differential interference contrast optics (Zeiss, IM 35, Oberkochen, Germany; or Nikon Diaphot, Tokyo, Japan). A permanent record was made either directly with photographic film or using a Newicon video camera coupled to an Image-1/AT (Universal Imaging Corp., West Chester, PA) imaging device, permitting us to frame average and background subtract.

**Intracellular Ca$^{2+}$ Measurements**

The distribution of free-intracellular Ca$^{2+}$ was determined using ratiometric ion imaging. Pollen tubes about 300 μm in length for *Lilium*, or 30 μm for *Nicotiana*, were pressure injected with fura-2-dextran (20 mg/ml in 100 mM KC1; Molecular Probes) (Miller et al., 1992; Pierson et al., 1994); occasionally cells, especially *Tradescantia*, were ionophoretically injected with free fura-2. Pollen tubes, appropriately loaded with indicator dye, were then analyzed with the ratiometric ion microscope system. Briefly, this is a Zeiss IM-35 inverted microscope that includes a highly regulated Hg vapor lamp as light source and a thermoelectrically cooled CCD (photomicro, Tucson, AZ) as fluorescence detector. A purpose-built filter slider allows us to rapidly switch excitation between 340 nm, the Ca$^{2+}$ sensitive wavelength, and 360 nm, the isobestic point. Images were acquired with either a 1:8 or a 1:4 exposure time for the 360- and 340-nm excitation wavelengths, respectively. The final 340:360 nm ratio images include a background subtraction step, as well as thresholding, in which regions of very low signal are set to black. The conversion of ratio values into absolute [Ca$^{2+}$] has been calculated according to well-established procedures (Bolsover et al., 1993) applied earlier for pollen tubes (Miller et al., 1992; Pierson et al., 1994).

**Extracellular Ca$^{2+}$ Flux Measurements**

Extracellular Ca$^{2+}$ flux was measured with an ion selective vibrating probe (Jaffe and Levy, 1987; Kuhlbrecher and Jaffe, 1990; Smith et al., 1994). We employed the same method as described in Pierson et al. (1994), programming the Ca$^{2+}$ selective electrode to vibrate with an amplitude of 9 to 10 μm, at a frequency of about 0.3 Hz, along its own axis but perpendicular to the pollen tube surface. The reading was a voltage difference output expressed in μV. Each final measurement, presented in Figs. 8 and 12, is the result of the average of 25 measurements taken close to the cell, minus 25 reference measurements, usually about 0 μV in magnitude, taken at distance of more than 50 μm from any cell. Figure 14 shows single data points. Positive values correspond to an influx and negative values to an efflux. When assuming an electrode efficiency of 50% (J. Kunkel, personal communication), an output value of 10 μV was calculated to be theoretically equivalent to a net absolute flux value of about 1.22 pmole/cm$^2$/sec, which corresponded to a current density of 1.93 pA/cm$^2$ (Pierson et al., 1994). Calculation and compilation of the data were done with Origin software.

**Growth Measurements**

In order to correlate the growth rate with Ca$^{2+}$ levels we determined the increase in pollen tube length between subsequent intracellular Ca$^{2+}$ ratio images by making direct measurements from the fluorescence ratio images on the monitor. Calibration of the image size revealed that there was a magnification factor of 17:20. Because of low noise in the images, increases in length could be determined with an error of less than about 0.2 to 0.4 μm. Growth measurements in...
combination with the vibrating probe were also conducted by projection of the microscopic video image on the monitor.

Because the combined growth and Ca\(^{2+}\) determinations above have relatively poor time resolution (0.5–1 min), we analyzed the growth characteristics at short time intervals (2–3 sec) using video microscopy. For these studies, pollen tubes, cultured similarly to those used for ratiometric imaging, were observed on an inverted Zeiss microscope with a 40x oil immersion lens (n.a. 1.3). In some instances, however, we varied the concentration of the agarose from 0.6 to 1.2%. The growing tubes were recorded on a MTI video camera (DAGE-MTI-65, Michigan City, IN) and displayed on a Sony Trinitron monitor (Sony Corp., Tokyo, Japan) connected to an Image-1/AT image analysis system. The Image-1/AT was set to automatically capture images at 1.92- to 3.36-sec intervals over 5-min periods. Serial images of each pollen tube were analyzed with a line scan function of pixel values, which permitted us to precisely determine the contour of the pollen tube and to accurately locate its tip spatially.

Graphs of the raw data of elongation rate (\(\mu m/sec\)) vs time were coherent but displayed "noise" inevitable from taking measurements close to the limit of the microscope's resolution (0.34 \(\mu m\)). We chose to smooth the graphs by taking adjacent averages, i.e., the average of each data point and its closest neighbor on each side. Pulse period was defined to be the time between two successive minimum velocities, seen as troughs in a graph of elongation rates vs time. Pulse amplitude was defined to be the maximum deviation within a given pulse period from the overall mean elongation rate. Tube length was set at the beginning of the time of data collection.

**Modulation of Pollen Tube Growth; Thermal Shock and Caffeine**

As a consequence of adhering the pollen tubes to the coverslip chamber we found that the brief exposure to the molten low gelling agarose (37–40\(^\circ\)C) followed by cooling (4\(^\circ\)C) was very effective at reversibly inhibiting pollen tube elongation (Pierson et al., 1993). We therefore adopted this protocol as an experimental procedure. Thus pollen tubes, which were embedded in the agarose layer, were exposed to 37–40\(^\circ\)C for 10 sec, followed immediately by cooling at 4\(^\circ\)C for 20 sec. Caffeine is also an extremely effective agent for reversibly inhibiting pollen tube growth. It was used in the culture medium at concentrations from 0.06 to 15 mM. For reversals, the caffeine was thoroughly removed and replaced with fresh, caffeine-free, culture medium.

Cells treated with either thermal shock or caffeine application were used in conjunction with ratiometric imaging and extracellular flux measurements to elucidate the changes in the Ca\(^{2+}\) gradient and extra-cellular influx that are associated with growth inhibition and recovery.

**RESULTS**

**Pollen Tubes of Different Species Possess a Tip-Focused Gradient**

Thus far, localized intracellular Ca\(^{2+}\) gradients have been observed primarily in Lilium (Obermeyer and Weisenseel, 1991; Rathore et al., 1991; Miller et al., 1992; Pierson et al., 1994), and occasionally in Agapanthus umbellatus (Malhó et al., 1994). To establish whether or not the steep Ca\(^{2+}\) gradient is a general feature of pollen tubes, we have examined a dicot, Nicotiana sylvestris, and another, unrelated, monocot, Tradescantia virginiana. The three species showed a nearly identical tip-focused Ca\(^{2+}\) gradient (Fig. 1). These observations also compared the use of fura-2–dextran (Lilium and Nicotiana) with free fura-2 (Tradescantia). Both forms of the dye produced similar results; however, the free fura-2 fluorescence intensity dropped precipitously during the first 10 min following injection (data not shown), indicating that the dye became rapidly sequestered or extruded from the cell (Callaham and Hepler, 1991; Haugland, 1992). Clearly, fura-2–dextran is the indicator of choice since it remains in the cytosol, permitting measurements on individual cells over periods as long as 5 hr. However, owing to dye dilution during cell enlargement there is a decline in the signal in long time recordings.

**Ratiometric Measurement Is Required to Detect the Tip-Focused Gradient**

Because of the marked change in shape of the pollen tube at its apex there is a dramatic increase in pathlength between the point at the tip of the tube and the place where the tube becomes cylindrical (Fig. 2). In Lilium this transition occupies the apical 15–20 \(\mu m\) of the tube. Line scan analysis of the fura-2–dextran fluorescence taken at the Ca\(^{2+}\) insensitive excitatory wavelength (360 nm) along a median line reveals that the fluorescence rises markedly in the apical 15 \(\mu m\) to a plateau (Fig. 2, trace A). Of particular interest, the fluorescence emitted when excited at the Ca\(^{2+}\) sensitive wavelength (340 nm) shows a very similar pattern, except for a small deviation reflecting the high apical Ca\(^{2+}\) domain (Fig. 2, trace B). Thereafter the intensity remains relatively constant over the next 50 to 100 \(\mu m\). When the ratio between 340 and 360 nm is calculated, these variations in pathlength disappear, and the steep, tip-focused gradient becomes strikingly evident (Fig. 2, traces C and D). Similarly there is a marked change in pathlength across the width of the tube from low values at the cortex to a maximum at the midpoint, again emphasizing the importance of performing ratiometric imaging to provide an accurate assessment of the free [Ca\(^{2+}\)].

These data also provide information concerning the packing of organelles and amount of accessible volume in the pollen tube. Inspection of the linear profile at the Ca\(^{2+}\)-independent wavelength (360 nm) (Fig. 2, trace A) reveals that, with the exception of the terminal 15–20 \(\mu m\) at the tip of the tube, the fluorescence value is remarkably constant, indicating that there is no variation in pathlength due to differential packing of organelles or vesicles. However, it is important to note that the accuracy of these single wavelength measurements depends critically upon the microscope being focused at the midplane of the pollen tube. Any deviation from this plane will cause the overall signal to decline. In previous work, for example, we reported that the accessible volume decreased in a tip-to-base manner (Miller et al., 1992); we now realize that this is in error due to a deviation of the focus from the midplane of the cell.

**Ca\(^{2+}\) Entry Occurs through a Limited Area at the Extreme Apex of the Tube Dome**

Acknowledging that the Ca\(^{2+}\) concentration is elevated at the tip of the tube, here we seek to determine more
exactly the locus of Ca\(^{2+}\) entry. Taking advantage of the high spatial resolution provided by our imaging system, we have been able to observe that the Ca\(^{2+}\) enters through a rather small region in the plasma membrane at the extreme apex of the tube dome (Fig. 3); this can be appreciated from analysis of the pseudocolours which appear as contour lines of decreasing diameter depicting highest Ca\(^{2+}\) levels. If the Ca\(^{2+}\) entered over the complete area of the dome at the tip, we would observe a high level of Ca\(^{2+}\) apparent across the cell cortex rather than concentric rings that focus toward a point.

To provide quantitative information on the origin point of Ca\(^{2+}\) entry that is less subject to color bias, we have quantitatively measured the fluorescence ratio values in selected areas immediately adjacent to the plasma membrane. When comparing the values in the cytoplasmic cortex immediately adjacent to the plasma membrane at the extreme of the tube dome and in flanking regions still within the dome (Fig. 4), again we find the highest values only within a limited region at the extreme apex. Finally, we made transverse line scans at the extreme apex and at intervals of 2.5, 5, and 40 \(\mu\)m back from the tip (Fig. 5). If there had been leakage of Ca\(^{2+}\) through the plasma membrane at points away from the apex itself, the transverse scans would appear "U" shaped. However, the results show that all scans are flat, indicating that the Ca\(^{2+}\) enters the tube exclusively at the extreme apex and may be limited to a patch of plasma membrane that is only 2.5 \(\mu\)m in diameter. The Ca\(^{2+}\) ions then diffuse in radial lines from this point; the concentration sharply declines according to a profile that fits a curve for an exponential decay (Fig. 2, trace D).

The Tip-High [Ca\(^{2+}\)] Fluctuates and Is Correlated with Growth Rate

Repetitive measurements of the same tube over brief intervals reveal that the [Ca\(^{2+}\)] at the highest point fluctuates (Fig. 6), a phenomenon that has been observed in virtually all cells that have been surveyed. The maximum value ranges from 0.75 \(\mu\)M to greater than 3 \(\mu\)M. These large fluctuations were first observed in measurements made at 1-min intervals, but they were also found in measurements taken at 15- and 30-sec intervals (data not shown). Further analysis shows that the rise in [Ca\(^{2+}\)] at the tip is mirrored by a concomitant rise in the growth rate, with the growth rate appearing to follow slightly the [Ca\(^{2+}\)] change (Fig. 7). Parallel studies using the vibrating electrode on 60 different cells reveal that there is a positive correlation, albeit weak, between the average magnitude of Ca\(^{2+}\) influx at the pollen tube tip and the average growth rate (Fig. 8).

Due to the fact that we are limited in the speed with which we can obtain ratio images, it has not been possible to determine the true period of the changes in intracellular Ca\(^{2+}\). Working on the assumption that these fluctuations will be correlated with growth, we focused specifically on the latter process using video images acquired at 2- to 3-sec intervals and analyzed using a coordinate tracking function in the Image 1A/T system. The results show for 10 pollen tubes 300–600 \(\mu\)m in length that the average growth rate is 0.2 \(\mu\)m/sec with the time between pulses ranging from 20 to 50 sec, whereas for 9 pollen tubes 700–1200 in length the average growth rate is 0.25 \(\mu\)m/sec with the time between pulses being regular at 22.8 – 2.5 sec. The change in rate between the low and high growth values varied from 1.3 to 3.8\%. Three specific examples are shown in Figs. 9A–9C. Two tubes (Figs. 9A and 9B) longer than 700 \(\mu\)m clearly show the dramatic, periodic oscillations, while a 400-\(\mu\)m tube (Fig. 9C) displays the erratic fluctuations, in which periods of relatively constant growth rate are punctuated with faster and slower excursions. While these observations are strictly directed to growth rates and changes therein, we think it is likely that rapid ratiometric imaging will reveal underlying [Ca\(^{2+}\)] changes, which are related to and may anticipate the growth rate changes. We are currently reconfiguring our ratiometric imaging microscope so that these measurements can be made.

Caffeine and Mild Temperature Shock Reversibly Dissipate the Ca\(^{2+}\) Gradient and Ion Influx; Recovery Correlates with Massive Ca\(^{2+}\) Entry

Caffeine application in the medium rapidly inhibits pollen tube growth, with concentrations of 1.5–3.0 \(\mu\)M being
FIG. 2. Line scan profiles through the median area of a flat growing pollen tube of Lilium loaded with fura-2-dextran. The raw data are shown at left; trace A is the fluorescence intensity expressed as a pixel value resulting from dye excitation at 360 nm (isosbestic point) and trace B is the intensity at 340 nm, the Ca\(^{2+}\) sensitive wavelength. The low light observed outside of the pollen tube tip in both traces A and B is due to scattering and is removed by thresholding before ratio determination. Trace C shows the ratiometric profile of B and A after subtraction of the background (the latter being taken at the side of the cell), thresholding (to eliminate weak signals), and multiplication with a standard factor of 100. Trace D (right) is the [Ca\(^{2+}\)] profile after conversion obtained from the ratio values in trace C according to a standard calibration (Miller et al., 1992; Pierson et al., 1994). Profile D fits the curve for an exponential decay.

quickly effective but reversible. At 3.0 mM 100% of the cells tested were inhibited, with an approximately 80% recovery when returned to a caffeine-free medium. Concentrations higher than 3.0 mM are increasingly less reversible and those lower than 1.5 mM are not inhibitory. Within the range 1.5–3.0 mM the effects of caffeine application are remarkably similar, and no systematic differences have been uncovered. Thus, for some observations these two concentrations have been used interchangeably. The effects of 1.5 mM caffeine can be observed in Fig. 10, which shows rapid inhibition of elongation just following the application of the alkaloid (Figs. 10B and 10C). Upon removal of caffeine (Fig. 10D), the tip of the tube swells, and organelles, which had been approaching the tip more closely, move into the swollen dome. Swelling continues (Fig. 10E), and the clear zone begins to become reestablished after which cylindrical growth resumes (Figs. 10F and 10G), but in a slightly different direction from the original axis of the tube.

When applied to cells loaded with fura-2-dextran, 1.5 mM caffeine caused a rapid dissipation of the tip-focused Ca\(^{2+}\) gradient (Fig. 11). Within 1 min after the addition of the alkaloid and at the moment of growth inhibition, we observed a high Ca\(^{2+}\) elevation back from the tip, which then appeared to travel as a wave basipetally several micrometers before it disappeared (Fig. 11B). Within the next 30 sec the [Ca\(^{2+}\)] became quite uniform throughout the length of the tube, but interestingly, it continued to decline, even after the removal of the caffeine, reaching a level throughout the tube below that observed before caffeine treatment (Figs. 11C and 11D). This particular tube was slow to recover; after an hour in caffeine-free culture medium it started to reform the tip-focused gradient (Fig. 11E) and then generated a large Ca\(^{2+}\) transient (Fig. 11F). Thereafter, the tube tip underwent extensive swelling before resuming directed growth (Fig. 11G). Figure 12 further reveals that caffeine application (3 mM) also virtually eliminated the extracellular Ca\(^{2+}\) distribution in a lily pollen tube. The linear traces on the right correspond to the ratio images shown on the left. (A) 30 sec before treatment, showing a normal tip-focused gradient. (B) 1 min after the addition of caffeine. The tip-focused gradient has diminished and now there is a large bolus of Ca\(^{2+}\) back from the tip. (C) 1 min, 30 sec after the addition of caffeine. The [Ca\(^{2+}\)] in the entire tube has dropped to basal levels. (D) 30 sec after the removal of caffeine. There is no growth and the overall [Ca\(^{2+}\)] is very low throughout the tube. (E) 68 min, 30 sec after the removal of caffeine. The tube is finally beginning to recover, and a tip-focused gradient has reemerged. (F) 72 min, 15 sec after the removal of caffeine. The tip begins to swell, and the [Ca\(^{2+}\)] undergoes a huge increase. Note in the line trace that the scale has been changed, when compared to the others, in order to show the magnitude of the Ca\(^{2+}\) change. (G) 86 min, 30 sec after the removal of caffeine. The tube is finally beginning to recover, and a tip-focused gradient has reemerged. (F) 72 min, 15 sec after the removal of caffeine. The tip begins to swell, and the [Ca\(^{2+}\)] undergoes a huge increase. Note in the line trace that the scale has been changed, when compared to the others, in order to show the magnitude of the Ca\(^{2+}\) change. (G) 86 min, 30 sec after the removal of caffeine. This particular tube underwent extensive swelling at the tip before it finally reestablished a tip-focused gradient and reinitiated cylindrical elongation. Bar, 20 μm.

FIG. 11. Effect of 1.5 mM caffeine application on the growth and Ca\(^{2+}\) distribution in a lily pollen tube. The linear traces on the right correspond to the ratio images shown on the left. (A) 30 sec before treatment, showing a normal tip-focused gradient. (B) 1 min after the addition of caffeine. The tip-focused gradient has diminished and now there is a large bolus of Ca\(^{2+}\) back from the tip. (C) 1 min, 30 sec after the addition of caffeine. The [Ca\(^{2+}\)] in the entire tube has dropped to basal levels. (D) 30 sec after the removal of caffeine. There is no growth and the overall [Ca\(^{2+}\)] is very low throughout the tube. (E) 68 min, 30 sec after the removal of caffeine. The tube is finally beginning to recover, and a tip-focused gradient has reemerged. (F) 72 min, 15 sec after the removal of caffeine. The tip begins to swell, and the [Ca\(^{2+}\)] undergoes a huge increase. Note in the line trace that the scale has been changed, when compared to the others, in order to show the magnitude of the Ca\(^{2+}\) change. (G) 86 min, 30 sec after the removal of caffeine. The tube is finally beginning to recover, and a tip-focused gradient has reemerged. (F) 72 min, 15 sec after the removal of caffeine. The tip begins to swell, and the [Ca\(^{2+}\)] undergoes a huge increase. Note in the line trace that the scale has been changed, when compared to the others, in order to show the magnitude of the Ca\(^{2+}\) change. (G) 86 min, 30 sec after the removal of caffeine. This particular tube underwent extensive swelling at the tip before it finally reestablished a tip-focused gradient and reinitiated cylindrical elongation. Bar, 20 μm.

FIG. 13. Effect of mild temperature shock (10 sec at 37–40°C followed by 20 sec at 4°C) on growth and Ca\(^{2+}\) distribution in a lily pollen tube. The linear profiles on the right correspond to the ratio images shown on the left. (A) 30 sec before thermal shock. The growing pollen tube displays a prominent tip-focused gradient. (B) 2 min after treatment. The gradient has dissipated and growth has stopped. (C) 4 min, 45 sec after treatment. The Ca\(^{2+}\) remains low. (D) 5 min, 45 sec after treatment, or 1 min after C. There is a large increase in intracellular Ca\(^{2+}\) as the tip-focused gradient reforms. (E) 6 min, 45 sec after treatment. The pollen tube tip swells. (F) 8 min, 15 sec after treatment. Tip swelling has continued, but localized growth is being reinitiated. Bar, 20 μm.
Tip-Localized Calcium Entry Fluctuates deviates as shown in Figs. 15A-15C, an observation that has been noted previously by Malhó et al. (1994) in studies on Agapanthus pollen. It is noteworthy, however, that the position of the tip-focused gradient denotes the position and direction of elongation (Figs. 15B and 15C).

DISCUSSION

The results provide greater detail than has been possible heretofore concerning the spatial distribution of the tip-focused $\text{Ca}^{2+}$ gradient. The exponential decay of the $\text{Ca}^{2+}$ gradient extending back from the tip indicates a substantial capacity of the apical cytoplasm to sequester or extrude the ion. When we compare these results with the ultrastructure of the terminal $20 \mu$m of the tube (Lancelle and Hepler, 1992), it becomes apparent that the sequestration must be mainly accomplished by the endoplasmic reticulum or the accumulating Golgi vesicles since there are no other organelles in the tip region of the tube. Given the evidence for $\text{Ca}^{2+}$ pumps on the ER (Evans, 1994), this organelle would appear to be the prime candidate for the sequestration activity and thus in creating the sharp gradient.

Not only is the gradient steep, but its source appears to be limited to a small area of the plasma membrane, possibly only $2.5 \mu$m in diameter or less, at the extreme apex of the tube. Comparative measurements of areas only a few micrometers from the extreme apical zone indicate that

![FIG. 4. $\text{Ca}^{2+}$ concentrations at various sites in the cortex of the tip of a growing lily pollen tube. In complete agreement with the pseudocolor image in Fig. 3, the highest values are only found in the extreme apex. Other cortical regions away from the apex show lower values.](image-url)

![FIG. 5. $\text{Ca}^{2+}$ concentrations at various distances from the extreme apex of a growing lily pollen tube determined from transverse scans of the ratio values. The data show that the $[\text{Ca}^{2+}]$ rapidly drops at increasing distances from the tip. Trace A, extreme apex; trace B, 2.5 $\mu$m; trace C, 5 $\mu$m; and trace D, 40 $\mu$m. Note that traces B-D are basically flat and not "U" shaped, indicating that there is no appreciable $\text{Ca}^{2+}$ entry at the cell periphery.](image-url)
the Ca\(^{2+}\) level dramatically drops, emphasizing the spatial restriction of the process of Ca\(^{2+}\) entry. These conclusions are pertinent to the process of growth since they suggest that vesicle fusion would similarly be restricted to this limited area. Further, as vesicles fuse and new plasma membrane becomes inserted, the old plasma membrane is swept rapidly to the flanks; for example, if the pollen tube is elongating at 0.25 μm/sec (15 μm/min) (Figs. 9A and 9B), an area of membrane 2.5 μm in diameter at the extreme apex would be displaced in less than 0.5 sec. Since Ca\(^{2+}\) entry no longer occurs away from the apex of the tip, by some mechanism the channels must either be removed or inactivated. It can be entertained that there is a special mechanism that selectively retains Ca\(^{2+}\) channels at the tip; however, we favor the idea that new channels are introduced with the fusion of Golgi vesicles at the tip and that the channels already present are displaced and inactivated as the membrane is swept aside.

It seems plausible that these channels are primarily stretch activated (Cosgrove and Hedrich, 1991; Ding and Pickard, 1993; Bush, 1995). While direct evidence for stretch activated channels in pollen tubes is lacking, the sensitivity of Ca\(^{2+}\) entry to hypertonic media (Pierson et al., 1994) and lanthanides (Málho et al., 1995) can be construed as support for their activity. In other tip growing systems, notably fungal hyphae, stretch activated channels have been recently identified using patch clamp recording (Zhou et al., 1991; Garrill et al., 1992; 1993; Levina et al., 1994), making it further reasonable to postulate their presence at the tip of the pollen tube. If we assume that the channels require a certain threshold of stretching in order to open, it is possible to explain their activation/inactivation profiles as a function of the surface expansion of the plasma membrane across the pollen tube dome. Pollen tubes have a hemispherical shape at their tip and it has been argued that the displacement of membrane would follow a cosine function, with a maximum at the extreme apex, falling to zero at the base of the dome (Green, 1974). Here we simply suggest that the putative mechanosensitive Ca\(^{2+}\) channels are regulated by a similar gradient, with the added proviso that their threshold is set at a high level such that only those channels undergoing maximal stretching will be open. With this view, open channels will be restricted to the extreme apex of the pollen tube, and as they are swept away from the apex they will experience decreasing, or subthreshold, stretching and accordingly will close. A process of rapid channel inactivation might help explain why virtually any treatment that blocks growth leads to a rapid dissipation of the tip-focused gradient (Pierson et al., 1994; observations herein). The results also clearly reveal that the tip-focused intracellular Ca\(^{2+}\) gradient fluctuates substantially in magnitude (fourfold or more) during normal tube elongations. Because

**FIG. 7.** Comparative plots of the Ca\(^{2+}\) ratio values and growth rates on the same tube indicate that they are correlated. Generally, as the [Ca\(^{2+}\)] increases so does the growth rate.

**FIG. 8.** Relationship between the extracellular Ca\(^{2+}\) influx close to the tip of lily pollen tubes and their growth rate. The regression line (solid line) corresponding to the following: y = A + Bx can be expressed either as a function of growth rate x (in μm/min) and voltage output y (μV), whereby A = 14.4 μV and B = 3.41 μV/min/μm, or as a function of growth rate x (in μm/min) and Ca\(^{2+}\) flux y (in pmole/cm²/sec), whereby A = 1.76 pmole/cm²/sec and B = 0.42 pmole/cm³. N = 60; r = 0.46; dotted line represents the 0.95 confidence limits.
Tip-Localized Calcium Entry Fluctuates

FIG. 9. Detailed analysis of the growth rates of lily pollen tubes of different overall lengths. Measurements at both high temporal resolution and high spatial resolution indicate, for tubes longer than 700 μm, that the growth oscillates with a period around 23 sec (A, B), while for shorter tubes the change in rate is more erratic with periods of even growth being punctuated by excursions of slower and faster growth (C).

these fluctuations are positively correlated with the rate of growth, the results support the conclusion that the magnitude of the tip-focused gradient controls the growth of the pollen tube; the higher the [Ca²⁺], the faster the rate of elongation. In previous work based on different techniques, intense accumulations of total Ca²⁺ (Jaffe et al., 1975), and pulses of net inward current (Weisenseel et al., 1975) have been reported in a small percentage of lily pollen tubes longer than 1 mm. Although their relationship with the fluctuations described herein is not established, these may be caused by a similar underlying mechanism. In the present study on the tip-focused gradient there are problems in determining the periodicity of the fluctuations since we have been constrained by a variety of factors including the difficulty of obtaining ratio images rapidly with our measuring system as presently configured. Nevertheless, we have been able to accurately measure the fluctuation in growth rate itself; tubes shorter than 700 μm show erratic changes in growth rate within intervals of 20–50 sec, whereas tubes longer than 700 μm show regular oscillations with a period of about 23 sec. These detailed growth measurements further show that the rate can vary as much as three- to fourfold, a result that stands in remarkably close agreement with the magnitude of Ca²⁺ change observed. While it seems likely

FIG. 10. Effect of 1.5 mM caffeine application and removal on the morphology of a lily pollen tube, viewed with differential interference contrast optics. (A) 3 min before the addition of caffeine. The pollen tube exhibits normal morphology with a prominent clear zone in the apex. (B) A few seconds before the addition of caffeine. (C) 3 min after the addition of 1.5 mM caffeine. Note that pollen tube growth ceased quickly and that the clear zone has diminished somewhat from B. (D) 3 min caffeine, followed by 20 min recovery in control medium. The pollen tube tip has begun to swell and numerous organelles have invaded the apical region. (E) 22 min in recovery. The clear zone is reemerging. (F) 25 min in recovery. The tube has shifted from global swelling to cylindrical elongation. (G) 29 min, 30 sec in recovery. Normal growth has resumed, although the tube is wider than it was initially in A. Bar, 10 μm.
that the rapid growth rate changes are anticipated by similar changes in intracellular $[\text{Ca}^{2+}]$, further work is needed to make the direct comparison. In these studies it will be important to elucidate the characteristics of the $\text{Ca}^{2+}$ oscillations since these may be a central aspect of cellular control as seems evident in animal systems (Tsien and Tsien, 1990).

Growth fluctuations in tip growing systems have recently gained attention from studies of both fungal hyphae (Lopez-Franco et al., 1994) and pollen tubes (Tang et al., 1992; Li et al., 1992, 1994; Pierson et al., 1995). Early studies from Castle (1940) revealed rapid fluctuations in Phycomyces, but more recently Lopez-Franco et al. (1994) have demonstrated both random and periodic fluctuations in several different fungal hyphae. Interestingly, the results for lily pollen closely resemble those for fungal hyphae. Among pollen tubes the most dramatic fluctuations that have been observed occur in Petunia, Nicotiana, and Gasteria where Pierson et al. (1995) report that the tube grows slowly for 0.5 to a few min and then during a period of 20-sec pulses forward at a rapid rate, before returning to slow growth. Of particular note, these periods of slow growth correlate with an annular deposition of both arabinogalactans (Li et al., 1992) and methylated pectins (Li et al., 1994) in the cell wall. It seems plausible that all these fluctuations are in part regulated by an internal tip-focused $\text{Ca}^{2+}$ gradient; however, the proof awaits further experimentation.

The presence of high intracellular $\text{Ca}^{2+}$ at the point of vesicle fusion adds weight to the idea that the ion controls the docking and/or fusion of the vesicles with the plasma membrane, and thereby the exocytotic growth process (Almers, 1990; Blackbourn et al., 1992; Creutz, 1992; Gilroy and Jones, 1992; Llinas et al., 1992; Zorec and Tester, 1992; Battey and Blackbourn, 1993; Blackbourn and Battey, 1993). It seems likely that these events are mediated by $\text{Ca}^{2+}$-binding proteins, for example, the annexins, which recent work reveals are localized in the tips of lily pollen tubes (Blackbourn et al., 1992). If we extrapolate from the in vitro data reported herein to the situation in vivo an intriguing question arises. Does the substantially greater growth rate (up to fourfold faster) (Janson, 1992) of the pollen tube in the style correlate with an equally elevated tip-focused intracellular $\text{Ca}^{2+}$ gradient? In other words, if the gradient in in vitro growing pollen tubes already exceeds 3 $\mu$M, perhaps even reaches 5 to 10 $\mu$M (Pierson et al., 1994; observations herein), might the gradient in pollen tubes growing in vivo reach 10–40 $\mu$M? While these values seem extremely high, they are not unreasonable. Recent studies on nerve terminals undergoing rapid vesicle fusion exhibit values from 100 to 200 $\mu$M (Llinas et al., 1992). These high levels also approach the half-maximal binding of $\text{Ca}^{2+}$ by annexin, which has been determined to be 50–150 $\mu$M (Blackbourn and Battey, 1993). Since it is known that some pollen tubes in the style grow at different rates, with the faster ones exhibiting a selective advantage regarding their success in fertilization (Snow and Spira, 1991), it becomes attractive to suggest that the magnitude of the tip-focused gradient contributes fundamentally to these variations. Of course, the extremely rapid growth of the tube in the style could be due to other factors, such as the interaction of the male gametophyte with the transmitting cells, in which the association of intracellular cytoskeletal elements with components of the extracellular matrix participate in moving the pollen tube forward (Sanders and Lord, 1992; Juah and Lord, 1995). Although technically difficult, it will be important in the future to determine $\text{Ca}^{2+}$ levels in pollen tubes growing in vivo.

FIG. 12. The extracellular $\text{Ca}^{2+}$ flux close to the tip of lily pollen tubes treated with 3.0 mM caffeine. Before caffeine (black bars) all eight tubes show an influx of $\text{Ca}^{2+}$ at the tip. After 10 min in 3.0 mM caffeine the influx is essentially eliminated (white bars). When the caffeine is removed and the tubes allowed to recover for 30 min, four of the eight tubes, including numbers 2, 4, 5, and 7 resumed growth. Only these tubes exhibited an appreciable influx of $\text{Ca}^{2+}$.

FIG. 14. Extracellular $\text{Ca}^{2+}$ flux at the tip of a lily pollen tube recovering from mild temperature shock. Influx is plotted against time. The dotted vertical lines indicate points where the electrode was repositioned. At (A) the growth is still inhibited and no influx is observed. However, within 100 sec the influx rises to a maximum (B) concomitantly with induction of tip swelling. The influx levels then drop to intermediary values (C, D) as the tube switches from swelling to directed growth. (E) A reference measurement.
The studies reported herein show that caffeine or mild thermal shock rapidly inhibit pollen tube growth, and further that this inhibition is correlated with a dissipation of the tip-focused Ca\textsuperscript{2+} gradient and an elimination of the extracellular influx. These observations thus are similar to our earlier findings using BAPTA buffers to block growth (Pierson et al., 1994). We were particularly intrigued by the caffeine results since considerable work in animal systems indicates that this alkaloid causes a release of Ca\textsuperscript{2+} (Endo, 1977; Callewaert et al., 1989; Galione and White, 1994). Often this condition is characterized by the elevation of Ca\textsuperscript{2+} from internal stores, quite likely the ryanodine receptor (Galione and White, 1994), together with subsequent inhibition of reuptake. As a consequence we had been on the lookout for elevations of Ca\textsuperscript{2+} following caffeine application, however, none was observed, although we did note an apparent wave of high Ca\textsuperscript{2+} traveling from the apex of the tube basipetally for several micrometers. However, this phenomenon had already been observed following BAPTA buffer injection, and thus is not specific to caffeine. Indeed, not only did the caffeine not cause an increase in Ca\textsuperscript{2+}, in agreement with previous studies on stamen hair cells (Keifer et al., 1992), instead it led to a slight overall decline in the basal level of the ion throughout the tube. Caffeine has been used extensively to perturb plant cell processes, such as cell plate formation (Bonsignore and Hepler, 1985; Hepler and Bonsignore, 1990; Battey and Blackbourn, 1993) and tracheary element differentiation (Roberts and Haigler, 1992), and although many authors implicate an interaction with the mechanism of Ca\textsuperscript{2+} homeostasis, these are the first studies on plant cells to provide direct evidence that Ca\textsuperscript{2+} is indeed modulated. The rapidity with which caffeine completely diminished the tip-focused gradient in pollen tubes raises the interesting question of whether this is the basis for its ability to inhibit other processes such as cell plate formation. On the other hand, perhaps the Ca\textsuperscript{2+} effect we observe is only secondary, with the primary action of caffeine being the inhibition or retardation of vesicle fusion, and the consequent inhibition of growth followed by dissipation of the tip-focused gradient due to insufficient activation of stretch-dependent ion channels.

The studies with caffeine and mild thermal shock, in addition to providing further evidence for the connection between the tip-focused Ca\textsuperscript{2+} gradient, the tip-directed Ca\textsuperscript{2+} influx, and tube elongation, have also made it possible to elucidate the growth recovery process and in particular to decipher the Ca\textsuperscript{2+} changes associated therewith. As is shown herein, recovery includes a swelling of the entire terminal region of the tube, together with a massive entry of Ca\textsuperscript{2+}. We suggest that the strain deformation caused by swelling activates stretch-dependent channels, and this accounts for the increases in Ca\textsuperscript{2+} entry (Ding and Pickard, 1993). How the tip converts from global swelling to directed cylindrical extension is less well defined, but may involve the chance accumulation or activation of a group of channels in a given locus, possibly brought about by the yielding of the wall to a greater extent within a local region. Whatever the controlling factor, it need not bear any relationship to the previous axis of growth since the tube can and often does elongate in a new direction. Although Malho et al. (1994) have reported that these changes in direction are caused by experimental conditions that elevate levels of Ca\textsuperscript{2+}, we find in contrast that any condition which inhibits growth can lead to new directions during the recovery phase. From the observations it appears that once a localized region in the swelling dome becomes defined, even partially, the positive feedback mechanism rapidly amplifies its activity, quickly converting it from a slightly preferred region of Ca\textsuperscript{2+} entry and vesicle fusion to one that dominates and dictates cylindrical extension. Viewed in this way, cell polarity would appear to be only loosely regulated, being defined by local perturbations that can self-amplify.

During the course of this study novel and important information concerning the use of fluorescent dyes in pollen tubes has emerged. Regarding pathlength, it is evident that the shape of the tube profoundly affects the fluorescent signal. However, it has also been thought that the zonation of the organelles, with small Golgi vesicles accumulated at the tip, and large amyloplasts back from the tip, would create different relative amounts of accessible cytoplasm. The studies herein performed on tubes that were entirely flat, aside from the changes in shape at the tip of the tube, indicate that the cylindrical portions contain a constant amount of accessible cytoplasm. The current observations showing that the path-length, once it reaches its maximum at 10–15 \(\mu\text{m}\) behind the tip, remains constant for at least the next 100 \(\mu\text{m}\), differ somewhat from that which we published earlier (Miller et al., 1992). Previously we had shown that the single wavelength signal declined toward the base of the tube. We now realize that it is absolutely essential for these measurements to be made on tubes that are completely flat; any deviation from the medial focal plane causes the signal to decline. However, when performing ratiometric analysis, variations of path-length are cancelled, permitting one to observe the Ca\textsuperscript{2+} concentration. The same is not true for single wavelength indicators, such as fluo-3 and Calcium Green, even when used with the confocal microscope (Herth et al., 1990; Franklin-Tong et al., 1993; Malho et al., 1994). This may explain why these studies have failed to detect a Ca\textsuperscript{2+} gradient in the tip and systematically showed the highest Ca\textsuperscript{2+} values in the central part of the pollen tube cylinder. It becomes imperative, therefore, in all studies where path-length is an issue to use ratiometric procedures.

In conclusion, these various aspects of Ca\textsuperscript{2+} behavior, including natural fluctuations, localized tip entry, and rapid channel inactivation, permit us to develop a model for pollen tube growth. We continue to support the idea of a positive feedback loop in which the high Ca\textsuperscript{2+}, brought about by the local activity of stretch-dependent channels, promotes vesicle fusion in restricted areas at the extreme apex of the tube, and thus tip growth (Steer, 1988; Harold, 1994; Hepler et al., 1994; Pierson et al., 1994; Feijo et al., 1995). An essential part of this scheme is the idea that changes in the extensibility of the cell wall at the tip occupy a prime position in the control of growth. Stated simply, when the wall yields to the turgor pressure there will be greater defor-
formation and stretching of the plasma membrane in that region, leading to increased Ca^{2+} entry, vesicle fusion, and growth. At the moment it is less clear how the periods of slow growth are brought about, but any condition that stiffens the wall and reduces its extensibility will reduce stretching of the plasma membrane, promote channel closure, and reduce Ca^{2+} entry.

These thoughts focus our attention more acutely on the cell wall, which in pollen tube tips is known to be composed largely of esterified pectin with smaller amounts of acidic pectin residues (Li et al., 1994). A change in the balance of these two components could markedly alter the stretching of the cell wall, with the esterified pectins being much more plastic and extensible than the acidic units, especially when the latter are linked by Ca^{2+} cross bridges (Grant et al., 1973). While the complexity of the wall opens the door to many possible factors as potential regulators of tip growth, the ability to monitor the magnitude and spatial distribution of the underlying Ca^{2+} gradients, and to correlate them with growth characteristics while probing cell wall properties, may make it possible to gain new insight about basic growth mechanisms in plant cells.

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