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# Free $Ca^{2+}$ gradient in growing pollen tubes of *Lilium*

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## Summary

**Fluorescence ratiometric imaging of *Lilium* pollen tubes loaded with the $Ca^{2+}$ indicator Fura-2 dextran has revealed a distinct elevation of free intracellular calcium ion concentration ($[Ca^{2+}]_i$) at the extreme tip of actively growing *Lilium* pollen tubes that declines to a uniform basal level of ~170 nM throughout the length of the tube. The calcium gradient occurs within the first 10-20 μm proximal to the tip. Experimental inhibition of tip growth, usually achieved through the injection of the $Ca^{2+}$ buffer 5,5′-dibromo BAPTA, results in the loss of the $[Ca^{2+}]_i$ gradient. Occasionally these inhibited cells reinitiate growth, and when they do so ratio imaging reveals that the tip gradient of free $[Ca^{2+}]_i$ re-emerges. The results presented here are very different from those previously published by revealing the presence of the $[Ca^{2+}]_i$ gradient that is restricted to the 10-20 μm adjacent to the tube tip. Further, these experiments demonstrate a strict correlation between the presence of a $[Ca^{2+}]_i$ gradient, and tip growth in *Lilium* pollen tubes.**

Key words: free $Ca^{2+}$ gradient, Fura-2 dextran, pollen tubes, polarity, tip growth.

## Introduction

The fundamental process involved in growth and development of pollen tubes is the acquisition and expression of polarity. This vital process in higher plants is necessary to deliver the male gametes to the egg apparatus and thus is one of the key events in the control of sexual reproduction. The possible connection between calcium and pollen tube growth has been recognized for many years (Brewbaker and Kwack, 1963; vanderWoude and Morré, 1974; Mascarenhas and Lafountain, 1972; Picton and Steer, 1983). It is well known, for example, that calcium in the culture medium is essential for tip growth (Steer and Steer, 1989). The spatial distribution of calcium ion channels is believed to lie almost exclusively in the tip region (Weisenseel and Jaffe, 1976), and agents that interfere with $Ca^{2+}$ uptake prevent elongation (Picton and Steer, 1985; Reiss and Herth, 1985).

Further studies have shown that calcium influx is localized at the tube tip (Weisenseel and Jaffe, 1976; Kühtreiber and Jaffe, 1990), a process that probably contributes to the elevated levels of total and membrane-bound calcium observed in this region (Reiss et al. 1983; Reiss et al. 1985). It has also been reported that free calcium is higher in the tip (Reiss and Nobiling, 1986; Nobiling and Reiss, 1987), but, due to experimental and technical difficulties with the measurement of calcium ion concentration in this compartment, there remain many questions about the existence, magnitude and spatial profile of this putative free calcium gradient.

In the present study we have reinvestigated the issue of free calcium gradients in pollen tubes. Using a newly developed form of the indicator, Fura-2, that has been covalently linked to dextran to prevent its sequestration into membrane compartments, we provide evidence for a very steep gradient in free cytoplasmic calcium that is focused at the tip of the growing pollen tube. We further show that dissipation of this gradient can be achieved experimentally with the injection of the calcium buffer 5,5′-dibromo BAPTA under which conditions the tubes stop growing. Reinitiation of growth can occur in previously inhibited cells, whereupon the calcium gradient re-emerges. These studies establish a strong connection between the free calcium gradient and the process of pollen tube elongation.

## Materials and methods

### *Pollen germination*

Pollen of *Lilium longiflorium* was sown and allowed to germinate in a solution of 10% sucrose, 1 mM $Ca^{2+}$, 0.16 mM boric acid, and 15 mM MES buffer, pH 5.5 (Lancelle and Hepler, unpublished data), in a 500 μl microfuge tube on a rotator. After germination the pollen was transferred to a culture chamber slide with a small drop of melted 2% agarose

in culture medium. The pollen/agarose mixture was then gently spread over the surface of the coverslip, creating a thin layer. The slide chamber with cells was then chilled for a few seconds in order to gel the agarose and thus firmly secure the tubes to the coverslip. The preparation was flooded with liquid culture medium and covered to keep the cells moist. The pollen tubes were allowed to grow to a length of approximately 300-400 μm before injection (tubes smaller than this were difficult to pressure inject sucessfully).

### *Fura-2 dextran injection and ratio imaging*

Pollen tubes were loaded with calcium indicator by pressure injecting a small volume of Fura-2 dextran (10,000 $M_r$), 20 mg/ml in 100 mM KCl (the Fura-2 dextran was kindly provided by Molecular Probes, Inc., Eugene, OR 97402, USA, 12-17-90, no lot number). Micropipettes were pulled from filamented 1.0 mm diameter glass (cat. no. 1B100F-4, WPI, Sarasota, FL 34240-9258, USA). The actual volume delivered was variable but gave adequate brightness to allow exposures of 50-100 ms at 365 nm excitation with our charge-coupled device camera. Only those injected cells showing normal, rapid streaming and elongation after a brief recovery period to allow uniform distribution of the dye were used for observation and experimental manipulation. Images of the fluorescence (500 nm long-pass filtered) excited at 365 nm and 334 nm, respectively, with 1:10 relative exposure times were acquired in rapid succession on a charge-coupled device imaging system operating in pseudo-frame-transfer mode. Background images composed of the fluorescence signal detected at the same plane of focus but just adjacent to the cells were acquired in the same way. Ratio images (334 nm/365 nm) were computed from background-subtracted images of the pollen tubes and displayed as pseudocolor modulated in brightness by the intensity of the relatively calcium-insensitive denominator (365 nm) image as previously described (Linderman et al. 1990). This produces a display in which colors indicate ratio levels and brightness is proportional to the dye concentration. Autofluorescence of the cell cannot be subtracted from the dye signal, since the cell is constantly elongating and changing in shape, and thus autofluorescence images taken before the introduction of Fura-2 dextran are not appropriate at later times. Fortunately, the autofluorescence from these cells is very even and much weaker than the Fura-2 dextran fluorescence (Fig. 3 illustrates relative signal amplitudes). To exclude areas with insignificant signal levels (background, vacuoles, wall autofluorescence), the ratio values were set to zero if the calcium-insensitive denominator values were below a threshold level set by the user. Line scan plots from ratio images were created on an Image-1 system (Universal Imaging Corporation, Media, PA 19063, USA).

### *Iontophoretic injection*

Iontophoretic injections were made by passing the specified current (regulated, ±1%) through a micropipette that had its tip filled with 5 mM 5,5′-dibromo BAPTA (Molecular Probes, Inc., Eugene, OR 97402, USA) titrated to 100 nM free [$Ca^{2+}$] or 10 mM KCl for the negative current controls. The iontophoretic micropipettes were drawn from filamented capillary tubing (described above) and typically had a resistance of 10-15 MΩ when filled with 3 M KCl for testing. Current source compliance voltage was monitored during injections to confirm the high conductance of the micropipettes.

### *Calibrations*

The response of the Fura-2 dextran (10,000 $M_r$) to [$Ca^{2+}$] was calibrated *in vitro* by ratio imaging uniform 165 μm thick layers of Fura-2 dextran (40 μg/ml) in 2.5 mM BAPTA, 2.5 mM HEPES (pH 7.0), 100 mM KCl and 60% (w/w) sucrose. The BAPTA buffer component of the mixture was set to various [$Ca^{2+}$] values by adjusting the ratio of calcium-free and calcium-bound BAPTA stocks. The sucrose was included to mimic the effects of cytoplasmic viscosity on the fluorescence ratio (Poenie, 1990), although this effect has not been characterized for the dextran-conjugated form of the dye.

### *Growth rates*

The growth rates of pollen tubes were recorded by video microscopy. Measurements were made prior to injection, then after pressure injection of Fura-2 dextran, and again after iontophoretic injection of either KCl or 5,5′-dibromo BAPTA. Growth rate values were determined on the Image-1 track point program (Universal Imaging Corporation, Media, PA 19063, USA) and were followed by 1-way analysis of variance with repeated measures (Afifi and Azen, 1972).

## Results

Fluorescence ratiometric analysis of dye-loaded growing tubes reveals a region with elevated $[Ca^{2+}]_i$ located near the tip (Fig. 1A), which drops off sharply within 10-20 μm. A plot of the ratio value along the length of the pollen tube in these images provides a quantitative measure of the steepness and magnitude of the $[Ca^{2+}]_i$ gradient (Fig. 1B). Through calibration we have determined that the basal level of $[Ca^{2+}]_i$ is ~170 nM, with the highest values at the extreme tip of the tube reaching 490 nM. These high values at the tip are most likely below the true peak values due to spatial resolution limitations (~2 μm) dominated by the 2 × 2 pixel binning used to obtain the images. Within the molecular dimensions of the plasma membrane at the tip itself the intracellular concentration of $Ca^{2+}$ might reach considerably higher values, particularly if the ion enters the tip through plasma membrane channels (Chad and Eckert, 1984).

To probe the function of this gradient, dye-loaded cells were injected with 5,5′-dibromo BAPTA, a $Ca^{2+}$ buffer that has been proposed to inhibit development by facilitating the diffusion of $Ca^{2+}$ and thus dissipating a concentration gradient of the ion (Speksnijder et al. 1989). Presumably, if the buffer has a $Ca^{2+}$ dissociation constant between the high and low concentration of the gradient then it will preferentially bind free $Ca^{2+}$ at regions of high concentration and quickly diffuse to regions of low concentration where the ion will be released. This process, referred to as "shuttle buffering", is thought to be the underlying mechanism by which BAPTA inhibits development of fucoid eggs (Speksnijder et al. 1989), and may be a generally applicable tool for probing $Ca^{2+}$ gradients in other systems (Pethig et al. 1989).

Iontophoretic injection of 5,5′-dibromo BAPTA into *Lilium* pollen tubes at 2 nA for 2 min stopped growth in 12 out of 15 cells. The sequence depicted in Fig. 1 includes an image of the cell just prior to injection of 5,5′-dibromo BAPTA (Fig. 1A,B), when the cell is

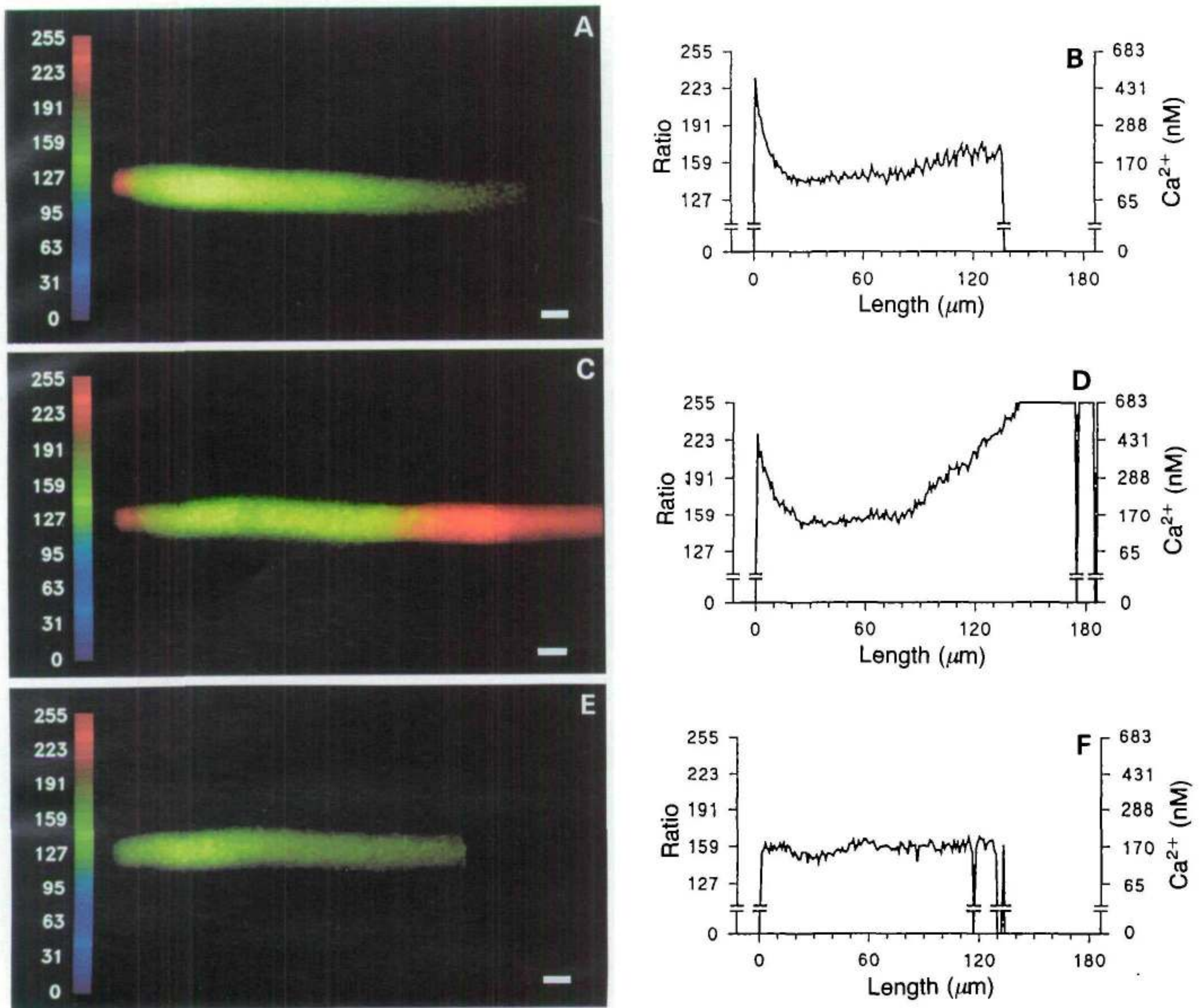


**Fig. 1.** Ratiometric images and plots of the ratio values along the length of the pollen tube indicate the $[Ca^{2+}]_i$ in growing and non-growing pollen tubes. (A) Ratiometric image of $[Ca^{2+}]_i$ in a growing pollen tube. (B) Plot of ratio values along the central axis of A indicating the presence of a calcium gradient in a growing pollen tube. The gradient occurs within the first 10-20 μm proximal to the tip and the $[Ca^{2+}]_i$ thereafter is at a relatively uniform basal level. (C) Ratiometric image of the same tube during iontophoretic injection of 5,5′-dibromo BAPTA for 2 min at 2 nA. (D) Plot of the ratio values for C showing the presence of a gradient and a large increase in $[Ca^{2+}]_i$ near the site of 5,5′-dibromo BAPTA injection. (E) Subsequent ratio image of growth inhibited pollen tube 17 minutes post-injection. (F) Plot of the ratio values for E depicting the loss of the $[Ca^{2+}]_i$ gradient during inhibition of growth. Bar, 10 μm.

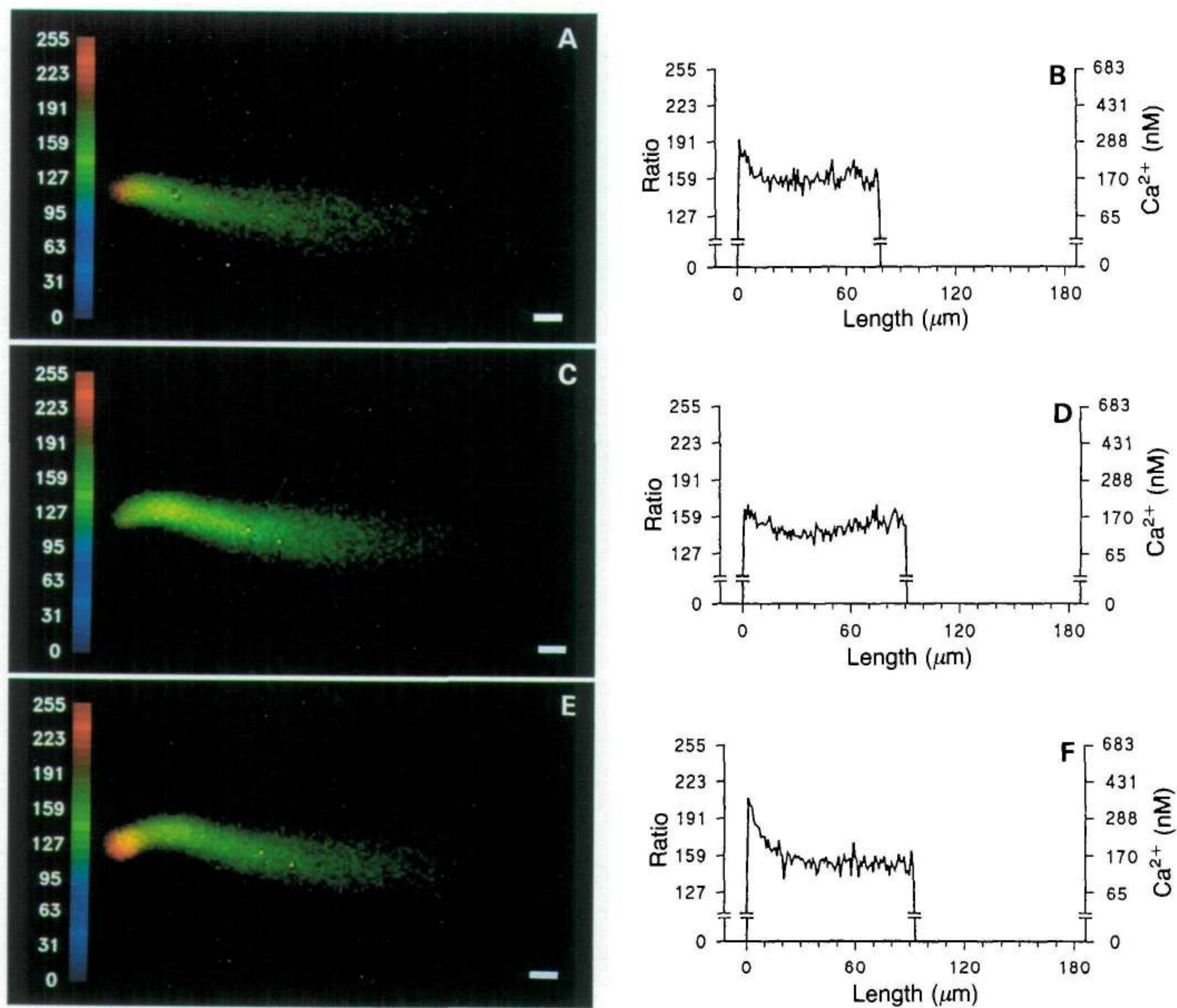


**Fig. 2.** Ratiometric images and plots depicting the recovery of growth in an inhibited pollen tube. (A,B) Ratiometric image and plot of a line of pixels in a growing pollen tube immediately after injection of 5,5′-dibromo BAPTA; the gradient at the tip is reduced. (C,D) Image and plot of the same cell after inhibition of growth. (E,F) Image and plot after the cell has re-established a gradient and is once again growing. Bar, 10 μm.

**Table 1.** *Pollen tube growth rates and statistical analysis*

A. *Growth rates*

| | (a) KCl treatment ($\mu$m/s) | (b) 5,5'-dibromo BAPTA treatment ($\mu$m/s) |
|---|---|---|
| Preinjection | 0.106±0.026 | 0.083±0.028 |
| Fura-2 dextran | 0.105±0.030 | 0.095±0.019 |
| KCl | 0.118±0.023 | – |
| 5,5'-dibromo BAPTA* | – | 0.000±0.000 |
| 5,5'-dibromo BAPTA† | – | 0.121±0.020 |

*5,5'-dibromo BAPTA-injected cells in which growth was inhibited.
†5,5'-dibromo BAPTA-injected cells in which growth was not inhibited.

B. *Statistical analysis: one way analysis of variance with repeated measures*

| | *n* | *F* | *d.f.* | Prob. |
|---|---|---|---|---|
| 1. Preinjection rates treatments (a) and (b) | 5 | 1.9 | (1,8) | 0.206 |
| 2. Fura-2 dextran rates treatments (a) and (b) | 5 | 0.43 | (1,8) | 0.531 |
| 3. KCl *vs* 5,5'-dibromo BAPTA (* above) | 5 | 200.4 | (1,8) | 0.000 |
| 4. KCl *vs* 5,5'-dibromo BAPTA († above) | 4 | 0.08 | (1,7) | 0.788 |

*n*, number of individual cells.
*F*, ratio of the mean square of between groups over the within groups; values greater than 4.0 are significant.
*d.f.*, degrees of freedom of numerator and denominator of the mean square.
Prob., probability that the *F* ratios of this size could have occurred by chance.

growing and has a typical gradient. During the injection (Fig. 1C,D) the gradient is still present at the tip; however, there is a large increase in the $[Ca^{2+}]_i$ near the site of the 5,5'-dibromo BAPTA injection. Even though the 5,5'-dibromo BAPTA had been titrated with $Ca^{2+}$ to a level near that of the resting cell in order to prevent extensive lowering of the intracellular $[Ca^{2+}]$, it nevertheless caused an increase in $Ca^{2+}$ at the injection site. Within 3-6 minutes, however, the elevated $Ca^{2+}$ at the injection site as well as that at the tip was reduced to basal levels (Fig. 1E,F) and the pollen tube was no longer elongating although a normal streaming rate was maintained. In several instances, pollen tubes inhibited by 5,5'-dibromo BAPTA recovered; i.e. they re-established a gradient and reinitiated growth (Fig. 2). The sequence shown in Fig. 2 depicts recovery, starting first with a growing cell immediately after injection of 5,5'-dibromo BAPTA showing a reduced gradient (Fig. 2A,B), followed by an image of the inhibited cell (Fig. 2C,D) and finally an image that reveals the growth of this tube and that the gradient has re-formed (Fig. 2E,F).

Microinjection of chloride, achieved by passing a negative current from a 10 mM KCl-filled micropipette, serves as a control for the negative current that is used to deliver 5,5'-dibromo BAPTA. These injections have no effect on the growth of the pollen tube (Table 1) or on the $[Ca^{2+}]_i$ gradient and only localized but very limited increases in $[Ca^{2+}]$ are observed at the injection site. With a different kind of injection, in which the pollen tube is pricked under conditions that cause the expulsion of a small volume of cytoplasm but do not generate an elevation of $[Ca^{2+}]_i$, we find that growth of the tube is often inhibited, and that the tip gradient is lost. Because this "wound-induced" stoppage of growth and loss of gradient can also be achieved with microneedles that do not contain any $Ca^{2+}$ indicator or buffer, we are forced to conclude that mechanisms other than shuttle buffering can result in the loss of the $[Ca^{2+}]_i$ gradient. These observations also cause us to entertain the possibility that the action of 5,5'-dibromo BAPTA in these experiments may be effected by a mechanism other than shuttle buffering. However, the loss of the $[Ca^{2+}]_i$ gradient at the tip always correlates with growth stoppage.

Although we are able to determine unequivocally whether or not pollen tubes are growing, owing to operational limitations with the imaging system we have not been able to record the growth rates of the imaged cells. To overcome this problem we have made growth measurements on a second population of pollen tubes, which, except for not being imaged, were treated identically to those that were. The results from five control cells show that neither pressure injection of Fura-2 dextran nor the iontophoretic injection of KCl at 2 nA for 2 min had any significant effect ($F > 4.0$) on the rate of growth (Table 1). Subsequently the growth rates of nine cells that had been iontophorectically injected with 5,5'-dibromo BAPTA, instead of KCl, at 2 nA for 2 min, were determined (Table 1). In complete agreement with the imaged cells we find that the results with 5,5'-dibromo BAPTA are bimodal; of the nine cells, five were completely inhibited, exhibiting growth rates of 0.00 $\mu$m/s, while the remaining four showed normal growth (Table 1). This apparent threshold effect achieved with 5,5'-dibromo BAPTA is of great interest to us and will be considered in detail in a forthcoming study.

## Discussion

The results show that growing pollen tubes possess a steep gradient of free calcium that is focused within the apical 10-20 $\mu$m of the tip. If this gradient is dissipated then pollen tube elongation stops. Further, inhibited tubes can reinitiate growth, whereupon the calcium gradient re-emerges. Taken together these results provide compelling evidence for suspecting a primary interaction between the free calcium gradient and the process of normal pollen tube elongation. It seems likely that the region of elevated calcium creates conditions favoring vesicle fusion. If the vesicles contain calcium channels, and if the activity or number of previously inserted calcium channels decays from the tip to the base of the tube, then the calcium-stimulated process of vesicle fusion establishes a positive feedback mechanism that reinforces further vesicle fusion at the tip and thus ensures the polar elongation of the tube (Steer and Steer, 1989).

Our observations on the $[Ca^{2+}]_i$ gradient in *Lilium* pollen tubes show the decline beginning at the extreme tip, with $Ca^{2+}$ reaching basal levels 20 $\mu$m proximally to the tip. This requires a calcium removal system to be active within this region. In *Tradescantia* pollen tubes a physiological examination of $Ca^{2+}$ sequestration activity failed to establish a role for the endoplasmic reticulum (ER), although mitochondria were thought to possess this activity (Steer and Steer, 1989). *Lilium* pollen tubes may have a different cytoplasmic organization than those of *Tradescantia*. Excellent images from rapidly frozen lily pollen tubes show the occurrence of mitochondria beginning 15-30 $\mu$m proximal to the tip while numerous profiles of the ER are found throughout the tip cytoplasm (Lancelle and Hepler, unpublished data).

If we model the growing pollen tube as a long rod with a $Ca^{2+}$ influx at one end (Kühtreiber and Jaffe, 1990) and a uniform distribution of $Ca^{2+}$ pumps along the remainder of the tube, the solution of the diffusion equation for the steady-state spatial distribution of $[Ca^{2+}]_i$ is $C = C_{basal} + C_{amp} \exp [-x(p/D)^{\frac{1}{2}}]$ (Carslaw and Jaeger, 1959). $C = [Ca^{2+}]_i$ as a function of distance $x$ from the growing end of the tip, $C_{basal}$ is the basal level of $[Ca^{2+}]_i$ (170 nM), $C_{amp}$ is the peak amplitude above the basal level of the $[Ca^{2+}]_i$ gradient at the tip (>320 nM), $p$ is the pumping capacity for $Ca^{2+}$ extrusion from the cytoplasm, and $D$ is the diffusion coefficient of $Ca^{2+}$ in the cytoplasm ($6 \times 10^{-6}$ $cm^2/s$) (Speksnijder et al., 1989). This model does not differentiate between plasma membrane pumps and intracellular calcium pumps of the ER or mitochondria. Our observation of a $[Ca^{2+}]_i$ gradient that begins declining at the extreme tip is consistent with this model if $p$ is interpreted as the ER pumping capacity and if ER-sequestered $Ca^{2+}$ is removed from the tip region. The ER located within the tip region in lily possibly functions as the calcium-removal system. Given that the measured characteristic distance of decay of the $[Ca^{2+}]_i$ gradient $[(D/p)^{\frac{1}{2}}]$ is approximately 8 $\mu$m, the calculated $Ca^{2+}$ flux at the growing tip of the pollen tube is $C_{amp}D(p/D)^{\frac{1}{2}} = 2.4$ $pmol/cm^2$ sec, which is equivalent to a current density of 460 $nA/cm^2$. These values are about 57% of those measured in growing tobacco pollen tubes (~4 $pmol/cm^2$ sec or ~800 $nA/cm^2$) (Kühtreiber and Jaffe, 1990), but are lower-limit estimates, since the measured peak amplitude of the $[Ca^{2+}]_i$ gradient, $C_{amp}$, is a lower-limit estimate.

The first demonstration of $Ca^{2+}$ uptake into the cytoplasm of pollen tubes was given by Jaffe et al. (1975), who showed a rapid incorporation of $^{45}Ca^{2+}$ focused toward the tip of the pollen tube. Two $Ca^{2+}$-binding components were identified, one at the tip wall and a cytoplasmic component that was located at the tip and not dispersed by cytoplasmic streaming. Proton-induced X-ray emission more recently has revealed a tip-to-base gradient in the total $Ca^{2+}$ content of pollen tubes (Reiss et al. 1983).

Several reports have probed the distribution of membrane-associated calcium in pollen tubes (Reiss and Herth, 1978; Polito, 1983) and other tip-growing cells (Reiss and Herth, 1979; Kropf and Quantrano, 1987) using the fluorescent dye chlortetracycline (CTC). When applied to pollen grains before tube emergence CTC staining produces a fine line of fluorescence at or just below the plasma membrane at the presumptive growth site. Quite similar observations have been reported at the new growth sites in the desmid *Micrasterias* (Meindl, 1982), and in fucoid embryos, which, when taken together, support the idea that the site of high calcium within growing or nascent growing zones is closely associated with the plasma membrane. However, in pollen, as germination proceeds, CTC fluorescence becomes associated with intracellular sites, notably the organelle/vesicle-rich zone near the tip of the tube (Polito, 1983). Thus concentration gradients of membrane-bound and membrane-associated calcium become superimposed, although further studies, which include an analysis of total calcium and phosphorus using proton-induced X-ray emission, indicate an enrichment of calcium in the membranes near the tip (Reiss et al. 1983). While it is attractive to speculate that the gradient in membrane-associated calcium reflects an underlying concentration gradient in free calcium, it must be realized that these two entities are quite different, with the free ion being present at a much lower concentration than that which is complexed or compartmentalized with membranes.

Because it is free calcium that ultimately participates in ion-dependent physiological reactions, it is the most important form of the ion to characterize with regard to its concentration and spatial location within the cell. The idea that a $[Ca^{2+}]_i$ gradient may be a feature of tip-growing cells in general remains attractive and has been the subject of studies on *Fucus* rhizoids (Brownlee and Pulsford, 1988) as well as pollen tubes (Reiss and Nobiling, 1986; Nobiling and Reiss, 1987; Herth et al. 1990). Of particular interest to the current work are the three studies that have reported a tip-to-base gradient in free $Ca^{2+}$ in pollen tubes of *Lilium longiflorum* (Reiss and Nobiling, 1986; Nobiling and Reiss, 1987; Herth et al. 1990). Using Quin2-acetoxymethylester as

a permeant indicator, Nobiling and Reiss (1987) allow that there is a gradual and linear decrease in the $[Ca^{2+}]_i$ from 90 nM at the tip to 20 nM at the base, some 350 μm from the tip. However, given the established problem of loading plant cells with ester dyes (Callaham and Hepler, 1991; Cork, 1986; Gilroy et al. 1986), with the likely possibility that these dyes once in the cytoplasm would be subjected to rapid compartmentation and/or extrusion from the cell (Callaham and Hepler, 1991; Cork, 1986; Malgaroli et al. 1987; Di Virgilio et al. 1990), and with the difficulty of using Quin2 as a ratiometric indicator (Grynkiewicz et al. 1985), there are reasons to question these results. Moreover, the 30 μm diameter spot size employed by Nobiling and Reiss (Nobiling and Reiss, 1987) would have prevented them from observing the gradient, which, as shown herein, appears to be fully expressed within the apical 20 μm. More recently, Herth et al. (1990), using Fluo-3 AM provide results similar to those obtained earlier with Quin2. However, given the lack of quantitative data these results cannot be assessed. Therefore, there remain major uncertainties about the existence, localization and magnitude of the free $[Ca^{2+}]_i$ gradient from previously published reports, although current, but unpublished, studies of Rathore et al. (1990) provide evidence for a $Ca^{2+}$ gradient in indo-1-loaded lily pollen tubes similar to that reported herein.

The results presented here are very different from those previously published by revealing the presence of a $[Ca^{2+}]_i$ gradient that is restricted to the 10-20 μm adjacent to the tube tip. We believe these differences are due to the favorable properties of Fura-2 dextran when compared to other indicators. To begin with, Fura-2 is markedly superior to Quin2 as a $Ca^{2+}$ indicator because of its brighter fluorescence (up to 30-fold), its major changes in excitation spectrum rather than just fluorescence intensity upon $Ca^{2+}$ binding, its slightly longer wavelengths of excitation, and its considerably improved selectivity for $Ca^{2+}$ over other divalent cations (Grynkiewicz et al. 1985). Further, because of its spectral shift, it has an important advantage over Fluo-3. However, perhaps the most important factor is the covalently coupled dextran, which prevents Fura-2 from becoming sequestered into various cytoplasmic organelles and compartments, in contrast to the AM ester or free anion form of the dye (Roe et al. 1990; Moore et al. 1990). Fura-2 dextran thus remains in the cytosol for hours permitting long-term recordings, and does not require the use of anion transport blockers, which themselves can alter cell function (Di Virgilio et al. 1990). A further favorable property is the fact that the dextran-coupled form of Fura-2 is a good chromophore, producing images that are at least 35 times the levels of background autofluorescence (Fig. 3). Finally, and importantly, Fura-2 dextran has no detectable effect on growth (Table 1) or streaming of the pollen tubes.

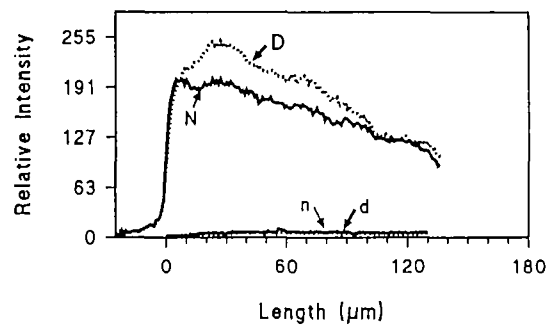


**Fig. 3.** Comparison of numerator and denominator values of a dye-loaded and autofluorescent cell. Since the tubes are continuously elongating, it is not possible to subtract an autofluorescence image of the cell acquired before dye loading. Numerator (N) and denominator (D) values for the dye-loaded cell shown in Fig. 1A have high relative intensity values whereas the numerator (n) and denominator (d) of the autofluorescent cell are almost negligible. This indicates that the Fura-2 dextran fluorescence accurately reports the $[Ca^{2+}]_i$ within the pollen tube without interference by autofluorescence. The values before the zero length mark are caused by fluorescence flare.

## Conclusions

The above experiments provide conclusive evidence that a steep $[Ca^{2+}]_i$ gradient exists at the extreme tip of elongating lily pollen tubes. All cells that were growing contained a gradient, whereas all non-growing cells did not. The experimental manipulation of cells with 5,5′-dibromo BAPTA and other conditions indicate that pollen tube elongation can be reversibly inhibited. Growing cells that were inhibited lost their gradient while those that recovered re-established their gradient. Given the reliability of the $Ca^{2+}$ detection procedure it now becomes possible to undertake more detailed analyses of the $[Ca^{2+}]_i$ gradient and its role in controlling pollen tube growth.

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