

Uncoupling secretion and tip growth in lily pollen tubes: evidence for the role of calcium in exocytosis

Stéphane J. Roy^{1,2,†}, Terena L. Holdaway-Clarke^{1,*†},
Grant R. Hackett^{1,3}, Joseph G. Kunkel¹, Elizabeth M. Lord²
and Peter K. Hepler¹

¹Biology Department, University of Massachusetts,
Amherst, MA 01003, USA,

²Department of Botany and Plant Science, University of
California, Riverside, CA 92521, USA, and

³623 South Main St, Great Barrington, MA 01230, USA

Summary

Cytoplasmic calcium concentration ($[Ca^{2+}]_i$) and extracellular calcium (Ca^{2+}_o) influx has been studied in pollen tubes of *Lilium longiflorum* in which the processes of cell elongation and exocytosis have been uncoupled by use of Yariv phenylglycoside ($(\beta\text{-D-Glc})_3$). Growing pollen tubes were pressure injected with the ratio dye fura-2 dextran and imaged after application of $(\beta\text{-D-Glc})_3$, which binds arabinogalactan proteins (AGPs). Application of $(\beta\text{-D-Glc})_3$ inhibited growth but not secretion. Ratiometric imaging of $[Ca^{2+}]_i$ revealed an initial spread in the locus of the apical $[Ca^{2+}]_i$ gradient and substantial elevations in basal $[Ca^{2+}]_i$ followed by the establishment of new regions of elevated $[Ca^{2+}]_i$ on the flanks of the tip region. Areas of elevated $[Ca^{2+}]_i$ corresponded to sites of pronounced exocytosis, as evidenced by the formation of wall ingrowths adjacent to the plasma membrane. Ca^{2+}_o influx at the tip of $(\beta\text{-D-Glc})_3$ -treated pollen tubes was not significantly different to that of control tubes. Taken together these data indicate that regions of elevated $[Ca^{2+}]_i$, probably resulting from Ca^{2+}_o influx across the plasma membrane, stimulate exocytosis in pollen tubes independent of cell elongation.

Introduction

The polarised growth of the pollen tube, which is essential for the delivery of the sperm cells to the egg apparatus, results from the continued fusion of Golgi vesicles at the extreme apex of the tube. The process provides new plasma membrane and cell wall components necessary for pollen tube elongation (Heslop-Harrison, 1987; Mascarenhas, 1993; Steer and Steer, 1989) and results

from a delicate balance between exocytosis of cell wall components and cell wall assembly.

Calcium (Ca^{2+}) is implicated as a fundamental element in the process of pollen tube growth (Picton and Steer, 1983). Tip growth is dependent on the existence of a $[Ca^{2+}]_i$ gradient focused at the tip of the elongating pollen tube (Malhó *et al.*, 1994; Miller *et al.*, 1992; Obermeyer and Weisenseel, 1991; Pierson *et al.*, 1994; Rathore *et al.*, 1991), which derives from extracellular Ca^{2+} (Ca^{2+}_o) entry at the tip (Malhó *et al.*, 1995; Pierson *et al.*, 1996). It has been postulated that growing pollen tubes have open Ca^{2+} channels in their tip (Malhó *et al.*, 1994; Pierson *et al.*, 1994) and that these channels rapidly inactivate as new ones are inserted during vesicle fusion (Pierson *et al.*, 1996).

In lily we have previously shown that arabinogalactan proteins (AGPs) are secreted into the pollen tube tip via Golgi vesicles (Jauh and Lord, 1996; Roy *et al.*, 1998). We have also shown that it is possible to uncouple exocytosis from pollen tube extension by blocking the incorporation of the newly synthesised cell wall components using Yariv phenylglycoside ($(\beta\text{-D-Glc})_3$), an agent that binds AGPs (Nothnagel, 1997; Roy *et al.*, 1998). This subtle change in the balance between exocytosis and elongation not only causes a build up of cell wall components during arrest of lily pollen tube extension but also results in a surprising pattern of disorganized exocytosis that may be a consequence of changes in the dynamics of $[Ca^{2+}]_i$. In this study we have used fluorescent ratio imaging and the non-invasive ion-selective vibrating probe to decipher the patterns of $[Ca^{2+}]_i$ and Ca^{2+}_o influx when pollen tube extension has been blocked but exocytosis still continues. Our results provide strong evidence that domains of high $[Ca^{2+}]_i$ are correlated with secretion and may be responsible for triggering it. That binding of a cell surface glycoprotein can so dramatically affect $[Ca^{2+}]_i$ and cell morphology is further indication that the cell wall-membrane interface is an active player in the process of polar growth.

Results

Morphological response to $(\beta\text{-D-Glc})_3$

Addition of $30\ \mu\text{M}$ $(\beta\text{-D-Glc})_3$ to growing lily pollen tubes caused considerable inhibition of elongation rates, as previously reported (Jauh and Lord, 1996), with growth stopping within 10 min.

The clear zone, that is the vesicle-rich area at the tip of the pollen tube, was unchanged and no large organelles such as mitochondria, amyloplasts, dictyo-

Received 18 March 1999; revised 1 June 1999; accepted 4 June 1999.

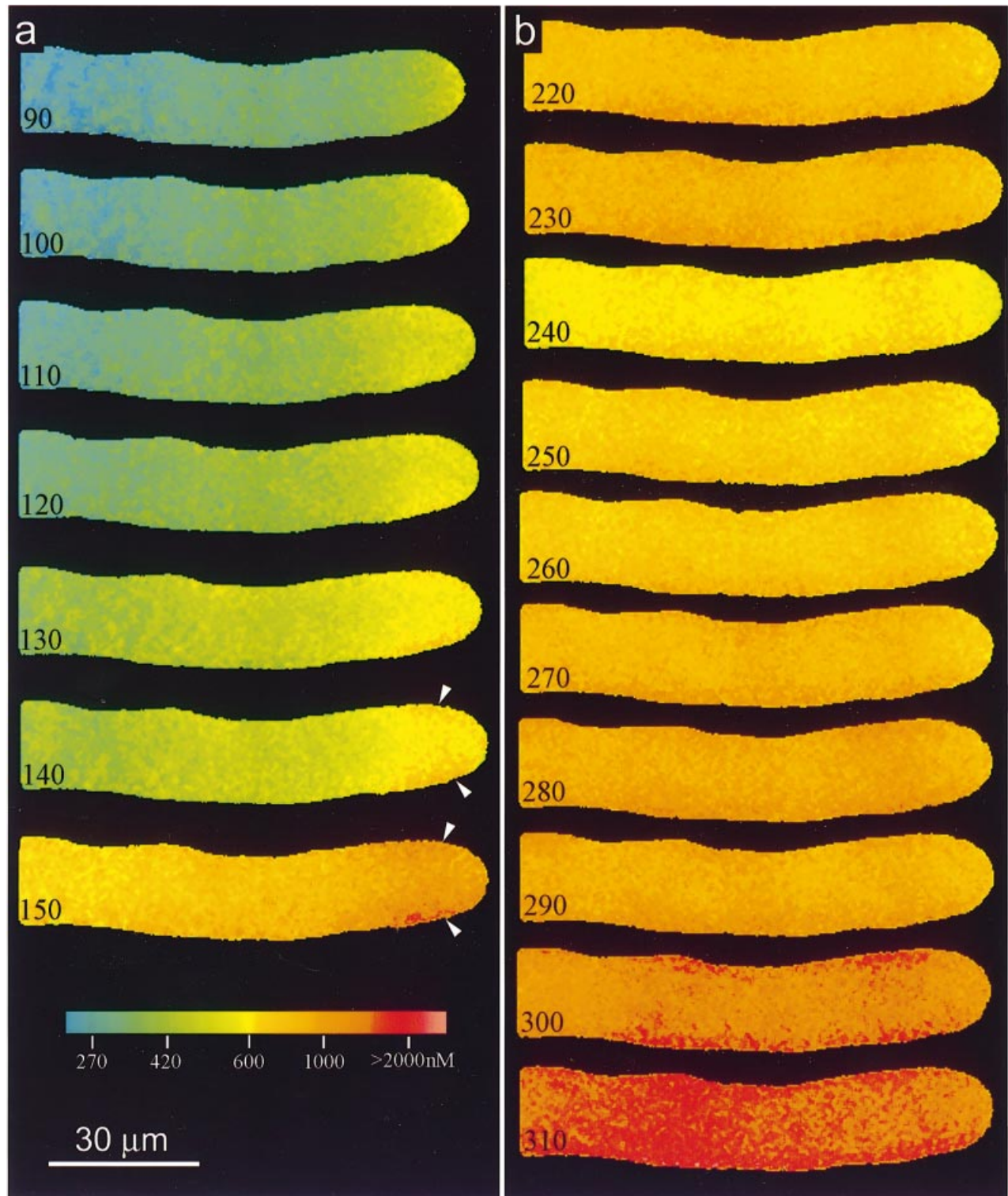
*For correspondence (fax +1 413 545 3243; e-mail terena@bio.umass.edu).

[†]These authors contributed equally to this study.

somes or lipid bodies penetrated it (data not shown). Often the clear zone became enlarged and streaming decreased significantly (data not shown).

(β-D-Glc)₃ modifies [Ca²⁺]_i at the pollen tube tip

Fluorescence ratiometric analysis of growing pollen tubes loaded with fura-2 revealed a steep tip-focused gradient of



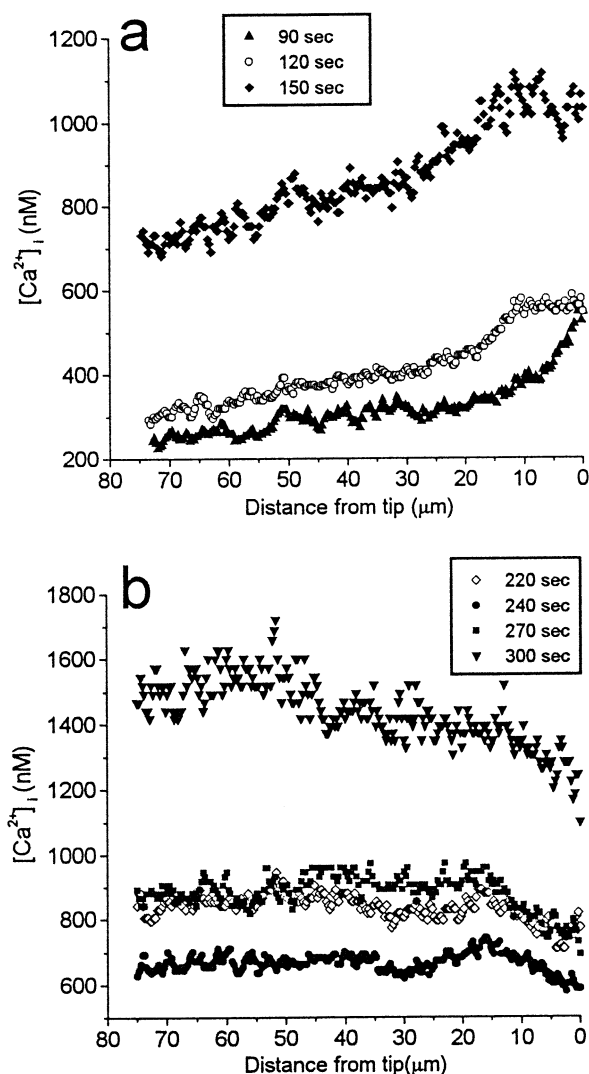


Figure 2. Line scan plots of $[Ca^{2+}]_i$ through the median part of the tube shown in Figure 1.

(a) Selected line scans of Figure 1(a) 90 s after (β -D-Glc) $_3$ treatment, there is a steep tip-focused $[Ca^{2+}]_i$ gradient (\blacktriangle). 120 s after (β -D-Glc) $_3$ treatment the region of high $[Ca^{2+}]_i$ has spread into the dome of the pollen tube tip and $[Ca^{2+}]_i$ back from the tip has increased slightly (\circ). 150 s after (β -D-Glc) $_3$ $[Ca^{2+}]_i$ has increased throughout the pollen tube and treatment the region of high Ca^{2+} has extended even further back from the tip and there is a global increase of $[Ca^{2+}]_i$ (\blacklozenge).

(b) Selected line scans of Figure 1(b) showing fluctuations in $[Ca^{2+}]_i$. Profile of $[Ca^{2+}]_i$ 220 s (\diamond), 240 s (\bullet), 270 s (\blacksquare) and 300 s (\blacktriangledown) after addition of (β -D-Glc) $_3$.

$[Ca^{2+}]_i$, located in the first 20 μ m of the apex, as previously reported (Figure 4, Pierson *et al.*, 1994). Ratiometric ion imaging reveals that elevated $[Ca^{2+}]_i$ followed treatment with 30 μ M (β -D-Glc) $_3$ in all pollen tubes observed ($n=10$). The exact time between the treatment and the response varied but in all cases pollen tube growth had ceased within 10 min. This variation was probably due to differences in diffusion rates of the solution of (β -D-Glc) $_3$ through the agarose in which the pollen tubes are embedded.

Figure 1 illustrates the typical change in $[Ca^{2+}]_i$ at the tip of the pollen tube after treatment with 30 μ M (β -D-Glc) $_3$. The sequence includes images acquired every 10 s starting 90 s after treatment. In this particular example, growth became progressively slower and by 220 s had stopped. Treated pollen tubes initially still possessed a steep, tip-focused $[Ca^{2+}]_i$ gradient ranging from 0.6 μ M at the extreme tip to a basal level of 0.25 μ M 20 μ m back from the tip (Figure 1a, 90 s and Figure 2a, \blacklozenge), similar to the control (data not shown; for a previously published example see Figure 4, Pierson *et al.*, 1994). Within minutes pollen tubes exposed to (β -D-Glc) $_3$ experience a global increase of $[Ca^{2+}]_i$ in the tip-most 100 μ m of the pollen tube. In addition to raised levels of basal $[Ca^{2+}]_i$ (β -D-Glc) $_3$ caused the region of elevated $[Ca^{2+}]_i$, normally confined to the very apex of the pollen tube, to be extended over a larger area of the tip (Figures 1a, 100–140 s) within which discrete domains of high $[Ca^{2+}]_i$ could also be seen on the flanks of the dome (Figure 1a, arrowheads, 140 s and 150 s).

$[Ca^{2+}]_i$ fluctuates in (β -D-Glc) $_3$ treated pollen tubes

The pollen tube imaged in Figure 1(a), was measured again in a second sequence (Figure 1b). The viability of the pollen tube was checked before and after the second sequence and showed cytoplasmic streaming and an intact clear zone at the tip. The sequence of images acquired every 10 s starting 220 s after treatment with (β -D-Glc) $_3$ reveal fluctuations in $[Ca^{2+}]_i$. Measurement of $[Ca^{2+}]_i$ 220 s after application of (β -D-Glc) showed the treated pollen tube had an average concentration of 0.8 μ M (Figure 1b, Figure 2b, 220 s). $[Ca^{2+}]_i$ was elevated within 80 μ m and perhaps further from the tip. It is possible that the modified gradient shown in Figures 1(a) and 2(a) was still present with lower $[Ca^{2+}]_i$ out of the field of view. Twenty seconds

Figure 1. Pseudocolor ratio images of Ca^{2+} distribution in a pollen tube treated with (β -D-Glc) $_3$.

A pollen tube about 300 μ m long was loaded with fura-2-dextran and after recovery was treated with 30 μ M (β -D-Glc) $_3$. Images were acquired every 10 s starting 90 s after treatment. Numbers are time in seconds after addition of (β -D-Glc) $_3$.

(a) A tip-focused $[Ca^{2+}]_i$ gradient occurs from 0.6 μ M at the tip to 0.25 μ M within 20 μ m from the tip (90 s). Between 100 s and 140 s the region of higher $[Ca^{2+}]_i$ at the tip spreads to encompass the whole dome of the pollen tube tip while further back in the tube $[Ca^{2+}]_i$ increases to around 420 nM. Note the highest levels of $[Ca^{2+}]_i$, indicated by orange colour (1 μ M) appear on the flanks of the pollen tube in the tip region (130–140 s). After 150 s exposure to (β -D-Glc) $_3$ the basic pattern of distribution is the same as at 140 s, with the regions of highest $[Ca^{2+}]_i$ at the flanks near the tip (arrowheads), but $[Ca^{2+}]_i$ is much higher throughout the visualised portion of the pollen tube.

(b) The pollen tube has now stopped growing and the overall $[Ca^{2+}]_i$ has increased substantially (1.5 μ M, 310 s). Note that $[Ca^{2+}]_i$ fluctuates and that higher $[Ca^{2+}]_i$ signals are present on the flanks.

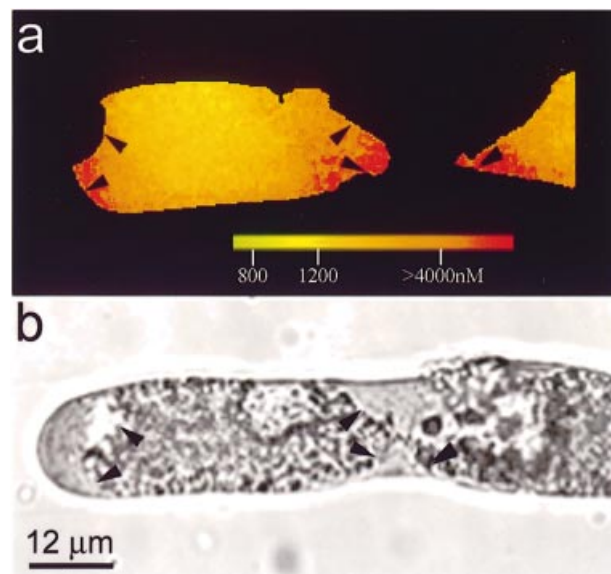


Figure 3. Pollen tube treated with $(\beta\text{-D-Glc})_3$ for 1 h and microinjected with fura-2-dextran.

(a) Ratio image showing elevated $[\text{Ca}^{2+}]_i$ throughout most of the visible part of the cell ($0.9\ \mu\text{m}$). Areas of higher $[\text{Ca}^{2+}]_i$ are evident on the flanks and the tip (arrowheads). High $[\text{Ca}^{2+}]_i$ signals outline the plasma membrane outside of which wall thickenings occur (compare with b).

(b) Light micrograph of the same pollen tube used for Ca^{2+} imaging. The tip region has been invaded by wall material. Some of the cytoplasm has been trapped by the cell wall ingrowths at the flanks of the tube. Areas of cell wall deposition (arrowheads) are adjacent to the regions of elevated $[\text{Ca}^{2+}]_i$, shown in (b).

later the pollen tube showed an overall $[\text{Ca}^{2+}]_i$ decrease (Figures 1b, 240 s) followed by an increase of $[\text{Ca}^{2+}]_i$ in the whole tube (Figures 1b, 250 s and later images). Line scans (Figure 2b) illustrate an apparent reversal of the tip-focused gradient within the $15\ \mu\text{m}$ behind the tip and fluctuations of $[\text{Ca}^{2+}]_i$. We did not attempt to evaluate the periodicity of the fluctuations in $[\text{Ca}^{2+}]_i$.

Domains of elevated $[\text{Ca}^{2+}]_i$ outline the zone of secretion

Pollen tubes treated with $(\beta\text{-D-Glc})_3$ for 1 h or more showed a basal $[\text{Ca}^{2+}]_i$ level of $0.8\ \mu\text{M}$, indicating that the ion concentration did not increase indefinitely (for example, Figure 3). Treated cells are apparently able to regulate $[\text{Ca}^{2+}]_i$, however, discreet domains of very high $[\text{Ca}^{2+}]_i$ were evident and usually associated with regions of marked accumulation of cell wall material (Figure 3, arrowheads). A $[\text{Ca}^{2+}]_i$ signal higher than $4\ \mu\text{M}$ was clearly detected along the plasma membrane outlining the accumulations of cell wall material (Figure 3b, arrowheads).

$(\beta\text{-D-Glc})_3$ does not stop the influx of Ca^{2+}_o at the tip

Ca^{2+}_o fluxes were measured in a growth medium that was low in Ca^{2+} and low in buffer (Holdaway-Clarke *et al.*,

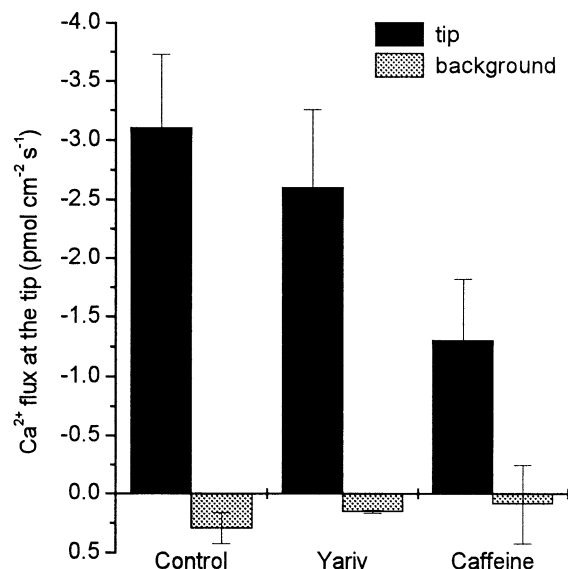


Figure 4. Ca^{2+}_o influx at the tip of pollen tubes treated with $30\ \mu\text{M}$ $(\beta\text{-D-Glc})_3$, 3 mM caffeine or washed in medium (control).

Pollen tubes treated with $(\beta\text{-D-Glc})_3$ maintain Ca^{2+}_o influx at the tip not significantly different to the influx in control tubes, but significantly different to the Ca^{2+}_o flux in caffeine-treated tubes.

1997). The non-invasive ion selective probe revealed a tip-directed inward Ca^{2+}_o current, as expected in growing pollen tubes (Figure 4). This influx was reduced when pollen tubes were treated with a growth-inhibiting solution of 3 mM caffeine as has been previously shown (Pierson *et al.*, 1996). In contrast, when pollen tubes were treated with $(\beta\text{-D-Glc})_3$ for less than 1 h the influx of Ca^{2+}_o persisted, despite the arrest of growth (Figure 4). The influx observed in $(\beta\text{-D-Glc})_3$ -treated pollen tubes is not significantly different to that measured in control tubes, but is significantly different to the flux in tubes treated with caffeine ($P > 0.05$, F-ratio test).

Discussion

$(\beta\text{-D-Glc})_3$ is the first agent known to us to arrest pollen tube elongation while simultaneously inducing elevated $[\text{Ca}^{2+}]_i$ through a broad region of the tube apex and maintaining Ca^{2+}_o influx at the tip. Using $(\beta\text{-D-Glc})_3$ we have been able to uncouple pollen tube extension and the elevated $[\text{Ca}^{2+}]_i$ at the tip, while the clear zone is maintained and secretion continues.

AGPs are selectively bound by $(\beta\text{-D-Glc})_3$ (Nothnagel, 1997; Yariv *et al.*, 1962). Evidence from experiments using $(\beta\text{-D-Glc})_3$ indicates that these proteoglycans play a role in plant cell extension, cell division and in plant developmental processes (Nothnagel, 1997; Serpe and Nothnagel, 1994; Thompson and Knox, 1998; Willats and Knox, 1996). AGPs have been found in stylar tissue (Clarke *et al.*, 1979) and may serve as a source of nutrition for growing pollen

tubes, or as a signalling molecule for pollen tube guidance (Cheung, 1995). Although $(\beta\text{-D-Glc})_3$ arrests pollen tube elongation in *Zea mays*, *Annona cherimoya* and *Lilium*, *Nicotiana tabacum* is insensitive to the reagent (Lord, unpublished data). AGPs have been localised to the plasma membrane of *in vivo* grown lily pollen tubes (Jauh and Lord, 1996; Roy *et al.*, 1998). It is not surprising that $(\beta\text{-D-Glc})_3$, which binds specifically to this component of the cell wall (for review see Nothnagel, 1997), inhibits cell extension, since wall yield threshold is a major factor determining the growth rate of plant cells (Cosgrove, 1986). The effect of $(\beta\text{-D-Glc})_3$ on $[Ca^{2+}]_i$, however, was not anticipated, given that in most situations cessation of pollen tube elongation is correlated with collapse of the tip-focused $[Ca^{2+}]_i$ gradient to basal levels (Pierson *et al.*, 1994).

Caffeine and $(\beta\text{-D-Glc})_3$ have markedly different effects on the ultrastructure and Ca^{2+} profile of pollen tubes despite the fact that both substances inhibit elongation. Pollen tube tips treated with caffeine for 1 h show collapse of the tip-focused $[Ca^{2+}]_i$ gradient, decreased Ca^{2+}_o influx at the tip and a slightly thickened cell wall at the apex of the tube (Lancelle *et al.*, 1997). In contrast, tubes exposed to $(\beta\text{-D-Glc})_3$ retain apical Ca^{2+}_o influx, have elevated $[Ca^{2+}]_i$ extending 100 μm back from the apex and large accumulations of cell wall material both at the tip and flanks of treated pollen tubes. (Jauh and Lord, 1996; Roy *et al.*, 1998; Figure 3). The large difference in wall volume at the pollen tube tip between the two treatments is compelling evidence that the rate of exocytosis is very much slower in caffeine than in $(\beta\text{-D-Glc})_3$. In pollen tubes treated with caffeine the zone of exocytosis appears to spread out from the apex indicating that there is a low basal level of exocytosis occurring constitutively (Lancelle *et al.*, 1997) and that the tip-focused $[Ca^{2+}]_i$ gradient simply biases exocytosis to the pollen tube apex. The persistence of exocytosis in $(\beta\text{-D-Glc})_3$ -treated pollen tubes (Jauh and Lord, 1996; Roy *et al.*, 1998) supports an intimate association between the presence of elevated $[Ca^{2+}]_i$ and secretion, a conclusion that holds for several other plant (Carroll *et al.*, 1998; Thiel *et al.*, 1994; Zorec and Tester, 1992), and animal systems (for review see Martin, 1997).

The overall increase of $[Ca^{2+}]_i$ in the $(\beta\text{-D-Glc})_3$ -treated pollen tubes may explain why these tubes exhibit slower cytoplasmic streaming than controls and account for the unusual phenomenon of concurrent arrest of elongation, maintenance of the clear zone and exocytosis. First, when $[Ca^{2+}]_i$ approaches 1 μM , as occurs in tubes exposed to $(\beta\text{-D-Glc})_3$ for more than a few minutes, cytoplasmic streaming is inhibited due to fragmentation of actin cables and the inhibition of myosin motor function (Kohno and Shimmen, 1987; Shimmen and Tazawa, 1982). Second, the dissipation of the gradient resulting from arrested growth by caffeine is usually accompanied by the occlusion of the clear zone (Pierson *et al.*, 1996) due to the

reformation of actin filaments at the tip (Lancelle *et al.*, 1997; Miller *et al.*, 1996). In $(\beta\text{-D-Glc})_3$ -treated pollen tubes, it is possible that a Ca^{2+} -dependent fragmentation of actin cables results in an intact or enlarged clear zone. Third, high $[Ca^{2+}]_i$ is also required for the aggregation, fusion and exocytosis of secretory vesicles with the plasma membrane (Battey and Blackbourn, 1993). The fact that elevated $[Ca^{2+}]_i$ is antagonistic to actin filaments and stimulates exocytosis accounts for the observed correlation between the presence of a clear zone and secretion at regions of elevated $[Ca^{2+}]_i$ gradient. The presence of high $[Ca^{2+}]_i$ domains at the points where cell wall material accumulates (Figure 3) further links elevated $[Ca^{2+}]_i$ and exocytosis and reveals that it is not the tip-focused gradient as such, that drives secretion but any domain of elevated $[Ca^{2+}]_i$. Clearly elevated $[Ca^{2+}]_i$ at the tip of the pollen tube and indeed exocytosis itself are not sufficient to induce pollen tube elongation.

The observed pattern of $[Ca^{2+}]_i$ elevation is difficult to explain by localised Ca^{2+} release from the ER in response to the interaction of $(\beta\text{-D-Glc})_3$ with the AGPs since ultrastructural analysis of cryofixed pollen tubes shows that the ER is evenly distributed throughout the tube cell (Lancelle and Hepler, 1992) and *in situ* visualisation of AGPs, to which $(\beta\text{-D-Glc})_3$ binds, shows that they outline the plasma membrane of the whole pollen tube (Roy *et al.*, 1998). The striking evidence of high $[Ca^{2+}]_i$ domains outlining the secretion centers is consistent with the proposition that the elevated $[Ca^{2+}]_i$ results from an increase of Ca^{2+}_o influx, and perhaps reduced extrusion via Ca^{2+} ATPases across the plasma membrane. The results of Serpe and Nothnagel (1994) indicate that $(\beta\text{-D-Glc})_3$ cross-links AGPs in the plasma membrane. It is possible that such cross-linking of plasma membrane elements may alter the function of membrane proteins directly, increasing the activity of Ca^{2+} influx channels or decreasing the efficiency of Ca^{2+} -ATPases.

There is strong evidence that the tip-focused $[Ca^{2+}]_i$ gradient in pollen tubes results from Ca^{2+} entry at the apical plasma membrane (Jaffe *et al.*, 1975; Malhó *et al.*, 1994; Malhó *et al.*, 1995; Messerli and Robinson, 1997). In our results, a Ca^{2+}_o influx similar to the control is maintained at the pollen tube tip after treatment with $(\beta\text{-D-Glc})_3$ (Figure 4), indicating that the strength of the Ca^{2+} 'sink' at the pollen tube apex is not significantly altered by exposure to $(\beta\text{-D-Glc})_3$. Previously we calculated that in untreated pollen tubes the Ca^{2+}_o influx required to support the tip-focused gradient was only a small fraction of the influx measured at the tip and that the majority of the Ca^{2+}_o current could be accounted for by binding of Ca^{2+} to de-esterified pectins in the newly secreted cell wall (Holdaway-Clarke *et al.*, 1997). Thus even a twofold increase in Ca^{2+} influx across the plasma membrane may not significantly increase the total flux measured.

It has been suggested that Ca^{2+} influx across the apical plasma membrane is regulated by stretch-activated channels (Feijó *et al.*, 1995; Malhó *et al.*, 1995; Pierson *et al.*, 1996). We see such channels as likely mediators of (β -D-Glc) $_3$ -induced changes in $[\text{Ca}^{2+}]_i$. Serpe and Nothnagel (1994) have shown that (β -D-Glc) $_3$ decreases the mobile fraction of probes that bind covalently to proteins and glycoconjugates of the plasma membrane, so reduced mobility of the plasma membrane proteins could result in tension in the lipid bilayer, which is known to open stretch-activated channels (Ramahaleo *et al.*, 1996). Elevated $[\text{Ca}^{2+}]_i$ would then increase exocytosis which in turn would increase the number of Ca^{2+} channels. We suggest inward stretching of the plasma membrane caused by accumulation of unincorporated cell wall components in the enlarged extracellular matrix, could induce a non-reversible deformation of the plasma membrane and accordingly activate stretch-activated Ca^{2+} channels. A positive-feedback loop could then be completed by increased $[\text{Ca}^{2+}]_i$ facilitating exocytosis which, in turn, provides more Ca^{2+} channels and cell wall material that would further stretch the membrane.

Experimental procedures

Pollen culture

Fresh pollen of *Lilium longiflorum* (Easter lily, Ace) or *Lilium formosanum* collected from plants grown at the University of California, Riverside or at the University of Massachusetts, Amherst or lily pollen kept frozen in liquid nitrogen was used for this study. Pollen was germinated under constant agitation for 60–90 min in one of two different germination media. For most experiments we used regular medium containing 7% sucrose, 160 μM H_3BO_3 , 15 mM 2N-morpholinoethane sulphonic acid (MES), 0.1 mM CaCl_2 and 1 mM KCl adjusted to pH 5.5 with KOH. A low Ca^{2+} , low buffer medium (LCLB) was devised for use with the extracellular Ca^{2+} -selective vibrating probe and consisted of 7% sucrose, 1.6 mM H_3BO_3 , 1.0 mM MES, 0.05 mM CaCl_2 , and 1 mM KCl, pH 6.0.

Pollen tubes were mounted for pressure injection or measurement with the non-invasive Ca^{2+} -selective vibrating probe, by mixing a small drop of concentrated germinating pollen with a drop of low-temperature gelling agarose diluted 1.2% in culture medium (type VII, Sigma) on the cover slip of a microscope slide chamber. The mixture was cooled quickly to 4°C for a few seconds and flooded with the culture medium.

Microinjection of fluorescent dye

Pollen tubes about 300 μm in length were pressure-injected with fura-2-dextran (40 mg ml^{-1} in deionized water, Molecular Probes, Inc., Eugene, OR, USA) using micropipettes pulled from filamented 1.0 mm diameter glass using a vertical puller (Model 700D, David Kopf Instruments, Tujunga, CA, USA). The tip of the micropipette was filled with the dye and the remainder with deionized water. A Narishige MO-103R micromanipulator (Narishige Scientific Instruments, Tokyo, Japan) was used to

position the micropipettes and impale the pollen tube 100 μm back from the tip.

Ratiometric imaging of $[\text{Ca}^{2+}]_i$

$[\text{Ca}^{2+}]_i$ distribution was determined by ratio ion imaging with the Ca^{2+} imaging system described in detail in Pierson *et al.* (1994). Briefly, it is composed of a Nikon inverted microscope that includes a highly regulated Hg vapour lamp as a light source and a charge-coupled device camera as a fluorescence detector. The purpose-built filter slider was used to present the two excitation wavelengths, namely the Ca^{2+} -insensitive or isosbestic wavelength (360 nm) and the Ca^{2+} -sensitive wavelength (340 nm). Images were acquired with a 1:5–1:10 exposure time for the 360 and 340 nm excitation wavelengths, respectively. Images were collected approximately every 10 s using purpose-written macros on PMIS (GKR Computer Consulting, Boulder, CO) image acquisition software. Background images were acquired in the same way after moving the cell out of the field of view. The final ratio images (340 nm/360 nm) were calculated from fluorescence images after background subtraction and thresholding, in which pixels with background-subtracted fluorescence at the Ca^{2+} -independent wavelength lower than a predefined threshold value, were displayed as black.

The absolute $[\text{Ca}^{2+}]_i$ was determined according to well established methods (Bolsover *et al.*, 1993). Briefly, ratio values were related to standardised Ca^{2+} concentrations obtained by imaging fura-2-dextran (40 mg ml^{-1}), 2.5 mM Hepes, pH 7.0, 100 mM KCl, 60% (w/w) sucrose in Ca^{2+} -free, Ca^{2+} -bound or equimix 2.5 mM BAPTA at the same exposure times used to acquire ratio images (Miller *et al.*, 1992; Pierson *et al.*, 1994, Pierson *et al.*, 1996).

Quantitative information on the ratio values and thus on $[\text{Ca}^{2+}]_i$ were determined by means of the line scan measurement function of the Image-1 program (Universal Imaging Corporation, West Chester, PA, USA).

Measurement of Ca^{2+}_o flux

A non-invasive vibrating Ca^{2+} -specific microelectrode was used to measure Ca^{2+}_o flux at the tips of lily pollen tubes, as described by Holdaway-Clarke *et al.* (1997). Briefly, electrodes were pulled from 1.5 mm glass capillaries, silanized with dimethyl-dichlorosilane (Sigma D-3879) and then backfilled with 100 mM CaCl_2 to a length of 15 mm from the tip. A 10–15 μm column of Ca^{2+} -selective liquid ion exchange cocktail (Fluka Chemie AG, Ca^{2+} Ionophore I, Cocktail A, 21048 Buchs, Switzerland) was then drawn into the tip of the electrode. Signals were measured with a purpose-built electrometer (Applicable Electronics, West Yarmouth, MA, USA), and data were acquired with the program 3DVIS (adapted by J.G. Kunkel from DVIS Version 6 described by Smith *et al.*, 1994).

Ca^{2+}_o fluxes were measured at the very tip of pollen tubes by positioning the electrode normal to the tangent at the tip, and vibrating the electrode back and forth along this line. The probe oscillated at 0.3 Hz, with an excursion of 10 μm . The difference in the voltages recorded at these two points is a measure of the Ca^{2+} flux. Calibration solutions of known $[\text{Ca}^{2+}]$ were used to determine the actual slope of the electrode (Nernst slope for Ca^{2+} = 29 mV/decade) by measuring the static (not vibrating) voltage in each solution. The actual $[\text{Ca}^{2+}]$ at a pollen tube tip was determined from the calibration values by measuring the static voltage very close to the pollen tube tip. Once the actual $[\text{Ca}^{2+}]_o$ at the tip is known, it is possible to convert voltage difference measurements (recorded in the vibrating mode) into Ca^{2+}_o flux units.

Growth inhibition of pollen tubes treated with (β -D-Glc) $_3$ or caffeine

(β -D-Glc) $_3$ was synthesised according to Serpe and Nothnagel (1994) and was a generous gift from G.Y. Jauh (University of California, Riverside, USA). In ratio-imaging experiments, pollen tubes that were actively growing after microinjection of fura-2-dextran were exposed to 30 μ M (β -D-Glc) $_3$ in culture medium. To visualise $[Ca^{2+}]_i$ distribution in pollen tubes that have been treated with (β -D-Glc) $_3$ for times longer than 1 h, we first treated the growing pollen tube with (β -D-Glc) $_3$ then mounted them on the microscope slide chamber, and finally loaded them with fura-2-dextran. Ca^{2+} flux at the tip of pollen tubes 300–800 μ m long were measured after the medium in the chamber was replaced with growth medium supplemented with either 30 μ M (β -D-Glc) $_3$ or 3 mM caffeine or growth medium alone (control).

Acknowledgements

This work was supported by National Science Foundation (NSF) grant MCB-93-04953 and MCB-96-01087 to P.K.H. and NSF grant IBN-92-06577 to E.M.L.

References

- Batley, N.H. and Blackbourn, H.D. (1993) The control of exocytosis in plant cells. *New Phytol.* **125**, 307–338.
- Bolsover, S.R., Silver, R.A. and Whitaker, M. (1993) Ratio imaging measurement of intracellular calcium and pH. In *Electronic Light Microscopy* (Shatton, D., ed.). New York: Wiley-Liss, pp. 181–210.
- Carroll, A.D., Moyen, C., Van Kesteren, P., Tooke, F., Batley, N.H. and Brownlee, C. (1998) Ca^{2+} , annexins, and GTP modulate exocytosis from maize root cap protoplasts. *Plant Cell*, **10**, 1267–1276.
- Cheung, A. (1995) Pollen–pistil interactions in compatible pollination. *Proc. Natl Acad. Sci. USA*, **92**, 3077–3080.
- Clarke, A.E., Anderson, R.L. and Stone, B.A. (1979) Form and function of arabinogalactans and arabinogalactan proteins. *Phytochemistry*, **18**, 521–540.
- Cosgrove, D. (1986) Biophysical control of plant cell growth. *Ann. Rev. Plant Physiol.* **37**, 377–405.
- Feijó, J.A., Malhó, R. and Obermeyer, G. (1995) Ion dynamics and its possible role during in vitro pollen germination and tube growth. *Protoplasma*, **187**, 155–167.
- Heslop-Harrison, J. (1987) Pollen germination and pollen tube growth. *Int. Rev. Cytol.* **107**, 1–78.
- Holdaway-Clarke, T.L., Feijó, J.A., Hackett, G.R., Kunkel, J.G. and Hepler, P.K. (1997) Pollen tube growth and the intracellular cytosolic calcium gradient oscillate in phase while extracellular calcium influx is delayed. *Plant Cell*, **9**, 1999–2010.
- Jaffe, L.A., Weisenseel, M.H. and Jaffe, L.F. (1975) Calcium accumulation within the growing tips of pollen tubes. *J. Cell Biol.* **67**, 488–492.
- Jauh, G.Y. and Lord, E.M. (1996) Localization of pectins and arabinogalactan-proteins in lily (*Lilium longiflorum* L.) pollen tube and style, and their possible roles in pollination. *Planta*, **199**, 251–261.
- Kohno, T. and Shimmen, T. (1987) Ca^{2+} -induced fragmentation of actin-filaments in pollen tubes. *Protoplasma*, **141**, 177–179.
- Lancelle, S.A., Cresti, M. and Hepler, P.K. (1997) Growth inhibition and recovery in freeze-substituted *Lilium longiflorum* pollen tubes: structural effects of caffeine. *Protoplasma*, **196**, 21–33.
- Lancelle, S.A. and Hepler, P.K. (1992) Ultrastructure of freeze-substituted pollen tubes of *Lilium longiflorum*. *Protoplasma*, **167**, 215–230.
- Malhó, R., Read, N.D., Trewavas, A.J. and Pais, M.S. (1994) Role of cytosolic free calcium in the reorientation of pollen tube growth. *Plant J.* **5**, 331–341.
- Malhó, R., Read, N.D., Trewavas, A.J. and Pais, M.S. (1995) Calcium channel activity during pollen tube growth and reorientation. *Plant Cell*, **7**, 1173–1184.
- Martin, T.F.J. (1997) Stages of regulated exocytosis. *Trends Cell Biol.* **7**, 271–276.
- Mascarenhas, J.P. (1993) Molecular mechanisms of pollen tube growth and differentiation. *Plant Cell*, **5**, 1303–1314.
- Messerli, M. and Robinson, K.R. (1997) Tip localized Ca^{2+} pulses are coincident with peak pulsatile growth rates in pollen tubes of *Lilium longiflorum*. *J. Cell. Sci.* **110**, 1269–1278.
- Miller, D.D., Callaham, D.A., Gross, D.J. and Hepler, P.K. (1992) Free Ca^{2+} gradient in growing pollen tubes of *Lilium*. *J. Cell Sci.* **101**, 7–12.
- Miller, D.D., Lancelle, S.A. and Hepler, P.K. (1996) Actin microfilaments do not form a dense meshwork in *Lilium longiflorum* pollen tube tips. *Protoplasma*, **195**, 123–132.
- Nothnagel, E.A. (1997) Proteoglycans and related components in plant cells. *Int. Rev. Cytol.* **174**, 195–291.
- Obermeyer, G. and Weisenseel, M.H. (1991) Calcium channel blockers and calmodulin antagonists affect the gradient of free calcium ions in lily pollen tubes. *Eur. J. Cell Biol.* **56**, 319–327.
- Picton, J.M. and Steer, M.W. (1983) Evidence for the role of Ca^{2+} ions in tip extension in pollen tubes. *Protoplasma*, **115**, 11–17.
- Pierson, E.S., Miller, D.D., Callaham, D.A., Shipley, A.M., Rivers, B.A., Cresti, M. and Hepler, P.K. (1994) Pollen tube growth is coupled to the extracellular calcium ion flux and the intracellular calcium gradient: Effect of BAPTA-type buffers and hypertonic media. *Plant Cell*, **6**, 1815–1828.
- Pierson, E.S., Miller, D.D., Callaham, D.A., van Aken, J., Hackett, G. and Hepler, P.K. (1996) Tip-localized calcium entry fluctuates during pollen tube growth. *Dev. Biol.* **174**, 160–173.
- Ramahaleo, T., Alexandre, J. and Lassalles, J.P. (1996) Stretch activated channels in plant cells. A new model for osmoelastic coupling. *Plant Physiol. Biochem.* **34**, 327–334.
- Rathore, K.S., Cork, J. and Robinson, K.R. (1991) A cytoplasmic gradient of Ca^{2+} is correlated with the growth of lily pollen tubes. *Dev. Biol.* **148**, 612–619.
- Roy, S., Jauh, G.Y., Hepler, P.K. and Lord, E.M. (1998) Effects of Yariv phenylglycoside on cell wall assembly in the lily pollen tube. *Planta*, **204**, 450–458.
- Serpe, M.D. and Nothnagel, E.A. (1994) Effects of Yariv phenylglycosides on *Rosa* cell suspensions: Evidence for the involvement of arabinogalactan-proteins in cell proliferation. *Planta*, **193**, 542–550.
- Shimmen, T. and Tazawa, M. (1982) Cytoplasmic streaming in the cell model of *Nitella*. *Protoplasma*, **112**, 101–106.
- Smith, P.J.S., Sanger, R.H. and Jaffe, L.F. (1994) The vibrating Ca^{2+} electrode: a new technique for detecting plasma membrane regions of Ca^{2+} influx and efflux. *Methods Cell Biol.* **40**, 115–134.
- Steer, M.W. and Steer, J.M. (1989) Pollen tube tip growth. *New Phytol.* **111**, 323–358.
- Thiel, G., Rupnik, M. and Zorec, R. (1994) Raising the cytosolic

Ca²⁺ concentration increases the membrane capacitance of maize coleoptile protoplasts: evidence for Ca²⁺-stimulated exocytosis. *Planta*, **195**, 305–308.

Thompson, H.J.M. and Knox, P.J. (1998) Stage-specific responses of embryogenic carrot cell suspension cultures to arabinogalactan protein-binding β -glucosyl Yariv Reagent. *Planta*, **205**, 32–38.

Willats, W.G.T. and Knox, J.P. (1996) A role for arabinogalactan-

proteins in plant cell expansion: evidence from studies on the interaction of β -glucosyl Yariv reagent with seedlings of *Arabidopsis thaliana*. *Plant J.* **9**, 919–925.

Yariv, J., Rapport, M.M. and Graf, L. (1962) The interaction of glycosides and saccharides with antibody to the corresponding phenylazo glycosides. *Biochem. J.* **85**, 383–388.

Zorec, R. and Tester, M. (1992) Cytoplasmic calcium stimulates exocytosis in a plant secretory cell. *Biophys. J.* **63**, 864–867.