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RESEARCH PAPER

Calcium gradients in conifer pollen tubes; dynamic properties differ from those seen in angiosperms

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Abstract

Pollen tubes are an established model system for examining polarized cell growth. The focus here is on pollen tubes of the conifer Norway spruce (*Picea abies*, Pinaceae); examining the relationship between cytosolic free Ca^{2+}, tip elongation, and intracellular motility. Conifer pollen tubes show important differences from their angiosperm counterparts; they grow more slowly and their organelles move in an unusual fountain pattern, as opposed to reverse fountain, in the tip. Ratiometric ion imaging of growing pollen tubes, microinjected with fura-2-dextran, reveals a tip-focused $[Ca^{2+}]_i$ gradient extending from 450 nM at the extreme apex to 225 nM at the base of the tip clear zone. Injection of 5,5′ dibromo-BAPTA does not dissipate the apical gradient, but stops cell elongation and uniquely causes rapid, transient increases of apical free Ca^{2+}. The $[Ca^{2+}]_i$ gradient is, however, dissipated by reversible perfusion of extracellular caffeine. When the basal cytosolic free Ca^{2+} concentration falls below 150 nM, again a large increase in apical $[Ca^{2+}]_i$ occurs. An external source of calcium is not required for germination but significantly enhances elongation. However, both germination and elongation are significantly inhibited by the inclusion of calcium channels blockers, including lanthanum, gadolinium, or verapamil. Modulation of intracellular calcium also affects organelle position and motility. Extracellular perfusion of lanthanides reversibly depletes the apical $[Ca^{2+}]_i$ gradient, altering organelle positioning in the tip. Later, during recovery from lanthanide perfusion, organelle motility switches direction to a reverse fountain. When taken together these data show a unique interplay in *Picea abies* pollen tubes between intracellular calcium and the motile processes controlling cellular organization.

Key words: Calcium, conifer, *Picea abies*, pollen tube.

Introduction

Pollen tubes, which deliver sperm cells to the egg for fertilization in higher plants, are an established model system for examining polarized cell growth. In angiosperms, pollen tube elongation is very fast (300–1500 μm h^{-1}) and is localized exclusively to the apex of the cell. These highly polarized cells possess a clear zone at their apex, which contains an inverted cone of secretory vesicles, and surrounding mitochondria, Golgi dictyosomes, ER, but lacking refractile amyloplasts and vacuoles. In addition, there is a highly organized array of actin microfilaments that consists of a fringe in the clear zone starting 1–5 μm back from the tip and confined largely to the cell cortex (Lovy-Wheeler *et al*., 2005). These microfilaments, together with myosin, drive cytoplasmic streaming and create the well-known pattern of reverse fountain flow in which vesicles and organelles flow towards the tip along the edge of the cell, and towards the base through the centre of the tube (Taylor and Hepler, 1997; Hepler *et al*., 2001). A prime function of cytoplasmic streaming is to transport the secretory vesicles to the apical clear zone, where eventually they will fuse with the plasma membrane. It can be appreciated, therefore, that elongation and cytoplasmic

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streaming are sensitive to alterations in microfilament organization (Heslop-Harrison and Heslop-Harrison, 1989; Gibbon *et al*., 1999). Microtubules, by contrast, are largely absent from the pollen tube apex (Lancelle and Hepler 1992; Pierson *et al*., 1986), and instead are present as a cortical fringe, distal to the actin fringe and thus several microns from the apex (Foissner *et al*., 2002; He and Wetzstein, 1995; Lovy-Wheeler *et al*., 2005). Their disruption has little effect on elongation in angiosperm pollen tubes (Heslop-Harrison *et al*., 1988; Åström *et al*., 1995).

The control of cell elongation and the underlying motile processes depends on several interacting factors. Of particular importance is the intracellular tip-focused calcium gradient, which appears to depend on an influx of extracellular calcium (Holdaway-Clarke *et al*., 1997; Messerli *et al*., 1999). Also notable is the pH gradient (Feijó *et al*., 1999), which appears to be driven by ATP-dependent proton efflux along the shank of the clear zone and passive proton influx at the apex (reviewed in Holdaway-Clarke and Hepler, 2003). Presumably these gradients play a crucial role in controlling vesicle fusion and secretion. The control of growth thus requires the co-ordinated activity of many factors, including channels and pumps to control ion fluxes and gradients, and actin binding proteins, which probably respond to local ionic conditions, to regulate the assembly and organization of microfilaments spatially. Candidates for the overarching regulator include the small GTPases, the Rops, which may co-ordinate and control different activities including ion fluxes and cytoskeletal assembly (Yang, 2002).

Against this backdrop it is important to consider the conifer pollen tube. To begin, it grows much slower than its angiosperm counterpart, with rates in culture of 20 μm h^{-1} for Norway spruce (Anderhag *et al*., 2000) compared with 300–1500 μm h^{-1} in culture for flowering plants (Pierson *et al*., 1996; Parton *et al*., 2001). The conifer pollen tube must elongate through several millimetres of megagametophyte tissue to reach the egg cell and, since the spermatogenous cells initially remain within the pollen grain, callose plugs cannot form to isolate the elongating apical region of the tube (Dawkins and Owens, 1993; Runions and Owens, 1999). The elongating conifer pollen tube tip does not contain an inverted cone of secretory vesicles. Instead a clear zone lacking amyloplasts but enriched in mitochondria and the endomembrane system extends 20–30 μm back from the tip. There is a demarcation running perpendicular to the tube axis between this clear zone and the amyloplasts in the rest of the pollen tube (de Win *et al*., 1996; Lazzaro, 1996). Organelles do not typically stream in a reverse fountain pattern in conifer pollen tubes. Instead the dominant pattern in *Pinus sylvestris* and *Picea abies* pollen tubes is a fountain, with organelles moving towards the tip in the tube centre and away from the tip along the cell cortex (de Win *et al*., 1996; Justus *et al*., 2004). This pattern coincides with, and is regulated by, an interplay between microtubules and microfilaments (Lazzaro, 1996, 1999; Anderhag *et al*., 2000). On the one hand microtubules control the positioning of organelles into and within the tip, and influence the direction of streaming by mediating microfilament organization (Justus *et al*., 2004). On the other hand microfilaments, presumably together with myosin, generate the force for motion, since their disruption completely stops growth and reduces organelle motility to Brownian motion. However, microtubule disruption also stops growth, alters organelle motility within the tip, and alters the organization of actin microfilaments. It is particularly noteworthy that propyzamide and oryzalin, anti-microtubule agents, cause the accumulation of membrane tubules or vacuoles in the tip that reverse direction and stream in a reverse fountain as microfilaments reorganize into pronounced bundles in the tip cytoplasm (Justus *et al*., 2004).

Given the unusual fountain streaming pattern, which is dependent on microtubules and microfilaments (Justus *et al*., 2004), and given the potential role that calcium plays in the regulation of the cytoskeleton (reviewed in Hepler *et al*., 2001; Reddy, 2001; Staiger and Franklin-Tong, 2003), it becomes important to understand the dynamics of cytoplasmic free calcium within the apex of elongating conifer pollen tubes. The questions are, therefore, whether conifer pollen tubes have a tip-focused $[Ca^{2+}]_i$ gradient, whether external calcium is required for germination and elongation, and what effect perturbation of cytoplasmic free calcium levels has on organelle motility within the tip. The results reveal that conifer pollen tubes indeed possess an intracellular gradient, but uniquely, the perturbation of this gradient elicits dramatic, tip-focused increases in cytoplasmic calcium. These conditions also modulate the pattern of streaming, with low $[Ca^{2+}]_i$ converting the normal fountain patterns into a reverse fountain pattern. Taken together these studies enlarge our understanding about the role of calcium in pollen tube growth.

Materials and methods

Pollen germination and morphology experiments

Pollen grains from *Picea abies* (Norway spruce, Pinaceae) were field-collected near Stockholm, Sweden and stored at −20 °C; they will remain viable for years. To measure the effects of $LaCl_3$, $GdCl_3$, verapamil, or caffeine on germination and elongation, stock solutions were made separately in distilled water and diluted as a concentration series into liquid germination media containing calcium (500 mM sucrose, 1 mM H_3BO_3, and 1 mM $CaCl_2$ in distilled water). The working concentration ranges for lanthanum (up to 100 μM), gadolinium (up to 100 μM), and verapamil (up to 1000 μM) were based on the effective concentrations for *Petunia hybrida* pollen tubes (Geitmann and Cresti, 1998). The concentration range for caffeine (up to 300 mM) was extended beyond the effective concentration (up to 30 mM) for *Lilium longiflorum* pollen tubes (Lancelle *et al*., 1997). To measure the requirement for external calcium on germination and elongation, pollen was incubated in paired experiments in liquid media (500 mM sucrose and 1 mM H_3BO_3 in distilled water) ±1 mM $CaCl_2$. For all experiments, pollen

grains were incubated in 2 ml of liquid media in tissue culture plates for 24 h at 30 °C, then examined on an inverted microscope equipped with a CCD camera connected to a Macintosh computer. For each treatment, ten Nomarski DIC images containing 3–5 pollen grains were digitally captured through the ×10 objective. Using IP Lab, these images were subsequently scored for germination and pollen tube length, measured from the aperture to the tube tip. Data were formatted in two-dimensional matrices of replicate (at least three per experiment) versus concentration and analysed in paired comparisons between each treatment and the control using Systat. Pollen tube length was analysed with Tukey's multiple comparison and germination frequency with Pearson's chi-square (Zar, 1984). Values were significant at $P < 0.05$.

Experiments on single cells and perfusion

For experiments on single cells, growing pollen tubes were embedded in a thin layer of agarose media (1.5% Sigma Type VII low melt agarose, 500 mM sucrose, 1 mM $CaCl_2$, and 1 mM H_3BO_3 in distilled water) on cover slip slides (Justus *et al*., 2004). Pollen tubes recovered for 1 h from the shock of transfer and were subsequently examined on an inverted microscope equipped for microinjection. Since *P. abies* pollen tubes grow slowly at 20 μm h^{-1} in culture (Anderhag *et al*., 2000), time-lapse DIC images (usually 600 frames at 1 fps) were captured throughout experimentation to ensure that only actively growing tubes were measured. To apply compounds around pollen tube tips externally, a large tip injection needle (diameter about 70 μm) was back-loaded with 5 μl of concentrated solutions of either $LaCl_3$, $GdCl_3$, verapamil, or caffeine in culture media with FITC-dextran (Justus *et al*., 2004). The needle tip was manipulated about 600 μm away from the tube and the complete 5 μl volume was then loaded into the agar media. The FITC-dextrans were detected by epifluorescence to ensure that the applied solution passed across the pollen tube tip. After diffusion of the 5 μl volume throughout the agar media and pollen tubes, the effective concentrations were 50 μM $LaCl_3$, 50 μM $GdCl_3$, 1000 μM verapamil, or 50 mM caffeine. Since the external application experiments above preclude treatment and recovery experiments; 50 μM $LaCl_3$, 50 μM $GdCl_3$, 1000 μM verapamil, or 50 mM caffeine in liquid germination media were also perfused across pollen tubes at 0.26 ml min^{-1}. In some experiments, perfusion and recovery was performed on fura 2-dextran loaded pollen tubes as described below.

Ratiometric calcium imaging

To measure cytoplasmic free calcium, pollen tubes were pressure injected (described in detail in Pierson *et al*., 1994; Holdaway-Clarke *et al*., 1997) with fura-2-dextran, a dual excitation, calcium sensitive dye. Briefly, 5 mg ml^{-1} fura-2-dextran (10 kDa; Molecular Probes) in 5 mM HEPES buffer (*N*-(2-hyrdoxyethyl)-piperazine-*N*′-(2-ethanesulphonic acid)), pH 7.0 was pressure injected through micropipettes made from borosilicate glass capillaries into pollen tubes elongating at 19.5 μm h^{-1} (n=37). A blunt needle was positioned on one side of a tube while an injection needle was placed on the opposite side, typically 100 μm behind the growing tip, near the vegetative nucleus. Following needle removal and recovery from injection, fluorescent images at excitation wavelengths of 340 nm (Ca^{2+}-dependent) and 380 nm (Ca^{2+}-independent) were captured sequentially with DIC images at 3 s intervals for up to 1 h using MetaFluor. The ratio images were converted to $[Ca^{2+}]$ values as previously described (Pierson *et al*., 1994; Holdaway-Clarke *et al*., 1997). Briefly, a calibration curve was established by imaging fura 2-dextran (40 μg ml^{-1}), 2.5 mM HEPES, pH 7.0, 100 mM KCl, and 60% sucrose in Ca^{2+}-free, Ca^{2+}-bound, or equimix 2.5 mM BAPTA (1,2-*bis* (2-aminophenoxy) ethane-*N*, *N*, *N*′, *N*′-tetra-acetic acid). Imaging of standards was performed under identical conditions and in parallel with pollen tubes. Using MetaFluor, cytoplasmic free Ca^{2+} was measured in pollen tube tips within elliptical regions of interest at the extreme tip, within the clear zone where fountain streaming occurs, and at the base of the clear zone where amyloplasts collect about 30 μm from the tip. A subset of fura 2-dextran-loaded pollen tubes were then pressure injected with a second needle filled with 100 mM 5,5′ dibromo-BAPTA in distilled water and examined. The final concentration of 5,5′ dibromo-BAPTA in the cytoplasm is approximately 1.5 mM based on a calculated dilution of 400 femtolitres of 100 mM 5,5′ dibromo-BAPTA into the cytoplasm of a 200 μm long pollen tube contiguous with its 50 μm diameter pollen grain.

Iontophoresis

Actively growing pollen tubes were also iontophoretically injected as previously described (Pierson *et al*., 1994). Briefly, borosilicate glass micropipettes were loaded up to the shank with 100 mM 5,5′ dibromo-BAPTA in distilled water. The micropipette was then filled with 100 mM K_2SO_4 and the needle holder with 3 M KCl. Pollen tubes that continued growing for 10 min after the initial micropipette insertion were iontophoretically injected with 100 mM 5,5′ dibromo-BAPTA in K_2SO_4 for 40 s with 0.5 nA of current that generated a 400 mV spike. This results in a calculated final concentration of approximately 0.5 mM 5,5′ dibromo-BAPTA throughout the pollen tube and grain (Pierson *et al*., 1994). Control pollen tubes were injected using the same parameters with 100 mM K_2SO_4 alone. All pollen tubes were examined for at least 10 min following iontophoretic injection.

Results

Conifer pollen tubes grew slowly at 19.5 μm h^{-1} *in vitro* (n=37) and exhibited a modest, 2-fold tip-focused cytosolic free calcium gradient (Fig. 1). At the extreme tip, the average $[Ca^{2+}]_i$ concentration was 450 nM and dropped to 225 nM at the base of the clear zone (n=7 measured tubes). This gradient fluctuated between 1.3–2.0-fold as tubes elongated and organelles moved in a fountain pattern into

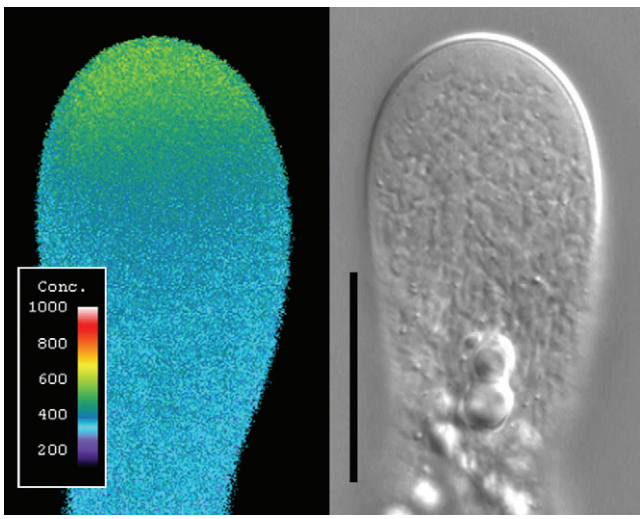


Fig. 1. Conifer pollen tubes have a modest tip-focused calcium gradient within the clear zone. This gradient pulsated from 1.3–2.0-fold over time as organelles streamed in a fountain pattern into the clear zone (a supplemental video can be found at JXB online). Scale bar=25 μm.

the clear zone at the tip (there is a supplemental video for Fig. 1 that can be found at JXB online). External calcium enhanced pollen tube elongation. Pollen tubes grown for 24 h in the absence of 1 mM $CaCl_2$ were significantly shorter (77 μm) compared with the controls (162 μm) grown in media containing 1 mM $CaCl_2$ (Table 1). The addition of external calcium was not required for germination, but the inclusion of 5 mM EGTA in calcium-free media caused a significant decline in germination frequency (Table 1) by chelating endogenous calcium from the pollen grain.

Modulation of the intracellular $[Ca^{2+}]_i$ status has a marked effect on cell structure and pollen tube elongation. Whereas control iontophoretic injections of K_2SO_4 alone (n=7) had no significant effect on growth rate (Fig. 2A, B) and did not alter cytoplasmic organization within the tip (Fig. 2B), the iontophoretic injection of the Ca^{2+} shuttle buffer 5,5′ dibromo BAPTA in K_2SO_4 significantly inhibited growth, with rates dropping from 11.4 to 0.6 μm h^{-1} (n=9). The presence of 5,5′ dibromo-BAPTA also changed the tip morphology, with small vesicles in the clear zone being replaced in 1 min by membranous tubules that migrated into the tip from the amyloplast region behind the tip (Fig. 2C). To examine the relationship between calcium and the change in organelle pattern further, several fura-2-dextran-loaded tubes were secondarily pressure-injected with 5,5′ dibromo-BAPTA (n=3). These experimental conditions, without causing an apparent decline in the tip-focused calcium gradient, elicited an immediate and repetitive increase in cytoplasmic free calcium at the apex (Fig. 3A, B). Apical cytoplasmic calcium rose from 300 nM

Table 1. *Effects of calcium dynamics on pollen germination and elongation*

The perturbation of calcium dynamics by externally applied caffeine, lanthanum, gadolinium, or verapamil inhibits elongation and germination. The exclusion of 1 mM Ca^{2+} from external media slows growth rate, but has no effect on germination until 5 mM EGTA is included in Ca^{2}-free media. Values marked with 'a' are significantly different from controls based on Tukey's multiple comparison test (tube length) and Pearson's Chi square (germination) at P <0.05; nd, not determined.

Compound	Concentration (μM)	Germination (%)	Sample size	Tube length (μm)	Sample size
Caffeine	0	82	205	233	146
	3000	80	193	200 a	150
	30 000	68 a	264	188 a	164
	300 000	61 a	386	120 a	231
$LaCl_3$	0	86	469	235	335
	1	87	383	216	213
	10	87	371	179 a	185
	50	57 a	303	97 a	75
	100	5 a	382	55 a	19
$GdCl_3$	0	86	469	235	335
	1	88	351	241	180
	10	80 a	403	197 a	204
	50	38 a	292	121 a	47
	100	2 a	421	75 a	7
Verapamil	0	90	447	201	297
	10	84 a	363	199	269
	100	80 a	417	169 a	275
	1000	59 a	401	55 a	235
$CaCl_2$	1000	81	528	162	269
	0	79	617	77 a	333
+0.4 mM EGTA	0	79	407	nd	nd
+1.0 mM EGTA	0	74	431	nd	nd
+5.0 mM EGTA	0	36 a	238	nd	nd

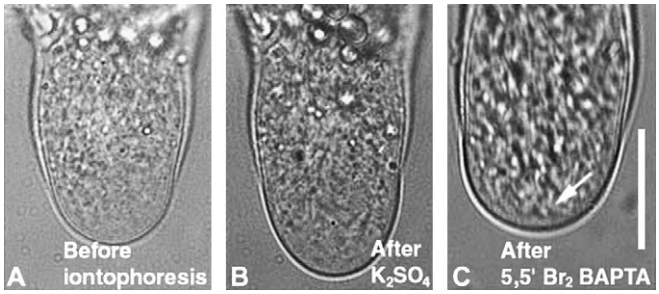


Fig. 2. Control pollen tubes (A) iontophoretically loaded with K_2SO_4 continued growing with no change in tip morphology (B). After iontophoretic loading of 5,5′ dibromo BAPTA, membranous tubules (arrow in C) migrated from the amyloplast region into the tip concurrent with the cessation of growth. Scale bar=25 μm.

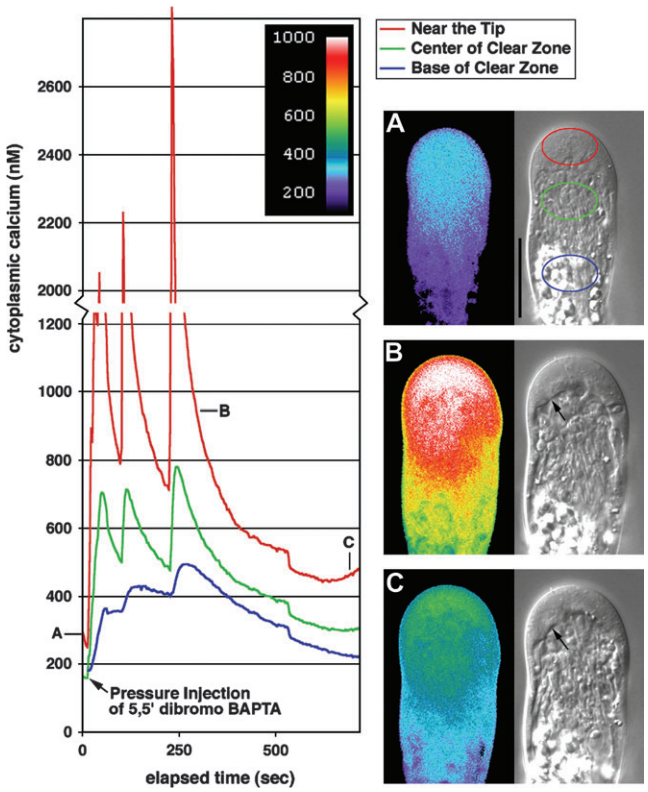


Fig. 3. The pressure injection of 5,5′ dibromo BAPTA into the cytoplasm caused multiple increases in cytoplasmic calcium at the tip. Vesicles (arrows) collect at the tip (B) concurrent with the calcium elevation. The 2-fold calcium gradient returns after these transient spikes, but the vesicles persist (C). During these experiments, organelles continued streaming in a reverse fountain but elongation stopped (a supplemental video can be found at JXB online). Scale bar=25 μm.

to above 2000 nM in 30 s, but within 10 min declined to 200–500 nM. Together with the abrupt increases in $[Ca^{2+}]_i$, there is a concomitant accumulation of vesicles in the extreme tip, together with a decrease in organelle streaming (Fig. 3B: a supplemental video can be found at JXB online). The tip-focused cytosolic free calcium gradient persisted after the transient apical spike of cytoplasmic calcium, and organelles continued streaming in a reverse fountain pattern (a supplemental video for Fig. 3 can be found at JXB online). However, the accumulation of vesicles beneath the plasma membrane persisted (Fig. 3C) and growth stopped.

Since caffeine alters tip-focused cytosolic free calcium gradients in angiosperm pollen tubes (Pierson *et al*., 1996), its effect on conifers was tested. It was found that the inclusion of 30 mM caffeine in the germination media significantly lowered germination frequency to 68%, versus 82% in controls, and that continuous exposure to 3 mM caffeine over 24 h inhibited elongation (Table 1). In separate experiments, (n=4) transient perfusion of caffeine across actively growing control pollen tubes dissipated the tip-focused cytoplasmic gradient (Fig. 4A, B). Initially, perfusion of 5 mM caffeine lowered the apical $[Ca^{2+}]_i$ from 350 nM to 175 nM, but 50 mM was required to abolish the gradient completely and to drop the basal cytosolic calcium levels below 150 nM (Fig. 4B). Once this threshold was passed, a transient increase in $[Ca^{2+}]_i$ occurred that restored cytoplasmic free calcium above 150 nM and caused the accumulation of vesicles beneath the plasma membrane and disrupted organelle motility (Fig. 4C: a supplemental video can be found at JXB online). If caffeine was removed by perfusion, the 2-fold cytosolic free calcium gradient was restored (Fig. 4D, E) and could be depleted again by the

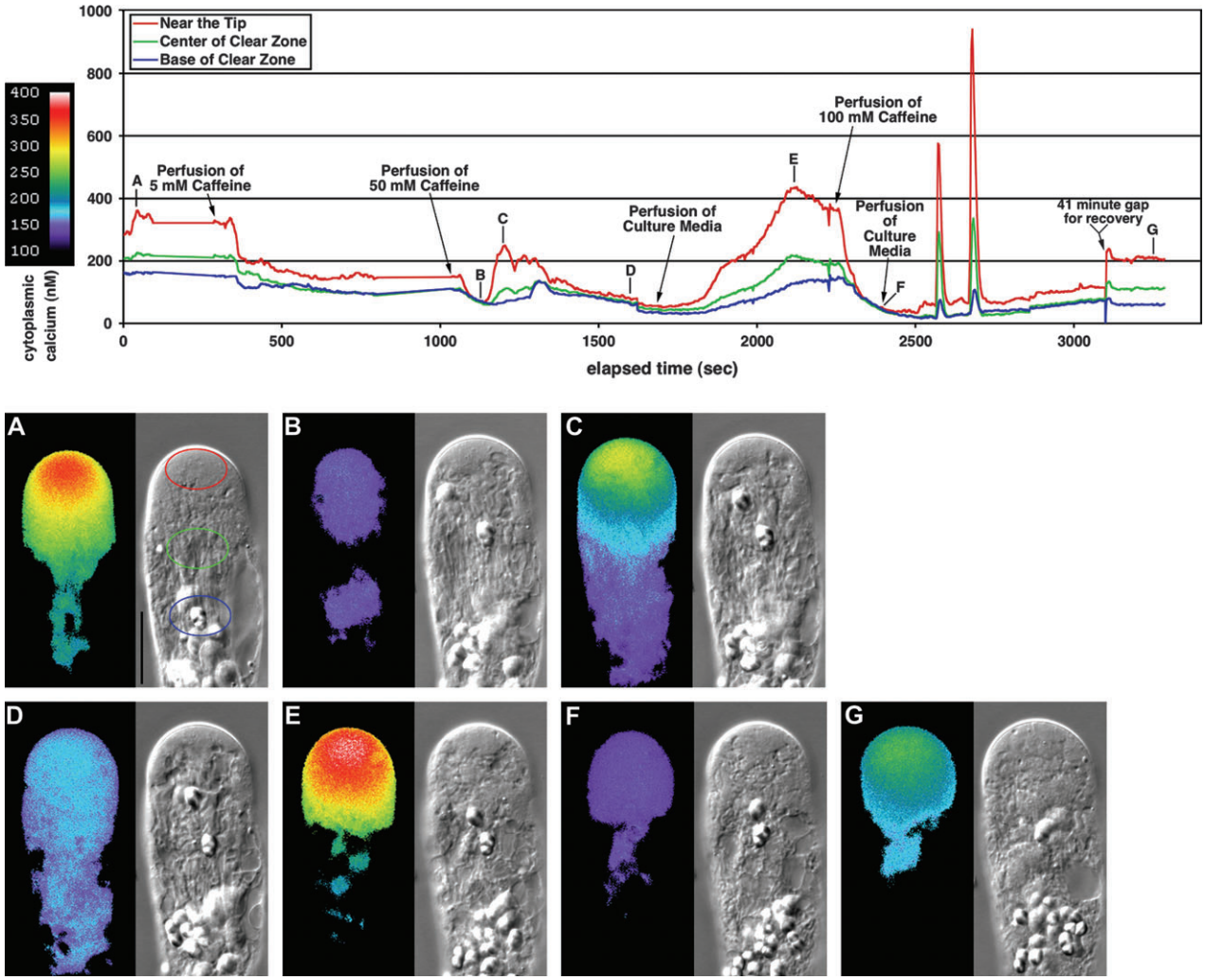


Fig. 4. When cytoplasmic calcium dropped below 150 nM following caffeine perfusion (B), a transient increase in calcium occurred (C), combined with the disruption of streaming and the reorganization of the tip ultrastructure. Dilution of caffeine restored the tip-focused calcium gradient (E) that could be depleted again by reintroduction of caffeine. After 40–50 min of recovery (F) the pollen tube elicited several transient spikes of calcium as it recovered its original tip-focused gradient and cell organization (G) and continued slowly elongating, but the streaming pattern did not fully recover (a supplemental video can be found at JXB online). Scale bar=25 μm.

reintroduction of caffeine. As transient calcium elevations occurred following either caffeine perfusion (Fig. 4C) or recovery (Fig. 4E), the 2-fold difference was maintained between calcium near the tip and at the base of the clear zone. After 40–50 min of recovery in normal perfusion media (Fig. 4F), pollen tubes elicited several transient spikes up to 900 nM of $[Ca^{2+}]_i$ as they ultimately recovered their original 2-fold calcium gradient and tip organization (Fig. 4G). These transient spikes were half the size of those elicited in response to 5,5′ dibromo-BAPTA. Although the fountain streaming pattern did not completely recover after caffeine experiments (a supplemental video for Fig. 4 can be found at JXB online), pollen tubes continued elongating at 10.3 μm h^{-1}, 50% of their original rate.

To probe the possibility that an influx of external calcium was responsible for the transient increases in apical cytosolic calcium, lanthanum (La^{3+}) and gadolinum (Gd^{3+}), which competitively block Ca^{2+} channels, were used. Elongation was significantly inhibited by the inclusion of 10 μM lanthanum or 10 μM gadolinium in media with 1 mM Ca^{2+} (Table 1). Germination was also significantly inhibited by 50 μM lanthanum and 10 μM gadolinium. Although pollen tubes were shorter when calcium uptake was inhibited, there were no other morphological changes over 24 h, including no increase in tip swelling nor in the frequency of branching (Fig. 5). External application or perfusion of 50 μM lanthanum (n=14) across elongating tips stopped growth and dissipated the tip-focused cytosolic free calcium gradient (Fig. 6A, B). However, the fountain streaming pattern was not initially affected (a supplemental video for Fig. 6 can be found at JXB online) and the tip-focused gradient was not restored once the cytosolic calcium levels equalized to 250 nM across the tip (Fig. 6B). When the cell was perfused with control media lacking lanthanum to clear the Ca^{2+} channels, calcium again entered the cell (Fig. 6C), generating a transient increase that was twice the magnitude of the initial gradient. At the peak of this transient, cytosolic calcium reached 1100 nM at the apex, but there was still a 2-fold gradient across the pollen tube. This transient rapidly declined to the original calcium levels (Fig. 6D). This calcium influx initially slowed organelle streaming in a fountain pattern, but after 30–40 min, organelle motility switched direction to a reverse fountain (Fig. 6E; a supplemental video can be found at JXB online). The extracellular application or perfusion of 50 μM gadolinium (n=12) also stopped growth and during the perfusion of control media, organelle motility switched direction to a reverse fountain.

To probe calcium influx further, verapamil, an organic inhibitor of Ca^{2+} channel activity, was used. Elongation was significantly inhibited by the inclusion of 100 μM verapamil in media with 1 mM Ca^{2+} and germination was significantly inhibited by 10 μM verapamil (Table 1). Similar to lanthanum and gadolinium, verapamil caused no other morphological changes over 24 h (Fig. 5). However, organelle motility responded differently to verapamil. External perfusion of 1000 μM verapamil (n=9) caused the rapid collection of vesicles at the extreme tip with a brief surge in elongation (Fig. 7A, B; a supplemental video can be found at JXB online). Small vacuoles exhibiting Brownian motion rapidly collected in the clear zone as organelle streaming stopped (Fig. 7B). After the brief elongation surge, growth stopped. During recovery from verapamil, the vesicle population at the extreme tip disappeared (Fig. 7C) as the small vacuoles reorganized into streaming endomembrane tubules (Fig. 7D), but elongation did not resume.

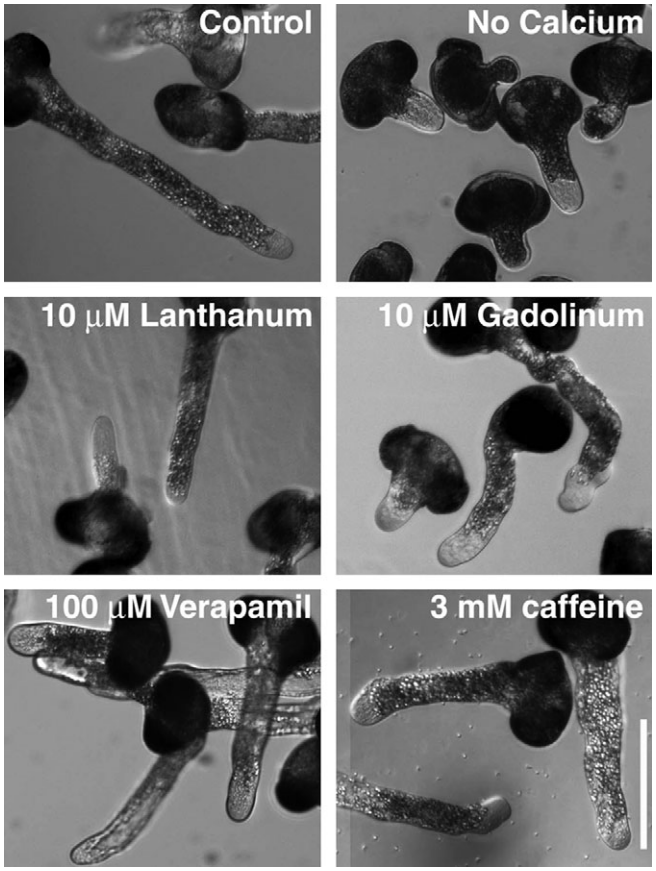
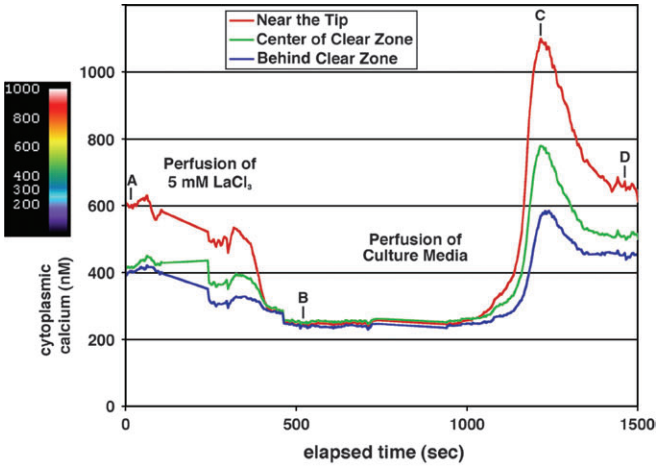


Fig. 5. The omission of external calcium slowed, but does not stop elongation. The inclusion of lanthanum, gadolinium, verapamil or caffeine all inhibited elongation with no other changes in morphology. Scale bar=100 μm.

Discussion

This study's results show that calcium is a major factor in the control of spruce pollen tube growth. While there are similarities with the calcium status in angiosperm pollen tubes there are important differences. Firstly, it is important to note that conifer pollen tubes possess a tip-focused intracellular gradient, which, although lesser in magnitude



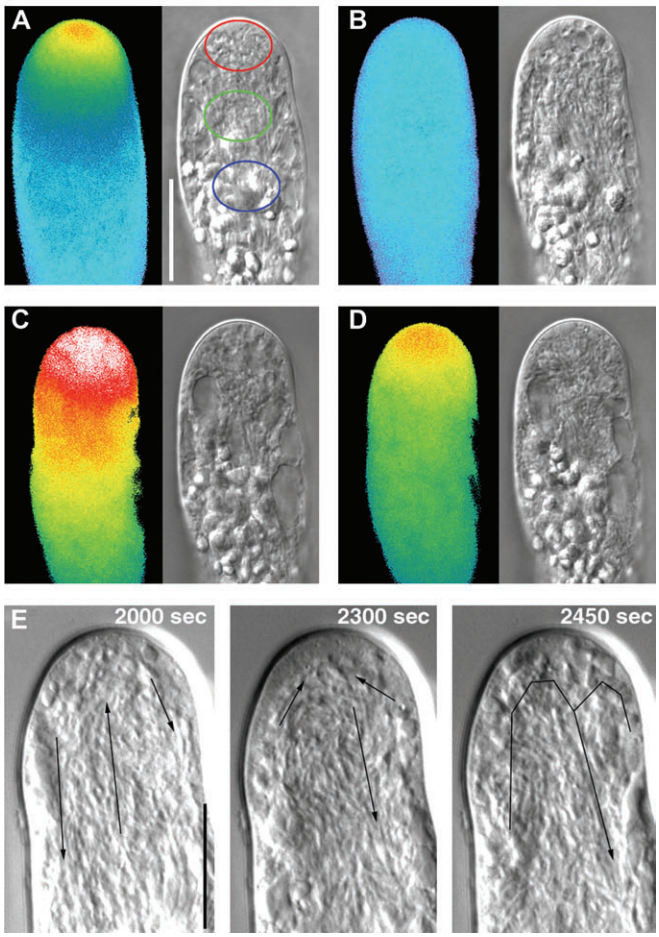


Fig. 6. Perfusion of $LaCl_3$ across pollen tube tips blocked the influx of calcium and depleted the gradient (B). However, tubes did not exhibit a transient elevation of apical calcium when the cytoplasmic level dropped below 150 nM. Once $LaCl_3$ was removed by perfusion, an influx of external calcium occurred (C) and cells recovered their original 2-fold calcium gradient (D). Organelles moved in a fountain pattern (arrows) throughout the $LaCl_3$ experiment, but 10–15 min after recovery of the calcium gradient, motility reversed direction to a reverse fountain (E: a supplemental video can be found at JXB online). Scale bars=25 μm.

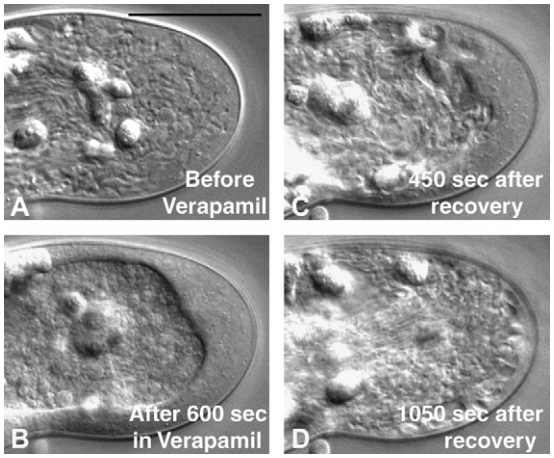


Fig. 7. Perfusion of verapamil across pollen tube tips (A) caused the rapid collection of vesicles at the extreme tip and numerous small vacuoles in the clear zone (B) exhibiting Brownian motion as organelle streaming stopped. During recovery, the vesicle population at the extreme tip disappeared (C) as the small vacuoles reorganized into streaming endomembrane tubules (D: a supplemental video can be found at JXB online). Scale bar = 25 μm.

than that observed in angiosperm pollen tubes, is nevertheless sufficient to activate some calcium-binding proteins. Of marked difference from angiosperm pollen tubes is the ability of different perturbing agents to elicit large increases of cytosolic free calcium within the apical domain of the spruce pollen tube. For example, 5,5′ dibromo-BAPTA, which does not appear to reduce the tip-focused gradient, nevertheless causes an unexpected immediate increase in calcium, with the apical levels transiently rising 5-fold or more above the normal observed peak of the gradient. By contrast, both caffeine and the lanthanides dissipate the tip-focused gradient as expected and, after some minutes following their removal, the resulting conditions lead to large, but transient increases of cytosolic free calcium within the apex.

Our hypothesis is that these transient elevations in apical $[Ca^{2+}]_i$ are due primarily to a rapid influx of extracellular calcium, which has not been observed in angiosperm pollen tubes. Although 5,5′ dibromo-BAPTA can elicit a single basipetal traveling wave of elevated intracellular calcium (Pierson *et al.*, 1994), it has not been shown to generate dramatic apical elevations in calcium. In lily pollen tubes, the dissipation of the gradient leads to an immediate growth arrest, but with some ongoing secretion. It seems plausible that the lily pollen tube wall quickly becomes too thick and inflexible for the cell to reverse the inhibition and, as a consequence, channels, particularly those that might be stretch activated (Dutta and Robinson, 2004), would not be able to reopen. By contrast, the slowly growing conifer pollen tube, which already possesses a thick, cellulose-enriched cell wall at the tip (Derksen *et al.*, 1999; Lazzaro *et al.*, 2003), retains its ability to react to different environmental or physiological conditions. Even though

5,5′ dibromo-BAPTA stops growth, the pollen tube can still respond positively by opening plasma membrane calcium channels. Lanthanum and gadolinium, in contrast to 5,5′ dibromo-BAPTA, compete for calcium binding sites on channels, and block extracellular entry of the ion (Malhó *et al*., 1995) which leads to the dissipation of the tip-focused gradient. This condition generates the low levels of calcium that are observed. When these trivalents are removed from the medium, it seems plausible that the cell responds to the low levels of cytoplasmic calcium by opening calcium channels on the plasma membrane and allowing large influxes. Even though there is no observed elevation in apical calcium until after the removal of lanthanides, intracellular calcium stored in the endoplasmic reticulum may also contribute to the observed transient increases. In particular, the most dramatic spikes occur in response to 5,5′ dibromo-BAPTA, which is unique in that it presumably shuttles calcium away from channels at the plasma membrane. This triggers a more rapid and extensive response that might include the release of calcium from the ER, which is enriched throughout the apical clear zone up to the plasma membrane of elongating spruce pollen tubes (Justus *et al*., 2004).

The effects of caffeine on pollen tube elongation remain difficult to explain. In flowering plant pollen tubes, caffeine disrupts vesicle zonation at the tip and stops elongation (Lancelle *et al*., 1997). It also dissipates the cytosolic free calcium gradient, but then in a manner similar to 5,5′ dibromo-BAPTA injection, triggers a transient rise in calcium that travels basipetally and disappears (Pierson *et al*., 1996). The spatial position of this transient rise in calcium (Pierson *et al*., 1996) is very different from the transient tip-focused calcium spike observed in conifer pollen tubes. Caffeine elevates cytoplasmic cAMP levels by inhibiting phosphodiesterase. The plant cell plasma membrane contains cyclic nucleotide gated calcium influx channels (Kurosaki, 1997; Lemtiri-Chlieh and Berkowitz, 2004) and elevated cAMP at the pollen tube tip promotes elongation (Moutinho *et al*., 2001; Rato *et al*., 2004). It is proposed that if caffeine is elevating cAMP at the pollen tube tip, this might open cAMP gated ion channels, leading to the observed elevation of apical calcium. Consistent increases in calcium occur following caffeine exposure and during the recovery from caffeine or lanthanide perfusion. These increases generate a calcium level at the tip that is transiently 2–3 times greater than the initial value, but rapidly declines towards the initial level. Interestingly, the cell maintains a 2-fold gradient throughout these response periods. Even at the peak of the transients the cell is rapidly sequestering calcium from the cytoplasm to maintain a 2-fold difference between calcium levels near the tip and at the base of the clear zone. There are different rapid spikes in apical calcium as cells finally recover from 40 min of repetitive caffeine perfusion. These spikes may occur as the cells reset their regulatory pathways.

Together with changes in the calcium status there are corresponding changes in the pattern of motility and the organization of the cytoplasmic inclusions in the apical domain. It was found that organelle streaming, a tip-focused cytosolic calcium gradient, and elongation driven by vesicle fusion are co-ordinated in normal pollen tubes, but there is no absolute requirement that the three occur simultaneously. Extracellular perfusion of lanthanides dissipates the tip-focused cytosolic calcium gradient and stops elongation but does not stop organelle streaming. Conversely, the injection of 5,5′ dibromo-BAPTA stops growth but does not stop organelle streaming or deplete the tip-focused calcium gradient. Instead, transient spikes of cytosolic calcium are triggered as growth stops and vesicles accumulate at the extreme tip. The details of the interactions between growth, organelle streaming, and calcium are unique to conifer pollen tubes. In flowering plants, 10 μM lanthanides dampen the pulsatile growth normally observed (Geitmann and Cresti, 1998) and 100 μM will stop growth and deplete the calcium gradient (Malhó *et al*., 1995). In flowering plants, 5,5′ dibromo-BAPTA causes a shift of larger organelles into the clear zone. The reverse fountain streaming changes to a circular or random pattern and vesicles accumulate at the extreme tip, although growth ceases (Pierson *et al*., 1994).

It is intriguing that, during recovery from lanthanide treatment, streaming changes direction to a reverse fountain pattern. This occurs after the return of the tip-focused calcium gradient. The reversal mimics that seen following microtubule disruption by propyzamide or oryzalin (Justus *et al*., 2004). This disruption reorganizes microfilaments, and this model is that microtubules position the microfilament network, thereby establishing the directional paths for myosin dependent motility (Justus *et al*., 2004). It is likely that proteins controlling motor activity or cytoskeletal organization are also being influenced by the depletion and return of the calcium gradient.

In conclusion, external calcium enhances germination and is required for the elongation of conifer pollen tubes. There is a 2-fold tip-focused gradient in cytoplasmic free calcium that extends from 450 nM at the extreme apex to 225 nM at the base of the clear zone. This gradient is much less than the 10-fold gradients measured with fura-2-dextrans in angiosperm pollen tubes (Holdaway-Clarke and Hepler, 2003), and it is perturbed by a calcium shuttle buffer, and depleted by caffeine, lanthanides, and verapamil. When the calcium gradient diminishes and the basal level in the cytoplasm is lowered, a transient tip-focused surge in cytoplasmic calcium occurs, restoring the gradient and triggering the accumulation of a large vesicle population at the plasma membrane. During recovery from lanthanide treatment, organelle motility within the tip switches direction from a fountain to a reverse fountain pattern. The threshold dependent elevation of cytosolic free calcium and the calcium-influenced reversal of motility

have not been observed in flowering plant pollen tubes. The dynamics of calcium metabolism in these slow growing conifer pollen tubes are unique.

Supplementary data

Supplementary videos are available at the Journal of Experimental Botany website, http://jxb.oxfordjournals.org/ and also at our laboratory website, http://www.cofc.edu/~lazzaro

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