Calcium gradient dependence of Neurospora crassa hyphal growth

Lorelei B. Silverman-Gavrila† and Roger R. Lew†

Department of Biology, York University, 4700 Keele Street, Toronto, Ontario, Canada M3J 1P3

INTRODUCTION

Polarized cell expansion, culminating in tubular extensions (tip growth), is a morphogenic process observed in all kingdoms, from bacteria to animals. In all the examples of tip-growing eukaryotic cells which have been studied so far, there is a relationship between tip extension and internal calcium gradients. For example, pollen tubes exhibit a cytoplasmic tip-high Ca\(^{2+}\) gradient during growth. The gradient is quite steep: [Ca\(^{2+}\)] is about 3-0 \(\mu\)M at the tip, decreasing to 0-2 \(\mu\)M about 20 \(\mu\)m behind the tip (Pierson et al., 1994). Inhibition of pollen tube growth by BAPTA injection correlates with dissipation of the cytoplasmic tip-high Ca\(^{2+}\) gradient and inhibition of tip-localized Ca\(^{2+}\) influx (Pierson et al., 1994). In addition, pulsations of the tip-localized Ca\(^{2+}\) concentration are correlated with pulsatile growth in pollen tubes (Pierson et al., 1996; Messerli & Robinson, 1997). During pulsatile pollen tube growth, growth precedes increased Ca\(^{2+}\) influx and pulsatile cytoplasmic Ca\(^{2+}\) increases by a few seconds (Messerli et al., 1999, 2000). This suggests that Ca\(^{2+}\) influx ‘senses’ tip expansion during growth; the response naturally lags behind tip expansion. Such a mechanism has been proposed to be mediated by stretch-activated Ca\(^{2+}\) channels localized at the tip. Stretch-activated Ca\(^{2+}\) channels have been characterized in the oomycete Saprolegnia ferax (Garrill et al., 1993), in which there is evidence for a correlation between growth rate and the magnitude of the Ca\(^{2+}\) gradient measured using ratio imaging of Ca\(^{2+}\) and pH-sensitive fluorescent dyes (Hyde & Heath, 1997).

Fungi also exhibit tip-high Ca\(^{2+}\) gradients during hyphal growth. Spatial cytoplasmic [Ca\(^{2+}\)] has been measured using quantitative dual dye (fluor-3 and Fura Red) ratio imaging (Silverman-Gavrila & Lew, 2000). Analogous to pollen tubes, injection of BAPTA dissipates the gradient and stops growth (Silverman-Gavrila & Lew, 2000). Unlike pollen tubes (Pierson et al., 1994), root hairs (Felle & Hepler, 1997) or S. ferax (Lew, 1999), there is no indication that Ca\(^{2+}\) influx at the growing tip is responsible for generating the tip-high gradient. Although Neurospora crassa does have stretch-activated Ca\(^{2+}\) channels (Levina et al., 1995), there is no net Ca\(^{2+}\) influx during hyphal growth (Lew, 1999) and direct manipulation of the membrane potential to modify the driving force for Ca\(^{2+}\) influx does not affect growth rate (Silverman-Gavrila & Lew, 2000). The gradient is generated and maintained internally by the concerted action of inositol 1,4,5-trisphosphate (IP\(_3\))-activated Ca\(^{2+}\) release from tip-localized vesicles (Silverman-Gavrila & Lew, 2002) and Ca\(^{2+}\)-ATPase-mediated sequestration into the endoplasmic

Abbreviations: BAPTA, 1,2-bis(orho-aminophenoxy)ethane-N,N,N',N' tetrapotassium acetate; CTC, chlortetracycline; fluo-3, 2,7-dichloro-6-hydroxy-3-oxo-9-xanthenyl-4'-methyl-22'-(ethylenedioxy)dianiline-\(\cdot\)N\(_2\'), \(\cdot\)N' tetraacetate acid; IP\(_3\), inositol 1,4,5-trisphosphate.

†Both authors contributed equally to the work.
reticulum behind the growing tip (Silverman-Gavrila & Lew, 2001). The location of the tip-localized vesicles is maintained by interaction with the actin cytoskeleton (Silverman-Gavrila & Lew, 2001).

Our objective in this paper is to explore the relation between the Ca\(^{2+}\) gradient and growth in the ascomycete N. crassa, and to identify a possible growth sensor responsible for generating the gradient to maintain continued growth. Does growth depend upon an absolute [Ca\(^{2+}\)] at the tip, or is it the steepness of the gradient that is required during growth? Our assessment is done in the context of spatial regulation of the Ca\(^{2+}\) gradient, and its relation to other aspects of the polar cytology of N. crassa hyphae. Random fluctuations of the Ca\(^{2+}\) distribution may generate localized regions of elevated Ca\(^{2+}\) to initiate tip growth. Based on stretch-activated production of diacylglycerol, we propose that activation of a tip-localized phospholipase C may ‘sense’ growth, initiating a cascade of events that maintains the Ca\(^{2+}\) gradient during continued hyphal growth.

**METHODS**

**Culturing.** The wild-type Neurospora crassa strain RL21a (FGSC no. 2219) was grown in 35 mm tissue culture dishes on solid substrate (2 %, w/v, gellan gum) containing 2 % sucrose and Vogel’s minimal medium (Vogel, 1956), and incubated at 28–30 °C for 14 h. Prior to experiments, the culture was flooded with BS [10 mM KCl, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM PIPES (pH adjusted to 5–8 with KOH) and the osmolality adjusted to 260 mosmol kg\(^{-1}\) with sucrose] (Levina et al., 1995).

**Ratiometric fluorescence imaging of cytoplasmic calcium.** Cytosolic [Ca\(^{2+}\)] was measured by ratio imaging the emission intensities of the Ca\(^{2+}\)-sensitive fluorescent dyes fluo-3 and Fura Red. They were loaded ionophoretically into the hypha. The electrophysiological techniques are described in detail elsewhere (Silverman-Gavrila & Lew, 2000, 2001). The micropipette was filled at the tip with 0-33 mM fluo-3 and 0-99 mM Fura Red (both as potassium salts; Molecular Probes) and backfilled with 3 M KCl. Hyphae were impaled about 35 μm behind the tip. Fluorescence imaging was performed using a Bio-Rad MRC-600 confocal apparatus with a krypton–argon mixed gas laser attached to a Nikon Optiphot 2 microscope (Silverman-Gavrila & Lew, 2000). Briefly, the dyes were excited at 488 nm using 10 % laser intensity (neutral density filter 1) and the emitted fluorescence was detected simultaneously at 522 nm (fluorescence intensity) and 510 nm (autofluorescence intensity). Ratio intensities were measured using 2-54 μm longitudinal transects within the cytoplasm of the hyphae in the software program NIH-Image. To correct for the smaller volume for each 0.22 μm filter, then scraped into a 1-5 ml Eppendorf tube containing 0-75 ml ice-cold chloroform/methanol (1:2, v/v), vortex-mixed and kept on ice for 15 min. Diacylglycerol extraction followed the protocol described in detail by Ramsdale & Lakin-Thomas (2000), adapted from Bligh & Dyer (1959). The diacylglycerol extracts were stored at −20 °C in chloroform containing 50 μM butylated hydroxytoluene ml\(^{-1}\). Mycelial dry weight was determined by washing mycelial debris from the initial extraction in methanol, drying overnight at 60 °C, then weighing. The lipids were measured using an HPLC technique modified from Bocckino et al. (1985). We used a Betalis silica-60 (5 μm particle size) (250 x 4-6 mm) column (ThermoHypersil-Keystone). Chromatography was performed using a BioCAD Sprint chromatography system (PerSeptiva Biosystems). The solvent was hexane/2-propanol/glacial acetic acid (250:2:5:0.025) (HIA) run at 3 ml min\(^{-1}\) at about 1200 p.s.i. (8280 kPa). Lipid samples (125 μl) were dried under N\(_2\) at 60 °C and redissolved in 500 μl HIA. After equilibration of the column with HIA, 100 μl samples were injected into the column. Lipids were detected by the A\(_{266}\). Diacylglycerol and ergosterol (Sigma-Aldrich) standards were used to identify HPLC peaks. All other reagents were obtained from Sigma-Aldrich and were HPLC grade.

**Data analysis.** The experiments (77 in all) were sorted by growth rate and mean Ca\(^{2+}\) gradients were calculated for subsamples (n=11 or n=7). This assured an even spread of growth rates. A statistical software package (SYSTAT, version 5.0) was used for linear and nonlinear regression analysis of the relation between growth rate and various aspects of the Ca\(^{2+}\) gradient. Best fits for various mathematical models were obtained by minimization of least squares, \[ \min \left( \sum (observed\_output - predicted\_output)^2 \right) \], with either a quasi-Newton or Simplex method (Wilkinson, 1988). Linear or exponential models were used as described in Results. Goodness of fit was assessed quantitatively with correlation coefficients and two-tail probabilities.
Calcium gradients and fungal tip growth

Hyphal growth dependence on Ca$^{2+}$ gradient steepness and magnitude

The steepness of the gradient can be quantified by using an exponential fit of [Ca$^{2+}$] versus distance from the tip. We used an exponential equation of the form $[\text{Ca}^{2+}] = [\text{Ca}^{2+}]_{\text{basal}} + [\text{Ca}^{2+}]_{\text{max}} e^{-d/\tau}$, where $[\text{Ca}^{2+}]_{\text{basal}}$ is the basal [Ca$^{2+}$], and is summed with $[\text{Ca}^{2+}]_{\text{max}}$ to approximate tip-localized [Ca$^{2+}$], $d$ is the distance from the tip, and $\tau$ is a measure of the steepness of the gradient; a small $\tau$ corresponds to a steep gradient (Table 1). Growth rate was poorly correlated with the steepness of the gradient (Fig. 3); the Pearson correlation coefficient was very small, $r^2 = 0.000$, $P = 0.462$. Rather than steepness per se, it is possible that growth rate depends upon the magnitude of the Ca$^{2+}$ gradient, either tip-localized [Ca$^{2+}$], or the difference between tip-localized and basal free cytoplasmic [Ca$^{2+}$] (Fig. 3). Similar correlations were found for both measurements of the [Ca$^{2+}$] gradient; the correlations, while small, were statistically significant. To assure that the choice of sample size ($n = 11$) did not cause a fortuitous correlation, the 77 individual experiments, sorted by growth rate, were recompiled into sample sizes of 7. No correlation was observed for growth versus gradient steepness ($r^2 = 0.078$, $P = 0.172$). The correlation coefficients for growth...
versus either tip-localized \([\text{Ca}^{2+}]\), or the difference between tip-localized and basal \([\text{Ca}^{2+}]\) and the gradient steepness (tau) were smaller due to increased variability with the smaller sample sizes \((r^2=0.23)\), but were still statistically significant \((P<0.05)\) (data not shown). As an additional check, data were compiled on the basis of experimental runs, 5–8 days of intensive fluorescence image acquisition. In this case, growth rate variability was high, but both tip-localized \([\text{Ca}^{2+}]\) and tip-localized versus basal free cytoplasmic \([\text{Ca}^{2+}]\) were correlated significantly with growth rate.

### Table 1. Data summary for cytoplasmic \([\text{Ca}^{2+}]\) gradients at the hyphal tip

Mean \([\text{Ca}^{2+}]\) from ratio images from different regions of the hyphae are tabulated for hyphae growing at the mean growth rates as shown. The difference between tip-localized and basal \([\text{Ca}^{2+}]\) and the gradient steepness (tau) are shown for the compiled growth rates.

<table>
<thead>
<tr>
<th>Growth rate ((\mu\text{m min}^{-1}))</th>
<th>Cytoplasmic free ([\text{Ca}^{2+}]) behind the hyphal tip (nM)</th>
<th>([\text{Ca}^{2+}]<em>{\text{apical}}-\text{Ca}^{2+}</em>{\text{basal}}) difference (nM)</th>
<th>tau ((\mu\text{m}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>sd</td>
<td>n</td>
<td>0–2.5 (\mu\text{m})</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>19</td>
<td>190</td>
</tr>
<tr>
<td>1.57</td>
<td>0.71</td>
<td>11</td>
<td>272</td>
</tr>
<tr>
<td>3.81</td>
<td>0.82</td>
<td>11</td>
<td>302</td>
</tr>
<tr>
<td>5.94</td>
<td>0.45</td>
<td>11</td>
<td>196</td>
</tr>
<tr>
<td>7.39</td>
<td>0.601</td>
<td>11</td>
<td>428</td>
</tr>
<tr>
<td>10.75</td>
<td>1.76</td>
<td>11</td>
<td>387</td>
</tr>
<tr>
<td>14.25</td>
<td>0.98</td>
<td>11</td>
<td>420</td>
</tr>
<tr>
<td>19.58</td>
<td>2.81</td>
<td>11</td>
<td>387</td>
</tr>
</tbody>
</table>

Fig. 3. Dependence of growth rate on the \([\text{Ca}^{2+}]\) gradient steepness, tip-localized \([\text{Ca}^{2+}]\), and the difference between apical and basal \([\text{Ca}^{2+}]\) \((\text{[Ca}^{2+}]_{\text{apical}}-\text{Ca}^{2+}_{\text{basal}})\). Linear best fits are shown, along with correlation coefficients and \(P\)-values. Steepness of the gradient was measured using best fits to an exponential equation, \([\text{Ca}^{2+}]_{\text{apical}}=\text{Ca}^{2+}_{\text{basal}}+\text{Ca}^{2+}_{\text{max}}\text{e}^{-d/\text{tau}}\). The higher the tau value, the less steep is the gradient. Tip-localized \([\text{Ca}^{2+}]\) \((\text{[Ca}^{2+}]_{\text{apical}})\) is the mean \([\text{Ca}^{2+}]\) 0–2.5 \(\mu\text{m}\) behind the hyphal tip. The difference between apical and basal \([\text{Ca}^{2+}]\) \((\text{[Ca}^{2+}]_{\text{apical}}-\text{Ca}^{2+}_{\text{basal}})\) was measured as the difference in \([\text{Ca}^{2+}]\) at 0–2.5 \(\mu\text{m}\) versus \([\text{Ca}^{2+}]\) at 10–20 \(\mu\text{m}\). Growth rates are shown as mean ± SD \([n=11, \text{except zero growth (}n=19\text{)}]\).
(0.37 > r² > 0.52, P < 0.002, data not shown); the gradient steepness was not (r² = 0.147, P = 0.213). Thus growth rate depends upon the magnitude of tip-localized [Ca²⁺], which must be higher than basal free cytoplasmic [Ca²⁺]. Growth is not correlated with the steepness of the Ca²⁺ gradient.

**Initial Ca²⁺ gradient generation**

Inhibitor effects on hyphal growth and the [Ca²⁺] gradient suggest that the Ca²⁺ gradient is generated and maintained by the action of an IP₃-activated Ca²⁺ channel releasing Ca²⁺ at the tip, and a Ca²⁺-ATPase sequestering Ca²⁺ behind the hyphal tip (Silverman-Gavrila & Lew, 2001). An IP₃-activated Ca²⁺ channel with an inhibitor signature consistent with such a role has been characterized biochemically (Silverman-Gavrila & Lew, 2002). But how is the gradient generated de novo and how is it regulated during hyphal expansion? The initiation of hyphal extension from spherical spores, either asexual or sexual, could result from the spontaneous appearance of localized regions of elevated [Ca²⁺] as a consequence of the vagaries of the Ca²⁺ random walk. A simplified test of this hypothesis is shown in Fig. 4. Starting from a homogeneous array, a random walk causes the appearance of a typical heterogeneous distribution of Ca²⁺, a model for the expected random distribution of Ca²⁺ within the cell. Within the array, regions of elevated Ca²⁺ do appear (Fig. 4a), some localized near the outer border of the array. Histogram analysis of the distribution reveals a subset of array elements containing 1.5–2-fold elevations of Ca²⁺ (Fig. 4b). Similar heterogeneous distributions, varying with time, are observed in the vesicular Ca²⁺ of a conidium (Fig. 4c) and cytoplasmic [Ca²⁺]basal in a hypha (Fig. 4d). Thus spontaneous localized Ca²⁺ elevations could be sufficient to trigger the cascade of events that result in hyphal initiation.

**Fig. 4.** Heterogeneous Ca²⁺ distributions. (a) A Ca²⁺ random walk was used to generate a model of the typical random distribution of calcium within the cell. Localized regions of elevated Ca²⁺ appear throughout the two-dimensional 64 by 64 array. For visualization, the image was blurred (Gaussian), and a linear contrast stretch was applied. (b) The histogram shows frequency versus array element intensity. A subset of the arrays contain elevated Ca²⁺ (lighter regions), as expected. Time-dependent changes in Ca²⁺ distribution are observed in CTC fluorescence imaging of vesicular Ca²⁺ in conidium (from top to bottom: 0, 40 and 125 s) (c) and basal cytosolic Ca²⁺ in a hypha imaged with fluo-3 and Fura Red (d). They too could result in localized regions of elevated [Ca²⁺]. For scale, the conidium is 4 μm in diameter, and the hyphal cytoplasmic free Ca²⁺ images are 4 μm wide.
Spatial correlations of vesicular and cytoplasmic Ca\textsuperscript{2+} gradients and hyphal cytology

Once hyphal growth is initiated, Ca\textsuperscript{2+} release from internal stores is the likely source of tip-localized cytoplasmic Ca\textsuperscript{2+} during continued hyphal growth. Therefore, we examined the spatial correlation between Ca\textsuperscript{2+} stores and cytoplasmic Ca\textsuperscript{2+} (Fig. 5). CTC fluorescence was used to determine the spatial distribution of Ca\textsuperscript{2+}-containing vesicles. The presence of Ca\textsuperscript{2+}-containing vesicles at the hyphal apex (Dicker & Turian, 1990) has been confirmed after fixation and electron microscopy (Torralba et al., 2001). The vesicles can be monitored during hyphal growth using CTC; their distribution is affected by treatment with inhibitors of either IP\textsubscript{3}-activated Ca\textsuperscript{2+} channels or phospholipase C (Silverman-Gavrila & Lew, 2002). To obtain a quantitative distribution of CTC fluorescence, it must be corrected for the smaller cell volume near the apex. The corrected Ca\textsuperscript{2+}-containing vesicle density exhibited a ‘steepness’, tau of about 1·2 \(\mu\)m. Using published data on vesicles destined for fusion at the expanding tip (Collinge & Trinci, 1974) and regions of maximal wall synthesis (Goody, 1971), we calculated best fits to exponential functions to determine tau, a measure of steepness. The value was 1·6 \(\mu\)m, very similar to the ‘steepness’ of hyphal diameter, tau = 2·2 \(\mu\)m, and CTC fluorescence steepness (1·2 \(\mu\)m). By comparison, the tip-high cytoplasmic [Ca\textsuperscript{2+}] gradient was not as steep. A subsample of total experiments with a ‘mean’ growth rate (6·3–14·0 \(\mu\)m min\(^{-1}\)) was used to examine spatial correlations. The tau value was 10·1 \(\mu\)m. One possible cause for a gradient of cytoplasmic free [Ca\textsuperscript{2+}] gentler than the vesicular Ca\textsuperscript{2+} gradient could be diffusion of Ca\textsuperscript{2+} away from the growing tip after Ca\textsuperscript{2+} release from the vesicular stores. To examine whether diffusion was a reasonable explanation for the gentler Ca\textsuperscript{2+} gradient, the gradient was fit to a model for the time dependence of concentration changes due to diffusion, of the general form \(C = \frac{M}{\{1 + 4D\tau\}^{1/2}} e^{-x^2/(4Dt)}\) (Crank, 1975), where \(M\) is the initial concentration, \(D\) is the diffusion

![Fig. 5. Calcium gradients in N. crassa hyphae: spatial correlations. (a) Cytosolic free [Ca\textsuperscript{2+}] (circles, \(\text{nM}\)) and hyphal diameter (squares, \(\mu\)m). For the Ca\textsuperscript{2+} gradient, an exponential best fit (thin line) yielded a tau of 10·1 \(\mu\)m. The thick line is a best fit to the diffusion equation (time dependence of concentration) (Fig. 6). Essentially, diffusion can explain the gentler gradient for calcium compared to hyphal diameter. Hyphal diameters were measured from images captured on a digital camera on a Zeiss Axioscope using a \(\times\) 100 water immersion objective. Note that the y-axis is reversed. The best-fit exponential yielded a tau of 2·2 \(\mu\)m. (b) CTC fluorescence intensity (circles, in arbitrary units) and fluorescence intensity corrected for hyphal volume (squares, arbitrary fluorescence units per unit hyphal volume). Fluorescence intensity was measured from confocal images of medial sections, similar to measurements of fluo-3/Fura Red images (Silverman-Gavrila & Lew, 2000). Longitudinal transects (2·54 \(\mu\)m wide) along hyphae (23 experiments) were averaged versus distance from the apex. Note the close correspondence between hyphal diameter (first panel) and CTC fluorescence corrected for hyphal volume. The tau value from an exponential best fit was 1·3 \(\mu\)m. For gradient steepness comparisons, exponential fits of wall vesicle density based on percentage volume (reported by Collinge & Trinci, 1974) and wall synthesis based upon radioautography of hyphae grown in the presence of a radioactively labelled precursor of walls (Goody, 1971) yield tau values of about 1·6 \(\mu\)m. Gradient steepness has also been reported for SNAREs based upon immunocytochemistry with an antibody to the yeast t-SNARE, Sso2p (Gupta & Heath, 2000). SNAREs are believed to play a role in ‘docking’ of vesicles at a site of fusion in expansion zones of plasma membrane. The best-fit exponential yielded a tau of 8·9 \(\mu\)m.
Calcium gradients and fungal tip growth

coefficient and \( t \) is the time. In aqueous solutions, the diffusion coefficient for \( Ca^{2+} \) varies with \([Ca^{2+}]\), but is about 775 \( \mu m^2 s^{-1} \) in dilute CaCl\(_2\) (Wang, 1953). Intracellular diffusion coefficients for \( Ca^{2+} \) are in the range 2–15 \( \mu m^2 s^{-1} \) (Al-Baldawi & Abercrombie, 1995; Nakatani et al., 2002). \( Ca^{2+} \) diffusion intracellularly is complicated by the complexity of the cytoplasm, especially the presence of \( Ca^{2+} \) buffers and transporters (Smith et al., 1998). To assess whether diffusion could explain the gentler \( Ca^{2+} \) gradient, the gradient from the subsample with ‘mean’ growth rate was initially fit to obtain an estimate of the diffusion coefficient at time 4 s (sufficient time for the hypha to extend about 0.6 \( \mu m \)). Then the diffusive gradients were calculated 1, 2, 4 and 8 s after the initial state, time zero, when all the calcium was located at the extreme tip (Fig. 6). The estimated diffusion coefficient was 6.3 \( \mu m^2 s^{-1} \), in the range of reported intracellular diffusion coefficients, and lower than published values for \( Ca^{2+} \) in aqueous solution. A smaller diffusion coefficient is reasonable, given the structural complexity of the growing apex, in which a variety of physical obstacles, proteins, vesicles and cytoskeleton would impede the free movement of \( Ca^{2+} \). Thus the gentler \( Ca^{2+} \) gradient can be explained on the basis of \( Ca^{2+} \) diffusion away from the tip after release from the vesicular \( Ca^{2+} \) stores, which would require 1–8 s.

**Hyphal growth ‘sensing’ and \( Ca^{2+} \) gradient maintenance**

During hyphal elongation, continued generation of the \( Ca^{2+} \) gradient must rely upon some mechanism which senses hyphal expansion. Tip-localized IP\(_3\) production has been implicated as the cause of IP\(_3\)-induced \( Ca^{2+} \) release from vesicular \( Ca^{2+} \) stores. Since IP\(_3\) is produced by the action of phospholipase C, we tested whether phospholipase C could be activated by membrane stretching, a natural consequence of the process of hyphal elongation. Rather than assay for IP\(_3\), we chose to assay the other product of phospholipase C activity, diacylglycerol, since extraction can be performed rapidly with minimal degradation of the diacylglycerol product. Diacylglycerol was identified based on the same retention time (15.5 ± 2.4 min; \( n = 29 \)) as diacylglycerol standards [1,2-dipalmitoyl-sn-glycerol, 15.3 ± 0.4 min (\( n = 2 \)); 1,2-dioleoyl-sn-glycerol, 14.6 ± 1.2 min (\( n = 5 \)) (Fig. 7). An ergosterol peak was also identified based on the same retention time as a standard [10.3 ± 1.5 min (\( n = 29 \)) compared to 10.1 ± 0.1 min (\( n = 3 \))], and a single peak when lipid extracts were mixed with the ergosterol standard. Ergosterol served as an internal control for normalization of diacylglycerol levels to mycelial dry weight. Phospholipase C inhibitors known to inhibit hyphal growth (Silverman-Gavril & Lew, 2002) depleted diacylglycerol significantly relative to the control (Table 2). Mild hypoosmotic shock (a 1 : 1 dilution with H\(_2\)O), which causes the growing tips to bulge and continue growth (data not shown), caused elevated diacylglycerol levels. Severe hypoosmotic shock (perfusion with H\(_2\)O), which causes growth to stop immediately (growth resumes eventually), caused diacylglycerol depletion.

**DISCUSSION**

Ratiometric fluorescence imaging is crucial for spatial measurements of the cytoplasmic \([Ca^{2+}]\) gradient in tip-growing organisms (Camacho et al., 2000). Dextran-conjugated dyes are reported to be less likely to become sequestered. However, they must be pressure-injected into

![Image](http://mic.sgmjournals.org)
the hyphae, which requires large aperture micropipettes and a higher probability that the hypha will be damaged. By ionophoresing free-acid dyes into the hyphae, we could use a smaller aperture micropipette, were able to control dye loading very efficiently, and obtain good quantification (Silverman-Gavrila & Lew, 2000). Dye sequestration was very rare, observed only in hypha which had been damaged. To examine the relation between cytoplasmic free \( [\text{Ca}^{2+}] \) and hyphal growth, we compiled the data into subsamples, each with a mean growth rate and gradient. We have demonstrated that the hyphal growth rate depends upon the tip-localized \( [\text{Ca}^{2+}] \), which must be elevated above basal \( [\text{Ca}^{2+}] \) behind the tip. Hyphal growth does not depend upon the steepness of the \( \text{Ca}^{2+} \) gradient. To initiate tip growth, random molecular motion may be sufficient to generate the gradient. Stretch-activated phospholipase C may act as the growth sensor, maintaining the gradient as hyphal growth continues.

**Ca\(^{2+}\) requirement for growth in fungi**

Fungal growth requires extracellular calcium. \( \text{Ca}^{2+} \) concentrations greater than 10–100 nM are required for hyphal extension to occur in *N. crassa* (Schmid & Harold, 1988) and *Fusarium graminearum* (Robson et al., 1991). Similar extracellular \( [\text{Ca}^{2+}] \) dependencies of growth are also observed for the oomycete *S. ferax* (Jackson & Heath, 1989) and root hairs (Schiefelbein et al., 1992). At low \( \text{Ca}^{2+} \) concentrations, hyphal morphology is aberrant: irregular hyphal width or bulbous spherical cells are observed. \( \text{Ca}^{2+} \) dependence of growth and morphology could be due to many different effects: some physical, such as \( \text{Ca}^{2+} \) cross-linking of wall components, some biochemical, such as \( \text{Ca}^{2+} \)-dependent enzymic activities and cytoskeletal rearrangement, and some physiological, such as signalling. Since basal cytoplasmic \( [\text{Ca}^{2+}] \) is similar to the minimal extracellular \( [\text{Ca}^{2+}] \) required for growth, a role in biochemistry, signalling or both is likely. Whether the tip-localized cytoplasmic \( [\text{Ca}^{2+}] \) is directly related to extracellular \( \text{Ca}^{2+} \) is not clear. In the spray mutant of *N. crassa*, the tip-high cytoplasmic \( [\text{Ca}^{2+}] \) gradient is the same as that in the wild-type (Bok et al., 2001), but vesicular \( \text{Ca}^{2+} \), measured with CTC, is absent (Dicker & Turian, 1990). The rescue of the slow growth phenotype by elevated extracellular \( \text{Ca}^{2+} \) has no effect on the electrical properties of the plasma membrane (Bok et al., 2001), yet causes the reappearance of vesicular calcium (Dicker & Turian, 1990). This implies that vesicular \( \text{Ca}^{2+} \) normally functions as an intermediate step in generation of the tip-high \( \text{Ca}^{2+} \) gradient, and that vesicular \( \text{Ca}^{2+} \) storage, but not cytoplasmic \( \text{Ca}^{2+} \), is more closely related to extracellular \( [\text{Ca}^{2+}] \). There is evidence for this in other organisms. In the oomycete *S. ferax*, Jackson & Heath (1989) reported elevated CTC fluorescence when hyphae were grown in high extracellular \( [\text{Ca}^{2+}] \). By contrast, yeast cytoplasmic \( [\text{Ca}^{2+}] \) is insensitive to extracellular \( [\text{Ca}^{2+}] \) from 0·1 \( \mu \text{M} \) to 10 mM (Halachmi & Eilam, 1993).

**Table 2. Diacylglycerol levels**

The diacylglycerol (DAG) levels, normalized to the ergosterol level, are shown as a percentage of the control (a control was run for each experiment). The treatments were mild or severe hypoosmotic stress, or inhibitors of phospholipase C, as shown. The values are mean \( \pm \text{SD} \) (n).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 (8)</td>
</tr>
<tr>
<td>Mild hypoosmotic (1:1 Vogel’s:H(_2)O)</td>
<td>123 ( \pm ) 24 (3)</td>
</tr>
<tr>
<td>Severe hypoosmotic (1:19 Vogel’s:H(_2)O)</td>
<td>54·5 ( \pm ) 22·3 (4)</td>
</tr>
<tr>
<td>3-Nitrocoumarin</td>
<td>43·9 ( \pm ) 29·6 (5)</td>
</tr>
<tr>
<td>Neomycin</td>
<td>40·3 ( \pm ) 12·4 (4)</td>
</tr>
<tr>
<td>U-73122</td>
<td>60·2 ( \pm ) 19·5 (4)</td>
</tr>
<tr>
<td>U-73343</td>
<td>81·9 ( \pm ) 2·8 (2)</td>
</tr>
</tbody>
</table>

**Internal generation of the Ca\(^{2+}\) gradient**

Tip-localized inward \( \text{Ca}^{2+} \) currents play a role in generation of the cytoplasmic tip-high \( \text{Ca}^{2+} \) gradient in pollen tubes (Pierson et al., 1994), root hairs (Schiefelbein et al., 1992; Felle & Hepler, 1997) and the oomycete *S. ferax* (Lew, 1999). Fungi (*N. crassa*) rely solely upon internal generation of the tip-high \( \text{Ca}^{2+} \) gradient (Lew, 1999; Silverman-Gavrila & Lew, 2000). Two distinct intracellular transporters maintain...
and generate the gradient: an IP3-activated Ca2+ channel (Silverman-Gavrila & Lew, 2002) localized to vesicles at the extreme apex of the growing hypha, releasing Ca2+ into the tip, and a Ca2+-ATPase sequestering Ca2+ behind the growing apex, into the endoplasmic reticulum (Silverman-Gavrila & Lew, 2001). One explanation for internal generation is that N. crassa is a terrestrial fungus, commonly found in burned over areas (Turner et al., 2001). The presence of sufficient external Ca2+ to maintain the tip-high Ca2+ gradient may not be assured, especially in aerial hyphae. Thus internal Ca2+ alone may be used to generate and maintain the Ca2+ gradient. The role of vesicular Ca2+ stores as the source of the elevated tip-localized [Ca2+] is supported by its direct dependence on extracellular [Ca2+], and the spatial correlation between Ca2+-containing vesicles, wall vesicles and wall synthesis. Once Ca2+ is released into the cytoplasm, it diffuses away from the tip.

**Comparison with other organisms**

For the oomycete S. ferax, analyses of the dependence of growth on the Ca2+ gradient relied upon ratio imaging of Ca2+-sensitive (fluor-3) and pH-sensitive (SNARF) fluorescent dyes (Hyde & Heath, 1997). The qualitative Ca2+ gradient was linear from 0 to 40 μm behind the tip. Growth was correlated with the difference between tip-localized Ca2+ and basal Ca2+. However, at higher growth rates, the growth rate became independent of the Ca2+ gradient. Ca2+ fluxes at the growing apex of S. ferax are independent of growth rate, although this may be due to interplay between Ca2+ influx and Ca2+ exocytosis (Lew, 1999). In a comparison of the Ca2+ gradient and root hair growth by Wymer et al. (1997), the steepness of the Ca2+ gradient was similar to that in N. crassa (tau values of 4–12 μm), and the growth rate was about 10-fold less. Both the tau values and the difference between apical and basal [Ca2+] were correlated with growth rate, based on datasets comparing 0, 0.5 and 1.5 μm min⁻¹ growth rates. Thus either the gradient steepness or the tip-localized [Ca2+] could account for root hair growth, while in fungi it is the elevated tip-localized [Ca2+] which is important.

Tip-growing organisms grow at very different rates. Root hairs grow at about 1 μm min⁻¹ while fungi and pollen tubes grow about 10-fold faster. Since the magnitudes of the cytoplasmic [Ca2+] gradient are similar, the kinetics of Ca2+ supply, either from internal stores or from the extracellular medium, or both, must vary to maintain a steady state Ca2+ gradient. Clearly, one important determinant of growth rate will be the rate of vesicle supply to the growing tip. In fungi, this would result in increased [Ca2+] at the tip, but only if Ca2+ release was activated by IP3 production. In other organisms, Ca2+ influx would elevate [Ca2+] directly.

**Spontaneous generation of gradient to initiate growth**

If elevated tip-localized [Ca2+] relative to basal [Ca2+] is the key factor regulating growth rate, initiation of the Ca2+ gradient will precede polar organization of cytological structures. Ca2+ is known to play a role in conidial germination in some fungal species (Osherov & May, 2001), but it is not known whether a [Ca2+] gradient precedes germination. In some organisms, Ca2+ elevation does precede the appearance of tip growth. For example, a localized region of elevated Ca2+ predicts the site of rhizoid formation in Pelvetia compressa (Pu & Robinson, 1998). In other organisms, the Ca2+ gradient appears after initiation of tip growth: increased Ca2+ appears only after bulge formation in root hair development (Wymer et al., 1997). Microinjection of Ca2+ into N. crassa hyphae is known to initiate branching (Silverman-Gavrila & Lew, 2000), and therefore should function in the initiation of tip growth. From a biochemical perspective, it may be reasonable that elevated [Ca2+] would be important for both initiation of tip growth and continued hyphal growth. For any enzymic activity regulated by Ca2+, we expect [Ca2+] dependence to correspond closely with cytoplasmic [Ca2+]. The higher the tip-localized [Ca2+], the greater the enzyme activity at the tip, resulting in faster growth. Thus enzymic activities important in hyphal growth should be activated by [Ca2+] 30–160 nM higher than the basal [Ca2+] of about 220 nM 10–20 μm behind the tip. This predicted [Ca2+] dependence may be useful as the enzymic mechanisms causing initiation of hyphal extension are examined in more detail. In N. crassa, Ca2+-calmodulin activates chitin synthase (Suresh & Subramanyam, 1997), cAMP phosphodiesterase (Tellez-Inon et al., 1985) and calcineurin (PP2B) (Prokisch et al., 1997) and binds to microtubule-associated proteins (Ortega-Perez et al., 1994), all potential elements of polar organization and growth. Calcineurin is of special interest, since it appears to function in morphogenesis (Fox & Heitman, 2002) and generation or maintenance of the vesicular Ca2+ gradient imaged with CTC (Prokisch et al., 1997). Calcineurin forms an immunoprecipitable complex with COT1 (Gorovits et al., 1999), a serine threonine kinase known to function in normal hyphal growth (cf. Dickman & Yarden, 1999). In addition to a role in polar organization (Torrailba & Heath, 2001), the Ca2+ gradient would cause localized vesicle fusion, either on its own (Hall & Simon, 1976), or in association with a plethora of vesicle fusion mediators (Gupta & Heath, 2000, 2002).

The initiation of the Ca2+ gradient could be spontaneous. That is, in the spherical conidium or ascospore, random redistribution of the Ca2+ molecules could transiently create a Ca2+ gradient. A simplified simulation of a Ca2+ molecule random walk (Fig. 4) does suggest that initiation of hyphal growth could be a consequence of random molecular motions.

Once generated, the Ca2+ gradient must be maintained during continued hyphal growth. We explored the possibility that stretch-activated phospholipase C could sense hyphal expansion, and increase tip-localized [Ca2+] to maintain hyphal growth.
Phospholipase C may be the growth sensor

Fungal growth normally relies upon an internal hydrostatic pressure which would generate a constant tension on the hyphal plasma membrane/wall interface. As the hypha expands, the tension would increase. A natural candidate for sensing of hyphal expansion would be tip-localized stretch-activated Ca\(^{2+}\) channels, as occurs in S. ferax (Garrill et al., 1993). However, we have been unable to demonstrate any role for stretch-activated Ca\(^{2+}\) channels in hyphal growth in N. crassa (Lew, 1999; Silverman-Gavrila & Lew, 2000), even though they do exist in the plasma membrane (Levina et al., 1995), distributed evenly along the hypha. Because IP\(_3\) plays a role in generation of the Ca\(^{2+}\) gradient from internal stores, it is possible that a stretch-activated phospholipase C (Kinnunen, 2000) could sense hyphal expansion, and cleave PIP\(_2\) to IP\(_3\) and diacylglycerol. Phospholipase C inhibitors do inhibit hyphal growth and modify the vesicular Ca\(^{2+}\) gradient similarly to inhibitors of the IP\(_3\)-activated Ca\(^{2+}\) channel (Silverman-Gavrila & Lew, 2002). In fact, mild hypoosmotic stress does elevate rapidly diacylglycerol levels in conidial germlings (Table 2). Hypoosmotic stress activates phospholipase C in the plasma membrane of Dunaliella salina, as indicated by elevated diacylglycerol levels within 30 s (Ha & Thompson, 1991). In this case, phospholipase C activation probably plays a role in volume regulation of this wall-less green alga. Mechanical stretching is also reported to increase IP\(_3\) via phospholipase C activation in coronary artery (Tanaka et al., 1994). Thus a stretch-activated phospholipase C is a possible mechanism for sensing hyphal expansion. If this is the case, it is possible that other aspects of the polar cytology of the growing fungal tip may be regulated by other intermediates of the phosphatidylinositol and inositol phosphate metabolic pathways. Certainly, phosphoinositides have been implicated in regulation of the cytoskeleton (Yin & Janmey, 2003) and cellular polarity (Kost et al., 1999).

ACKNOWLEDGEMENTS

This research was funded by a discovery grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) (R.R.L.) and an Ontario Graduate Scholarship in Science and Technology (OGSST) and Ontario Graduate Scholarship (OGS) (L. B. S.-G.). Special thanks to Dr P. L. Lakin-Thomas for her advice and assistance in diacylglycerol extractions and HPLC analysis, and to the two anonymous reviewers for their thoughtful criticisms and helpful comments.

REFERENCES


Calcium gradients and fungal tip growth


