QUANTITATION OF CYTOPLASMIC CALCIUM IN GROWING *SAPROLEGNIA FERAX* HYPHAE USING INDO-1 AND TWO-PHOTON CONFOCAL MICROSCOPY

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Abstract

Ca^{2+} concentration was determined for the tip, 5 µm and 10 µm intervals between 10 to 80 µm from the tip of growing *S. ferax* hyphae using the Ca^{2+}-sensitive fluorochrome Indo-1. Results from this study suggest that there is a statistically significant difference between ratiometric readings, reflecting Ca^{2+} concentration, 0 to 30 µm from the tip, of two categories—'slow' and 'fast' growing hyphae. Although 'slow' and 'fast' growing hyphae exhibited tip-high Ca^{2+} concentration, slower growing hyphae exhibited a secondary peak 10 µm distal to the tip. The overall Ca^{2+} concentration, 0–30 µm from the tip, was higher in fast growing hyphae than in slow growing hyphae. The means of the ratiometric readings at 40 µm were not statistically significantly different between slow and fast growing hyphae. The increase in Ca^{2+} concentration at the base of growing branching hyphae also supports suggestions that there is a relationship between Ca^{2+} and hyphal growth.

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Introduction

Calcium ions (Ca^{2+}) have been implicated in a wide range of cellular responses including branching, sporulation, tip growth, zoospore motility and cytokinesis (Hyde 1998, Hyde & Heath 1995, Jackson & Hardham 1996, Pitt & Ugalde 1984). There are several lines of evidence to indicate that Ca^{2+} regulates tip growth including the concentrated expression of stretch-activated Ca^{2+} channels at the tip (Garrill *et al.* 1993, Jackson & Heath 1993), Ca^{2+} regulation of the cytoskeleton and vesicle fusion rates (Jackson & Heath 1993), and the orientation of germinating *S. ferax* cysts towards a Ca^{2+}-transporting ionophore, A23187 (Hyde & Heath 1995). During tip growth there is localized transport and fusion of cytoplasm and individual organelles at the tip (McKerracher & Heath 1986). The fine tubules, which make up the vacuolar reticulate structure in the growing tip of *S. ferax* hyphae, may allow expansion and movement of this organelle to occur while maintaining contact with the large vacuole in the more mature regions of hyphae (Allaway *et al.* 1997). There is some suggestion from selective staining of the vacuolar reticulum with Ca^{2+} dependent markers that the structure may be involved in the sequestering of Ca^{2+} (Allaway *et al.* 1997, Jackson & Heath 1993).

A tip-high gradient of cytoplasmic Ca^{2+} has been identified by Hyde and Heath (Hyde & Heath 1997) using Fluo-3 and SNARF, and by Garrill *et al.* (1993) using Indo-1 and the patch-clamp technique. How growth rate correlates with $[Ca^{2+}]i$ at discrete locations, particularly at the growing tip, has not been established. In this paper, Indo-1 and a highly sensitive two-photon confocal scanning microscope were used to determine the concentration of Ca^{2+} at discrete locations along the length of slow and fast growing *S. ferax* hyphae. The advantage of this technique is that it allows minor changes in concentration to be detected by measuring the wavelength shift in Indo-1 emission with changing intracellular Ca^{2+} concentration. The technique used in this study minimized background and autofluorescence and therefore allowed differences in intracellular Ca^{2+} concentration to be detected more accurately.

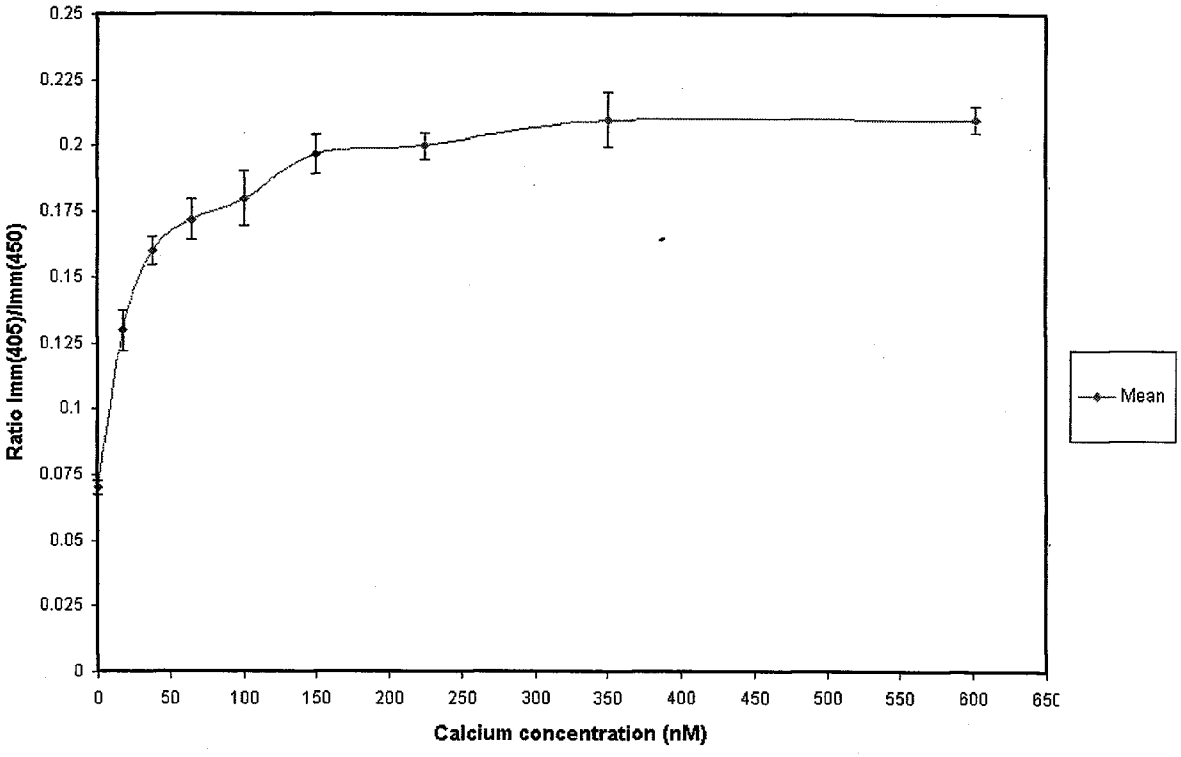
The 405/480 nm emission ratio method for the Ca^{2+} sensitive ratiometric indicator, Indo-1 was used to measure the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$). Upon Ca^{2+} binding, Indo-1 produces changes in fluorescence intensity and a shift in emission from 480 nm to 405 nm (Haugland 1996, Millot *et al.* 1995). The Indo-1 probe is conjugated with an acetyl-methyl ester (AM) residue to form a neutral Indo-1/AM molecule. In this esterified form Indo-1/AM is able to pass through the cellular membranes while minimising cellular disruption (Millot *et al.* 1995).

Materials and Methods

Maintenance of *S. ferax* cultures

The method outlined in Allaway *et al.* (1997) was used in the preparation of *Saprolegnia ferax* (Gruith.) Thuret. *Saprolegnia ferax* (H302) was obtained from stock cultures kept in the Research School of Biological Sciences, Canberra. In brief, the cultures were initially grown on GYPS agar (Beakes & Gay 1981) at 23°C in darkness until the plates were confluent. Small agar cubes of culture were sectioned and incubated in GYPS liquid medium (GYPSL) [5 g D-glucose, 0.5 g peptone, 0.5 g KH_2PO_4, 0.15 g $MgSO_4.7H_20$ and 0.05 g Yeast extract in 1L dH_20]. Hyphal segments (approx. 0.2 mm in length) were cut aseptically and incubated in GYPSL in sterile watch glasses. By 18–20 hrs the cultures were 10–15 mm in diameter and ready for Indo-1/AM loading.

Figure 1. Intracellular calcium calibration in *S. ferax* (0–602 nM).
The mean ratio of emission increased with increasing $[Ca^{2+}]i$. Indications of saturation occurred at 150 nM with limited increase in subsequent ratios. The ratio increase was more pronounced from 0 to 17 nM and 17 nM to 38 nM. From 38 nM to 150 nM the increase in emission ratio was less pronounced. The change in emission ratio from 0 nM to 38 nM was greater than between 38 nM and 225 nM.



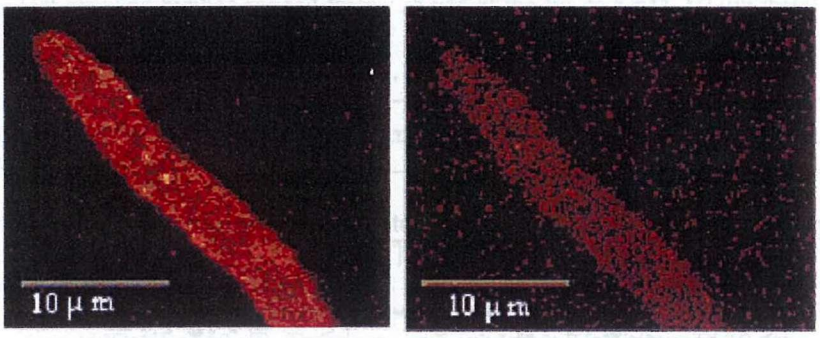
Indo-1/AM loading and specimen mounting

The cultures were rinsed once with GYPSL and incubated in the dark at room temperature with approximately 200 µl of 10 µg/ml Indo-1 AM ester (Molecular Probes Inc.) in GYPSL (pH5) for 30 min. They were kept submerged, in order to minimise disturbance and possible hyphal damage, then gently washed with fresh GYPSL followed by three partial rinses over 10 min. A coverslip was then gently slid under each culture to lift it. The coverslip was then inverted onto a slide over teflon spacers (Heath 1988) so that hyphae were not squashed. The mounted cultures were then left to recover normal morphology and growth. Using the Leica TCP SPII differential interference contrast facility, only viable hyphae exhibiting growth, the presence of cytoplasmic streaming, characteristic vacuolar reticulum and typical general hyphal morphology (Allaway *et al.* 1997, Rees *et al.* 1994) were measured in this study. Hyphal growth measurements were taken before laser irradiation by using an ocular micrometer. Only growing hyphae were used in this study. Growth was determined by measuring the distance travelled by hyphal tips over five minutes. The growth rates of all hyphae were recorded and the median growth rate (5.75 µm/min.) was used to separate slow growing hyphae (less than or equal to 5.75 µm/min.) and fast growing hyphae (greater than 5.75 µm/min.).

Two independent point measurements were taken for each distance (0–80 µm) along the length of eight randomly chosen hyphae in each of three cultures. The measurements for each distance were used to calculate the mean ratio of emission at 405 and 480 nm for that distance. The Student's t-test (paired two sample for

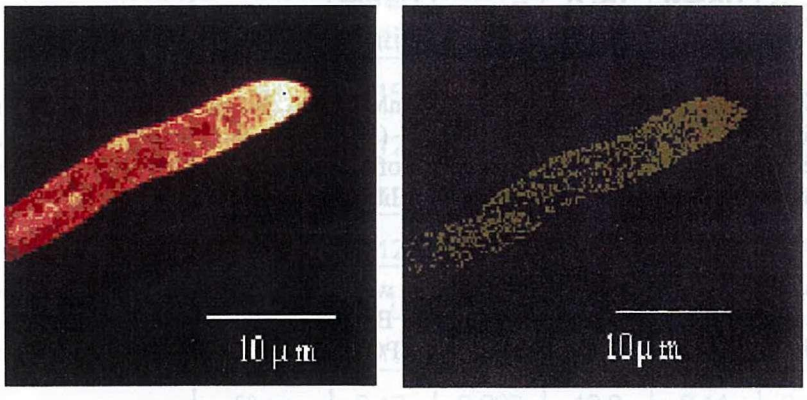
means) was used for ratiometric results obtained for different distances from the tip. The null hypothesis was that there was no significant difference between the ratiometric means for fast and slow growing hyphae at 0, 5, 10, 30, 40 or 50 µm from the tip, at a significance level of $p = 0.05$.

Figure 2. Intracellular calibration of Indo-1.



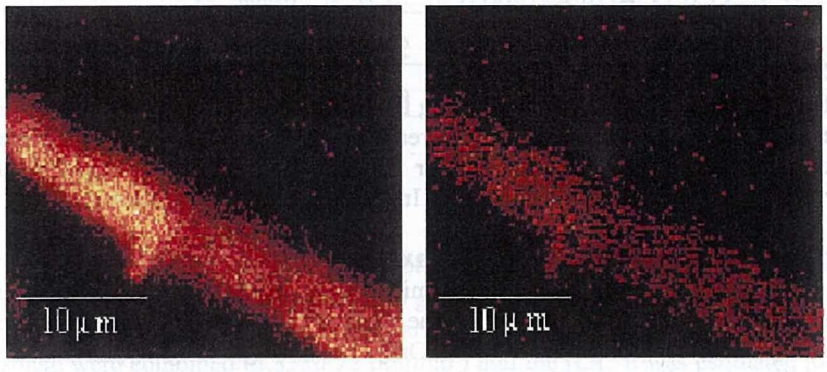
The intracellular $[Ca^{2+}]i$ was equilibrated with the extracellular $[Ca^{2+}]i$ using calibration buffers. Stable emission at 405 nm and 480 nm respectively along the hypha indicated an even distribution of Ca^{2+}. The intensity of emission increased uniformly along the hyphae with increasing Ca^{2+} calibration buffers. Sequestration of Indo-1 was indicated by random increases in emission along the length of the hyphae.

Figure 3. Emission of Indo-1 in a fast growing hypha.



Binding of Indo-1 to Ca^{2+} results in an emission shift from 480 nm to 405 nm respectively. Emission at 405 nm increased towards the tip indicating increased binding to Indo-1. By 5 µm Indo-1 emission decreased and continued to do so further from the trip. Emission levels reflect level of Ca^{2+} binding. No sequestering of Indo-1 was noted.

Figure 5. *Saprolegnia ferax* hyphal bud.

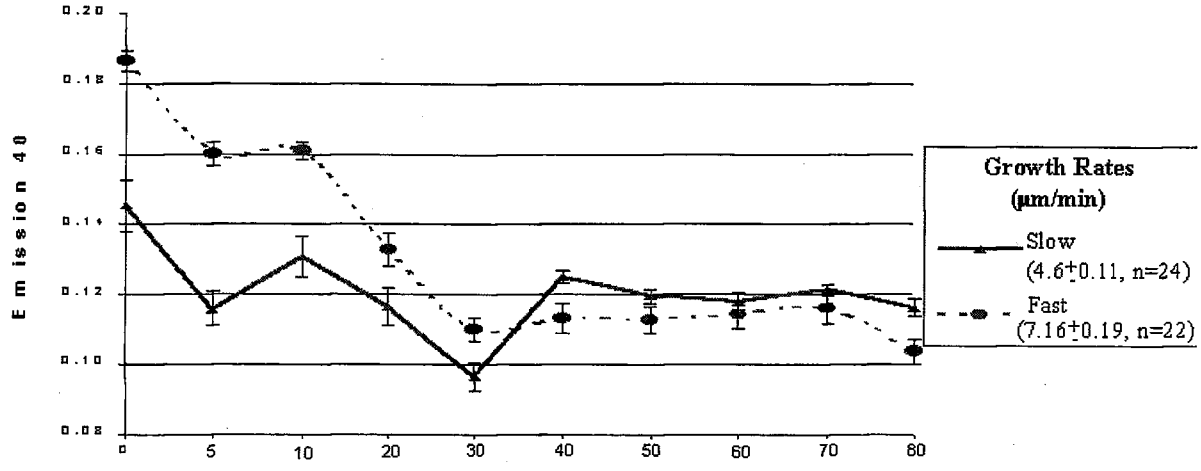


Ratiometric measurements (0.16±0.01) at the base (3–5 µm) of the hyphal bud ($n = 5$) indicated an increase in $[Ca^{2+}]i$ to 38 nM. Based on these limited results it is difficult to determine whether the increase in $[Ca^{2+}]i$ initiates budding or results from budding. The $[Ca^{2+}]i$ calculated at the base of the bud (3–5 µm) is the same as that for fast growing hyphae 5 µm from the tip.

Intracellular Calibration

Calibrated solutions of known Ca^{2+} concentration were obtained in kit form as a range of premixed Ca/EGTA (0 to 10.0 mM with corresponding free Ca^{2+} values ranging from 0 to 3900 nM from BioScientific Pty Ltd (Gymea, Australia). The pH was maintained at 7.2. Intracellular $[Ca^{2+}]i$ was equilibrated with the extracellular $[Ca^{2+}]i$ (Millot *et al.* 1995). The walls were permeabilised with 0.05% Triton-X (Sigma) in GYPSL for 10 min. (20°C) (Wang & Zhou 1999), washed three times with GYPSL and then incubated in the dark at room temperature with calibration buffer containing 10 µg/ml of Indo-1 for 30 min. The hyphae were mounted on teflon strips as described previously. The mean ratio of emission at 405 and 480 nm was calculated from measurements taken at random distances along the length of five hyphae randomly chosen in each of three cultures.

Figure 4. Emission ratio of *S. ferax* at different growth rates.
Measurements of fast growing hyphae (7.2±0.91 µm/min.) indicated higher ratiometric readings than slow growing hyphae (4.6±0.56 µm/min.), particularly between 0–20 µm. Emission ratios correlate directly with $[Ca^{2+}]i$. A secondary peak at 10 µm was only observed in slow growing hypha. The significant trough at 30 µm in slow growing hyphae may be due to continued retraction of Ca^{2+}.



Autofluorescence

Autofluorescence was determined for each culture by the addition of 20 mM $MnCl_2$ (Sigma Aldrich, Castle Hill, Australia) in calibration buffer or GYPSL as described by Monteith *et al.* (1997) to quench Indo-1 fluorescence. The background-corrected ratios were calculated by the subtraction of autofluorescence (10–20%) from the mean ratio of emission at 405 and 480 nm. $[Ca^{2+}]i$ was determined by using a calibration curve (Figure 1).

Microscope

A Leica TCS SP2 MP two photon confocal laser scanning microscope was used to measure Indo-1 emission intensity (Exc. 730 nm, PMT1 Band 385–410 nm, Gain 900V; PMT2 Band 455–495 nm, Gain 900V; Scan mode-xyz; Format 512 × 512; Pinhole[AV] airy; Objective-HCX PL APO 63x/1.20 W CORR). Images were saved as files.

Results

Intracellular calibration of Indo-1

Intracellular $[Ca^{2+}]i$ was equilibrated with the extracellular $[Ca^{2+}]i$ using the calibration buffers (λ1/λ2). Figure 1 indicates a gradual increase in the emission ratio as concentration increased up to 17 nM. A direct correlation between increasing ratio and increasing $[Ca^{2+}]i$ was not evident for concentrations above 150 nM as the saturation level was reached. Only hyphae not showing sequestration of Indo-1 were included (Figure 2).

Influence of growth rate on the calcium gradient along *S. ferax* hyphae

Laser irradiation of hyphae with the two-photon confocal scanning microscope did not appear to cause any deleterious morphological effects or inhibit growth. Viability of the hyphae was checked by differential interference contrast microscopy before and after laser irradiation. Only one fast growing hypha failed to maintain all the indicators of viability, including growth, cytoplasmic streaming, an undisturbed vacuolar reticulum and general hyphal morphology. The hypha was therefore excluded from the data. The images were obtained from 4 irradiating scans, each taking approximately 5 seconds. The mean preimaging growth rate was 5.83±0.22 µm/min. (n = 46). The relationship between calcium gradient and growth rate was investigated by sub-dividing the population of measured hyphae, using the growth median (5.75 µm/min.), into 'slow' and 'fast' growing hyphae with mean growth rates of 4.6±0.11 µm/min. (n = 24) and 7.16±0.19 µm/min. (n = 22) respectively. In growing hyphae, ratiometric values increased towards the tip of the hypha (Figure 3). Differences in emission ratio which reflect $[Ca^{2+}]i$ were observed for slow and fast growing hyphae. These were statistically significant at 0 µm ($t = -6.1$, $df = 21$, $p = 5 \times 10^{-6}$), 5 µm ($t = -7.5$, $df = 21$, $p = 2.4 \times 10^{-7}$), 10 µm ($t = -6.7$, $df = 21$, $p = 1.4 \times 10^{-6}$) and 30 µm ($t = -3.2$, $df = 21$, $p = 4 \times 10^{-3}$) from the tip (Figure 4), but not at 40 µm ($t = 1.8$, $df = 17$, $p = 0.08$) or 50 µm ($t = 1.3$, $df = 17$, $p = 0.2$). No differences were detected between slow and

fast growing hyphae at 60 µm, 70 µm and 80 µm distal to the tip. Using Figure 1, $[Ca^{2+}]i$ was calculated for slow and fast mean growth rates (Table 1). Calculated calcium levels for slow growing hyphae ranged from 28 nM at the tip, to a 13 nM plateau 40 µm to 80 µm distal to the tip. Slow growing hyphae exhibited a secondary peak of 17 nM at 10 µm, which was not evident at 10 µm in fast growing hyphae (Figure 4). Calculated calcium levels for fast growing hyphae ranged from 125 nM at the tip, to 8 nM, 80 µm distal to the tip. In fast growing hyphae, calcium levels decreased from 125 nM at the tip to 38 nM, 5–10 µm distal to the tip. A further decline in calcium levels was observed at 20 µm (17 nM), 30 µm to 60 µm (11 nM) and 80 µm (8nM).

Emission ratios for hyphal buds 3–5µm in length (n = 5) were also measured and the $[Ca^{2+}]i$ calculated to be 38 nM (Figure 5).

Table 1. Calcium concentration estimates for slow and fast growing *S. ferax* hyphae.
In slow growing hyphae (n = 24), $[Ca^{2+}]i$ decreased from 28 nM at the tip to 8 nM at 30 µm. A secondary increase in $[Ca^{2+}]i$ at 10 µm (17 nM) was noted. From 40 to 80 µm there was no change in $[Ca^{2+}]i$. In fast growing hyphae (n = 22), the $[Ca^{2+}]i$ changed more steeply from 125 nM at the tip to 11 nM at 30 µm. There was no secondary increase in $[Ca^{2+}]i$ at 10 µm. There was little change in $[Ca^{2+}]i$ beyond 10 µm.

	Slow Growth Rates (4.6±0.56µm/min)			Fast Growth Rates (7.16±0.91µm/min)		
Location	Mean Ratio	SE	[Ca] (nM)	Mean Ratio	SE	[Ca] (nM)
tip	0.15	0.007	28.0	0.19	0.003	125
5µm	0.12	0.005	13.0	0.16	0.003	38
10µm	0.13	0.006	17.0	0.16	0.002	38
20µm	0.12	0.005	13.0	0.13	0.004	17
30µm	0.1	0.004	8.0	0.11	0.003	11
40µm	0.13	0.002	13.0	0.11	0.005	11
50µm	0.12	0.002	13.0	0.11	0.004	11
60µm	0.12	0.002	13.0	0.11	0.004	11
70µm	0.12	0.002	13.0	0.12	0.004	13
80µm	0.12	0.002	13.0	0.1	0.003	8

Discussion

Using Indo-1/AM ester and the two-photon confocal laser scanning microscope it was shown that slow (4.6±0.11 µm/min.) and fast (7.16±0.19 µm/min.) growing *S. ferax* hyphae have tip-high $[Ca^{2+}]i$ gradients of around 28 and 125 nM respectively. To allow comparison with the literature ratiometric readings of slow and fast growing hyphae were combined (5.83±0.22 µm/min.) and the $[Ca^{2+}]i$ was estimated to be 65 nM (0.17±0.005) at the tip. Using Fluo-3 and SNARF, Hyde & Heath (1997) estimated a tip-high concentration of 76 nM. The results from this study suggest that estimates of $[Ca^{2+}]i$ near the tip (0–30 µm) are influenced by growth rate.

There are several lines of evidence that support the theory that the tip-high $[Ca^{2+}]i$ influences growth (Hyde & Heath 1997, Levina *et al.* 1994). During growth, the Ca^{2+} sensitive filamentous actin cap of an *S. ferax* hypha undergoes cytoplasmic contraction and strengthening (Jackson & Heath 1993). The concentration of stretch-activated Ca^{2+} channels at the tip is mediated by an actin-dependent system (Garrill *et al.* 1993, Levina *et al.* 1994). Similar observations of tip-high $[Ca^{2+}]i$ gradient have been demonstrated in growing pollen tubes, root hairs and *S. ferax* and *Achlya* spp. hyphae (Jackson *et al.* 2001, Obermeyer & Weisenseel 1991, Reiss & Heath 1979, Yuan & Heath 1991). The distribution of mitochondria, endoplasmic reticula and Golgi bodies located approximately 8 µm distal to the tip may also contribute to a tip-high $[Ca^{2+}]i$ gradient exhibited by *S. ferax*

(Garrill *et al.* 1993). In pollen tubes, the high concentrations of mitochondria and endoplasmic reticula at the tip have been implicated as Ca^{2+}-sequestering structures (Jackson *et al.* 2001). The buffering capacity of mitochondria and other organelles located in specific regions of growing hyphae needs to be further investigated using the patch-clamp or similar techniques. The secretory vesicles observed at 1–8 μm in *S. ferax* using chlortetracycline (Yuan & Heath 1991), may be associated with the fine tubules that form the vascular reticulum at the tip of the hypha (Allaway *et al.* 1997).

In this study, hyphal growth ranged between 4 and 8.5 μm/min. Subdivision of growth rates at the median into 'slow' and 'fast' growing hyphae made it possible to observe statistically significant differences between emission ratios of slow and fast growing hyphae 0 to 30 μm from the tip. In contrast, there was no significant difference in emission ratios at 40 or 50 μm from the tip. Similarly, no differences in emission ratios were observed 60, 70 and 80 μm from the tip (Figure 4). The higher ratiometric readings of fast growing hyphae (0 to 30 μm) suggest a direct correlation between $[Ca^{2+}]i$ in *S. ferax* and growth. In *S. ferax* hyphae $[Ca^{2+}]i$ was estimated from the tip (0, 5, 10, 20 and 30 μm) to be 28, 13, 17, 13 and 8 nM in slow growing hyphae and 125, 38, 38, 17 and 11 nM in fast growing hyphae (Table 1). The results suggest that Ca^{2+} gradient increases as growth rate increases. A similar correlation between growth rate and $[Ca^{2+}]i$ has been observed by Hyde & Heath (1997).

It is possible that the observed differences in Ca^{2+} distribution in slow and fast growing hyphae may be due to the retraction of Ca^{2+} from the vacuolar reticulum at the tip into the central vacuole. The central vacuole is a potential calcium sink (Allaway *et al.* 1997) located approximately 10–20 μm distal to the tip. The continuing retraction of Ca^{2+} into the central vacuole may account for the irregularity in ratiometric readings 10–40 μm from the tip of slow growing hyphae. Whether $[Ca^{2+}]i$ influences growth or vice versa is not clear from these results. A larger sample size may provide a clearer indication of the relationship between growth and $[Ca^{2+}]i$.

Unlike *S. ferax*, $[Ca^{2+}]i$ in *Neurospora* appeared to peak 10 μm from the tip using non-ratiometric Ca^{2+} dye, Fluo-3 (Jackson & Heath 1993). A similar observation was made using ratioed Fluo-3 and SNARF-1 in *Neurospora* (Levina *et al.* 1994). The potential sources of Ca^{2+} in *Saprolegnia* and *Neurospora* could provide a possible explanation for these differences. In *Saprolegnia* the Ca^{2+} source is more likely to be from the tip-localized stretch-activated Ca^{2+} channels (Garrill *et al.* 1993), whereas in *Neurospora*, the subapical stores of Ca^{2+} may contribute to the peak at around 10 μm (Levina *et al.* 1994).

The observed decrease in $[Ca^{2+}]i$ 5 μm distal to the tip may be due to the lack of distinction in emission readings of the vacuolar reticulum, the central vacuole, and the surrounding cytoplasm. Subcellular measurements of plants have shown that the central vacuole $[Ca^{2+}]i$ levels were three to four times higher than the surrounding cytoplasm (Gilroy *et al.* 1993). The $[Ca^{2+}]i$ gradient declined gradually from the tip and plateaued 40–70 μm distally. This is unlike Hyde & Heath's (1997) observation of a steep decline in $[Ca^{2+}]i$ at 40 μm. The central vacuole extending throughout this region, is most likely complemented by increasing numbers of nuclei (Yuan & Heath 1991).

In developing branches of the hyphae there were indications of elevated levels of Ca^{2+} (38 nM) at the base of 3–5 μm long hyphal buds (Figure 5). The presence of 'hotspots' or localized elevated readings of Ca^{2+} early in branching may act as a special cue or morphogen and define the axis of polarity (Hyde & Heath 1995, Jackson *et al.* 2001). Evidence supporting Ca^{2+} as a morphogen in the growing tip include its regulation of actin, stretch-activated Ca^{2+} channels, cytoskeletal changes and vesicle fusion (Garrill *et al.* 1993, Jackson & Heath 1993). There is, however, inconclusive evidence to indicate that Ca^{2+} is essential for the earlier stages of branch emergence (Jackson *et al.* 2001). It is not clear from these preliminary observations whether the elevated levels of Ca^{2+} trigger budding or whether another factor initiates Ca^{2+} accumulation.

It has been observed with Fluo-3 and SNARF that subapically $[Ca^{2+}]i$ was higher in the periphery of *S. ferax* hyphae than in the centre (Hyde & Heath 1997). The Ca^{2+} sequestering mitochondria located along the wall (Yuan & Heath 1991) may contribute to differential spatial distribution of $[Ca^{2+}]i$ from the middle of the hypha to the periphery. Results with Indo-1 were not able to demonstrate changes in the distribution of Ca^{2+} along the width of *S. ferax* hyphae in subapical locations. $[Ca^{2+}]i$ was, however, not measured beyond 80 μm where mitochondria localization was noted (Yuan & Heath 1991). The central vacuole, 80 to 100 μm distal to the tip, takes up a large proportion of the hypha. The restriction of the mitochondria to the periphery suggests the low $[Ca^{2+}]i$ exhibited in this area may reflect the $[Ca^{2+}]i$ present in the central vacuole alone.

Evidence from this study suggests that the growing hyphae of *S. ferax* have a tip-high gradient of Ca^{2+}. There appears to be a correlation between growth rate and Ca^{2+} gradient, particularly 0 to 30 μm from the tip. The increase in ratiometric reading 10 μm from the tip in slow growing hyphae may reflect physiological and spatial changes in the Ca^{2+} gradient during growth.

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