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Ca^{2+} Gradients in Hyphae and Branches of *Saprolegnia ferax*

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Hyde, G. J., and Heath, I. B. 1997. Ca^{2+} gradients in hyphae and branches of *Saprolegnia ferax. Fungal Genetics and Biology* 21, 000–000. Hyphae of *Saprolegnia ferax* maintain a tip-high gradient of cytoplasmic Ca^{2+} which is essential to tip growth. The gradient extends further along the periphery than the center of the hyphae, consistent with an extended region of Ca^{2+} influx. Both cytoplasmic [Ca^{2+}] and the gradient's slope are higher in faster growing hyphae, indicating greater exocytosis or apical extensibility in response to elevated [Ca^{2+}]. Conversely, UV-induced elevation of apical [Ca^{2+}] to levels little above those of faster growing hyphae transiently halted growth, showing that the relationship between growth and apical [Ca^{2+}] is applicable within a narrow range. UV elevated [Ca^{2+}] concentrated in central cytoplasm, a putative storage region, consistent with maintenance of observed gradients formed without exogenous Ca^{2+} from intracellular stores. Branch formation did not show elevated [Ca^{2+}] levels in the subtending hyphae, but a gradient formed as the branch developed, indicating either an unknown initiator or subdetectable [Ca^{2+}] initiation concentrations which are less than those needed for tip maintenance.

Index Descriptors: tip growth; Ca^{2+}; branching; *Saprolegnia;* ratio imaging.

Tip growth is one of the most successful cellular adaptations, being the dominant growth mode of the estimated 1.5 million extant fungal species (Hawksworth, 1991); it also occurs in plant cells such as pollen tubes and root hairs. Tip growth permits the exploration of extensive environments, which is made possible by three attributes of the tip: first, it can continually advance, while retaining links with a distant nutrient source; second, it extrudes a wall that provides both penetrative capacity and a protective shell against which internal forces can be exerted; and third, it can alter its direction of growth in response to environmental cues. These activities depend upon the cooperative involvement of the cytoskeleton, turgor pressure, ion channels and integrin-like proteins in the plasma membrane, vesicle production, wall extensibility, and cytoplasmic ion gradients (Harold, 1990; Heath, 1990; Kropf, 1992; Miller *et al.*, 1992; Jackson and Heath, 1993; Kropf, 1994; Malho *et al.*, 1994; Pierson *et al.*, 1994; Kaminskyj and Heath, 1995).

Several lines of argument indicate a central, coordinating role for cytoplasmic Ca^{2+} in tip growth. Ca^{2+} ions are well suited to such a role, given their ubiquitous functions as second messengers in eukaryotic cells. Experimentally, Ca^{2+} regulation of the cytoskeleton and vesicle fusion rates is well known (Hepler and Wayne, 1985; Gomperts, 1986; Jackson and Heath, 1993). Long-term tip growth requires external Ca^{2+} and patent, stretch-activated plasma membrane Ca^{2+} channels (Jackson and Heath, 1989; Garrill *et al.*, 1993; Jackson and Heath, 1993). Also, production of various tip-growing systems can be polarized by gradients of Ca^{2+} or Ca^{2+}-regulating ionophores (Robinson and Cone, 1980; and references in Hyde and Heath, 1995).

However, because of technical difficulties (Read *et al.*, 1992), the required studies of the distribution and behavior

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of cytoplasmic Ca^{2+} in hyphae have been limited. Only four studies have achieved adequate Ca^{2+} indicator dye loading, (Garrill *et al.*, 1993; Jackson and Heath, 1993; Levina *et al.*, 1995; Yuan *et al.*, 1995), and the results have indicated differences between species. In *Saprolegnia*, use of the ratiometric dye Indo-1 indicates that $[Ca^{2+}]$ is highest at the very tip of the hypha (Garrill *et al.*, 1993), while the fluorescence of the nonratiometric Ca^{2+} dye, Fluo-3, peaks 3–9 μm back (Jackson and Heath, 1993; the data of Yuan *et al.*, 1995, was not at sufficient resolution to determine this feature). It has been argued that the subapical position of the Ca^{2+} peak suggested by Fluo-3 is artifactual, resulting from a high cytoplasm to organelle ratio in this region (Jackson and Heath, 1993). However, even when Fluo-3 was ratioed against SNARF-1, in the *Neurospora* study, the Ca^{2+} peak still appeared behind the tip (Levina *et al.*, 1995). It has been proposed that the latter difference is real and that the two species may have different sources for the Ca^{2+} gradient: release of Ca^{2+} from subapical stores (Levina *et al.*, 1995) and ion flow through tip-localized plasma membrane Ca^{2+} channels (Garrill *et al.*, 1993). Thus these two organisms might rely on dissimilar sources of Ca^{2+}, but the differences may be due to the approaches used to detect cytoplasmic Ca^{2+}.

In the present study we further explore the nature and significance of patterns of cytoplasmic Ca^{2+} distribution within *Saprolegnia* hyphae. We have studied *Saprolegnia* using the same methodology that provided the *Neurospora* results (Levina *et al.*, 1995) and used both much larger sample sizes than those used in previous studies and the single wavelength Ca^{2+} dye, Calcium Green. We have also examined the relationship between hyphal growth rate and Ca^{2+} distributions, the effects of changing external Ca^{2+} and pH, and the relative levels of Ca^{2+} in developing branches. The results indicate that previously reported differences between *Neurospora* and *Saprolegnia* are not methodologically based, that the Ca^{2+} gradient is regulatory, and that, taken together with previous *Neurospora* results, hyphae may use internal stores of Ca^{2+} to maintain the gradient when external Ca^{2+} is limiting.

MATERIALS AND METHODS

Culturing, Dye Loading, and Mounting of Hyphae

Hyphae of *Saprolegnia ferax* (Gruith) Thuret (ATCC No. 36051) were grown on strips of dialysis tubing on an agar medium (OM; Heath and Greenwood, 1968). After about 12 h of growth, portions of the strips were transferred to embryo cups containing approximately 1 mL of liquid OM, buffered to pH 4.0 with citrate buffer. The culture was left to adapt to the low pH medium for 18–24 h, after which it was covered with 180 μL of solution containing between 140 and 280 μ*M* of one of two Ca^{2+}-sensitive dyes (Fluo-3, pentapotassium salt; Teflabs, Austin, TX, henceforth abbreviated to Fluo; or Calcium Green-1, hexapotassium salt; Molecular Probes, Eugene, OR) and/or 175–350 μ*M* of the Ca^{2+}-insensitive, pH-sensitive dye, carboxy SNARF-1 (Molecular Probes; henceforth abbreviated to SNARF). The dye-loading mixtures for each sample of culture were made from concentrated dye stock solutions (in dH_2O), further diluted with some of the pH 4.0 OM in which the sample had been growing. After a further 20–24 h, the culture was briefly washed several times in another embryo cup with either buffered (pH 4.0) or unbuffered OM (pH 5.5) or a modified growth medium (pH 5.5, MOM; Garrill *et al.*, 1993). It was then placed in a slide chamber (Heath, 1988) in buffered or unbuffered OM or MOM, where it was maintained for approximately 20–40 min prior to measuring. Some samples were mounted in Ca^{2+}-free MOM, containing 50 m*M* BAPTA (K^+ salt, Teflabs). In these experiments, the slides, coverslips, and embryo cups used for washing samples were prewashed in concentrated HCl and 1 m*M* EGTA. The free $[Ca^{2+}]$ in the BAPTA-containing mounting medium was estimated using a modified version of software supplied by Drs. Jean Youatt and Ian McKinnon (Youatt and McKinnon, 1992).

Estimation of $[Ca^{2+}]$ within Hyphae

Most experiments used hyphae dual-loaded with Fluo and SNARF. Images of these dyes' fluorescence were acquired using the twin photomultipliers of a Bio-Rad MRC600 confocal microscope, fitted with a krypton–argon laser and K1 and K2 filter blocks. The latter was modified by replacing the 585-nm barrier filter with a band pass filter transmitting at 607 nm (bandwidth at 50% peak transmission is 20 nm; Omega Optical, Brattleboro, VT). Fluorescence images from cells excited at 488 nm were detected simultaneously in the photon counting mode via an oil immersion, 1.4 NA, 60X objective and analyzed with Bio-Rad's Comos and SOM software. All images were obtained without a neutral density filter and with the pinhole wide open. The number of scans for each image was kept to the minimum (typically about 10, depending on dye loading) required to acquire a useable image, to

reduce total exposure and photobleaching. The 522-nm filter of the K2 block selected the Ca^{2+}-sensitive Fluo emissions (F) and the 607-nm filter selected the isobestic emissions of SNARF (S). It is important to understand that at this wavelength, the SNARF emissions are independent of variations in both Ca^{2+} and H^+ concentrations; thus variations in the ratio between the intensities of the two dyes should indicate variations in Ca^{2+} concentrations, assuming that there are no variations in concentrations of the two dyes. Ratio images (F/S) were created on an Image-1 system (Universal Imaging Corp., West Chester, PA). Some images were acquired using the standard photomultiplier mode of the confocal microscope, but no quantitative measurements were made from these.

Measurements of hyphal growth rates were made with an ocular micrometer (1 μm/div) immediately before and sometimes also after recording their fluorescence images. The laser irradiation of the hyphae needed to acquire the images was not deleterious; in 16 cases a hypha was imaged two or more times with an average of 9.6 scans per image. The preimaging growth rates increased from 1.69 μm/min (±2.57, range 0–8) to 2.69 μm/min (±1.99, range 1–8) with a mean of 6.32 min between images. All of these hyphae continued (or started) to grow at the same or higher rates except one which declined from 7 to 6 μm/min. The fastest growing member of the population was recorded at 8 μm/s before imaging and at the same rate 50 s after the end of imaging, prior to the next imaging.

The relative intensities of F and S were assessed in two ways. First, measurements were made, using the raw photon-counting-acquired paired images, of the average pixel intensity in boxes (cf Levina *et al.*, 1995) placed at the tip and 5, 10, and 40 μm back from the tip of the hypha. These measurements were normalized for the number of scans that were used to acquire each image pair; then the mean values of autofluorescence (which averaged 30% of the total signal) at the tip, 5, 10, and 40 μm at the Fluo and SNARF wavelengths in 49 unloaded growing hyphae, were subtracted. Nongrowing hyphae had levels of autofluorescence similar to those of these growing hyphae. Histograms of pixel intensities of the autofluorescence images were examined for obvious anomalies such as plateauing or sharp vertical cut-offs to ensure that the full signal was being recorded. Images of dye-loaded and autofluorescent cells were acquired at the same instrument settings. Mathematical calculations and statistical analysis were performed using the Macintosh Statview package.

Second, ratio imaging was performed, after transfer of the F and S images to separate buffers in the Image-1 software. To facilitate visual comparison of the final ratio images, one or both of the F and S images were adjusted by multiplying or dividing the intensity of each image pixel by constant factors to produce a F/S ratio of approximately 1:4 at the 40-mm point. This means that comparisons of visual F/S ratios are meaningful within, but not between, individual hyphae. However, when a single hypha was manipulated, before and after after images were treated identically for valid comparison.

Images of Calcium Green fluorescence were acquired on photon counting mode, using the normal FITC filter combination of the Bio-Rad MRC600.

To estimate levels of crosstalk between the filter blocks, hyphae loaded only with either Fluo or SNARF were imaged using the opposite filter combinations.

Values preceded by ± in the text, and the error bars in the figures, are all standard errors of the indicated means.

UV Irradiation

Hyphae, unloaded or loaded with Fluo and SNARF, were irradiated for 30 s with 330- to 380-nm light from the Nikon UV-2B filter block. The intensity of the beam was adjusted by varying the size of the iris diaphragm between the mercury light source and the filter. High intensities killed the cells; thus a lower intensity was chosen to cause slowing or temporary cessation of growth.

RESULTS

Dye Loading

Optimal dye loading was a compromise between achieving sufficient dye to give a useable signal and avoidance of dye sequestration. The latter is a serious problem in hyphae since even dextran-conjugated dyes rapidly become sequestered into intracellular compartments when microinjected at high concentration (Read *et al.,* 1992). Concentrations of 140–280 μ*M* Fluo and Calcium Green and 175–350 μ*M* SNARF gave fluorescence images which showed the desirable exclusion of dye from vacuoles and nucleus-like regions (Figs. 1, 2, 4, and 8) and intensities which were two to three times above autofluorescence. Higher concentrations gave higher intensities, but the dyes

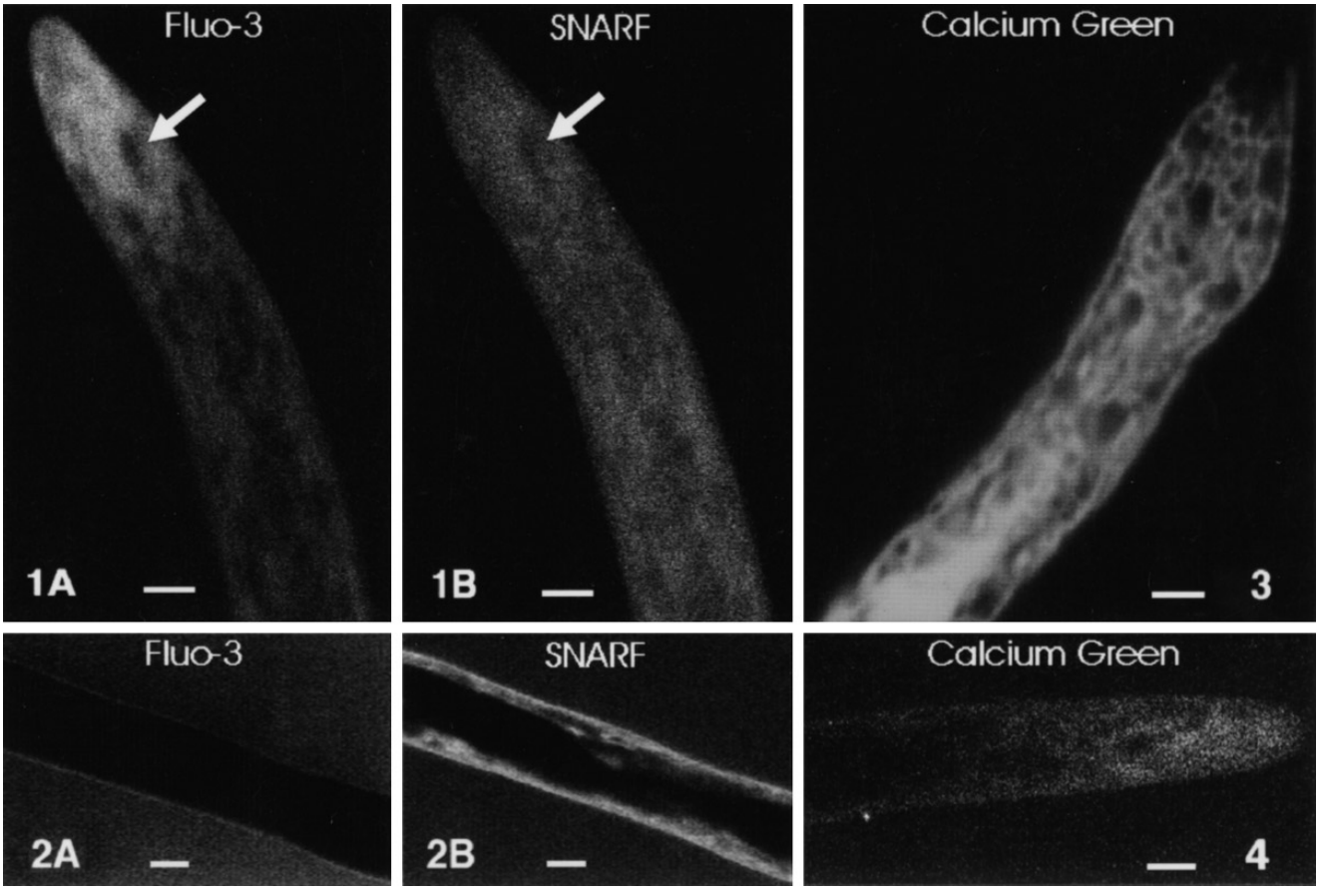


FIG. 1. Successful dual loading of Fluo (A) and SNARF (B) results in cytoplasmic localization of the dyes, shown here in two simultaneous confocal microscope images of a single hypha growing under standard conditions. Large organelles, probably nuclei, exclude the dye, giving rise to obvious shadows (arrows). Fluo fluorescence is higher near the tip. A color ratio image of this hypha is shown at the bottom left in Fig. 6. Bar, 5 μm.

FIG. 2. When loading was successful, both Fluo (A) and SNARF (B) were also excluded from the vacuole in distal portions of the hypha. Vacuole exclusion of the cytoplasm to the hyphal periphery, as shown in B, cannot explain the peripheral extension of the apical $[Ca^{2+}]$ gradients (Fig. 6) since this degree of vacuolation is not present until much further back than the ± 40 μm analyzed in Fig. 6. Some cytoplasmic strands are seen in B, loaded with SNARF. Bar, 5 μm.

FIG. 3. Sequestration of Calcium Green in a reticulate network and the vacuole, resulting from incubation in too high concentration of dye. Similar patterns were seen in hyphae highly loaded with Fluo and SNARF. Bar, 5 μm.

FIG. 4. Hypha optimally loaded with Calcium Green, growing under standard conditions. Fluorescence is not sequestered into tubues (cf. Fig. 3) and is highest near the tip, indicating that $[Ca^{2+}]$ is also higher in this region. Bar, 5 μm.

were clearly sequestered (Fig. 3). At any specific dye concentration, there was always interhyphal variability in the amount of dyes taken up.

Hyphal Ca^{2+} Distribution in Growing Hyphae

Because fluorescence of the optimal concentrations of loaded dyes was not greatly above background, it was necessary to use both the normal ratioed visual images of single hyphae and statistically analyzable population data to assess the validity of the indicated $[Ca^{2+}]$. Furthermore, when we analyzed the individual Fluo and SNARF data, we found unexpected variations which indicate the need to present, and explain, both ratio and individual dye data.

Since the major parts of tip morphogenesis are located in the most apical approximately 10 μm of the hyphae, we focused our quantitative analysis there and compared the mean fluorescence intensities in this region with those 40

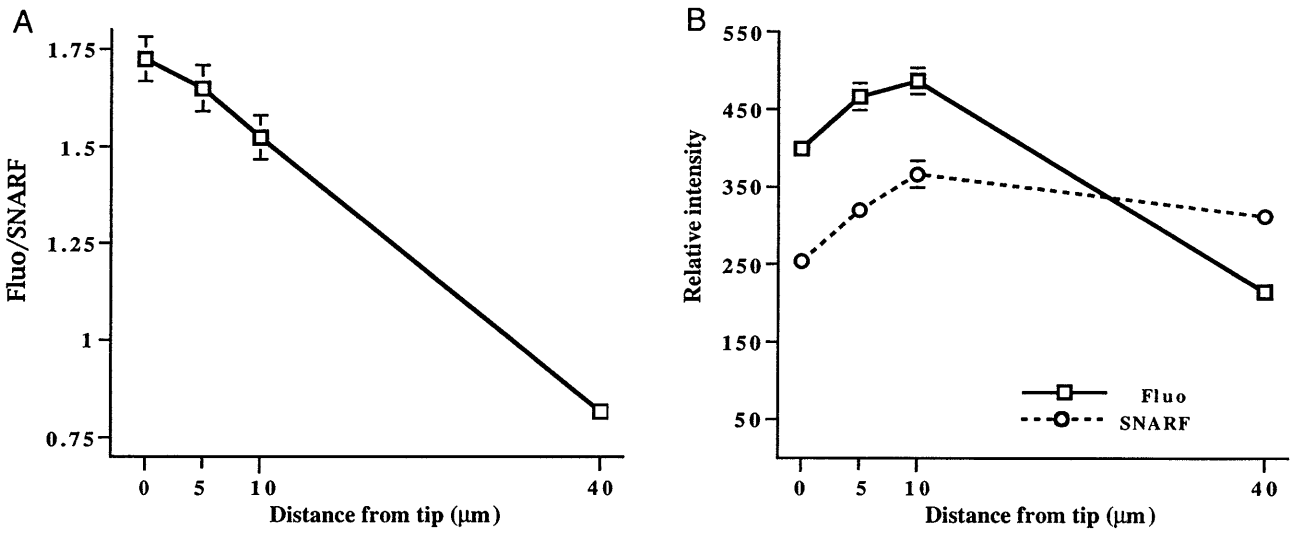


FIG. 5. (A) The means of the individual ratios of intensities of fluorescence due to Fluo and SNARF along the length of hyphae ($n = 133$). The mean ratios at the four points were significantly different with $F/S_{tip} > F/S_{5\ \mu m} > F/S_{10\ \mu m} > F/S_{40\ \mu m}$ (ANOVA, repeated measures test, $P = 0.0001$; mean ranking by Fisher's PLSD and Scheffe's F test, $P = .05$). The line indicates a tip high gradient of cytoplasmic free $[Ca^{2+}]$. (B) The mean relative intensities (in arbitrary units) of Fluo and SNARF at the same four points shown in A. Hyphae growing under standard conditions.

μm back, which is well behind the main zone of tip shape change. In 133 growing hyphae, the mean F/S ratios, corrected for autofluorescence, declined behind the apex (Fig. 5A). Using the calibration in Levina *et al.* (1995), these ratios (Fig. 5A) indicate 76, 70, 62, and 31 n*M* Ca^{2+}, respectively. The F/S ratio at the tip was greater than at 5, 10, and 40 μm in 77, 86, and 100% of hyphae, respectively. The dye curve for SNARF (measured at an ion concentration insensitive wavelength; Fig. 5B) indicated that dye concentration was not uniform in the tips. SNARF was most abundant 10 μm back from the tip, declined substantially toward the apex, probably due to known variations in organelle distributions in these hyphae (Heath and Kaminskyj, 1989), and declined only gradually subapically, thus indicating the need for ratio imaging. The Ca^{2+}-sensitive Fluo curve showed a similar peak at 10 μm, but declined less steeply toward the tip and more steeply subapically, giving the ratio gradient, which is most simply explained as an indication of variation in cytoplasmic $[Ca^{2+}]$. The fluorescence image of Calcium Green was consistent with the Fluo, SNARF, and Ca^{2+} distributions; it too showed a tip high gradient, presumably due to tip high Ca^{2+}, which peaked around 10 μm behind the tip, presumably due to a higher dye concentration (Fig. 4).

Individual hyphae viewed as the ratio of the paired Fluo and SNARF intensities in each pixel also showed a tip high Ca^{2+} gradient (Fig. 6), consistent with the quantitative data. However, a conspicuous additional feature of these images is the shape of the higher Ca^{2+} concentrations, which consistently extend further subapically along the sides of the hyphae than in their centers.

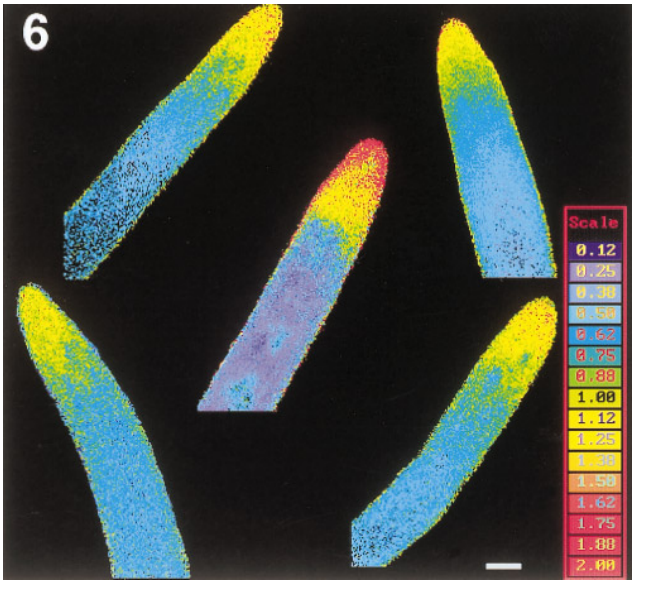


FIG. 6. Five typical pseudocolored F/S ratio images of growing hyphal tips. The scale bar shows how the colors relate to the F/S ratio, and, indirectly, the $[Ca^{2+}]$. The lower left hypha is the same one shown in Fig. 1. Hyphae growing under standard conditions. Bar, 5 μm.

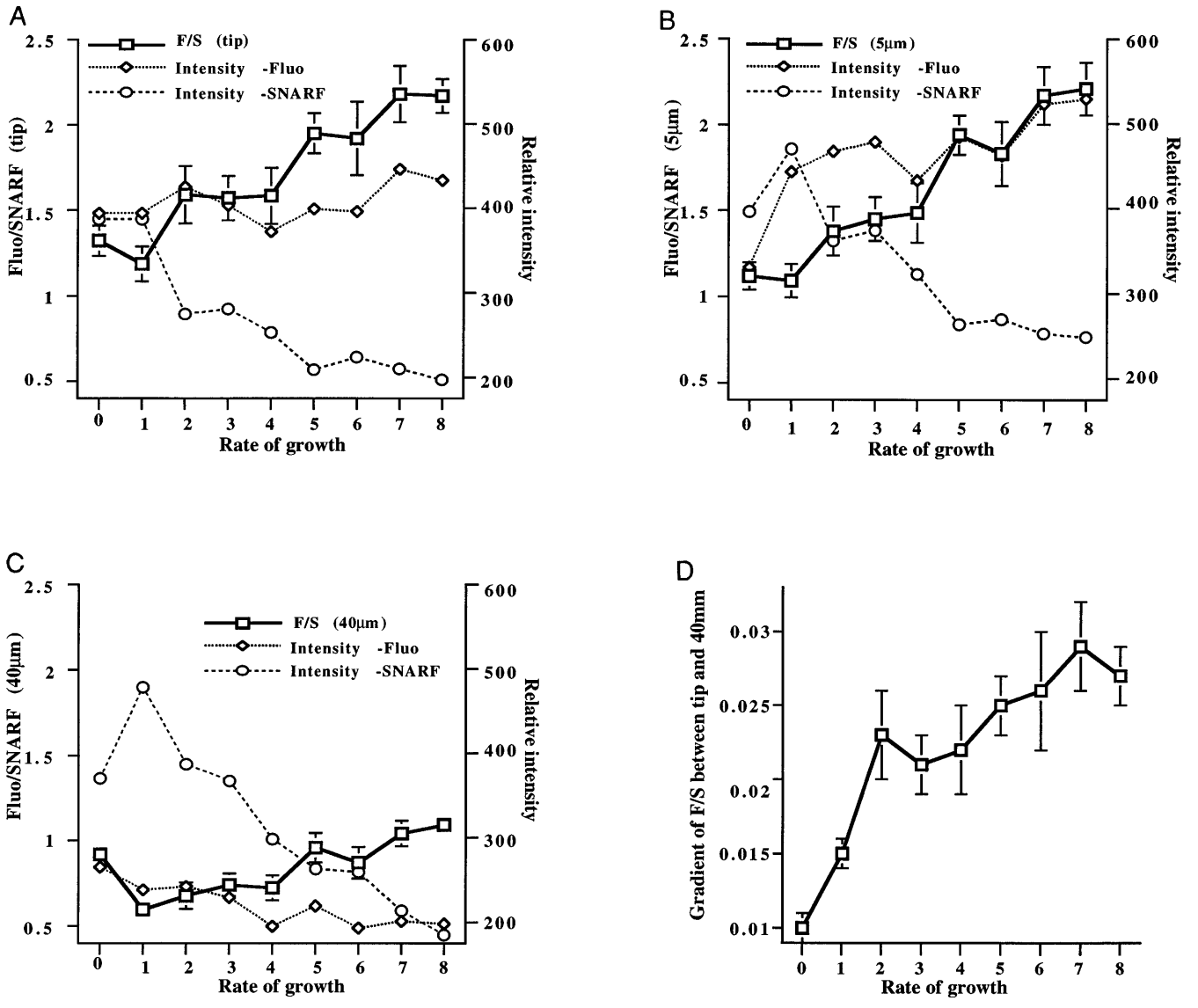


FIG. 7. Correlation between the F/S ratios and hyphal growth rate. The 133 hyphae analyzed in Fig. 5 were divided into eight groups, based on growth rate (1.0–1.9, 2.0–2.9, 3.0–3.9, 4.0–4.9, 5.0–5.9, 6.0–6.9, 7.0–7.9, and >8.0 μm/min, $n = 19$; 18, 18, 18, 18, 14, 18, and 10, respectively). The F/S ratio and the relative intensities (in arbitrary units) of F and S for the eight groups are shown at the tip (A) and at 5 μm (B) and 40 μm (C). The data for 10 μm are not shown, but were intermediate between those for 5 and 40 μm. All points show mean values; for clarity SEM are only shown on the bold F/S curves. The slopes of the gradients of the F/S lines joining the 0- and 40-μm points at each growth rate also correlate with growth rate (D). All four panels also include points (0 rate) for the nongrowing hyphae shown in Figs. 8A and 8B.

Growth Rate Correlates with Changes in [Ca^{2+}] and Gradients

Separation of the above data into groups based on growth rates [within any population of hyphae there is always considerable spontaneous variability in growth rates (Kaminskyj *et al.*, 1992; Jackson and Heath, 1990)] indicates that faster growing hyphae have both higher [Ca^{2+}] at all measured points, especially at the tips, and steeper slopes to their gradients (Fig. 7). Analysis of the separate Fluo and SNARF values indicates that the differences are dominated by growth-rate-dependent decreases in the SNARF values. Presumably this decrease is due to dye dilution by the greater rate of cytoplasm synthesis in the

faster growing hyphae; the absence of a similar decrease in the Fluo intensities is due to compensatory increases in Ca^{2+}-stimulated fluorescence of the presumably lower concentration of Fluo. Alternative explanations are considered under Discussion.

The F/S ratios for nongrowing hyphae indicated lower apical [Ca^{2+}] and a much shallower gradient, with most of the decline between the tip and 10 μm (Fig. 8A). In fact, the majority of these hyphae did not show any gradient or even had a slight tip low gradient. Only 13% showed a gradient as steep as, or steeper than, the mean gradient for the growing hyphae. The differences in the indicated Ca^{2+} gradients between growing and nongrowing hyphae were dominated by differences in the Fluo intensities (Fig. 8B), indicating that they are indeed reporting *in vivo* Ca^{2+} conditions. Ratio images of single hyphae (Figs. 8C to 8D) were also consistent with the aggregated data. All of the observed nongrowing hyphae were on the same slides as the growing ones; thus there was no systematic bias in the data gathering conditions, nor did the nongrowing hyphae look particularly "sick"; they were ones which transiently ceased growth for unidentified reasons and typically occur in colonies.

Effect of Medium pH on Ca^{2+} Gradients

Reducing the pH of the mounting medium from 5.5 to 4.0 reduced hyphal growth rates from 4.6 ± 0.36 to 3.6 ± 0.26 μm/min (n = 64 and 37, respectively) and both overall [Ca^{2+}] and the gradient slopes (Fig. 9). The differences in F/S ratios derive from lower Fluo intensities (Fig. 9B); the differences between the SNARF intensities in these populations was comparable to, but not quite as great as, those found for similar growth rate differences shown in Fig. 7, which reinforces the conclusion that the acid-grown hyphae do indeed have different Ca^{2+} levels and gradients. The reasons for the differences are uncertain, but suggest pH influence on intracellular Ca^{2+} behavior.

Hyphal Ca^{2+} Gradients in Ca^{2+}-Free Media

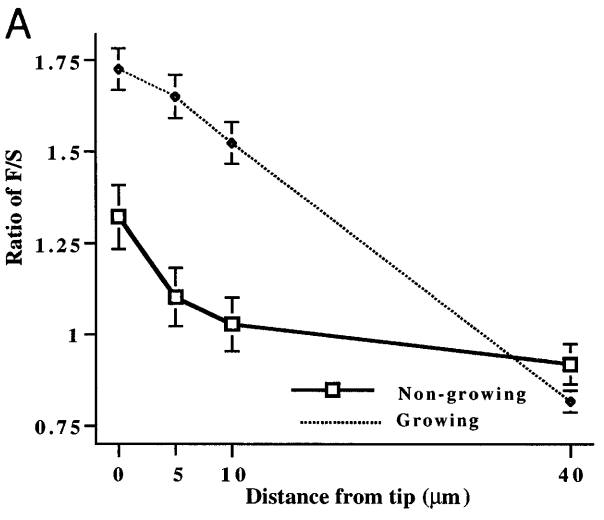
Reducing the level of exogenous Ca^{2+} to approximately 10^{-11} *M* with 50 m*M* BAPTA (assuming a possible 10 μ*M* of contaminating Ca^{2+}) slowed hyphal growth to 2.3 ± 0.27 μm/min (n = 17). However, the hyphae still showed a Ca^{2+} gradient (Fig. 10A) which was apparently steeper, peaked at 10 μm rather than at the tip, and had more than double the indicated [Ca^{2+}]. This gradient was still evident in hyphae examined more than 1 h after mounting. The differences in indicated [Ca^{2+}] were primarily attributable to dramatic reductions in SNARF intensities, relative to normal hyphae growing at the same rates (Fig. 10B), suggesting that the changes may be an artifact due to BAPTA- or low exogenous Ca^{2+}-induced differential loss of SNARF from the hyphae. Because the Fluo curve was of normal intensity, but 2.8 times steeper between the tip and 10 μm, the BAPTA-treated hyphae probably contain normal levels of Ca^{2+}, but with a real shift of peak [Ca^{2+}] subapically.

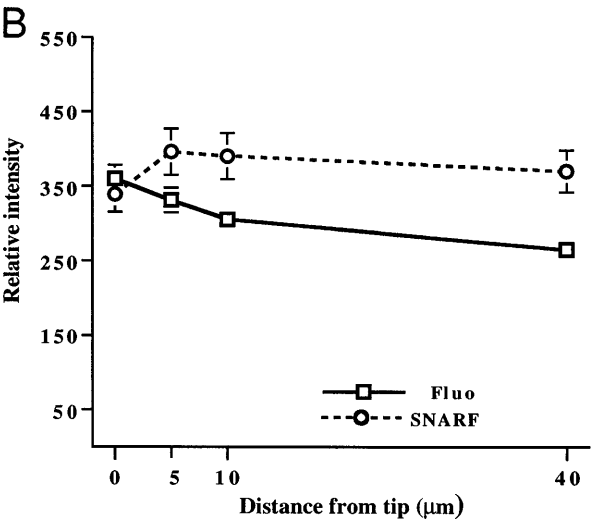
UV Irradiation Raises [Ca^{2+}]

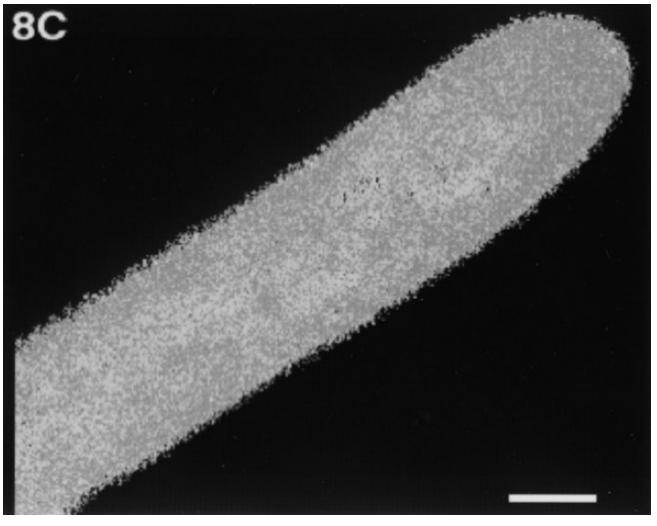
Irradiation of hyphae with 30 s of UV light caused dramatic increases in overall [Ca^{2+}], especially at 5 and 10 μm (Figs. 11A and 11C–11F; Table 1). The gradient was similar to normal in shape and slope, but appeared less concentrated around, and did not extend so far subapically along, the plasma membrane (compare Figs. 6 and 11D). The quantitative changes were attributable to increases in Fluo intensities, and the SNARF values remained little altered (Fig. 11B), indicating that the F/S values are truly reporting Ca^{2+} fluxes, which appear to be strongest in the 5- to 10-μm region. Hyphae irradiated with UV light either stopped growing or their growth rates were reduced (Table 1). Hyphae that stopped recovered and resumed growth (Figs. 11C–11F), often with a more spherical tip than normal (Fig. 11E).

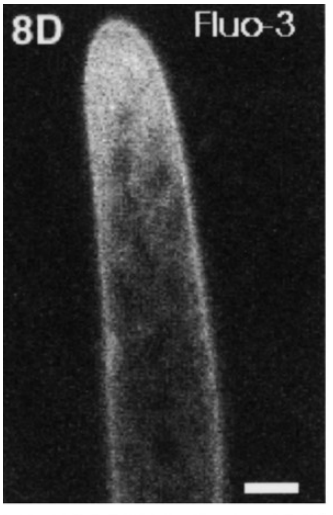
The above results raised the possibility that laser irradiation used for routine image acquisition (488 nm) might itself lead to rises in Ca^{2+}. Observations of four dual-loaded hyphae showed that typical measurement irradiations had no effect on SNARF intensities, and only 4 and 7% increases in Fluo levels at the tip and 10 μm, respectively. Routine data acquisition and UV irradiation also had no effect on autofluorescence intensities.

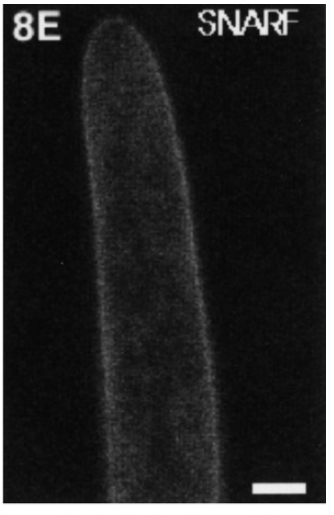
FIG. 8. Nongrowing hyphae have a weaker gradient of [Ca^{2+}]. The bold curve in A shows the mean F/S curve of 59 nongrowing hyphae. The mean F/S ratios were significantly higher at the tip than at 40 μm (two-tailed, paired t test, P = 0.001). The broken line curve from normally growing hyphae (see Fig. 5A) is included for comparison. (B) The relative intensities (in arbitrary units) of F and S at the same four points in the nongrowing hyphae. (C) A ratio image of a nongrowing hypha with little or no [Ca^{2+}] gradient. (D and E) The Fluo and SNARF fluorescence of a single hypha, which although not growing, had an obvious Ca^{2+} gradient. Eight minutes after D and E were taken, this hypha had begun to grow (F,G) and still had a [Ca^{2+}] gradient. Bar, 5 μm.

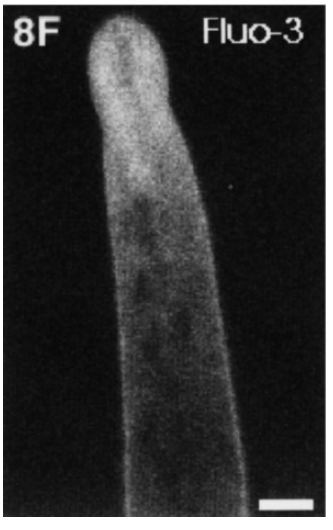


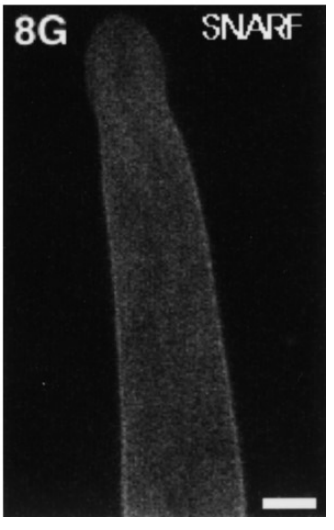












Ca^{2+} Distribution in Developing Branches

Eight newly forming branches were imaged as they developed from subapical regions of hyphae. Elevated [Ca^{2+}] were confined to the developing branches, as opposed to the subtending hyphae, from the earliest stages detected, but as the branches elongated, they developed longer gradients similar to those in hyphae (Figs. 12A–12D). Limited scanning of unbranched hyphae in regions of potential branch formation did not reveal any F/S "hotspots."

DISCUSSION

Studies on the Ca^{2+} gradients of hyphae are rare, no doubt partly due to the problem of sequestration of even the dextran-conjugated indicator dyes (Read *et al.,* 1992). At present there are only three reports, which have been obtained with different methods and species and which have come to significantly different conclusions concerning both the nature of the gradients and their role in tip growth.

Previous work on *Saprolegnia* (Garrill *et al.,* 1993; Jackson and Heath, 1993; Yuan *et al.,* 1995) used a single ratio dye, presented no ratio images of hyphae (and thus could not provide a detailed analysis of the intracellular distribution of Ca^{2+}), and concluded that the tip high gradient was very steep, peaking right at the apex and declining precipitously within about 5 μm. These hyphae also contain a tip high gradient of stretch-activated Ca^{2+}-transmitting channels whose activity is essential to tip growth (Garrill *et al.,* 1993). In contrast, use of the dual dye ratio technique showed that *Neurospora* contains a tip high gradient which peaks 3 μm behind the tip, appears to be less steep, and does not have its stretch-activated channels in a tip high gradient, and blockage of these channels, or alteration of the ion motive force across the plasma membrane, has little impact on growth (Levina *et al.,* 1995). Given the technical differences between these studies, further characterization of the apparent differences between species is desirable before conclusions can be drawn about interspecies differences in the way in which Ca^{2+} participate in the tip growth process.

The present dual dye ratioing demonstration of a Ca^{2+} gradient peaking at the extreme tip confirms the previous report for *Saprolegnia* (Garrill *et al.,* 1993) and emphasizes the difference between *Saprolegnia* and *Neurospora.* However, the *Saprolegnia* gradient reported by the dual dyes is shallower than previously indicated and clearly extends further back along the plasma membrane than it does through the center of the hyphae. This extension presumably indicates Ca^{2+} influx from the plasma membrane and sequestration toward the central and subapical cytoplasm, consistent with the loss of the gradient when the stretch-activated Ca^{2+}-transmitting channels are blocked (Garrill *et al.,* 1993). This indicates that influx extends much further back than the 2.5-μm diameter apical circle recently described in pollen tubes (Pierson *et al.,* 1996). Such extension is consistent with the presence of channels in subapical protoplasts (Garrill *et al.,* 1993) and indicates significant variation in the Ca^{2+} influx regions of tip growing cells. This variation may explain the more hemispherical shape of the pollen tube tips compared with *Saprolegnia,* suggesting that the extent of the apical Ca^{2+} influx zone directly contributes to tip morphogenesis. The difference in extent of the influx zone does not relate to variations in growth rates because both pollen tubes (Pierson *et al.,* 1996) and dye-loaded *Saprolegnia* hyphae have similar growth rates. A further indication of variation is that the Ca^{2+} gradient pattern in *Neurospora* does not extend back along the sides of the hyphae and is most prominent in the center of the hyphae, as predicted if it is produced from internal stores rather than influx through the apical plasma membrane (Levina *et al.,* 1995).

The relationships between growth rates and both the apical [Ca^{2+}] (other than the simple presence or absence of a gradient in growing or nongrowing hyphae) and the steepness of its gradient are previously unreported for hyphae. Because parts of these relationships are dominated by decreasing SNARF intensities at the higher growth rates, a differential loss of SNARF relative to Fluo cannot be ruled out. Differential permeability (SNARF higher) and dye loss at higher pH values (6.0 versus 4.2, compared with 5.5 used here) have been reported in *Neurospora* (Levina *et al.,* 1995) and can be induced in *Saprolegnia* (see above), but these seem unlikely to differ in hyphae simply growing at different rates in the same medium. The times for hyphal rinsing prior to observation of the growth rates (and thus time available for dye loss) are large relative to the times during which the hyphae are actually observed to be growing (~30 min versus ~5 min), and individual hyphal growth rates fluctuate substantially during short time periods (Kaminskyj *et al.,* 1992) so that fast and slow growers will have varied their rates prior to observation. Certainly the steeper gradients in the faster hyphae cannot be ascribed to differential dye loss because the greater decrease in SNARF intensities at the 40-μm point relative to the apical points in the faster hyphae

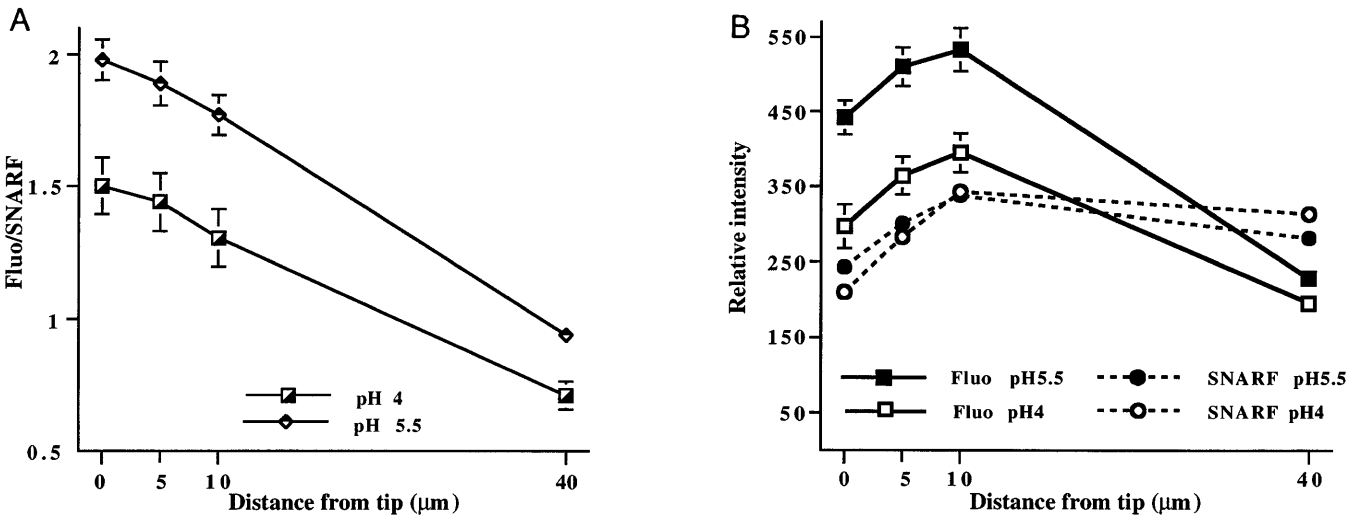


FIG. 9. Effect of mounting medium pH on the F/S gradient. (A) Both the overall height and gradient of the F/S curve are lower for hyphae mounted in medium of pH 4 ($n = 37$) than in that of pH 5.5 ($n = 91$). (B) These results are dominated by the Fluo values. For clarity, only the SEM of the Fluo values are shown; those of the SNARF values were approximately 50% lower.

predicts converse shallower gradients at higher growth rates. We conclude that the changes in $[Ca^{2+}]$ and gradients indicated by the ratio data are indeed reflections of the *in vivo* situation.

A similar relationship between $[Ca^{2+}]$ and growth has been recently shown for pollen tubes by Pierson *et al.* (1996), who suggested that the higher concentrations may be responsible for the predictable higher rates of exocytosis. However, the relationship is not direct. We have not attempted calibration of our F/S ratios because of a

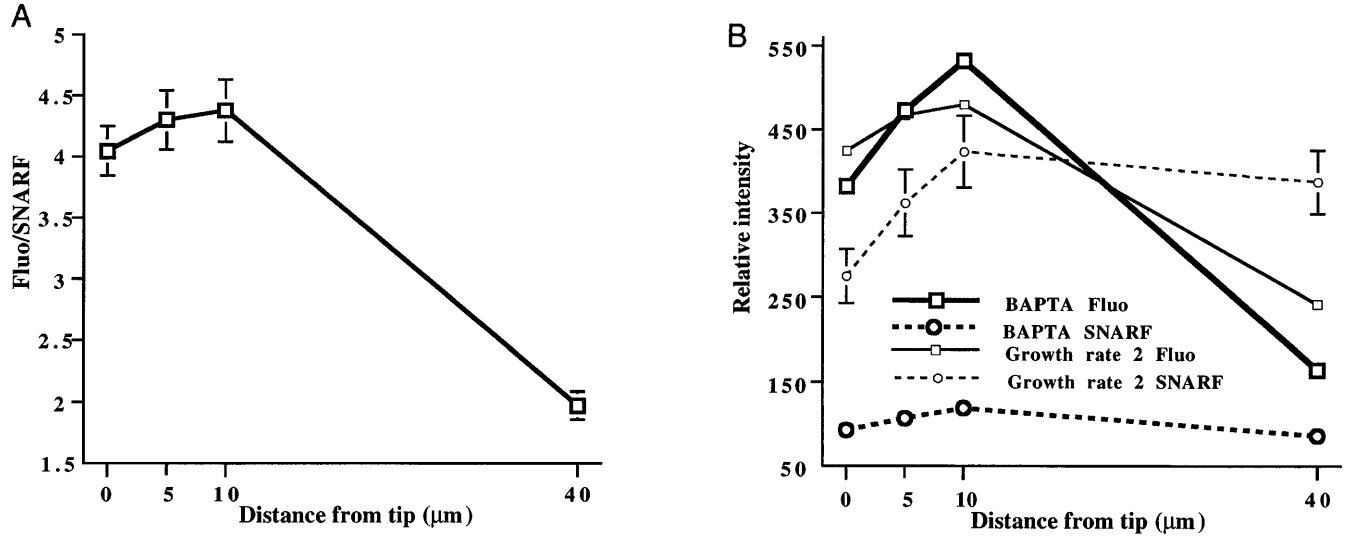


FIG. 10. Effect of depleting medium $[Ca^{2+}]$ on the F/S gradient. (A) Hyphae bathed in medium containing 50 m*M* BAPTA still had an F/S gradient, but the peak is shifted back behind the tip ($n = 17$) (cf. Fig. 5A). The mean F/S ratios at 5 and 10 μm are significantly greater than that at the tip ($P = 0.01$; Fisher's PLSD and Scheffe's *F* test). (B) Comparison of the individual Fluo and SNARF curves of these BAPTA-bathed hyphae with the similar means of the 2 μm/min hyphae (the average growth rate of the BAPTA-bathed hyphae) growing in normal medium shown in Fig. 7. For clarity, we have only shown the SEM of one line; the others were all lower.

number of well-known factors (Floto *et al.,* 1995), including the observed variations in loading of one or both dyes in different hyphae. However, assuming a relationship between F/S ratios and $[Ca^{2+}]$ comparable to those reported in *Neurospora* hyphae (Levina *et al.,* 1995), one can estimate that the approximately seven-fold difference in growth rates shown in Fig. 7 correlates with only a three-fold difference in apical $[Ca^{2+}]$ (Table 1). Furthermore, the UV irradiations caused apical Ca^{2+} levels to substantially exceed those found in even the fastest growing hyphae, yet the response was slowing of growth. These indications of a nonsimple relationship between cytoplasmic $[Ca^{2+}]$ and growth rates are in agreement with previous observations of the greater sensitivity of growth rate, versus hyphal Ca^{2+} levels (as indicated by chlortetracycline staining), to dramatic differences in medium $[Ca^{2+}]$ (Jackson and Heath, 1989; Yuan and Heath, 1991). Adaptation of the morphogenic cellular targets of the Ca^{2+} gradients, so that they respond to a gradient but do not depend on any specific concentration within a tolerated range, provides an attractive explanation of these observations. Thus rapid change, such as that following UV-mediated fluxes, deviates too far from the acceptable so that there is transient disruption of the delicate balance of growth regulating factors, but time permits correction or adaptation so that growth can continue with a new or reestablished gradient.

Our observations that UV irradiation and Ca^{2+} gradient maintenance in Ca^{2+}-free medium both elevate $[Ca^{2+}]$ more at about 10 μm behind the tip than at the tip indicate that *Saprolegnia* can maintain its critical Ca^{2+} gradients from internal Ca^{2+} stores, as postulated for *Neurospora* (Levina *et al.,* 1995). The 10-μm region is enriched in membrane-associated Ca^{2+} (Jackson and Heath, 1989; 1993; Yuan and Heath, 1991; and references in Herth *et al.,* 1990) and also mitochondria (Heath and Kaminskyj, 1989), endoplasmic reticulum (Yuan and Heath, 1991), and tubular vacuoles (Allaway *et al.,* 1997), all of which are likely Ca^{2+} stores (Jackson and Heath, 1993; Allaway *et al.,* 1997).

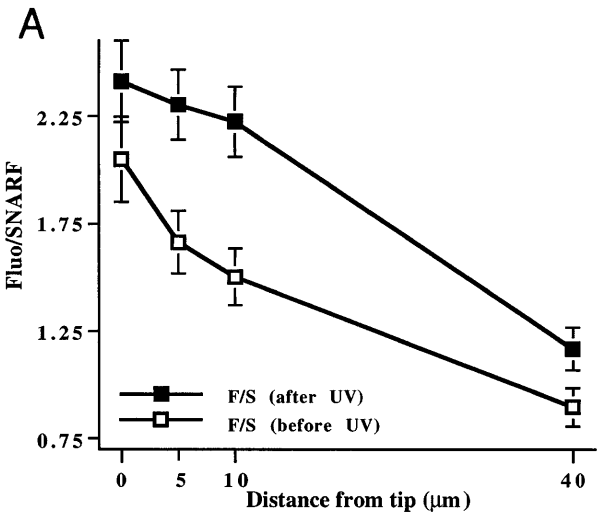
If internal stores are important in situations of Ca^{2+} depletion, their ability to function as a source is unlikely to last indefinitely. This may explain why *Saprolegnia* hyphae grown in Ca^{2+}-free media cease growing after 12–24 h (Jackson and Heath, 1989). The slower growth of *Saprolegnia* hyphae in Ca^{2+}-free media also suggests that the utilization of internal stores may not be as effective as influx via plasma membrane channels (Jackson and Heath, 1989). The ability of some other tip-growing systems to grow in Ca^{2+}-free media (e.g., fucoid zygotes; Kropf, 1992) might also be attributable to utilization of internal stores.

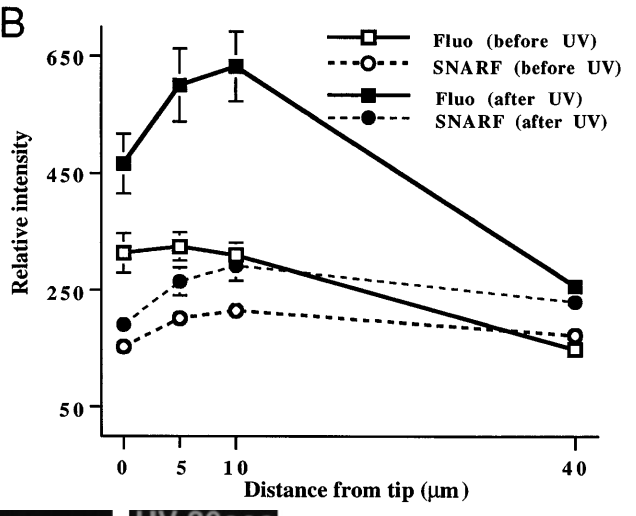
Our present observation of apparent release of Ca^{2+} from internal stores by UV irradiation is the first direct demonstration of the process previously inferred to be occurring both in *Saprolegnia* (Jackson and Heath, 1992) and in *Basidiobolus* (McKerracher and Heath, 1986) and as such lends support to the indications of the role for this ion in regulation of diverse hyphal processes not neccessarily directly related to tip growth itself.

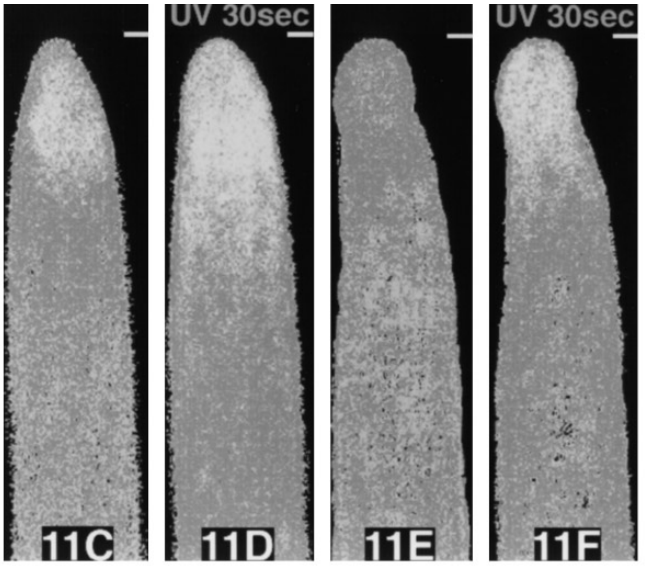
The present observations are the first to relate Ca^{2+} gradients to branching. While it is impossible to prove the absence of branch-initiating Ca^{2+} "hot spots" in the mature hyphae, the fact that the earliest detected branches have elevated Ca^{2+} levels restricted to the protrusion of the incipient branch indicates that these elevations were probably never present in the cytoplasm of the main hypha. The mechanisms of branch initiation remain a mystery; the only informative data are the observations that current entry into the future site of branch formation typically precedes protrusion, but continuation of such a current is not essential for continued tip growth (Kropf *et al.,* 1983). A plausible model compatible with both our data and those of Kropf *et al.* is that a yet unidentified stimulus (possibly Ca^{2+}, but at a concentration too low for us to detect) causes an initiating localized activation of plasma membrane channels which permits ion and thus current influx. Since the current reaches its maximum ~20 min prior to branch emergence, yet we detected no elevation of Ca^{2+} ions in the cytoplasm prior to emergence, the current is most probably carried by ions other than Ca^{2+}, consis-

FIG. 11. Effect of UV irradiation. (A) Thirty seconds of UV irradiation caused a great increase in the mean F/S levels, especially at 5 and 10 μm ($n = 12$). (B) This increase was dominated by rises in Fluo intensities. (C and D) The F/S ratio image before and 2.5 min after UV irradiation of a growing hypha. This hypha stopped growing, but resumed growth 20.5 min (E) into the experiment, at which time UV irradiation again caused an increase in the F/S ratio (F). Bar, 2.5 μm.

FIG. 12. Ca^{2+} levels in a developing branch. (A) The F/S ratio image at the earliest stage detected; (B–D) the same branch at the indicated times. Bar, 5 μm.







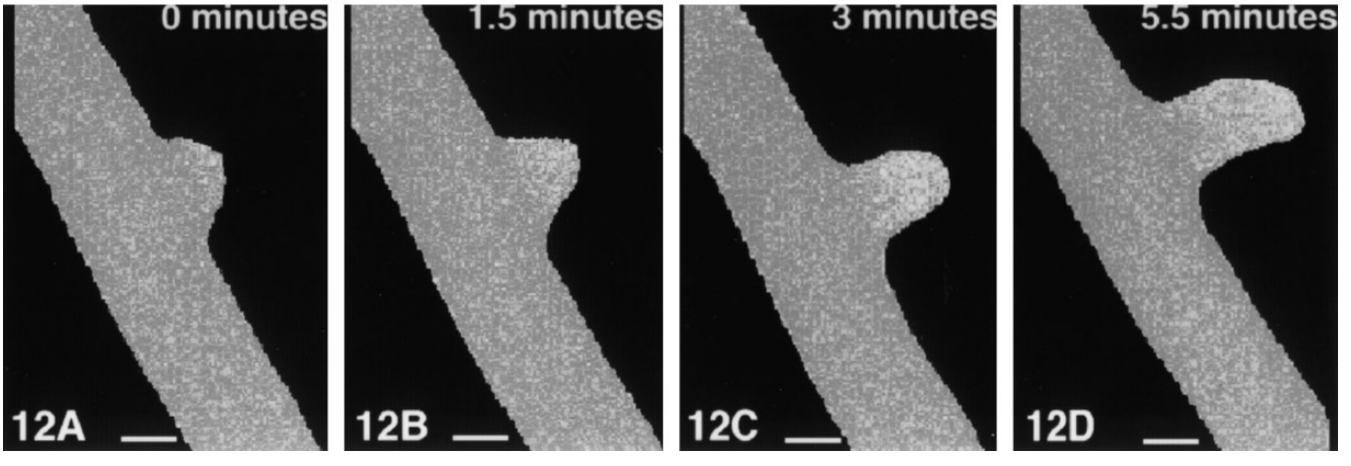


TABLE 1
Estimated [Ca^{2+}] (n*M*) in Hyphae under Different Conditions

Condition	Figure No.	Distance from tip (μm) 0	5	10	40
Maximum growth	7, 7+ μm/min	120	120	107	40
Mean growth	5A	76	70	62	31
Minimum growth	7, 1–2 μm/min	41	39	37	24
Nongrowing	8A	51	41	37	33
Before UV irradiation	11A, 6 μm/min	105	71	61	34
After UV irradiation	11A, 1 μm/min	149	135	113	43

Note. All values are derived from the F/S ratios in the indicated figures and the calibration curve in Levina *et al.* (1995). These values are only approximate since likely variations in concentrations of Fluo and SNARF under the different conditions will alter the calibrations. However, they are sufficiently accurate for the purposes used.

tent with the suggestion of H^+ by Kropf *et al.* (1983). Variations in [H^+] cause diverse effects on hyphal organization, including changes in the cytoskeleton (Bachewich and Heath, 1997). One such effect may be to localize and or activate plasma membrane Ca^{2+} channels concomitantly with wall softening. Extension of the plasma membrane would then activate stretch-activated Ca^{2+} channels (Garrill *et al.*, 1993), giving rise to the observed initial localized build up of Ca^{2+} in the branch initial. Subsequently, Ca^{2+} would operate in a positive feedback loop. The high Ca^{2+} level would favor tip-localized fusions of wall-forming vesicles, which, by bringing more Ca^{2+} channels to the branch tip, would perpetuate the cycle, leading to increased Ca^{2+} influxes and the observed increasing Ca^{2+} gradients and growth rates until other limiting factors would come into play and produce the equilibrium growth rates and gradients. Continued growth in the absence of inward current may be explained by either masking counter ion fluxes (Kropf *et al.*, 1983; Harold and Caldwell, 1990) or transient reliance on the internal Ca^{2+} stores discussed above.

With regard to nongrowing hyphae, the previous study of *Saprolegnia* reported that these had no Ca^{2+} gradients (Garrill *et al.*, 1993), as is the case for nongrowing pollen tubes (Miller *et al.*, 1992). This study supports the previous results for the majority of nongrowing hyphae, but the 13% with gradients similar to those of growing hyphae were probably growing too slowly for us to detect or were either just ceasing or starting growth; such hyphae may have not been detected in the previous *Saprolegnia* study due to the small sample size used (Garrill *et al.*, 1993).

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