

Roles of Ca^{2+} in hyphal and yeast-form growth in *Candida albicans*. Growth regulation by altered extracellular and intracellular free Ca^{2+} concentrations

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The dimorphic fungus *Candida albicans* has both a yeast form and a hyphal form. When yeast-form cells were starved and then transferred to a *N*-acetylglucosamine medium, the formation of true hyphae from the unbudded yeast-form cells was induced. Removal of Ca^{2+} from the medium with EGTA inhibited hyphal formation by 50%, resulting in only thin and short hyphae. Externally applied excess Ca^{2+} ($>10^{-2}$ M) also affected the hyphal formation, resulting in formation of pseudohyphae. This effect required a high concentration of Ca^{2+} but was Ca^{2+} -specific. Deprivation of Ca^{2+} also inhibited yeast-form growth. Interestingly, such cells had abnormally wide bud necks and became defective in cell separation. To measure cytosolic free Ca^{2+} , fura-2 was introduced into hyphal cells by electroporation. Its normal value was estimated to be about 100 nM. The electroporation caused transient elevation of cytosolic free Ca^{2+} concentration and transient cessation of hyphal growth. There was a close correlation between the timing of recovery of Ca^{2+} concentration and that of the resumption of hyphal growth. Our results demonstrate the importance of extracellular and intracellular free Ca^{2+} for the growth of *C. albicans*.

Key Words—calcium ion; cytokinesis; fura-2; probenecid; tip growth.

Candida albicans (Robin) Berkhout is a dimorphic fungus, which grows in two different morphological forms, a yeast form and a hyphal form. In the yeast form, cells proliferate by budding as a unicellular organism, while in the hyphal form they grow as filaments to make multicellular mycelia. Conversion between the two forms depends on various environmental conditions including temperature, pH and chemical constituents of the medium (Odds, 1988; Shepherd et al., 1985). Recently, the mechanisms of the conversion and the resultant hyphal growth have been studied extensively by physiological, biochemical and molecular biological approaches (Gow, 1994).

In eukaryotic cells, Ca^{2+} plays essential roles in various cellular functions, including cell differentiation, motility, division, proliferation, vesicular transport, exocytosis and tip growth (Beckers and Balch, 1989; Campbell, 1983; Hepler and Wayne, 1985; Jackson and Heath, 1993; Kropf, 1992; Miller et al., 1992; Toescu et al., 1992). In *C. albicans*, the involvement of Ca^{2+} in the transition from the yeast form to the hyphal form has been pointed out by several authors. Treatment of cells with various calmodulin inhibitors, or the Ca^{2+} ionophore A23187, which causes leakage of intracellular Ca^{2+} to the medium, inhibited hyphal formation, implying that Ca^{2+} acts as a positive regulator for hypha formation

(Paranjape and Datta, 1990; Paranjape et al., 1990; Roy and Datta, 1987; Sabie and Gadd, 1989). On the other hand, hyphal formation occurred in synthetic media to which no calcium salt had been added and even in the presence of EGTA (Simonetti et al., 1974; Walker et al., 1984). In addition, enhancement of Ca^{2+} uptake resulted in the inhibition of hyphal formation (Holmes et al., 1991). These lines of evidence suggest a non-essential or inhibitory role of Ca^{2+} . Therefore, the exact role of Ca^{2+} in hyphal formation of *C. albicans* remains unclear. Furthermore, the role of Ca^{2+} in yeast-form growth of this fungus has not been reported.

In this work, we examined the role of extracellular Ca^{2+} in both hyphal and yeast-form growth by manipulating Ca^{2+} concentration in the medium. We also measured Ca^{2+} uptake and cytosolic free Ca^{2+} concentration. We found that the change of cytosolic free Ca^{2+} correlates with the change of hyphal growth. We report here the effects of extracellular and intracellular Ca^{2+} on *C. albicans* growth, as well as cytosolic free Ca^{2+} concentration of this fungus.

Materials and Methods

Strain *Candida albicans* IFO1385 was used throughout this study. This strain is derived from the type strain of

this species, CBS562 (Suzuki et al., 1986).

Induction of hyphal formation Cells were precultured at 37°C in a medium consisting of 1% (w/v) yeast extract (Difco, Detroit, MI), 1% (w/v) Bacto-peptone (Difco) and 2% (w/v) D-glucose, which is a slight modification of a common yeast medium YPD (Sherman et al., 1986). We call this modified YPD medium simply YPD. The cells were grown overnight in the yeast-form to the stationary phase, then starved in distilled water at 37°C for 5 h. The resulting cells, most of which were round unbudded cells, were suspended in a mycelium-forming medium (MFM) at a density of $4\text{--}5 \times 10^6$ cells/ml and cultured at 37°C to induce hyphal formation. The composition of MFM was 0.9% yeast extract, 0.9% Bacto-peptone, 0.23% *N*-acetyl-D-glucosamine, 18 mM 2-(*N*-morpholino)ethanesulfonate (MES), pH 5.0 or 7.0. The pH was adjusted to 5.0 in the experiments using excess Ca^{2+} , because the addition of calcium chloride at higher pH caused precipitation in the medium, while it was adjusted to 7.0 in the Ca^{2+} -deprivation experiments, because the chelating activity of EGTA is low in acidic conditions. The pH was adjusted with hydrochloric acid or sodium hydroxide after autoclaving the medium. When MFM was supplemented with calcium chloride, ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), probenecid (Sigma, St. Louis, MO) or other substances indicated in the text, the pH was adjusted after the addition of these reagents.

Determination of the percentage of hyphal cells After the induction of hyphal formation, small aliquots of the culture were taken every 20 min, briefly sonicated to disperse cell clumps, put on hemocytometers and observed under a phase-contrast microscope at the magnification of $\times 400$. More than 300 cells were counted for each culture. Four cell types were recognized in the populations of *C. albicans* cultures: unbudded round cells, budded yeast-form cells, pseudohyphal cells and true hyphal cells (Fig. 1) (Odds, 1988). The last three types had

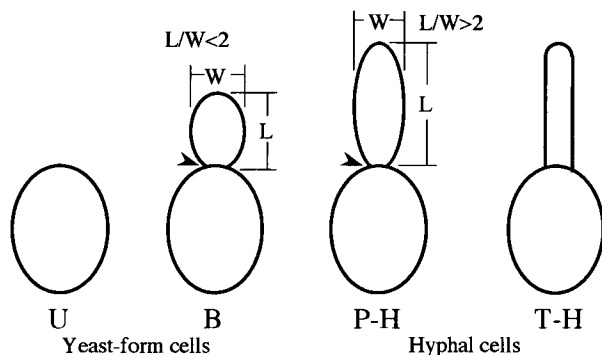


Fig. 1. Definition of cell types.

An unbudded round cell (U) has no projection. A budded yeast-form cell (B) has a short and wide ($L/W < 2$) projection with a constriction (arrowhead) at its base. A pseudohyphal cell (P-H) has a long and narrow ($L/W > 2$) projection with a constriction. A true hyphal cell (T-H) has a projection without constriction. U and B were classified as yeast-form cells, while P-H and T-H were classified as hyphal cells. L, length of projection; W, width of projection.

projections on the mother cells. When there was no constriction at the base of the projection, we defined the cell as a true hyphal cell. When the projection had a constriction at its base and a length/width ratio greater than two, we judged the cell to be a pseudohyphal cell. If the ratio was smaller than two, we judged the cell to be a budded yeast-form cell. We calculated the percentage of hyphal cells as the sum of the percentages of true hyphal and pseudohyphal cells.

Measurement of ^{45}Ca accumulation Cells were pre-incubated in MFM, pH 7.0, for 60 min to form hyphal cells. The percentage of hyphal cells was 80–90% at the end of the preincubation. Then $^{45}\text{CaCl}_2$ (NEZ-013; Du Pont-NEN Research Products, Wilmington, DE) was added to the cultures at a final radioactivity of 0.19 MBq/ml (5 mCi/ml). The specific radioactivity of ^{45}Ca in MFM was 80–90 dpm/pmol Ca. At 0, 10 and 30 min after the addition of ^{45}Ca , samples (1 ml) were taken in duplicate and filtered on MF-Millipore membrane filters (Type HA, pore size 0.45 μm ; Nihon Millipore, Tokyo) presoaked in 5 mM CaCl_2 . The filters were washed five times with 5 ml of the same solution and dried under an incandescent lamp. The radioactivity retained on the filters was counted with a scintillation mixture, Ready Protein (Beckman Japan, Tokyo), in a liquid scintillation counter (Aloka, Tokyo). Linearity of the accumulation of radioactivity was checked, and the rate of Ca accumulation was calculated from the average values of the duplicates.

To measure ^{45}Ca accumulation during yeast-form growth, cells were grown in YPD medium at 37°C to the mid to late log phase (2×10^6 to 2×10^7 cells/ml). $^{45}\text{CaCl}_2$ was added to the cultures at a final radioactivity of 0.19–0.37 MBq/ml (5–10 mCi/ml), and the accumulation of ^{45}Ca was measured by the same method as for hyphal growth. The specific radioactivity of ^{45}Ca in YPD was 70–150 dpm/pmol Ca.

Measurement of Ca content of the media and the estimation of their free Ca^{2+} concentration of media Ca contents of MFM, YPD and 200 mM MES solution were measured by use of an inductively coupled plasma atomic emission spectrometer (SPS1,200A; Seiko Instruments, Chiba, Japan). Free Ca^{2+} concentration in the presence of EGTA was calculated by the following equations, which are based on the theories of chemical equilibrium (Ogawa and Kitazawa, 1975; Oiki and Okada, 1987):

$$[\text{freeCa}^{2+}] = \frac{\sqrt{(K'[\text{EGTA}] - K'[\text{total Ca}] + 1)^2 + 4K'[\text{total Ca}]} - K'[\text{EGTA}] + K'[\text{total Ca}] - 1}{2K'}$$

$$\text{where, } K' = \frac{1}{\alpha} 10^{11-0.02(t-20)}$$

$$\alpha = 1 + 10^{9.46-pH} + 10^{18.31-2pH} + 10^{20.99-3pH} + 10^{22.99-4pH}$$

t: temperature of the medium ($^{\circ}\text{C}$), pH: pH of the medium

Measurement of cytosolic free Ca^{2+} concentration Cytosolic free Ca^{2+} concentrations in individual *C. albicans* cells were measured with fura-2, by modifying the

method developed for *Saccharomyces cerevisiae* Hansen cells (Iida et al., 1990b). Hyphal cells of *C. albicans* were washed, suspended in distilled water, and adhered to a 24 × 24 mm coverslip coated with 25 mg/cm² concanavalin A (Type IV, from *Canavalia ensiformis* DC.; Sigma). After a brief wash with distilled water, the coverslip was placed on the body of an electroporation chamber to form the top wall, being held in place with petroleum jelly (Fig. 2). The chamber was filled with 400 μM fura-2 (Molecular Probes, Eugene, OR) dissolved in distilled water, and one pulse of 2,000 V/cm was applied for 5 msec using an electroporation power supply (Gene Transfer, model GT-11; M&S Instrument, Osaka, Japan). By this method, fura-2 was loaded into about 30% of cells with cell viability of more than 80% as estimated by the methylene blue staining (Iida et al., 1990b). Immediately after the electroporation, the chamber was washed and filled with MFM by perfusion, and set on the stage of a microscope to start measurement. We chose only viable cells for the measurement, judging viability from the phase-contrast image of the cell: a dead or damaged cell showed an abnormally high contrast of the outline of the cell or the vacuole.

Details of the system for the microphotometry of Ca²⁺ concentration using fura-2 were described previously (Iida et al., 1990b). Briefly, a pair of fluorescent images of fura-2-loaded cells, illuminated with 340 nm and 380 nm light, respectively, were obtained by use of an epifluorescence microscope equipped with a Fluor 100/1.30 Oil Ph4DL objective (Nikon, Tokyo). The images were analyzed with an ARGUS-100 image processor (Hamamatsu Photonics, Hamamatsu, Japan) to display Ca²⁺ concentration as a pseudo-color image. The loading of fura-2 and the subsequent measurement of Ca²⁺ concentration were performed in a temperature-controlled room at 37°C.

Results

Effect of Ca²⁺ deprivation on hyphal growth In this work we induced transition from yeast-form cells to

hyphal cells by starvation of YPD-grown yeast-form cells in water and then transferring them into MFM containing *N*-acetylglucosamine as an inducer of hyphal growth. After the starvation, more than 95% cells became unbudded round cells. In normal MFM, such cells started to form hyphae at about 20 min after the transfer, and the percentage of hyphal cells finally reached 80–95% at 60–90 min (Fig. 3). All hyphae formed under these conditions were true hyphae (Fig. 4).

To examine the requirement of external Ca²⁺ for hyphal formation, we removed free Ca²⁺ from MFM by addition of 10 mM EGTA. This treatment markedly inhibited the hyphal formation (Fig. 3). The final percentage of hyphal cells was reduced to 50% of the control, and the hyphae formed were thin and short (Fig. 5). The addition of 10 mM calcium chloride together with 10 mM EGTA canceled the inhibition by EGTA, confirming that the effect of EGTA was due to the removal of free Ca²⁺ from the medium and not a side-effect of EGTA (Figs. 3, 6).

To assess the efficacy of EGTA, we measured total Ca content of MFM by inductively coupled plasma atomic emission spectroscopy. The content was 136 μM, while the concentration of free Ca²⁺ of the medium containing 10 mM EGTA was calculated to be 6 nM at 37°C. Since cytosolic free Ca²⁺ concentration of *C. albicans* cells is about 100 nM (this paper), the extracellular concentration of free Ca²⁺ in the presence of EGTA is lower than the intracellular one, and the uptake of Ca²⁺ into the cell must be inhibited. Therefore, our results show that prevention of Ca²⁺ uptake by addition of EGTA partially inhibited hyphal formation in *C. albicans*.

Effect of excess Ca²⁺ on hyphal growth To assess the effect of excess extracellular Ca²⁺, we added various concentrations of calcium chloride to MFM (data not shown). High concentrations (>10⁻² M) of calcium chloride retarded hyphal formation and lowered the final percentage of hyphal cells. Fifty percent inhibition, as judged by the final percentage of hyphal cells, was observed at 60 mM (Fig. 7). The effect was Ca²⁺-specific, because the addition of sodium chloride, magnesium

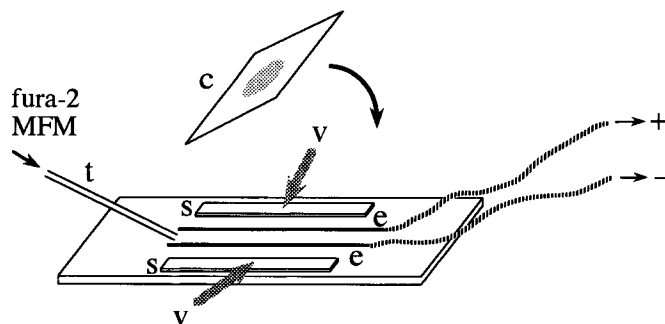


Fig. 2. Construction of an electroporation chamber.

A pair of platinum-wire electrodes (0.3 mm in diam, e), set 2 mm apart, are glued on a microscopic glass slide together with two glass spacers (0.4 mm in thickness, s) to form the body of the chamber. A concanavalin A-coated coverslip (c), on which *C. albicans* cells have been attached, is placed on the body and sealed with petroleum jelly (v) to form the top of the chamber. A fura-2 solution or MFM is injected into the chamber through a needle connected to a silicone tube (t). If solutions are to be exchanged, an older solution is drained from the side opposite to the tube by imbibition into filter paper. This chamber is directly set on a stage of microscope after the electroporation to measure fluorescence of cells loaded with fura-2.

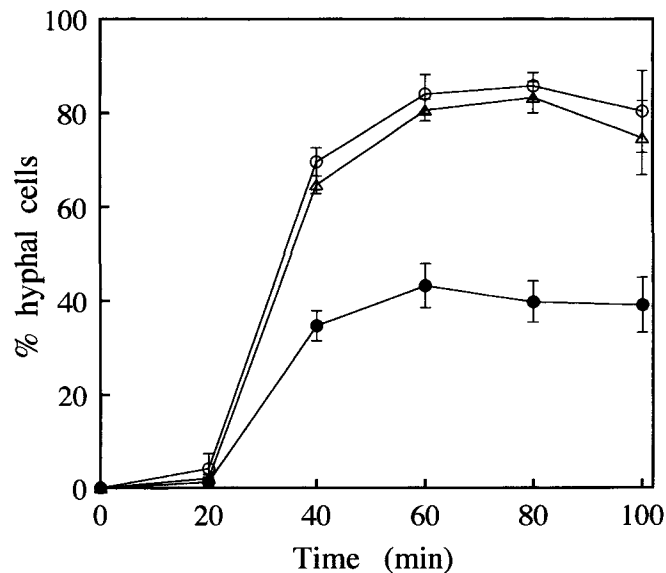
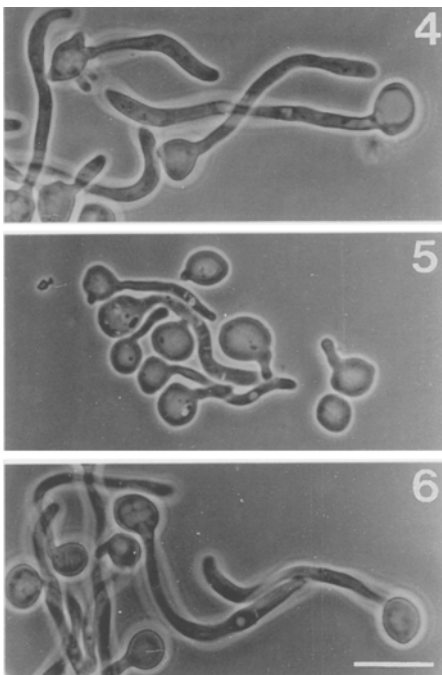


Fig. 3. Time course of hyphal formation in MFM, pH 7.0, with (closed circles) or without (open circles) 10 mM EGTA, or with both 10 mM EGTA and 10 mM CaCl₂ (triangles). Each value represents the mean \pm SD from triplicate cultures.



Figs. 4–6. Effect of Ca²⁺-deprivation on the morphology of hyphal cells.

Phase-contrast micrographs were taken at 100 min after the induction. 4. MFM. 5. MFM+10 mM EGTA. 6. MFM+10 mM EGTA+10 mM CaCl₂. The pH of all media was adjusted to 7.0. Bar, 10 μ m.

chloride or sorbitol at an osmotic concentration equivalent to that of 60 mM calcium chloride had no effect on hyphal formation (Fig. 7).

The hyphal cells formed in the presence of excess

Ca²⁺ were morphologically distinct from control cells (Figs. 8, 9). The hyphae had restrictions at their bases and were classified as pseudohyphae. Thus, in the presence of high concentration of Ca²⁺, pseudohyphae were formed instead of true hyphae.

Effect of Ca²⁺ concentration on yeast-form growth To check the effect of Ca²⁺ deprivation on yeast-form growth, exponentially growing yeast-form cells in YPD were transferred into YPD+20 mM MES, pH 7.0, containing 10 mM EGTA and cultured at 37°C. Because YPD+10 mM MES had the same Ca content as MFM, its free Ca²⁺ in the presence of 10 mM EGTA was thought to be as low as that of MFM+10 mM EGTA. The yeast-form cells grew well for several hours after the transfer, but then growth gradually slowed and by 9 h it stopped completely (Fig. 10). This growth inhibition was reversed by the simultaneous addition of 10 mM CaCl₂ (Fig. 10). Most cells in the growth-inhibited culture had abnormally wide bud necks and formed clumps, indicating a defect in the process of cell separation (Fig. 11).

The addition of excess calcium chloride slightly affected the yeast-form growth. We measured the rate of yeast-form growth at 37°C in YPD+20 mM MES, pH 5.0, to which calcium chloride was added at concentrations up to 100 mM. Even 100 mM addition had only the effect of inhibiting the growth rate in the exponential phase by 40%. No morphological abnormality was observed in these cells under the light microscope (data not shown).

Measurement of ⁴⁵Ca accumulation To check the possibility that Ca²⁺ influx is activated during hyphal formation, we compared the rates of ⁴⁵Ca accumulation in yeast-form and hyphal form growth. The accumulation rate in the mid-log phase in yeast-form cells grown in YPD medium at 37°C was 0.28 \pm 0.06 pmol Ca/min/10⁶

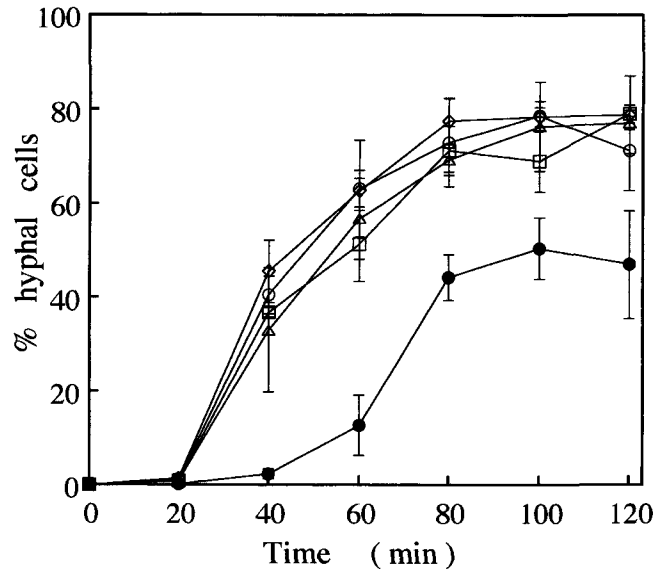
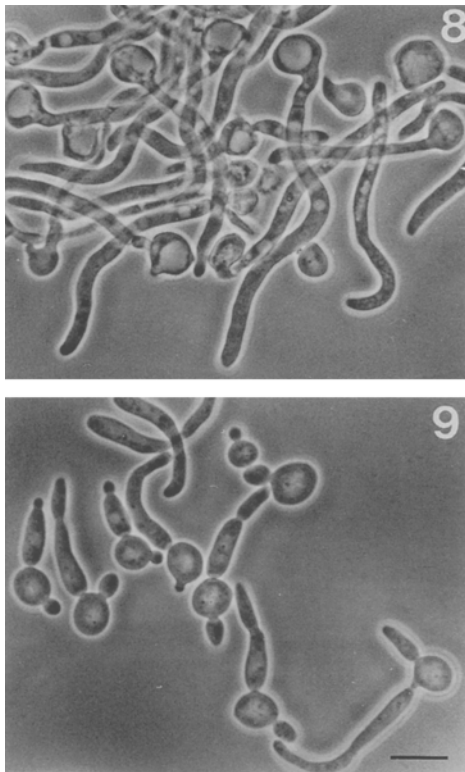


Fig. 7. Time course of hyphal formation in MFM, pH 5.0, with various substances added at 180 mOsm. Each value represents the mean \pm SD from triplicate cultures. Open circles, no addition; closed circles, +60 mM CaCl₂; triangles, +60 mM MgCl₂; squares, +90 mM NaCl; diamonds, +180 mM sorbitol.



Figs. 8, 9. Effect of excess CaCl₂ on the morphology of hyphal cells.

Cells at 120 min after the induction were observed under a phase-contrast microscope. 8. MFM. 9. MFM+60 mM CaCl₂. The pH of all media was adjusted to 5.0. Bar, 10 μ m.

cells (mean \pm standard deviation, n=4), while the average rate in the hyphal cells from 60 to 90 min after

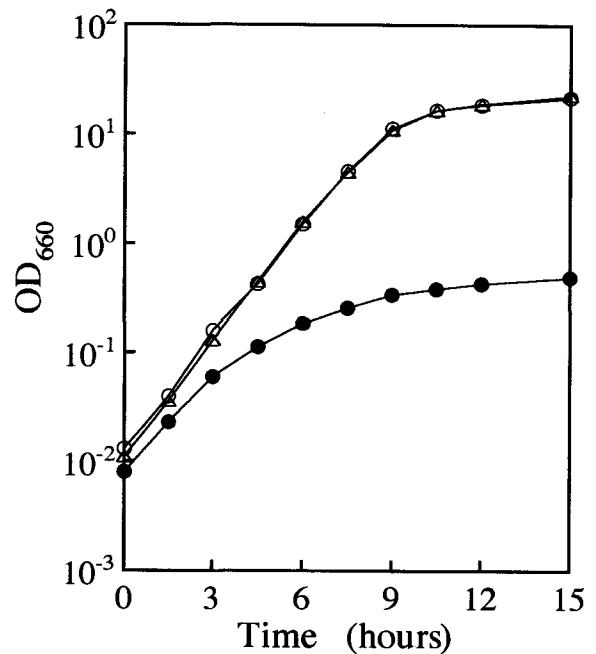


Fig. 10. Effect of EGTA on the yeast-form growth. Cells were precultured in YPD to the mid-log phase at 37°C, then transferred at time 0 into YPD+20 mM MES, pH 7.0, with (closed circles) or without (open circles) 10 mM EGTA, or with both 10 mM EGTA and 10 mM CaCl₂ (triangles). Cell growth at 37°C was monitored by OD₆₆₀. Each value represents the mean from triplicate cultures (SD was always less than 10% of the mean).

the induction in MFM, pH 7.0, at 37°C was 0.50 \pm 0.08 pmol Ca/min/10⁶ cells (n=2). Thus, the rate of Ca accumulation per cell was 1.8 times higher for

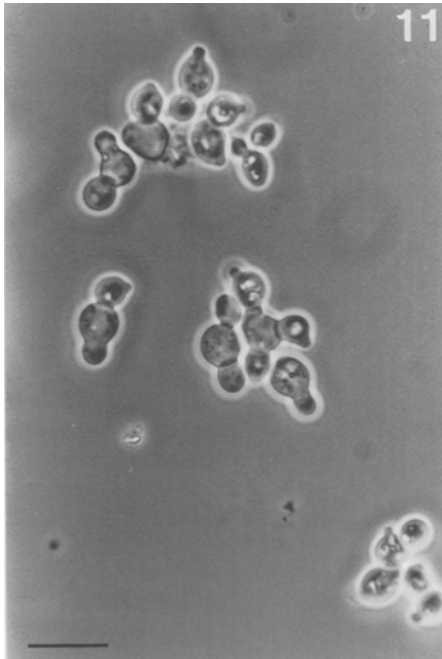


Fig. 11. Effect of Ca^{2+} -deprivation on the morphology of yeast-form cells.

Cells were cultured in YPD+20 mM MES+10 mM EGTA, pH 7.0, for 12 h (see the legend of Fig. 10 for details). Bar, 10 μm .

the cells in hyphal growth than for the cells in yeast-form growth. However, the averages of cell surface area were estimated to be 286 μm^2 for hyphal cells and 121 μm^2 for yeast-form cells. Therefore, the rate of Ca accumulation per unit cell surface area was almost the same in the two types of cells. The result indicates that there is no marked activation of Ca^{2+} influx during hyphal growth.

Measurement of cytosolic free Ca^{2+} concentration

Cytosolic free Ca^{2+} concentration in hyphal cells was measured using a fluorescent Ca^{2+} -indicator, fura-2. Because the cell membrane is impermeable to this indicator, it was loaded into the cells by electroporation. Cytosolic Ca^{2+} concentration was measurable for up to 10 min after the electroporation, but thereafter measurement was hampered by translocation of fura-2 into vacuoles. Successive measurements on individual cells revealed that Ca^{2+} concentrations were high (400–1,400 nM) immediately after the electroporation, then decreased rapidly and settled to about 100 nM within 10 min (Fig. 12). It is likely that the electroporation formed pores in the cell membrane allowing external Ca^{2+} to enter the cells, and after the pores had been sealed the ion was pumped out of the cell or sequestered into a cellular compartment to restore Ca^{2+} homeostasis. Thus the normal cytosolic free Ca^{2+} concentration of hyphal cells of *C. albicans* is estimated to be as low as the settled concentration, about 100 nM. No longitudinal gradient of Ca^{2+} concentration along the hyphae was found in a total of 18 hyphal cells (Fig. 12, portions la-

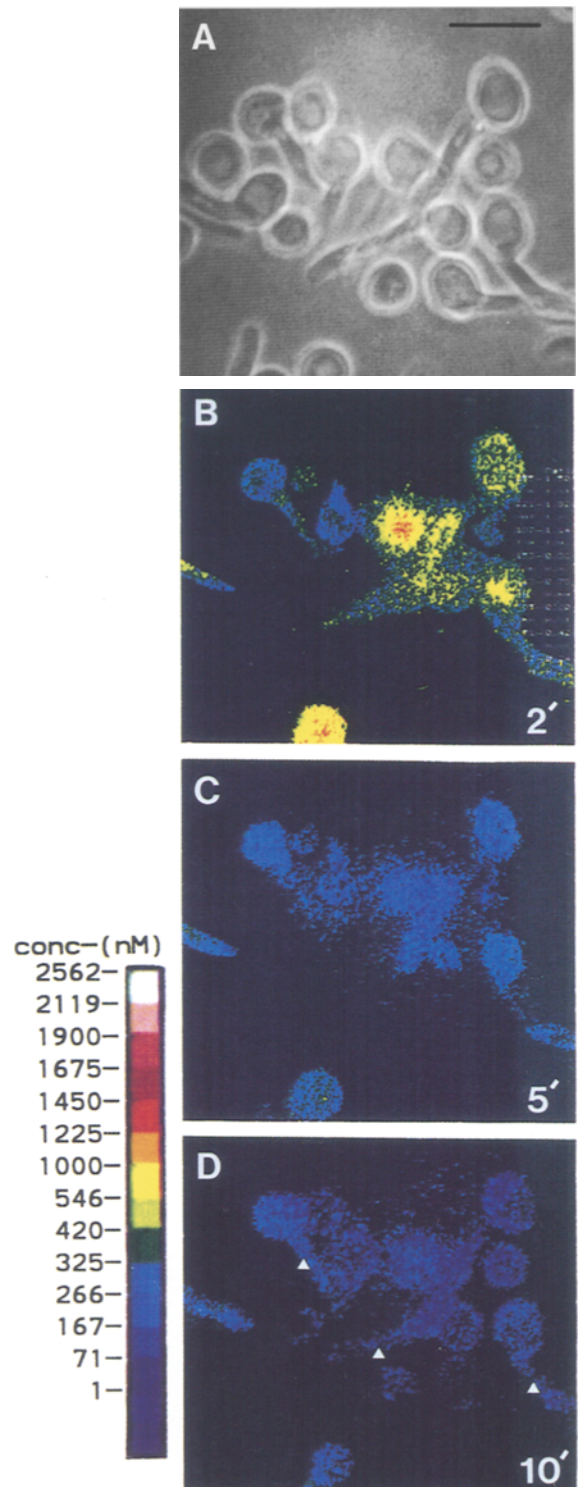


Fig. 12. Cytosolic free Ca^{2+} concentrations of individual hyphal cells.

Hyphal cells at 60 min after the induction in MFM, pH 7.0, were loaded with fura-2 by electroporation. Fluorescence ratios (340/380 nm) were measured at 2 min (B), 5 min (C) and 10 min (D) after the electroporation. The ratios are coordinated with pseudo-color hues and calibrated in terms of nanomolar Ca^{2+} . The phase-contrast image of the same field is shown in A. Triangles, tubular portions of hyphal cells. Bar, 10 μm .

beled with triangles).

Effect of electroporation on hyphal growth As electroporation caused the transient elevation of cytosolic free Ca²⁺ concentration, it is probable that the electroporation affected the hyphal growth. Thus we took successive photographs of the same hyphal cells after the electroporation (Fig. 13). The cells in the electroporation chamber (Fig. 2) were observed after the electroporation in the same way as in the measurement of Ca²⁺ concentration, except that phase-contrast images were observed instead of fluorescence images. About 10% of cells showed no growth after the electroporation (Fig. 13A, cell labeled with a white arrowhead). Because cell viability judged by methylene blue staining was 80–90% after the electroporation, these non-growing cells are thought to represent non-viable cells. About 30% of the cells showed hyphal growth as early as 3–10 min after the electroporation (Fig. 13A, hyphae labeled with white arrows). Many of these cells formed a knot-like portion (Fig. 13C, black arrows) at the position that had been the tip of the hypha at the moment of electroporation. This

indicates that smooth extension of the hypha was disturbed by the electroporation. Hyphal growth in these cells was probably interrupted by the electroporation but resumed within 10 min. The remaining 60% of the cells terminated growth of the existing hyphae and formed new hyphae from the basal part of the cells (Fig. 13D, hyphae labeled with black arrowheads). Observation of the fluorescence of the cells after the phase-contrast observation confirmed the loading of fura-2 in at least some of the cells whose original hyphae or newly formed hyphae were growing.

Effect of probenecid on translocation of fura-2, Ca²⁺ concentration and hyphal growth Our measurement of the free cytosolic Ca²⁺ concentration was hindered by the fairly rapid translocation of fura-2 from cytosol to vacuoles. We tried to inhibit this process by treating cells with probenecid. Probenecid is an inhibitor of organic anion transport and has been reported to inhibit the transport of organic anion dyes, Lucifer Yellow CH and fluorescein isothiocyanate (FITC) (O'Driscoll et al., 1991; Wright et al., 1992).

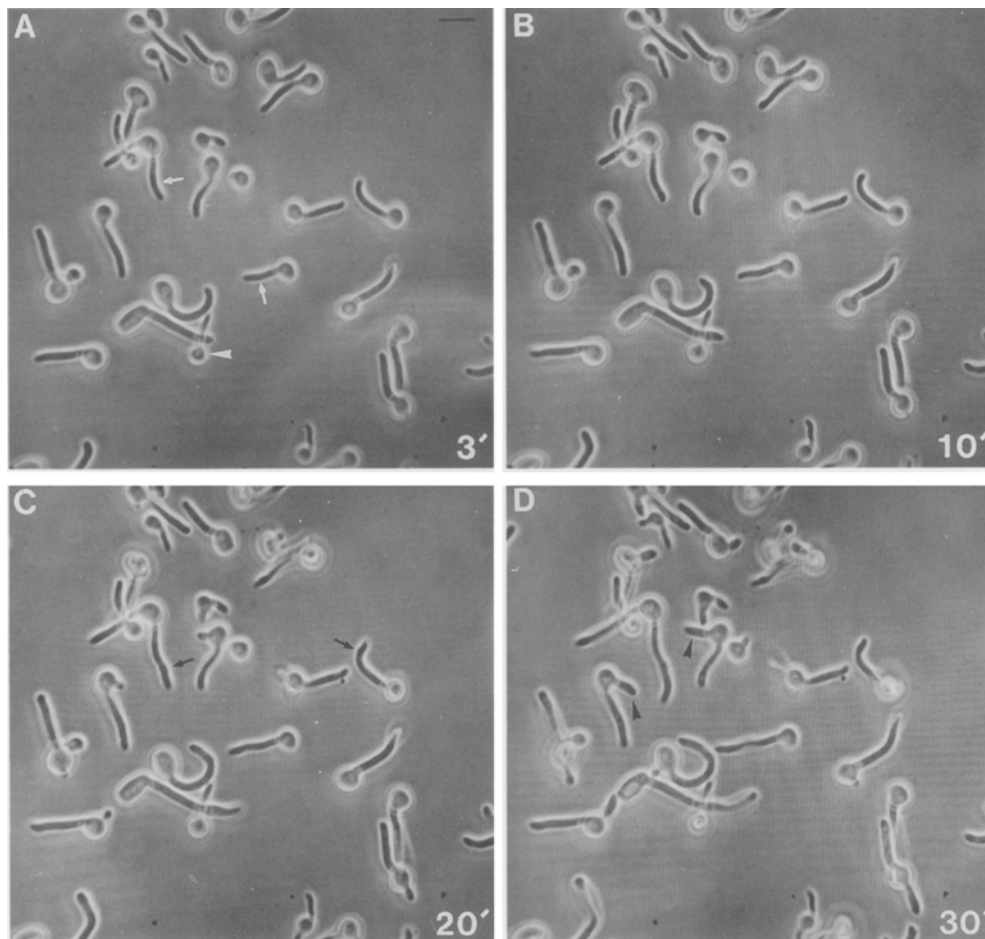


Fig. 13. Effect of electroporation on the extension of hyphae.

Hyphal cells were subjected to electroporation under the same conditions as in Fig. 12. The cells were incubated in the electroporation chamber for a further 30 min. Phase-contrast images of the same individual cells were taken at the indicated times after the electroporation (A–D). White arrows, hyphae that increased in length between 3 and 10 min after the electroporation; white arrowhead, cell showing no growth; black arrowheads, newly formed hyphae; black arrows, knot-like portions. Bar, 10 μ m.

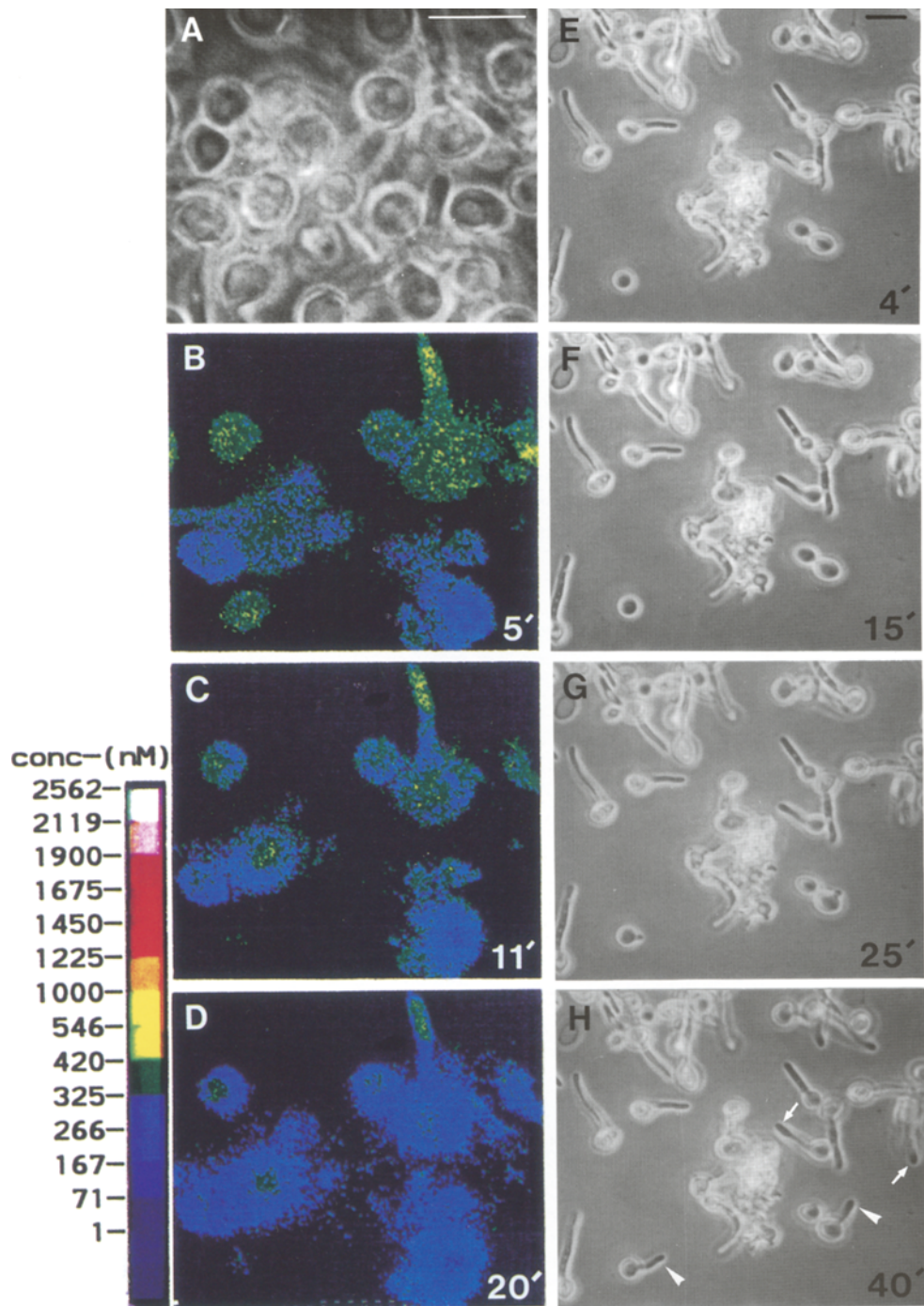


Fig. 14. Effect of probenecid on cytosolic free Ca^{2+} concentrations and on extension of hyphae. Hyphal cells were induced in MFM, pH 7.0, containing 10 mM probenecid. After 60 min cells were loaded with fura-2 by electroporation. Ca^{2+} concentrations (B–D) and hyphal growth (E–H) were monitored in MFM with 10 mM probenecid at 5 (B), 11 (C), 20 (D); 4 (E), 15 (F), 25 (G) and 40 min (H) after the electroporation. Panel A shows a phase-contrast image for B–D. Arrows, hyphae resuming extension; arrowheads, newly formed hyphae. Bars, 10 μm .

When hyphal cells were induced in MFM containing up to 10 mM probenecid, no influence on hyphal formation was observed at 60 min (data not shown). When these cells were loaded with fura-2, the transfer of fura-2 into vacuoles was delayed in the presence of 10 mM

probenecid and the dye remained in cytosol up to 20 min after the electroporation (Fig. 14A–D).

Transient elevation of Ca^{2+} was observed in the presence of probenecid as in the absence of this drug (Fig. 14B). Unexpectedly the recovery from the eleva-

tion of Ca²⁺ was also delayed by probenecid: the recovery was still incomplete at 11 min after the electroporation (Fig. 14C). Even at 20 min the concentration was about 200 nM, higher than the normal concentration of 100 nM. When we monitored the hyphal growth of individual cells after the electroporation in the presence of probenecid, the recovery of hyphal growth after interruption by electroporation was also retarded (Fig. 14E–H). After more than 20–25 min, some cells resumed growth of their existing hyphae (Fig. 14H, arrows) or started growth of the new hyphae (Fig. 14H, arrowheads). Most cell did not resume growth during the observation (Fig. 14H, cells without labels). Thus probenecid inhibited both recovery of Ca²⁺ concentration and resumption of hyphal growth after the electroporation, indicating a correlation between the cytosolic free Ca²⁺ concentration and the hyphal growth.

Discussion

Effect of Ca²⁺ deprivation In this work we showed that the removal of free Ca²⁺ from the medium by addition of EGTA partially inhibits the hyphal formation of *C. albicans*. Walker et al. (1984) also reported partial inhibition of hyphal formation by EGTA. The reason why the inhibition is partial is not clear, but it is possible that a significant amount of Ca²⁺ may be bound fairly tightly to the cell wall thus resisting removal by EGTA and providing a limited source of Ca²⁺, whose influx allows some cells to form short thin hyphae. In fact, it was reported that the yeast cell wall binds a large amount of Ca (Borbolla and Peña, 1980).

Ca²⁺ deprivation inhibited both the hyphal growth and the yeast-form growth. The effect of Ca²⁺ deprivation on hyphal growth was evident immediately after the treatment. On the other hand, its effect on yeast-form growth appeared only gradually, accompanied by a large increase of cell mass. Thus it is thought that the hyphal growth was inhibited directly by the failure of Ca²⁺ influx into the cell, while the yeast-form growth was inhibited by the deficiency of the intracellular pool of Ca²⁺ that resulted from the increase of cell mass without supply of Ca²⁺. The Ca²⁺ influx required for hyphal growth is, however, thought to be relatively small, because ⁴⁵Ca uptake was not markedly activated during hyphal growth. In *Saccharomyces cerevisiae*, yeast-form growth is highly resistant to the deficiency of extracellular Ca²⁺, but removal of the intracellular pool of Ca²⁺ results rapidly in cessation of cell growth (Iida et al., 1990a). Therefore, Ca²⁺ influx does not seem to be required directly for yeast-form growth in either *C. albicans* or *S. cerevisiae*.

Prolonged culture of yeast-form *C. albicans* cells in Ca²⁺-depleted medium gave a phenotype with a wide bud-neck and a defect in cell separation. One of the targets of Ca²⁺ may be chitin metabolism, because polyoxin D, an inhibitor of chitin synthase, causes the formation of wide bud-neck and the defect in cell separation in *C. albicans* (Hilenski et al., 1986). Another target may be chitin breakdown, because disruption of the chitinase gene leads to defects in cell separation in *S. cerevisiae* cells

(Kuranda and Ribbins, 1991). Unfortunately, no information is available about regulation of these enzymes by Ca²⁺.

Effect of high concentration of Ca²⁺ Our result showed that a high concentration of extracellular Ca²⁺ delayed the transition to the hyphal form. A similar result has been reported by Holmes et al. (1991). We found that high concentration of Ca²⁺ in the medium induced the formation of pseudohyphal cells. Compared with a true hyphal cell, a pseudohyphal cell is less slender, suggesting that its growth is less polarized. It is plausible that when external Ca²⁺ enters the cell, it disturbs intracellular homeostasis of Ca²⁺ and inhibits the polarized hyphal cell growth. Jackson and Heath (1993) reported that external Ca²⁺ had the effect of raising cytosolic free Ca²⁺ concentration in the oomycete *Saprolegnia*. Thus our result may be explained as follows: excess Ca²⁺ in the medium entered the cell and disturbed intracellular Ca²⁺ homeostasis; and this disturbance resulted in disruption of the normal cell polarity required for true hyphal growth and led to the formation of pseudohyphal cells. Otherwise, Ca²⁺ may work as a regulatory factor that switches the mode of growth between the true hyphal state and the pseudohyphal state.

Intracellular Ca²⁺ concentration and hyphal growth Measuring cytosolic free Ca²⁺ concentration gives valuable information about the role of Ca²⁺ in various cellular phenomena (Gryniewicz et al., 1985; Monck et al., 1992). However, as far as we know, there has been no report about cytosolic free Ca²⁺ concentration in *C. albicans*. Our results show that the normal cytosolic free Ca²⁺ concentration of hyphal cells of *C. albicans* is about 100 nM. This value coincides with a typical concentration of cytosolic free Ca²⁺ in other non-excited eukaryotic cells (Iida et al., 1990b).

Electroporation caused a transient rise of Ca²⁺ as well as a transient interruption of hyphal growth. Furthermore, electroporation induced the formation of new hyphae at the base of existing hyphae, suggesting the disruption of cell polarity in these cells. Thus it is possible that disturbance of Ca²⁺ homeostasis by electroporation may disrupt cell polarity and thereby inhibit hyphal growth. Electroporation forms pores with diameters of several nanometers, which are likely to allow the free passage of various other substances besides Ca²⁺ (Joeris and Brunstedt, 1991). Therefore, we cannot simply ascribe the inhibition of hyphal growth by electroporation to the disturbance of Ca²⁺ homeostasis. Nevertheless, we found an intimate correlation between the rise of Ca²⁺ concentration and the interruption of hyphal growth, because the treatment of cells with probenecid delayed the recoveries of both Ca²⁺ concentration and growth.

In various tip-growing cells, an intracellular tip-high Ca²⁺ gradient generated by Ca²⁺ influx from the tip is thought to play an essential role in the tip-growth (Connor, 1986; Jackson and Heath, 1993; Jaffe et al., 1974; Kropf, 1992; Miller et al., 1992; Schiefelbein et al., 1992). In the ascomycete *Neurospora* and the oomycete *Saprolegnia*, tip-high gradients of membrane-as-

sociated Ca^{2+} and cytosolic free Ca^{2+} have been observed (Jackson and Heath, 1989; Schmid and Harold, 1988). Our measurement of cytosolic free Ca^{2+} concentration using fura-2, however, gave no evidence for a tip-high Ca^{2+} gradient in the hyphal cell of *C. albicans*. Because electroporation caused transient cessation of hyphal growth, it is possible that we observed only non-growing hyphae, and that Ca^{2+} gradients may exist in growing hyphae. We could not measure cytosolic free Ca^{2+} concentration after we had checked the growth of a hypha because of rapid translocation of fura-2 into vacuoles. Thus, improvement of the method is needed to elucidate this point.

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