Dual Roles for Calcium Ions in Apical Growth of *Neurospora crassa*

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We report initial attempts to define the role of Ca^{2+} in the polarized extension of *Neurospora crassa*. Growth of the organism was diminished in media containing less than 1 mM- Ca^{2+} ; extension was more severely impaired than biomass synthesis, resulting in the formation of stubby, bulbous hyphae, even of spherical cells. Reduced extension and abnormal morphology were correlated with the loss of surface-bound Ca^{2+} , probably associated with the cell wall. Intracellular Ca^{2+} may be represented by material that fluoresces brightly in the presence of chlortetracycline. Punctate fluorescent bodies and diffuse fluorescence were both arrayed in a longitudinal gradient, maximum apically. Addition of the calcium ionophore A23187 induced dissipation of the fluorescence; concurrently, the hyphae lost as much as one half of their Ca^{2+} content. Extension continued almost unabated, but multiple branches quickly emerged from the apex. The observations suggest that a cytoplasmic Ca^{2+} gradient is not required for polarized extension, but may play a role in ensuring the dominance of the apex.

INTRODUCTION

Our present understanding of the role of Ca^{2+} in the polarization of tip-growing cells rests primarily on evidence from plants and algae (reviews: Hepler & Wayne, 1985; Schnepf, 1986; Krauss, 1987). Key findings include the uptake of Ca^{2+} into the apex of pollen tubes and fucoid rhizoids (Jaffe *et al.*, 1975*a, b*; Robinson & Jaffe, 1975); the dependence of extension on Ca^{2+} and its inhibition by reagents thought to block Ca^{2+} uptake (Picton & Steer, 1983, 1985; Reiss & Herth, 1985; Kropf & Quatrano, 1987); and the demonstration of cytoplasmic Ca^{2+} gradients, maximum apically (Jaffe *et al.*, 1975*b*; Reiss & Herth, 1978, 1979; Reiss *et al.*, 1985; Brownlee & Wood, 1986; Kropf & Quatrano, 1987; Nobiling & Reiss, 1987). Ca^{2+} ions are thought to play a central role in regulating apical extension. For example, Picton & Steer (1982) proposed that Ca^{2+} ions flow into the apex; the locally elevated level of cytosolic Ca^{2+} confines the exocytosis of precursor vesicles to the apical cap. Immediately behind the apex, Ca^{2+} is sequestered in storage vesicles. The reduced level of post-apical free Ca^{2+} would elicit relaxation of the microfilament meshwork that subtends and supports the tip, allowing the apex to extend. The evidence presently available is broadly consistent with this hypothesis.

Regarding the fungi, information is both sparse and scattered. Ca^{2+} ions appear to be generally required for growth and sporulation (Silver, 1977; Pitt & Ugalde, 1984; Ohya *et al.*, 1986), but their function is unclear. There is evidence that Ca^{2+} ions help maintain the ionic permeability of the plasma membrane (Slayman, 1965; Van Brunt *et al.*, 1982) and stabilize cell walls (Dow & Rubery, 1975). A role in the regulation of apical extension is suggested by the

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Abbreviations: CTC, chlortetracycline; FRIP, Fries-PIPES medium; NPN, *N*-phenyl-1-naphthylamine.

observation that calcium ionophores induce branching in *Neurospora* and *Achlya* (Reissig & Kinney, 1983; Harold & Harold, 1986); and also by the gradient of intracellular Ca^{2+} that Reiss & Herth (1979) noted in *Achlya*. Calcium channels have been documented only in *Blastocladiella* (Caldwell *et al.*, 1986). The experiments described here suggest that Ca^{2+} ions exert dual effects, in the cytoplasm and in the cell wall.

METHODS

Organisms and growth media. *Neurospora crassa* RL21A was maintained on Vogel's medium as described by Davis & de Serres (1970). Mass cultures were grown on FRIPI medium, a modified Fries' medium (Davis & de Serres, 1970) containing 160 μM -EGTA and 500 mM-PIPES, adjusted to pH 7.0 with NaOH. The basal Ca^{2+} level of this medium, prior to the addition of Ca^{2+} , was 38.6 μM ; 36 μM - Ca^{2+} was derived from the Fries' medium salts (determined by use of an Orion calcium electrode) and 2.6 μM - Ca^{2+} was contributed by the PIPES (information supplied by the manufacturers). The Ca^{2+} concentration was adjusted by adding CaCl_2 ; free Ca^{2+} concentrations were calculated, with due attention to pH and Mg^{2+} , by use of the dissociation constants published by Caldwell (1970) and a BASIC program (J. Schmid, W. J. A. Schreurs & D. Krauss-Varban, unpublished).

Calcium dependence of growth. FRIPI medium (500 ml) was inoculated with 5×10^5 conidia ml^{-1} , and incubated at 25 °C with rotation at 200 r.p.m. Rates of biomass increase and extension were monitored during the exponential phase of growth, 14–27 h after inoculation. Biomass was measured as the dry weight of mycelium harvested from samples by filtration through Nuclepore filters (pore size, 3 μm); filters were washed with distilled water. Extension was monitored by the contour length of the hyphae at a magnification of 200–400 $\times$; 36 hyphae were averaged for each time point. Ten samples were collected during the exponential phase for measurements of biomass and of extension; the results were plotted and the rates of increase were determined from the slope of each line. In some experiments, macromolecule synthesis was also measured: protein was assayed according to Bradford (1976), following extraction for 5 min with 2 M-NaOH at 95 °C, RNA by the orcinol method (Dawson *et al.*, 1986), and DNA according to Labarca & Paigen (1980).

Extension of individual hyphae was monitored in growth chambers under a Zeiss IM-35 microscope. Hyphae were grown on FRIPI medium without EGTA. Small agar plugs were inverted in a drop of FRIPI medium containing 100 μM - Ca^{2+} , placed on the bottom of the chamber. The chamber was then continuously flushed with FRIPI + 100 μM - Ca^{2+} at 0.4 ml min^{-1} . When a hypha emerged from under the plug, its extension was monitored for at least 60 min. The medium was then exchanged for FRIPI + 0.12 μM - Ca^{2+} , and extension was observed for another 60 min.

Effects of the calcium ionophore A23187. Individual hyphae were monitored as above, except that the organisms were grown on Fries' medium (900 μM - Ca^{2+}). Chambers were flushed with Fries' medium supplemented with 0.2% dimethyl sulphoxide, with or without 100 μM -A23187.

Fluorescence microscopy. Hyphae were grown on Fries' agar plates and introduced into growth chambers as described above. The medium in these experiments was Fries' supplemented with 100 μM -chlortetracycline (CTC) or 20 μM -*N*-phenyl-1-naphthylamine (NPN) in 0.1% dimethyl sulphoxide. CTC fluorescence was observed under a Zeiss IM-35 microscope by use of a BP400–440 nm excitation filter, an FT460 nm beam splitter and an LP470 nm emission filter. NPN fluorescence was observed with the same emission filter, a 380/9 nm excitation filter and a 405 nm beam splitter (the latter two from Omega Optical, the others from Zeiss). The light source was a HBO 50 W super-pressure mercury lamp (Osram). Photographs were taken with Kodak Trix pan film, sensitivity enhanced to 1600 ASA by the use of Acufine developer.

Calcium content. Fries' medium or FRIPI medium of known Ca^{2+} concentration, supplemented with $^{45}\text{Ca}^{2+}$ (70–900 kBq ml^{-1}) was inoculated with $3\text{--}5 \times 10^6$ conidia ml^{-1} and the cultures were grown on a rotary shaker as described above. Duplicate samples (0.1 ml) were collected on Millipore filters (3 μ pore size) and washed twice with 5 ml cold buffer (hyphae grown on Fries' medium were washed with 10 mM-MES/Tris, pH 6; hyphae grown on FRIPI were washed with 10 mM-PIPES/500 mM-NaCl, pH 7). One set of samples was washed with Ca^{2+} -free buffer, as a measure of total cell-associated $^{45}\text{Ca}^{2+}$. The other was washed with buffer supplemented with 5 mM- CaCl_2 , as a measure of 'intracellular' $^{45}\text{Ca}^{2+}$, i.e. that portion not readily exchangeable. The difference (after correction for radioactivity bound to the filters) was taken to represent $^{45}\text{Ca}^{2+}$ associated with the surface.

Reagents. Chlortetracycline (trade name, aureomycin) was purchased from Serva Chemicals, NPN from Eastman Kodak. A23187 and buffers were purchased from Sigma.

RESULTS

Extension of N. crassa hyphae requires Ca^{2+}

Growth of *N. crassa* hyphae was strongly dependent upon the concentration of free Ca^{2+} in the medium; maximal biomass production required approximately 1 mM- Ca^{2+} (Fig. 1). Given the

Fig. 1. Effect of extracellular Ca^{2+} concentration on growth of *N. crassa*. Conidia were inoculated into FRIP medium containing various concentrations of Ca^{2+} ; rates of biomass production (●) and extension (○) were determined during the exponential phase of growth. This is one of three experiments that gave essentially identical results.

multiple and well-established roles of Ca^{2+} in physiological regulation, this result was not unexpected. But we find it noteworthy that Ca^{2+} limitation had more drastic effects on the rate of hyphal elongation than on the rate of biomass increase (Fig. 1). The involvement of Ca^{2+} in extension was also apparent from the morphology of the hyphae (Fig. 2). With decrease in the extracellular Ca^{2+} concentration, the hyphae became wider as well as shorter, ultimately producing stubby bulbous rods. At $0.1 \mu\text{M}\text{-Ca}^{2+}$, approximately 20% of the population lost the capacity for polarized growth, producing spherical cells up to $20 \mu\text{m}$ in diameter. Organisms growing at the extremes of the range, 5 mM- and $0.1 \mu\text{M}\text{-Ca}^{2+}$, were in a state of balanced growth, as judged by the coordinated increase of protein, RNA and DNA with dry weight. Extension of *N. crassa* hyphae required the continuous presence of external Ca^{2+} . When individual hyphae were monitored for the effects of Ca^{2+} deprivation, extension was inhibited by at least 80% within 5 min (data not shown). Under the conditions used to examine individual hyphae, growth frequently ceased altogether, whereas elongation of hyphae in mass cultures merely slowed; we did not pursue the reason for this difference.

In an effort to determine whether Ca^{2+} ions exert their effects on the external surface or in the cytoplasm, we measured the amounts of total and surface-bound $^{45}\text{Ca}^{2+}$ in hyphae grown at various concentrations of $^{45}\text{Ca}^{2+}$. Fig. 3 shows that the steep decline in the rate of extension below $1 \text{ mM}\text{-Ca}^{2+}$ corresponded to a sharp drop in surface-bound Ca^{2+} . The amount of intracellular Ca^{2+} , like the production of biomass, was less drastically diminished by lowering of the extracellular Ca^{2+} concentration.

We are aware that the FRIP medium used in the above experiment is far from optimal. The heavy buffering and use of pH 7 were unavoidable if the free Ca^{2+} concentration was to be controlled over a wide range, for EGTA is rather ineffective below pH 6.8 and the free Ca^{2+} concentration becomes extremely sensitive even to minute changes in pH (Caldwell, 1970). Extension of *N. crassa* hyphae in the standard Fries' medium also required Ca^{2+} , but this medium proved unsuitable for detailed studies.

Intracellular distribution of Ca^{2+}

Since current models often propose a role for Ca^{2+} gradients in polarized growth, we examined the spatial distribution of Ca^{2+} in *N. crassa* hyphae. Attempts to measure the

Fig. 2. Morphology of *N. crassa* grown at various Ca^{2+} concentrations. Growth conditions as for Fig. 1; photomicrographs taken after 19 h of growth. Bars, 50 μm .

Fig. 3. Ca^{2+} content of hyphae grown at various concentrations of extracellular $^{45}\text{Ca}^{2+}$. Conditions as in Fig. 1. Intracellular (●) and surface-bound (○) calcium were measured as described in Methods. One of two essentially identical experiments.

distribution of free Ca^{2+} by the use of fura-2/AM, indo-1/AM or quin-2/AM all failed, because the esterified dyes were either excluded or else not cleaved. We therefore used CTC, which forms a fluorescent complex with Ca^{2+} . The fluorescence intensity increases further when the complex binds to membranes. Since Ca^{2+} ions in the complex are in equilibrium with those of the cytosol, CTC fluorescence gives some indication of the spatial distribution of total intracellular Ca^{2+} , the bulk of which is bound to membrane surfaces or organelles (Caswell, 1979; Blinks *et al.*, 1982; Kauss, 1987). We observed a clear apical gradient of CTC fluorescence (Fig. 4). The gradient was visible in every one of several hundred growing hyphae we examined; inhibition of growth by cycloheximide or sodium azide led to dissipation of the gradient within 1 h (data not shown). Young germlings (16–35 μm in length) did not exhibit a clear gradient: the apical region always displayed enhanced fluorescence, but there were also areas of increased

Fig. 4. The gradient of intracellular Ca^{2+} displayed by CTC fluorescence. Bar, 20 μm .

Fig. 5. Distribution of intracellular membranes displayed by NPN fluorescence. Bar, 20 μm .

Fig. 6. Effect of the ionophore A23187 on CTC fluorescence. The times after addition of A23187 are indicated on the photomicrographs. The bright spots on the 20 min photomicrograph are caused by A23187 precipitated onto the surface of the hypha. Bar, 20 μm .

fluorescence behind the apex and in the conidium (data not shown, because we could not obtain satisfactory photomicrographs of germlings).

CTC fluorescence does not depend on Ca^{2+} alone, but is affected by membrane density and Mg^{2+} ions as well (Caswell, 1979; Blinks *et al.*, 1982). The distribution of intracellular membranes was assessed with the fluorescent probe NPN (Ballard *et al.*, 1972). The pattern was variable; only rarely did we observe enhanced apical fluorescence with NPN, and no clear gradient was ever seen (Fig. 5). We can thus exclude the possibility that CTC fluorescence

Fig. 7

Fig. 7. Effect of the ionophore A23187 on the Ca^{2+} content of *N. crassa*. Hyphae were grown overnight on Fries' medium without added Ca^{2+} (contamination level, $36 \mu\text{M}$), plus $^{45}\text{Ca}^{2+}$. At 0 min the suspension was divided. One half (a) received only dimethylsulphoxide (final concentration 0.2%), the other (b) received A23187 in dimethylsulphoxide (final concentrations $100 \mu\text{M}$, 0.2%). Samples were collected at intervals thereafter. The rise in $^{45}\text{Ca}^{2+}$ content is due to continued growth of the organisms. This is one of five very similar experiments.

Fig. 8

Fig. 8. Effect of the ionophore A23187 on the extension of individual hyphae. *N. crassa* was grown in chambers as described in Methods; at 0 min, the medium was exchanged for medium supplemented with $100 \mu\text{M}$ -A23187.

reports a gradient of membrane distribution, but some contribution from Mg^{2+} cannot be ruled out. We shall proceed on the premise that CTC displays the distribution of Ca^{2+} , chiefly that sequestered in organelles.

Does extension require an undisturbed intracellular distribution of Ca^{2+} ?

If hyphal extension required a cytoplasmic gradient of free Ca^{2+} , the process should be vulnerable to perturbation of that gradient. A number of Ca^{2+} antagonists, namely La^{3+} ($40 \mu\text{M}$), Gd^{3+} ($40 \mu\text{M}$), Nifedipine ($100 \mu\text{M}$) and Verapamil ($100 \mu\text{M}$), had little or no effect on extension rates or on tip shape; however, they also did not perturb the gradient of CTC fluorescence, nor did they inhibit $^{45}\text{Ca}^{2+}$ uptake.

The effects of the calcium ionophore A23187 ($100 \mu\text{M}$) were more remarkable. Upon addition of the ionophore, the CTC fluorescence gradient started to disperse immediately: after 10 min, fluorescence was much reduced and its intensity was but slightly enhanced at the tip; after 20 min, no more than a weak uniform fluorescence remained (Fig. 6). The ionophore also immediately affected the Ca^{2+} content of the hyphae, reducing it to 25–60% of its original value within 20 min (Fig. 7). The latter effect was transitory: within 40–60 min, the hyphae recovered their Ca^{2+} complement. We are not certain whether the normal pattern of CTC fluorescence was also re-established, because of interference from the blue fluorescence of the ionophore, which was progressively incorporated by the cells in the course of the experiment. The effects of A23187 on hyphal extension and morphology were considerably milder. Extension was only

Fig. 9. Effect of the ionophore A23187 on the morphology of the apical region. The hyphae illustrated here were photographed 10 min after the addition of ionophore under the conditions of Fig. 8. Bar, 20 μ m.

slightly affected for the first 30 min following addition of the ionophore; it then slowed or even ceased for 20–30 min (Fig. 8). The ionophore also induced the emergence of multiple apical branches, some of which appeared as early as 5 to 10 min after addition (Fig. 9). Apart from distortion of the original tip during the initial stages of branching, hyphal morphology appeared reasonably normal.

DISCUSSION

Ca^{2+} ions are required for the growth of *N. crassa*, but it appears that polarized extension has a higher requirement for Ca^{2+} than does biomass increase (Fig. 1). In particular, at external concentrations of free Ca^{2+} below 100 μ M, biomass increase was almost normal but extension was seriously impaired and morphological abnormalities were becoming apparent (Fig. 2). At this level of Ca^{2+} the 'intracellular' Ca^{2+} content was still high, whereas the amount of Ca^{2+} associated with the surface was less than a tenth of that seen at 5 mM extracellular Ca^{2+} (Fig. 3). These results suggest that surface-bound Ca^{2+} ions serve an essential function in generating the normal hyphal morphology. In future, it may be possible to learn whether external Ca^{2+} ions are mainly needed to ensure the functioning of the cytoplasmic membrane, to confer mechanical strength upon the wall, or are involved in its assembly.

Intracellular Ca^{2+} ions, in *N. crassa* as in other eukaryotic organisms, probably participate in multiple regulatory functions, some of which require the hyphae to maintain a low level of free cytosolic Ca^{2+} . The recent report that mutants defective in the transport of Ca^{2+} into a vacuolar fraction are subject to inhibition by extracellular Ca^{2+} (Cornelius & Nakashima, 1987) supports this presumption. It seems likely, then, that CTC fluorescence mainly reports the distribution of membrane-bound compartments that sequester the bulk of the Ca^{2+} complement of the hyphae. The morphology of the CTC-bright bodies is quite unlike that of the large, spherical vacuoles; they may correspond to endoplasmic reticulum (Carafoli, 1987).

Judging by CTC fluorescence, sequestered Ca^{2+} in *N. crassa* is distributed along a pronounced gradient, maximum apically (Fig. 4). We were unable to investigate the distribution of free Ca^{2+} , but by extrapolation from recent work with pollen tubes and *Fucus* (Nobiling & Reiss, 1987; Brownlee & Wood, 1986) we would expect that to exhibit a similar distribution. The question is whether such gradients play a causal role in polarized extension, and the effects of A23187 supply a hint. The ionophore elicited an immediate loss of Ca^{2+} from the hyphae, apparent both as a decline in the $^{45}\text{Ca}^{2+}$ content and as the loss of CTC fluorescence. The loss of Ca^{2+} occurred despite the presence of $900\ \mu\text{M}\text{-Ca}^{2+}$ in the medium. It is not clear just what the ionophore does; one possibility is that it affects the permeability of the calcium storage organelles more severely than that of the plasma membrane, so that the hyphae manage to maintain a relatively normal level of free cytosolic Ca^{2+} .

Be this as it may, the dissipation of the CTC fluorescence and the massive calcium fluxes observed with $^{45}\text{Ca}^{2+}$ suggest a significant perturbation of intracellular Ca^{2+} gradients. The disturbance probably extends to the spatial distribution of free cytosolic Ca^{2+} , though we have no direct evidence of that. Despite this perturbation of Ca^{2+} gradients, the hyphae continued to extend in a polarized manner and their appearance was approximately normal. In fact, during the first 20 min following ionophore addition, the majority of the hyphae continued to extend at the normal rate. The effects of A23187 are far from being understood and must be regarded with some suspicion. Nevertheless, the lack of immediate and severe inhibition of extension by A23187 argues against the hypothesis that a spatial gradient of Ca^{2+} guides apical extension. The major morphological consequence of A23187 addition was the rapid appearance of apical branches (Fig. 9). A simple and exceedingly tentative interpretation is that Ca^{2+} gradients are not required to polarize extension, but may be involved in assuring the predominance of a single tip.

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