Vacuoles Play a Decisive Role in Calcium Homeostasis in *Neurospora crassa*

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Mutants of the filamentous fungus *Neurospora crassa* which grow well only in a medium in which the concentration of calcium is lower than in the medium used for culture of the wild-type strain were isolated by use of the ionophore A23187 and medium which contained a high concentration of calcium. These mutants showed a significantly lower uptake of calcium into their vacuoles than the wild-type, while other activities associated with the transport of calcium across several other types of membrane were not affected. The results indicated an essential role for the vacuoles in the regulation of the cytosolic concentration of calcium.

INTRODUCTION

In addition to its well-known participation in muscle contraction, calcium plays a crucial role in a variety of other cellular processes, such as secretion, intracellular transport and movement, and the communication of signals (Marme, 1985; Rasmussen *et al.*, 1984). Whereas the cytosolic concentration of calcium in a non-activated cell is in the range 10^{-8} – 10^{-6} M, the extracellular concentration typically exceeds this value by several orders of magnitude (Bygrave, 1978; Rasmussen *et al.*, 1984; Williamson & Ashley, 1982). Because calcium in high concentrations is toxic to the cell, an effective system for the regulation of the intracellular concentration of calcium is required. This system consists of a number of calcium pumps which are located in the plasma membrane as well as in the membranes of various intracellular organelles, such as the mitochondria and the endoplasmic reticulum. These organelles serve as compartments for the storage of calcium, and are able to take up and release calcium ions. With respect to the mechanism of calcium transport, two general systems can be distinguished. One kind of pump uses the energy derived directly from the hydrolysis of ATP, while the other involves an antiporter which exchanges calcium for another ion, for example H^+ or Na^+ . The transport of calcium across the plasma membrane of *Neurospora crassa* has been shown to be linked to an electrogenic ATPase by an H^+/Ca^{2+} antiporter (Stroobant & Scarborough, 1979). However, it remained to be determined whether or not this pump is the only system used to maintain the calcium content of *N. crassa* at a constant, low level. In order to establish the nature of the decisive transport system, we undertook the isolation of mutants with phenotypic differences in the metabolism of calcium.

Calcium is important for the function of the circadian clock which regulates the conidiation rhythm in *N. crassa* (Nakashima, 1984). Isolation of mutants with phenotypic differences in the metabolism of calcium should contribute to an increased understanding of the relationship between clock function and the metabolism of calcium. We thus used the band (*bd*) strain of *N. crassa* for isolation of mutants, and succeeded in isolating two whose growth is optimum at lower concentrations of calcium than are present in the medium which supports growth of the wild-

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Abbreviation: DCCD, *N,N'*-dicyclodihexylcarbodiimide.

type. We then compared the transport of calcium in various other organelles from these mutants with the corresponding activities in the wild-type.

METHODS

Culture conditions. Stock cultures were maintained in Horowitz complete medium (Horowitz, 1947), under continuous light at 25 °C. Conidia were inoculated into Vogel's medium (Vogel, 1956) which contained sucrose as indicated in the descriptions of individual experiments.

Mutagenesis and mycelial growth. The wild-type band (*bd*) strain was used in all experiments. Two mutants, *ca-19* and *ca-31*, which also had the *bd* mutation, were isolated by the method described by Davis & De Serres (1970), with slight modifications. Conidia of the *bd* strain of *N. crassa* were exposed to UV light for 4 min, and then cultured in Vogel's medium containing 2% sucrose, 1 µM-A23187 and 5 mM-CaCl₂. At various times, growing hyphae were removed by filtration through glasswool. After 3 d, conidia that had not germinated were washed once with distilled water and inoculated into sorbose complete medium (Malling, 1966) and cultured for 2 d at 26 °C in the dark. Individual colonies were then transferred to Horowitz complete medium and the effects of external calcium concentration on mycelial growth were determined.

For determination of growth rate, conidia (2×10^6) were inoculated into 20 ml of culture medium which contained Vogel's salts and 2% sucrose. After removal of the culture medium by paper filtration under suction, mycelia, still on the filter paper, were treated with 5 ml cold 10% (w/v) TCA. They were put into 5 ml 0.5 M-NaOH and incubated at 90 °C for 30 min. Samples of the extracts were used for assays of protein concentration.

Isolation of organelles. For growth of the *bd* wild-type strain Vogel's medium containing 2% sucrose and 1 mM-CaCl₂ was inoculated with 5.7×10^9 conidia l⁻¹.

For growth of the mutants *ca-19* and *ca-31* Vogel's medium containing 2% sucrose and 10 µM-CaCl₂ was inoculated with 5.7×10^9 and 11.4×10^9 conidia l⁻¹, respectively. After growing for 23 h in continuous light at 25 °C on a reciprocal shaker, the mycelia were harvested by filtration through cheesecloth. The organelles were isolated essentially by a combination of the methods of Cramer *et al.* (1983) for mitochondria and vacuoles and of Borgeson & Bowman (1983) for plasma membrane vesicles and microsomes. Buffer (10 mM-HEPES/NaOH, pH 7.45) containing 1 M-sorbitol, 1 mM-Na₂ATP and 1 mM-phenylmethylsulphonyl fluoride was used throughout the homogenization and washing procedures. Briefly, cells were disrupted by means of a bead-beater (Biospec, Bartlesville, Okla., USA). The glass beads were removed by filtration of the homogenate. The filtrate was centrifuged at 1000 *g* and the resulting supernatant filtered again through a Whatman 934-AH filter. This filtrate was centrifuged for 30 min at 14000 *g*. Organelles were isolated by two different centrifugation steps: the 14000 *g* pellet was layered onto a discontinuous sucrose gradient (1.2 M and 1.6 M-sucrose buffered at pH 7.4) and centrifuged for 2 h at 43000 *g* to separate mitochondria and vacuoles; the 14000 *g* supernatant was layered onto a continuous sucrose density gradient (14–42% sucrose, w/w, buffered at pH 7.4) and centrifuged for 2 h at 94000 *g* to separate plasma membranes and endoplasmic reticulum. Immediately thereafter the fractions were analysed for protein content (Bradford, 1976) and assayed for calcium transport activity.

Determination of calcium uptake by isolated cell fractions. This was measured using 20 µg vacuoles, 25 µg plasma membrane vesicles, 50 µg mitochondria or 50 µg microsomes (as determined by the protein content of each fraction). A 100 µl assay mixture (pH 7.4) was incubated with 7.4 kBq ⁴⁵Ca for 2 min (vacuoles) or 5 min (other fractions) at 25 °C. The assay mixtures contained the following: for the vacuolar fractions, 10 mM-MgSO₄, 5 mM-Na₃N₃, 1 mM-Na₃VO₄, 10 mM-ATP, 0.4 mM-CaCl₂; for the mitochondrial fraction, 10 mM-MgSO₄, 5 mM-K₂HPO₄, 1 mM-Na₃VO₄, 100 mM-KNO₃, 10 mM-ATP, 0.1 mM-CaCl₂; for the plasma membrane vesicles, 10 mM-MgSO₄, 10 mM-NaSCN, 5 mM-Na₃N₃, 100 mM-KNO₃, 10 mM-ATP, 0.1 mM-CaCl₂; and for the microsomes, 10 mM-MgSO₄, 5 mM-Na₃N₃, 100 mM-KNO₃, 10 mM-ATP, 0.1 mM-CaCl₂. Uptake was stopped by filtering the mixture through a Millipore AAWP filter and washing with 6 ml ice-cold preparation buffer. Radioactivity was determined in a liquid scintillation counter. As observed in preliminary experiments, calcium transport was ATP-dependent but was reduced by only 60% when ATP was omitted from the test mixture, which may be due to residual ATP in the preparation buffer. For this reason zero-time controls were used as blanks.

Other methods. The concentration of calcium in the various fractions was determined by atomic absorption spectroscopy. Arginine content was measured by the method of Van Pilsum *et al.* (1956) and vacuolar H⁺-ATPase activity by the method of Bowman (1983).

Chemicals. Nucleotides and enzymes were purchased from Sigma, ⁴⁵CaCl₂ from Amersham and A23187 from Calbiochem.

RESULTS

Isolation of mutants and mycelial growth

Mutants that could grow only in a medium which contained a low concentration of calcium were isolated from conidia that did not grow in the culture medium which contained a high

Fig. 1. Distribution of ratios of growth rates among progeny of a cross between *ca-19* and *ca-19+* (a) and between *ca-31* and *ca-31+* (b). Mutant strains were backcrossed with the *bd* strain and progeny were isolated. Samples of conidia suspended in distilled water were inoculated into medium that contained Vogel's salts without calcium, 2% (w/v) sucrose, and 10 μM or 1 mM - CaCl_2 and were cultured for 1 d with shaking. Mycelia were collected on filter paper and the dry wt was measured.

concentration of calcium and the ionophore A23187. Two mutants, *ca-19* and *ca-31*, were isolated from a total of several thousand colonies. The mutations segregated as single nuclear genes when they were backcrossed to the wild-type *bd* strain. Fig. 1 illustrates the 1:1 segregation among random spores. The fluctuations in the ratio of growth of mutant strains were due to the very poor growth in the culture medium which contained 1 mM -calcium.

Mycelial growth of the mutant strains *ca-19* and *ca-31*, as compared to the *bd* wild-type strain, was greatest when the calcium concentration in the medium was low (Fig. 2). The optimum calcium concentration for the mutants was about 10 μM , whereas growth of the wild-type was optimum at a 100-fold higher concentration. At optimum calcium concentrations, the growth rates of the mutant strains in the exponential phase were almost equal to that of *bd* wild-type strain (Fig. 3).

Uptake of calcium by fractionated cellular organelles

Calcium uptake by vacuoles was far greater than that by the other organelles (Table 1). In the mutant strains *ca-19* and *ca-31*, calcium uptake by vacuoles was much lower than in the *bd* wild-type strain. This difference is the only statistically significant difference between the wild-type and the mutants (1% level, Student's *t*-test). The other systems involved in calcium transport did not seem to play as prominent a role (quantitatively) and were not altered in the mutants. The transport of calcium into vacuoles from the wild-type did not change significantly when the wild-type was cultured in medium with only 10 μM - CaCl_2 (as used for the growth of the mutants), so that induction of the transport systems by high concentrations of calcium can be ruled out.

Other features of vacuoles of the wild-type strain

Properties other than calcium uptake were also altered in isolated vacuoles of the mutants *ca-19* and *ca-31* as compared to the wild-type. The calcium and arginine contents of the vacuolar fraction, as well as the activity of the vacuolar H^+ -ATPase, were decreased in the mutants (Table 2). Fig. 4 shows that calcium uptake by vacuoles required magnesium ions: that part of

Fig. 2

Fig. 3

Fig. 2. Dependence of mycelial growth of different strains on the external concentration of calcium. Conidia (2×10^6) were inoculated into 20 ml culture medium with different concentrations of CaCl_2 and cultured for the time required for the fastest growing culture in a given series to achieve a cell mass of about 5 mg protein per flask. This time was about 24 h for the wild-type (○), 30 h for ca-19 (●) and 36 h for ca-31 (▲).

Fig. 3. Mycelial growth rate at the optimum concentration of calcium for different strains. The procedures were the same as described in the legend to Fig. 1. Mycelia were harvested at different times as indicated on the abscissa. The concentration of calcium in the culture medium for each strain was 1 mM for the *bd* wild-type (●) and 10 μM for the mutants ca-19 (○) and ca-31 (□).

Table 1. Calcium uptake by isolated organelles

Values are means of *n* experiments $\pm$ SE.

Organelle	Mutant	<i>n</i>	Calcium uptake [nmol min ⁻¹ (mg protein) ⁻¹]
Vacuoles	<i>bd</i>	13	112.0 $\pm$ 23.0
	ca-19	9	21.0 $\pm$ 5.6
	ca-31	6	17.0 $\pm$ 3.6
Mitochondria	<i>bd</i>	13	2.1 $\pm$ 0.3
	ca-19	9	1.9 $\pm$ 0.3
	ca-31	6	1.8 $\pm$ 0.2
Plasma membrane vesicles	<i>bd</i>	15	5.6 $\pm$ 1.1
	ca-19	9	5.6 $\pm$ 0.9
	ca-31	6	6.4 $\pm$ 1.0
Microsomes	<i>bd</i>	13	3.4 $\pm$ 0.8
	ca-19	9	2.9 $\pm$ 0.5
	ca-31	6	2.9 $\pm$ 0.3

transport apparently independent of Mg^{2+} (about 25% of the control) may be due to residual magnesium ions present in the vacuolar fraction as supposed by Zerez *et al.* (1986) for vacuolar arginine transport. Azide and vanadate reduced calcium uptake only slightly but the effect of the proton transport inhibitors (Bowman & Bowman, 1982; Solioz, 1984) *N,N'*-dicyclohexylcarbodiimide (DCCD), NO_3^- and SCN^- was much greater (Fig. 4).

Fig. 4. Effects of different compounds on the uptake of calcium by vacuoles of the wild-type *bd* strain. The control value of calcium uptake was taken as 100%. MgSO_4 was included in all reaction mixtures except when its own effect was determined. DCCD was added 1 h before the start of the incubation and its effect was measured in the presence of azide and vanadate.

Table 2. Comparison of the vacuolar fractions of *N. crassa* *ca-19* and *ca-31* with the wild-type

Calcium and arginine contents and ATPase activity were determined in isolated vacuolar fractions of the *bd* wild-type and the mutants, and were compared to calcium transport (cf. Table 1). The values for the *bd* wild-type were each set to 100; these absolute values were $112 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ (calcium uptake), $4.12 \text{ mg (g protein)}^{-1}$ (calcium content), $2.94 \text{ nmol (g protein)}^{-1}$ (arginine content) and $0.11 \mu\text{mol P}_i \text{ min}^{-1} (\text{mg protein})^{-1}$ (ATPase activity).

Strain	Calcium transport	Calcium content	Arginine content	ATPase activity
<i>bd</i> wild-type	100	100	100	100
<i>ca-19</i>	19	10	32	39
<i>ca-31</i>	15	6	23	50

DISCUSSION

Our results show that in *N. crassa*, vacuoles isolated from the wild-type *bd* strain have a much greater ability to transport calcium ions than vacuoles isolated from mutants which can only grow at low calcium concentrations. This difference indicates that, in *N. crassa*, the vacuolar uptake of calcium is responsible for sequestering excess and hazardous amounts of free calcium from the cytosol. Therefore, mutants with a reduced ability to store calcium in the vacuole will suffer from a toxic excess of cytosolic calcium when grown in a medium which contains a high concentration of calcium.

Cytosolic calcium in animal cells is maintained at constant levels by the action of various pumps in the plasma membrane, in the mitochondrial membranes and in the endoplasmic reticulum (Bygrave, 1978; Carafoli & Crompton, 1978). Higher plants also have calcium pumps located in the membranes of various organelles (Buckhout, 1983; Dieter & Marme, 1980; Hager & Hermsdorf, 1981; Kreimer *et al.*, 1985; Rasi-Caldogno *et al.*, 1982; Schumaker & Sze, 1985). Recently, vacuolar systems for the transport of calcium have been detected in oat (Schumaker & Sze, 1985), carrot (Bush & Sze, 1986) and red beet (Blumwald & Poole, 1986). Yeast vacuoles also contain calcium transport systems (Ohsumi & Anraku, 1983; Eilam *et al.*, 1985; Okorokov *et al.*, 1985). Vacuoles are organelles that are possibly analogous to the lysosomes of animal cells in that they are the site of intracellular digestion; they also contain significant amounts of metabolites such as arginine and other basic amino acids. Arginine is transported into yeast vacuoles by an H^+ /amino acid antiporter, the energy being applied by H^+ -ATPase-dependent ATP hydrolysis (Ohsumi & Anraku, 1983). Vacuoles in *N. crassa* are also used as storage compartments for

arginine (Vaughn & Davis, 1981), and we showed that vacuoles in the wild-type *bd* strain contain an increased amount of arginine compared to vacuoles of mutants with reduced ability to transport calcium. In yeast, the uptake of calcium by vacuoles is coupled to the H^+ -ATPase in the same way as is arginine uptake (Ohsumi & Anraku, 1983). The vacuolar H^+ -ATPase of *N. crassa* has recently been partially purified (Bowman *et al.*, 1986). Because the effects of various compounds on the uptake of calcium by vacuoles of *N. crassa* (Fig. 4) are characteristic of a vacuolar H^+ -ATPase, it is possible that, in *N. crassa* the vacuolar transport of calcium is also coupled to a proton-translocating ATPase. This is supported by the recent observation of Zerez *et al.* (1986) that arginine uptake by vacuolar membrane vesicles of *N. crassa* also seems to depend on vacuolar H^+ -ATPase activity.

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