Calcium involvement in dimorphism of *Ophiostoma ulmi*, the Dutch elm disease fungus, and characterization of calcium uptake by yeast cells and germ tubes

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Exogenous Ca^{2+} , at concentrations up to 5 mM, induced partial germ tube formation in *Ophiostoma* (= *Ceratocystis*) *ulmi* in media normally supporting growth in the yeast-like phase. The calmodulin inhibitors calmidazolium (R24571) and trifluoperazine (TFP), and the Ca^{2+} ionophore, A23187, suppressed germ tube formation in germ-tube-inducing medium without affecting yeast-like growth. R24571 was the most effective inhibitor, giving almost complete suppression at 3 μM . Addition of excess Ca^{2+} (up to 5 mM) did not reverse the inhibitory action of R24571 and only ~10% of yeast-like cells formed germ tubes on addition of Ca^{2+} in the presence of 20 μM -TFP or 15 μM -A23187. Intracellular cAMP increased on incubation with R24571 and A23187, possibly as a result of inhibition of the cAMP phosphodiesterase. The exogenous supply of the calcium-binding agents methylhydroxybenzoate (MHB) and EGTA also suppressed germ tube formation under inducing conditions. These results confirm an involvement of Ca^{2+} in the yeast–mycelium transition of *O. ulmi*. Yeast-like cells and germ tubes of *O. ulmi* exhibited metabolism-dependent Ca^{2+} uptake which was reduced in the absence of glucose, or by the presence of KCN, the ATPase inhibitors *N,N'*-dicyclohexylcarbodiimide (DCCD) and diethylstilboestrol (DES), and the protonophoric uncoupler DNP, indicating dependence on the electrochemical proton gradient across the plasma membrane generated by the H^+ -ATPase. Germ tubes exhibited greater sensitivity to inhibitors of Ca^{2+} uptake than yeast-like cells, while Ca^{2+} uptake was competitively inhibited by Mg^{2+} , Mn^{2+} and Zn^{2+} . R24571 and A23187 inhibited Ca^{2+} uptake by germ tubes although TFP stimulated uptake in comparison to control cells. Ca^{2+} uptake by both cell types conformed to Michaelis–Menten kinetics at concentrations below ~200 μM but deviated strongly above this concentration. Kinetic analysis of Ca^{2+} uptake by yeast-like cells and germ tubes, at Ca^{2+} concentrations below 100 μM , revealed that both cell types possessed Ca^{2+} transport systems of similar specificity, with K_m values ranging between ~15 and 25 μM , although germ tubes always exhibited greater Ca^{2+} uptake than yeast cells under similar experimental conditions, possibly a consequence of increased vacuolar compartmentation.

Introduction

Calcium is an important element in the growth, metabolism and differentiation of several fungi and yeasts (Pitt & Kaile, 1990). Exogenous calcium can affect sexual and asexual reproduction and patterns of hyphal extension and branching in several fungi (Pitt & Ugalde, 1984; Schmid & Harold, 1988; Jackson & Heath, 1989;

Robson *et al.*, 1991a), including the induction of conidiation in *Penicillium* spp. (Ugalde & Pitt, 1986; Pitt *et al.*, 1988; Pitt & Kaile, 1990) and germ tube formation in di- and polymorphic fungi, e.g. *Ceratocystis* (*Ophiostoma*) *ulmi* (Muthukumar & Nickerson, 1984) and *Candida albicans* (Sabie & Gadd, 1989).

There is increasing evidence that cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_c$) plays a significant role in signal transduction in fungi, as in other eukaryotic cells (Miyakawa *et al.*, 1989; Pitt & Kaile, 1990; Trinci *et al.*, 1990; Iida *et al.*, 1990; Magalhães *et al.*, 1991). Since Ca^{2+} is toxic at high concentrations, cells utilize a complex system of sequestrative and homeostatic mechanisms which maintain $[\text{Ca}^{2+}]_c$ at a low level. Although $[\text{Ca}^{2+}]_c$ is maintained

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Abbreviations: $[\text{Ca}^{2+}]_c$, cytosolic free Ca^{2+} ; DCCD, *N,N'*-dicyclohexylcarbodiimide; DES, diethylstilboestrol; DNP, dinitrophenol; MHB, methylhydroxybenzoate; R24571, calmidazolium; TFP, trifluoperazine.

around 10^{-7} M in eukaryotic cells, the opening of ligand- or voltage-gated Ca^{2+} channels occurs in response to a variety of external stimuli, such as hormones, allergens, cell surface-adhering antibodies, membrane depolarization, light or mechanical pressure (Miyakawa *et al.*, 1985; Tachikawa *et al.*, 1987; Pitt & Kaile, 1990). On such stimulation, $[\text{Ca}^{2+}]_i$ can increase to approximately 10^{-5} M. During such an increase, Ca^{2+} can bind to intracellular calcium-binding proteins, which interact in turn with target proteins and stimulate their enzymic or biological activity. Calmodulin is probably the most important regulatory calcium-binding protein in eukaryotic cells and can act as a regulator of Ca^{2+} -dependent adenylate cyclase (Cheung *et al.*, 1985) and cAMP phosphodiesterase, thereby providing a molecular link between the two second messengers, Ca^{2+} and cAMP (Wright *et al.*, 1986). Calmodulin has been detected in a range of filamentous and dimorphic fungi, and yeasts from diverse taxonomic groups (Gomes *et al.*, 1979; Grand *et al.*, 1980; Hubbard *et al.*, 1982; Cox *et al.*, 1982; Salgado-Rodriguez *et al.*, 1986; Muthukumar *et al.*, 1985, 1986, 1987; Laccetti *et al.*, 1987). A Ca^{2+} -calmodulin interaction has previously been reported to be necessary for germ tube and mycelial development in *Ceratocystis ulmi* (Muthukumar & Nickerson, 1984, 1985) and *Candida albicans* (Sabie & Gadd, 1989) probably through calmodulin-dependent protein phosphorylation (Roy & Datta, 1987; Paranjape *et al.*, 1990), as in the entomopathogen *Metarhizium anisopliae* (St Leger *et al.*, 1989, 1990) and *Zoophthora radicans* (Magalhães *et al.*, 1991). More recently, indirect evidence has been presented for the existence of an inositol lipid signalling system, which may effect elevation of intracellular $[\text{Ca}^{2+}]_i$ (Berridge & Irvine, 1984), in *O. ulmi* (Brunton & Gadd, 1991).

As well as $[\text{Ca}^{2+}]_i$ regulation by calcium-binding proteins, transport systems for Ca^{2+} located on the plasma and vacuolar membranes have a major role in $[\text{Ca}^{2+}]_i$ homeostasis in fungi (Miller *et al.*, 1990; Iida *et al.*, 1990). Ca^{2+} efflux from the cytosol may result from operation of a $\text{Ca}^{2+}/\text{H}^+$ antiport, the electrochemical proton gradient ($\Delta\tilde{\mu}_{\text{H}^+}$) being generated by the plasma membrane H^+ -ATPase (Eilam, 1982; Slayman *et al.*, 1990) although recent work with *Neurospora crassa* has implied Ca^{2+} efflux by means of a $\text{H}^+/\text{Ca}^{2+}$ -ATPase as in plant cells (Miller *et al.*, 1990). In yeasts and fungi, vacuoles have a major function in maintaining cytoplasmic Ca^{2+} homeostasis and act as the main Ca^{2+} -sequestering organelles (Eilam *et al.*, 1985) transporting Ca^{2+} across the vacuolar membrane by $\text{Ca}^{2+}/\text{H}^+$ antiport (Ohsumi & Anraku, 1983; Okorokov *et al.*, 1985; Cornelius & Nakashima, 1987; Miller *et al.*, 1990). However, Ca^{2+} transport in dimorphic fungi and yeasts has been studied in relatively little detail, most work concentrating on *Saccharomyces cerevisiae* (Nieuwenhuis

et al., 1981; Eilam *et al.*, 1985; Eilam & Othman, 1990) although energy-dependent transport of Ca^{2+} has been described in some filamentous fungi, e.g. *N. crassa* and certain *Penicillium* spp. (Pitt & Kaile, 1990).

This paper confirms an involvement of calcium in the yeast-mycelium transition of *Ophiostoma ulmi*, the Dutch elm disease fungus, and provides further evidence for a Ca^{2+} -calmodulin interaction being involved in morphogenesis. The uptake of Ca^{2+} from low and high external concentrations during growth and germ tube formation is also described, including kinetic characteristics of Ca^{2+} transport, and the effects of Ca^{2+} ionophores, calmodulin antagonists and other divalent metal cations.

Methods

Organism, media and cultural conditions. *Ophiostoma (Ceratocystis) ulmi* NRRL 6404, kindly provided by Dr J. J. Ellis (US Department of Agriculture, Midwest Area Northern Regional Research Centre, Peoria, IL 616045, USA), was routinely maintained on malt extract agar (Lab M) at 25 °C. For liquid cultures supporting growth in the yeast phase, a defined liquid medium, pH 6.8, of the following composition was used (g l^{-1}): glucose, 20.0; proline, 1.15; KH_2PO_4 (anhydrous), 2.72; $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 5.22; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.12; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0022; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.004; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.004; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.0004 and supplemented with the following vitamins ($\mu\text{g l}^{-1}$): biotin, 20; pyridoxine.HCl, 200; thiamin.HCl, 200. Starter cultures were prepared by loop-inoculating 50 ml liquid culture medium and incubating at 25 °C on a rotary shaker ($100 \text{ cycles min}^{-1}$). For experiments, 100 ml medium was inoculated from late stationary-phase cultures to an initial cell density of approximately $2 \times 10^6 \text{ ml}^{-1}$ and incubated as previously described. When desired, the liquid medium was supplemented with 1% (w/v) yeast extract (Difco) in order to induce germ tube formation (Brunton & Gadd, 1989). Where required, filter-sterilized calmodulin inhibitors, calmidazolium (R24571) and trifluoperazine (TFP), the calcium ionophore A23187 and the calcium-binding agents methylhydroxybenzoate (MHB) and EGTA were added to the appropriate final concentrations. TFP, A23187, MHB and EGTA were made up as aqueous stock solutions whereas the lipophilic compound R24571 was dissolved in dimethyl sulphoxide (DMSO) and added to the medium with vigorous mixing. The final concentration of DMSO in the assay medium was <0.5% (v/v) and this did not affect cell growth.

Calcium uptake by non-growing cell suspensions. Yeast cells and germ tubes were harvested by centrifugation (5 min, 1200 g) from the liquid medium described. Cells were washed twice with, and finally resuspended in, 5 mM-PIPES buffer, pH 6.5, to a cell density of $\sim 10^7 \text{ ml}^{-1}$. Glucose was added to a final concentration of 70 mM and the suspensions were incubated for 2 h at 25 °C on a magnetic stirrer. Where required KCN, *N,N'*-dicyclohexylcarbodiimide (DCCD), diethylstilboestrol (DES), dinitrophenol (DNP), calcium ionophore A23187, calmodulin inhibitors R24571 and TFP, and the calcium-binding agents EGTA and MHB, were added to the appropriate concentrations 10 min before addition of ^{45}Ca . Ca^{2+} uptake was initiated by the addition of an appropriate concentration of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ together with a final concentration of $0.27 \mu\text{M}$ - ^{45}Ca (final activity approximately 8 kBq ml^{-1}). Samples (1 ml) were removed at intervals, rapidly filtered through cellulose nitrate membrane filters (0.45 μm , 25 mm diam.), and washed three or four times with ice-cold $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ of a concentration greatly in excess ($\geq 10 \times$) of that being

used for the uptake study. The filters were dried, transferred to scintillation vials and counted in Ecoscint A (National Diagnostics) scintillation fluid using a Packard 300 CD scintillation counter.

Other methods. Cells were counted using a modified Fuchs-Rosenthal haemocytometer. Dry weights of washed cell pellets, obtained by centrifugation (5 min, 2000 g), were determined by means of tared aluminium foil cups dried to constant weight at 105 °C. Cyclic AMP was determined using a cAMP-binding assay kit (Amersham) as previously described (Brunton & Gadd, 1989).

Chemicals. All chemicals used were of the highest purified grade available. R24571, TFP, EGTA, KCN and DCCD were obtained from Sigma. Ionophore A23187 was obtained from Boehringer. $^{45}\text{CaCl}_2$ was obtained from Amersham. MHB was kindly provided by Dr S. W. Edwards, Department of Biochemistry, University of Liverpool.

Results

Induction of germ tube formation in *O. ulmi* by exogenous calcium

The exogenous supply of Ca^{2+} to the culture medium, up to a final concentration of 5 mM, resulted in germ tube formation by *O. ulmi*. This was maximal at a final concentration of 1 mM- CaCl_2 , with approximately 22% of the population showing germ tube formation after 28 h (Table 1). At concentrations above 1 mM- CaCl_2 some inhibition of this response was observed (Table 1). CaCl_2 concentrations greater than 5 mM resulted in precipitation of $\text{Ca}_3(\text{PO}_4)_2$ in the medium. In Ca^{2+} -unsupplemented medium, growth of *O. ulmi* was exclusively yeast-like.

Effect of the calmodulin inhibitors R24571 and TFP, and the Ca^{2+} ionophore A23187, on germ tube formation by *O. ulmi*

Calmodulin inhibitors and the Ca^{2+} ionophore were added to the cells together with 1% (w/v) yeast extract which, by itself, induces a complete yeast-mycelium transition in *O. ulmi* NRRL 6404 (Brunton & Gadd,

Fig. 1. Effect of excess exogenous CaCl_2 on the inhibition of germ tube formation in *O. ulmi* by the anti-calmodulin drugs R24571 (a) and TFP (b) and by the calcium ionophore A23187 (c). The compounds were used in conjunction with 1% (w/v) yeast extract, which effects a complete yeast-mycelium transition in *O. ulmi*. Concentrations used were: (a) ○, 0; ●, 3 μM; △, 3 μM plus 10 mM- CaCl_2 ; (b) ○, 0; ●, 20 μM; △, 20 μM plus 10 mM- CaCl_2 ; (c) ○, 0; ●, 15 μM; △, 15 μM plus 10 mM- CaCl_2 . The bars in this and succeeding Figures indicate the standard error of the mean (SEM) of individual points (three replicates).

Table 1. Effect of exogenous CaCl_2 on germ tube formation by *O. ulmi* in defined liquid medium

Germ tube formation was assessed after 28 h incubation at 25 °C; the initial inoculum size was 2×10^6 cells ml^{-1} from a culture growing exclusively as yeast-like cells. Values shown are $\pm$ SEM (three replicate determinations); ND, not detected.	
$[\text{Ca}^{2+}]$ (mM)	Germ tube formation (%)
0	ND
0.1	13.4 ± 0.6
0.5	19.2 ± 0.8
1.0	21.8 ± 1.2
2.0	20.0 ± 1.0
5.0	16.8 ± 0.8

1989, 1991). The concentrations of the inhibitors and ionophores used in this work did not inhibit yeast-like growth of *O. ulmi*. R24571 was the most effective inhibitor, a concentration of 3 μM being sufficient to almost completely suppress germ tube formation (Fig. 1a). TFP caused a similar degree of suppression, approximately 5% germ tube formation being observed in the presence of 20 μM-TFP (Fig. 1b). A23187 was less effective, although germ tube formation was reduced approximately 50% by a concentration of 10 μM (Fig. 1c). The addition of excess CaCl_2 , to concentrations greater than 5 mM, did not reverse the inhibitory action of R24571 and only a maximum of 10% of yeast cells formed germ tubes on addition of CaCl_2 in the presence

Fig. 2. Intracellular cAMP levels during growth of *O. ulmi* in the presence of 3 μM -R24571 (○) and 15 μM -A23187 (●). The compounds were used in conjunction with 1% (w/v) yeast extract. In the presence of 1% (w/v) yeast extract, cAMP increased, with maximum levels coinciding with maximum germ tube formation; in the absence of yeast extract (yeast-like growth) cAMP levels remained approximately constant (Brunton & Gadd, 1989).

of 20 μM -TFP or 15 μM -A23187 (Fig. 1). The addition of 10 mM- CaCl_2 alone had no discernible effect on the complete yeast-mycelium transition induced by 1% (w/v) yeast extract (result not shown).

Levels of intracellular cAMP were monitored during exposure to R24571 and A23187 and, in both cases, an increase was observed. An intracellular level of approximately 35 pmol cAMP (mg dry wt)⁻¹ was recorded after 28 h incubation with 3 μM -R24571, whereas A23187 at 10 μM produced a lesser response, with a maximum 25 pmol cAMP (mg dry wt)⁻¹ after 24 h incubation (Fig. 2). Intracellular cAMP remained approximately constant in unsupplemented medium without yeast extract whereas a marked increase in intracellular cAMP, with the maximum level coinciding with, or just before, the time of maximum germ tube formation occurred in the presence of 1% (w/v) yeast extract (Brunton & Gadd, 1989). Extracellular cAMP was assayed for but was not detected.

Effect of the calcium binders MHB and EGTA on germ tube formation by *O. ulmi*

Calcium-binding agents were added to the cells together with 1% (w/v) yeast extract. In the presence of 1 mM-MHB or 5 mM-EGTA, germ tube formation was reduced to approximately 10% after 16 h incubation (Fig. 3). Further mycelium development from the germ tubes was inhibited by all concentrations of MHB used; in the presence of EGTA at concentrations below 2 mM

Fig. 3. Effect of the calcium binders MHB (a) and EGTA (b) on germ tube formation by *O. ulmi*. The compounds were used in conjunction with 1% (w/v) yeast extract. Final inhibitor concentrations used were: (a) ○, 0; ●, 100 μM ; △, 500 μM ; ▲, 1000 μM ; (b) ○, 0; ●, 0.5 mM; △, 1.0 mM; ▲, 2 mM; □, 5 mM.

mycelium formation was still evident, but above this concentration only germ tubes were produced.

Ca²⁺ uptake by different cell developmental stages and the effect of metabolic inhibitors, calmodulin inhibitors and Ca²⁺ ionophore A23187

Uptake of Ca^{2+} was examined in yeast cells and germ tubes, as induced by 1% (w/v) yeast extract, harvested

Fig. 4. Uptake of Ca^{2+} by different-aged yeast cells of *O. ulmi* from $10 \mu\text{M}$ - CaCl_2 in 5 mM -PIPES buffer, pH 6.5, in the presence of 70 mM -glucose (○), in the absence of glucose (●) and in the presence of $200 \mu\text{M}$ -KCN (△).

Table 2. Inhibition of Ca^{2+} uptake in yeast cells and germ tubes of *O. ulmi* by DCCD, DES and DNP

Cells were harvested after 12 h incubation in the absence (yeast cells) or presence (germ tubes) of 1% (w/v) yeast extract and suspended in 5 mM -PIPES buffer, pH 6.5, containing 70 mM -glucose, to a cell density of $\sim 10^7 \text{ ml}^{-1}$. The Ca^{2+} concentration was $10 \mu\text{M}$. Ca^{2+} uptake values were determined after 40 min incubation and expressed relative to the control, taken as 100%. Control values ($\pm \text{SEM}$) of Ca^{2+} uptake for yeast cells and germ tubes were 15.5 ± 0.4 and $146.5 \pm 5.0 \text{ pmol (mg dry wt)}^{-1}$ respectively.

	Inhibition of Ca^{2+} uptake (%)	
	Yeast cells	Germ tubes
Control	0	0
$100 \mu\text{M}$ -DCCD	20.5 ± 2.6	40.1 ± 2.7
$100 \mu\text{M}$ -DES	24.4 ± 3.2	42.4 ± 3.4
$50 \mu\text{M}$ -DNP	37.8 ± 1.9	76.9 ± 2.7

after different times. In both cell types, the uptake of calcium from $10 \mu\text{M}$ - CaCl_2 showed a similar pattern, with increased Ca^{2+} uptake being exhibited by 12 h cells as compared with those harvested at 4, 8 or 16 h

Fig. 5. Uptake of Ca^{2+} by different-aged germ tubes of *O. ulmi*, as induced by 1% (w/v) yeast extract, from $10 \mu\text{M}$ - CaCl_2 in 5 mM -PIPES buffer, pH 6.5, in the presence of 70 mM glucose (○), in the absence of glucose (●) and in the presence of $200 \mu\text{M}$ -KCN (△).

incubation (Figs 4 and 5). Reduced Ca^{2+} uptake was observed in the absence of glucose and in the presence of KCN, indicating that Ca^{2+} uptake was metabolism-dependent although the relative sensitivity of Ca^{2+} uptake to these conditions varied between cell types and between different cell ages (Figs 4 and 5). Ca^{2+} uptake by yeast cells and germ tubes was also inhibited by DCCD and DES, which indicated that Ca^{2+} uptake was dependent on plasma membrane ATPase activity (Table 2). The inhibition of Ca^{2+} uptake in both cell types by DNP also indicated that uptake was dependent on the transmembrane proton gradient generated by the plasma membrane H^+ -ATPase (Table 2). Germ tubes exhibited greater sensitivity to these compounds than yeast cells (Table 2).

Fig. 6. Uptake of Ca^{2+} by 12 h germ tubes of *O. ulmi*, as induced by 1% (w/v) yeast extract, from $10\ \mu\text{M}$ - CaCl_2 in 5 mM-PIPES buffer, pH 6.5 (○) and in the presence of $3\ \mu\text{M}$ -R24571 (●), $20\ \mu\text{M}$ -TFP (▲) and $15\ \mu\text{M}$ -A23187 (△).

Uptake of Ca^{2+} by germ tubes (induced by 1%, w/v, yeast extract) from $10\ \mu\text{M}$ - CaCl_2 was partially inhibited when the cells were pre-incubated with $3\ \mu\text{M}$ -R24571 or $15\ \mu\text{M}$ A23187 (Fig. 6). Ca^{2+} uptake was reduced approximately 50% by A23187, whereas R24571 produced a smaller inhibition of approximately 20%. TFP stimulated Ca^{2+} uptake (Fig. 6).

Kinetics of Ca^{2+} uptake by yeast cells and germ tubes of *O. ulmi*

Ca^{2+} uptake by both cell types increased as the external CaCl_2 concentration increased; uptake from all CaCl_2 concentrations examined was consistently higher in 12 h yeast-extract-induced germ tubes than in yeast-like cells of a similar age (Figs 7 and 8). The affinities of yeast-like cells and germ tubes for Ca^{2+} uptake were assessed using Michaelis-Menten kinetic analysis over a range of external CaCl_2 concentrations (10 – $1000\ \mu\text{M}$) in the presence of glucose. Rates of Ca^{2+} uptake were derived by linear regression on experimental points taken

Fig. 7. Kinetics of energy-dependent Ca^{2+} uptake by 4 h (○) and 12 h (●) yeast cells of *O. ulmi* from various CaCl_2 concentrations in 5 mM-PIPES buffer, pH 6.5. The rates were derived by linear regression from individual uptake curves and the data transformed according to Woolf (Morris, 1974). $[S]$ is the external Ca^{2+} concentration; v is the rate of Ca^{2+} uptake [$\text{nmol min}^{-1} (\text{mg dry wt})^{-1}$].

between 1 and 10 min and the data were transformed according to Woolf. It appeared that Ca^{2+} uptake was by means of more than one transport system in both cell types and both cell ages (Figs 7 and 8). Ca^{2+} uptake conformed to Michaelis-Menten kinetics at concentrations below approximately $200\ \mu\text{M}$, but deviated strongly above this concentration (Figs 7 and 8). Using Ca^{2+} uptake values obtained for CaCl_2 concentrations less than $100\ \mu\text{M}$, the K_m and $V_{\max}$ values of Ca^{2+} uptake by 4 h yeast cells were calculated to be $19.2\ \mu\text{M}$ and $9.1 \times 10^{-4}\ \text{nmol min}^{-1} (\text{mg dry wt})^{-1}$ respectively. For 12 h yeast cells, the K_m value was $14.6\ \mu\text{M}$ and the $V_{\max}$ value was $5.4 \times 10^{-3}\ \text{nmol min}^{-1} (\text{mg dry wt})^{-1}$. The K_m and $V_{\max}$ values of Ca^{2+} uptake by 4 h germ tubes were $25.3\ \mu\text{M}$ and $8.3 \times 10^{-4}\ \text{nmol min}^{-1} (\text{mg dry wt})^{-1}$ respectively; 12 h germ tubes gave K_m and $V_{\max}$ values of $20.5\ \mu\text{M}$ and $1.44 \times 10^{-2}\ \text{nmol min}^{-1} (\text{mg dry wt})^{-1}$.

Effect of divalent cations on Ca^{2+} uptake by germ tubes of *O. ulmi*

Fig. 9 shows the effect of Mg^{2+} , Mn^{2+} and Zn^{2+} on Ca^{2+} uptake by germ tubes, induced by 1% (w/v) yeast extract,

Fig. 8. Kinetics of energy-dependent Ca^{2+} uptake by 4 h (○) and 12 h (●) germ tubes of *O. ulmi*, as induced by 1% (w/v) yeast extract, from various CaCl_2 concentrations in 5 mM-PIPES buffer, pH 6.5. The rates were derived by linear regression from individual uptake curves and the data transformed according to Woolf.

from 10 μM - CaCl_2 . Uptake of Ca^{2+} by germ tubes was inhibited at all concentrations of MnCl_2 and ZnCl_2 , over 50% inhibition resulting at concentrations up to 20 μM . MgCl_2 was the least potent inhibitor of calcium uptake by germ tubes, maximum inhibition resulting at approximately 50 μM - MnCl_2 (Fig. 9).

The inhibitory effect of Mg^{2+} , Mn^{2+} and Zn^{2+} on the affinity of germ tubes to take up Ca^{2+} was further assessed over a range of CaCl_2 concentrations (10–100 μM) in the presence of glucose and the desired concentration of divalent cation. Rates of Ca^{2+} uptake were derived from individual determinations carried out over 10 min; data were transformed according to Lineweaver and Burk. As the concentration of the inhibiting divalent cation was increased, the slope of the double reciprocal plot increased, but continued to give the same value for $V_{\max}$, resulting in an increase in the K_m . In the presence of the inhibitory divalent cations, the $V_{\max}$ remained constant at approximately $1.25 \times 10^{-2} \text{ nmol min}^{-1} (\text{mg dry wt})^{-1}$. In the presence of

Fig. 9. Effect of divalent metal ions on the rate of Ca^{2+} uptake by 12 h germ tubes of *O. ulmi*, as induced by 1% (w/v) yeast extract, from 10 μM - CaCl_2 in 5 mM-PIPES buffer, pH 6.5. The rates were derived by linear regression from individual uptake curves. The control rate was calculated to be $(0.45 \pm 0.02) \times 10^{-2} \text{ nmol min}^{-1} (\text{mg dry wt})^{-1}$. ○, MgCl_2 ; ●, MnCl_2 ; △, ZnCl_2 .

20 μM - and 100 μM - MgCl_2 , the K_m was increased from 18.2 μM (control value) to 33.3 μM and 40.0 μM respectively; 20 and 100 μM - ZnCl_2 gave K_m values of 21.8 μM and 25.0 μM respectively, while 50 μM - and 100 μM - MnCl_2 increased the K_m to 45.5 μM and 62.5 μM respectively.

Discussion

Exposure of stationary-phase yeast cells of *O. ulmi* to Ca^{2+} concentrations up to 5 mM resulted in some degree of germ tube formation. Muthukumar & Nickerson (1984) reported that 85% of a population of *O. ulmi* NRRL 6404 cells formed germ tubes after addition of 20 mM- CaCl_2 to the growth medium. However, we could not confirm these results and found that extensive precipitation occurred at CaCl_2 concentrations above 5 mM. Although only a partial germination response was induced by exogenous CaCl_2 in our study, the other results presented nevertheless confirm a requirement for a Ca^{2+} -calmodulin interaction in the yeast-mycelium transition of *O. ulmi* (Muthukumar & Nickerson, 1984). The addition of the calcium-binding agents EGTA and MHB to a yeast-extract-supplemented growth medium, normally supporting mycelial growth, almost completely prevented the yeast-mycelium transition. MHB has been

reported to inhibit Ca^{2+} enhancement of trypsin activity, which indicates that MHB has the ability to interfere with intracellular free Ca^{2+} levels; the ability of MHB to bind Ca^{2+} was confirmed using a Ca^{2+} electrode (Edwards *et al.*, 1989).

TFP is commonly used to demonstrate inhibition of calmodulin-dependent stimulation of enzymic processes. Roy & Datta (1987) showed that TFP prevented germ tube formation in *C. albicans* at 37 °C, which indicated that Ca^{2+} and calmodulin were involved in the morphogenetic transition. TFP also inhibits calmodulin activity in *Mucor rouxii* (Salgado-Rodriguez *et al.*, 1986). The results presented here are in broad agreement with these findings: germ tube formation in *O. ulmi* was almost completely suppressed by TFP whereas yeast-like growth was unaffected. The calmodulin inhibitor R24571 and the calcium ionophore A23187 also had an inhibitory effect on germination. These inhibitory actions could not be reversed by the addition of excess CaCl_2 , which is in agreement with the suggestion that calmodulin inhibitors act on the Ca^{2+} -calmodulin complex and not on free calmodulin (Prozialeck & Weiss, 1985). Inhibition of the *C. albicans* yeast-mycelium transition by R24571 was also not relieved by excess exogenous CaCl_2 (Sabie & Gadd, 1989). However, Muthukumar & Nickerson (1984, 1985) reported that inhibition of *O. ulmi* germination by TFP and several other calmodulin inhibitors was reversed by further addition of CaCl_2 . Levels of intracellular cAMP increased during growth of *O. ulmi* in the presence of R24571 and A23187. Calmodulin can act as a regulator of Ca^{2+} -dependent cAMP phosphodiesterase and also of adenylate cyclase and it is therefore possible that the presence of calmodulin inhibitors prevented activation of the phosphodiesterase, thereby causing a build-up of cAMP within the cell.

Ca^{2+} uptake by yeast cells and germ tubes of *O. ulmi* followed a similar pattern to Ca^{2+} uptake in *Penicillium cyclopium* (Ugalde & Pitt, 1986) and *Penicillium notatum* (Pitt *et al.*, 1988) and other divalent cations in yeasts (Borst-Pauwels, 1981; White & Gadd, 1987; Sabie & Gadd, 1990). The presence of KCN or the absence of glucose inhibited metabolism-dependent uptake of Ca^{2+} by both cell types. The ATPase inhibitors DCCD and DES inhibited Ca^{2+} uptake by both cell types of *O. ulmi*, indicating that uptake was dependent on plasma membrane ATPase activity. The inhibition of Ca^{2+} uptake by the protonophoric uncoupler DNP confirmed previous reports that the driving force for cation uptake is the electrochemical proton gradient and is in agreement with DNP inhibition of Ca^{2+} uptake in *S. pombe* (Boutry *et al.*, 1977). However, the effect of DES on calcium transport, similar to that of DNP, may also reflect an effect on ATP levels rather than a

requirement for a transmembrane proton gradient (Serrano, 1980).

Germ tubes showed significantly greater Ca^{2+} uptake than yeast cells under the same conditions. Gow & Gooday (1984) studied germ tube formation in dimorphic *C. albicans* and found that in germ-tube-inducing media, the cells developed large vacuoles; it was suggested that there was little biosynthesis of cytoplasm during germ tube formation and that cell growth involved enlargement of the vacuole to maintain the turgor of the cell during hyphal extension. Vacuoles of yeast and other fungi are now known to possess a Ca^{2+} -transporting system which leads to storage of Ca^{2+} in the vacuole (Ohsumi & Anraku, 1983; Eilam *et al.*, 1985; Okorokov *et al.*, 1985; Miller *et al.*, 1990). Differences in vacuolar volume between the two types of cell may contribute to the great differences observed in Ca^{2+} uptake by yeast-like cells and germ tubes of *O. ulmi*.

In previous studies, calmodulin antagonists which are inhibitors of the yeast plasma membrane H^+ -ATPase have been found to stimulate Ca^{2+} uptake in yeasts (Borst-Pauwels *et al.*, 1986a). Here, TFP stimulated Ca^{2+} uptake in germ tubes of *O. ulmi*. This is in agreement with the reported enhanced uptake of Ca^{2+} by TFP in *S. cerevisiae* (Saavedra-Molina *et al.*, 1983; Borst-Pauwels *et al.*, 1986b; Eilam & Chernichovsky, 1988) and also Sr^{2+} in the same organism (Borst-Pauwels *et al.*, 1986a). The results presented here showed that R24571 and ionophore A23187 inhibited uptake of Ca^{2+} by germ tubes of *O. ulmi*. In contrast, Borst-Pauwels *et al.* (1986a) reported that R24571 enhanced Sr^{2+} influx in *S. cerevisiae*, as did other calmodulin inhibitors. However, such effects may be concentration dependent and non-specific (Borst-Pauwels & Theuvsen, 1985). The initial rate of Ca^{2+} uptake in *S. cerevisiae* was strongly inhibited by ionophore A23187 (Ohsumi & Anraku, 1983).

Ca^{2+} uptake by yeast cells and germ tubes conformed to Michaelis-Menten kinetics at Ca^{2+} concentrations below 100 μM , but above this concentration, deviations were apparent. Kinetic studies over a range of Ca^{2+} concentrations from 1 to 100 μM revealed that for each cell type, as the age of the cell increased, the number of sites available for Ca^{2+} on the cell membrane, as indicated by the V_{max} values, increased. However, the affinity of yeast cells and germ tubes for Ca^{2+} under the same conditions, as indicated by the K_m values, were similar. There are several other reports on the kinetic parameters of Ca^{2+} uptake by other yeasts. The K_m for Ca^{2+} uptake in *S. cerevisiae* has been reported to be as low as 1.9 μM (Borbolla & Pena, 1980; Borbolla *et al.*, 1985) or as large as 0.6 mM (Armstrong & Rothstein, 1967). Boutry *et al.* (1977) estimated the K_m for Ca^{2+} uptake by *Schizosaccharomyces pombe* to be 44 μM , while Ugalde & Pitt (1986) found that the K_m and V_{max} were

1.8 mM and 8.9 nmol min⁻¹ (mg dry wt)⁻¹ respectively in *P. cyclospium*.

Mg²⁺, Zn²⁺ and Mn²⁺ all had an inhibitory effect on the uptake of Ca²⁺ by germ tubes of *O. ulmi*, which may indicate a common transport mechanism and/or that the Ca²⁺ transport system can exhibit a significant affinity for other divalent cations (Boutry *et al.*, 1977). Ohsumi & Anraku (1983) studied the effect of metal cations on Ca²⁺ transport by *S. cerevisiae* and reported that Mg²⁺ was essential for Ca²⁺ uptake. However, under similar experimental conditions, Zn²⁺ was found to be a strong inhibitor of Ca²⁺ uptake while Mn²⁺ had no effect. It was thought that in *S. cerevisiae*, Zn²⁺ inhibited Ca²⁺ uptake into the vacuole (Ohsumi & Anraku, 1983; Eilam *et al.*, 1985). Magnesium and zinc have also been reported to influence a yeast-mycelium transition in other dimorphic fungi. Alsina & Rodriguez-Del Valle (1984) suggested an inhibitory role for zinc and magnesium in the regulation of the yeast-mycelium transition in *Sporothrix schenckii*. Inhibition of mycelium development by Zn²⁺ has also been reported in *C. albicans* (Soll *et al.*, 1981; Sabie & Gadd, 1990) and *Histoplasma capsulatum* (Pine & Peacock, 1958), while a requirement for Mg²⁺ has been demonstrated for germ tube production in *C. albicans* (Walker *et al.*, 1984). All three divalent cations increased the K_m but did not cause a change in the V_{max} . This was indicative of the germ tubes showing a decreased affinity for Ca²⁺ in the presence of the inhibitory cations. However, the unaltered V_{max} indicated that at sufficiently high concentrations of Ca²⁺, the same maximum initial rate of uptake was displayed as exhibited by the uninhibited reaction at lower Ca²⁺ concentrations. These results showed that the divalent cations inhibited Ca²⁺ uptake in a competitive manner. Ca²⁺ uptake by *Schiz. pombe* (Boutry *et al.*, 1977) was competitively inhibited by divalent cations in the order Sr²⁺ > Mn²⁺ > Co²⁺ > Mg²⁺.

In conclusion, this study confirms the involvement of a Ca²⁺-calmodulin interaction in the yeast-mycelium transition of *O. ulmi* and presents the first detailed account of Ca²⁺ uptake by different cell types of this organism. Kinetic analysis of Ca²⁺ transport at concentrations <100 µM revealed that yeast cells and germ tubes possessed Ca²⁺ transport systems of similar specificity, although germ tubes always showed greater Ca²⁺ uptake than yeast cells under similar experimental conditions, possibly a consequence of differences in vacuolar volume. As mentioned previously, the vacuole is of significance as a Ca²⁺ store in fungi (Cornelius & Nakashima, 1987; Eilam & Chernichovsky, 1988; Miller *et al.*, 1990) and there is now increasing evidence for the existence of an inositol lipid signalling system, possibly dependent on vacuolar and/or influx-related elevation of [Ca²⁺]_i, in *O. ulmi* (Brunton & Gadd, 1991) and certain

other fungi (Trinci *et al.*, 1990; Robson *et al.*, 1991*b, c*). In view of the observation that elevation of [Ca²⁺]_i in the mating pheromone response pathway of *S. cerevisiae* is dependent on Ca²⁺ influx rather than release from internal stores (Iida *et al.*, 1990), further detailed research on the relationship between Ca²⁺ fluxes and signal transduction in dimorphic and filamentous fungi is highly desirable.

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