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 *Kₚ* 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; 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\(^{2+}\) ([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 [Ca\(^{2+}\)]\(_{c}\) at a low level. Although [Ca\(^{2+}\)]\(_{c}\) is maintained...
around 10⁻⁷ M in eukaryotic cells, the opening of ligand- or voltage-gated Ca²⁺ 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, [Ca²⁺], can increase to approximately 10⁻⁵ M. During such an increase, Ca²⁺ 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 Ca²⁺-dependent adenylate cyclase (Cheung et al., 1985) and cAMP phosphodiesterase, thereby providing a molecular link between the two second messengers, Ca²⁺ 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 Ca²⁺-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), 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 [Ca²⁺] ([Berridge & Irvine, 1984], in *O. ulmi* (Brunton & Gadd, 1991).

As well as [Ca²⁺], regulation by calcium-binding proteins, transport systems for Ca²⁺ located on the plasma and vacuolar membranes have a major role in [Ca²⁺]ₖ homeostasis in fungi (Miller et al., 1990; Iida et al., 1990). Ca²⁺ efflux from the cytosol may result from operation of a Ca²⁺/H⁺ antiport, the electrochemical proton gradient (Ãp was) being generated by the plasma membrane H⁺-ATPase (Eilam, 1982; Slayman et al., 1990) although recent work with *Neurospora crassa* has implied Ca²⁺ efflux by means of a H⁺/Ca²⁺-ATPase as in plant cells (Miller et al., 1990). In yeasts and fungi, vacuoles have a major function in maintaining cytoplasmic Ca²⁺ homeostasis and act as the main Ca²⁺-sequestering organelles (Eilam et al., 1985) transporting Ca²⁺ across the vacular membrane by Ca²⁺/H⁺ antiport (Ohsumi & Anraku, 1983; Okorokov et al., 1985; Cornelius & Nakashima, 1987; Miller et al., 1990). However, Ca²⁺ 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 Ca²⁺ 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 Ca²⁺-calmodulin interaction being involved in morphogenesis. The uptake of Ca²⁺ from low and high external concentrations during growth and germ tube formation is also described, including kinetic characteristics of Ca²⁺ transport, and the effects of Ca²⁺ 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, 200; proline, 1.15; KH₂PO₄ (anhydrous), 2.72; KH₂PO₄, 5H₂O, 5.22; MgSO₄, 7H₂O, 0.12; FeSO₄, 7H₂O, 0.0022; ZnSO₄, 7H₂O, 0.004; MnSO₄, 4H₂O, 0.004; CuSO₄, 5H₂O, 0.0004 and supplemented with the following vitamins (μg 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 cycles min⁻¹). For experiments, 100 ml medium was inoculated from late stationary-phase cultures to an initial cell density of approximately 2 x 10⁶ 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 A23 187 and the calcium-binding agents methylhydroxybenzoate (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 ~10⁷ 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 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.

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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. 45CaCl2 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 Ca2+ 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-CaCl2, with approximately 22% of the population showing germ tube formation after 28 h (Table 1). At concentrations above 1 mM-CaCl2 some inhibition of this response was observed (Table 1). CaCl2 concentrations greater than 5 mM resulted in precipitation of Ca3(PO4)2 in the medium. In Ca2+-unsupplemented medium, growth of O. ulmi was exclusively yeast-like.

Effect of the calmodulin inhibitors R24571 and TFP, and the Ca2+ ionophore A23187, on germ tube formation by O. ulmi

Calmodulin inhibitors and the Ca2+ 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, 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 CaCl2, 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 CaCl2 in the presence of yeast extract, which effects a complete yeast–mycelium transition in O. ulmi. Concentrations used were: (a) O, 0; ●, 3 μM; △, 3 μM plus 10 mM-CaCl2; (b) O, 0; ●, 20 μM; △, 20 μM plus 10 mM-CaCl2; (c) O, 0; ●, 15 μM; △, 15 μM plus 10 mM-CaCl2. 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 CaCl2 on germ tube formation by O. ulmi in defined liquid medium

<table>
<thead>
<tr>
<th>[Ca2+] (mM)</th>
<th>Germ tube formation (%)</th>
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<tbody>
<tr>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>0.1</td>
<td>13.4 ± 0.6</td>
</tr>
<tr>
<td>0.5</td>
<td>19.2 ± 0.8</td>
</tr>
<tr>
<td>1.0</td>
<td>21.8 ± 1.2</td>
</tr>
<tr>
<td>2.0</td>
<td>20.0 ± 1.0</td>
</tr>
<tr>
<td>5.0</td>
<td>16.8 ± 0.8</td>
</tr>
</tbody>
</table>

Fig. 1. Effect of excess exogenous CaCl2 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) O, 0; ●, 3 μM; △, 3 μM plus 10 mM-CaCl2; (b) O, 0; ●, 20 μM; △, 20 μM plus 10 mM-CaCl2; (c) O, 0; ●, 15 μM; △, 15 μM plus 10 mM-CaCl2. The bars in this and succeeding Figures indicate the standard error of the mean (SEM) of individual points (three replicates).
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 mycelium formation was still evident, but above this concentration only germ tubes were produced.

**Ca$^{2+}$ uptake by different cell developmental stages and the effect of metabolic inhibitors, calmodulin inhibitors and Ca$^{2+}$ ionophore A23187**

Uptake of Ca$^{2+}$ was examined in yeast cells and germ tubes, as induced by 1% (w/v) yeast extract, harvested
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Fig. 4. Uptake of Ca²⁺ by different-aged yeast cells of O. ulmi from 10 μM-CaCl₂ 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 μM-KCN (△).

Table 2. Inhibition of Ca²⁺ uptake in yeast cells and germ tubes of O. ulmi by DCCD, DES and DNP

<table>
<thead>
<tr>
<th>Inhibition of Ca²⁺ uptake (%)</th>
<th>Yeast cells</th>
<th>Germ tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>100 μM-DCCD</td>
<td>20.5 ± 2.6</td>
<td>40.1 ± 2.7</td>
</tr>
<tr>
<td>100 μM-DES</td>
<td>24.4 ± 3.2</td>
<td>42.4 ± 3.4</td>
</tr>
<tr>
<td>50 μM-DNP</td>
<td>37.8 ± 1.9</td>
<td>76.9 ± 2.7</td>
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</table>

after different times. In both cell types, the uptake of calcium from 10 μM-CaCl₂ showed a similar pattern, with increased Ca²⁺ uptake being exhibited by 12 h cells as compared with those harvested at 4, 8 or 16 h incubation (Figs 4 and 5). Reduced Ca²⁺ uptake was observed in the absence of glucose and in the presence of KCN, indicating that Ca²⁺ uptake was metabolism-dependent although the relative sensitivity of Ca²⁺ uptake to these conditions varied between cell types and between different cell ages (Figs 4 and 5). Ca²⁺ uptake by yeast cells and germ tubes was also inhibited by DCCD and DES, which indicated that Ca²⁺ uptake was dependent on plasma membrane ATPase activity (Table 2). The inhibition of Ca²⁺ 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).
Uptake of Ca\(^{2+}\) by germ tubes (induced by 1\% (w/v) yeast extract) from 10 \(\mu\)M-CaCl\(_2\) was partially inhibited when the cells were pre-incubated with 3 \(\mu\)M-R24571 or 15 \(\mu\)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 \(\mu\)M) in the presence of glucose. Rates of Ca\(^{2+}\) uptake were derived by linear regression on experimental points taken 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 \(\mu\)M, but deviated strongly above this concentration (Figs 7 and 8). Using Ca\(^{2+}\) uptake values obtained for CaCl\(_2\) concentrations less than 100 \(\mu\)M, the \(K_m\) and \(V_{max}\) values of Ca\(^{2+}\) uptake by 4 h yeast cells were calculated to be 19.2 \(\mu\)M and 9.1 \(\times\) 10\(^{-4}\) nmol min\(^{-1}\) (mg dry wt\(^{-1}\)) respectively. For 12 h yeast cells, the \(K_m\) value was 14.6 \(\mu\)M and the \(V_{max}\) value was 5.4 \(\times\) 10\(^{-3}\) nmol min\(^{-1}\) (mg dry wt\(^{-1}\)). The \(K_m\) and \(V_{max}\) values of Ca\(^{2+}\) uptake by 4 h germ tubes were 25.3 \(\mu\)M and 8.3 \(\times\) 10\(^{-4}\) nmol min\(^{-1}\) (mg dry wt\(^{-1}\)) respectively; 12 h germ tubes gave \(K_m\) and \(V_{max}\) values of 20.5 \(\mu\)M and 1.44 \(\times\) 10\(^{-2}\) nmol min\(^{-1}\) (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,
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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.

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 \((0.45 \pm 0.02) \times 10^{-2}\) nmol min\(^{-1}\) (mg dry wt\(^{-1}\)). ○, MgCl\(_2\); ●, MnCl\(_2\); △, ZnCl\(_2\).

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 \(V_{\text{max}}\), resulting in an increase in the \(K_m\). In the presence of the inhibitory divalent cations, the \(V_{\text{max}}\) remained constant at approximately \(1.25 \times 10^{-2}\) nmol min\(^{-1}\) (mg dry wt\(^{-1}\)). In the presence of 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-cell 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 specific (Borst-Pauwels & Theuvenet, 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 \(V_{\text{max}}\) 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 \(V_{\text{max}}\) were
1.8 mM and 8.9 nmol min⁻¹ (mg dry wt)⁻¹ respectively in *P. cyclopium*.

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 *Kₘ* but did not cause a change in the *Vₘₚ*. This was indicative of the germ tubes showing a decreased affinity for Ca²⁺ in the presence of the inhibitory cations. However, the unaltered *Vₘₚ* 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⁺]ₙ in *O. ulmi* (Brunton & Gadd, 1991) and certain other fungi (Trinci et al., 1990; Robson et al., 1991 a, c). In view of the observation that elevation of [Ca²⁺]ₙ 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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### References


Calcium involvement in Ophiostoma ulmi dimorphism


