Ca\textsuperscript{2+}-ATPase-driven calcium accumulation in \textit{Ustilago maydis} plasma membrane vesicles

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INTRODUCTION

Calcium is well known as a second messenger in eukaryotic cells. Responses to various signals result in its release into the cytoplasm from either intracellular stores (e.g. sarcoplasmic reticulum) or via Ca\textsuperscript{2+} channels at the plasma membrane (Berridge, 1993). However, in resting cells it is necessary to maintain low cytoplasmic Ca\textsuperscript{2+} concentrations in the range 0.05–1 \textmu M, against extracellular concentrations of 1 mM. In plant cells, this is achieved by intracellular Ca\textsuperscript{2+} pumps located in different organelles. For example, there are P-type Ca\textsuperscript{2+}-ATPases in the plasma membrane (Carnelli \textit{et al.}, 1992) and in the endoplasmic reticulum (Wimmers \textit{et al.}, 1992). Although this method of regulating intracellular Ca\textsuperscript{2+} seems to be reasonably well established for animals and plants, evidence for such a system in fungal cells has not yet been found. To date, there has only been a partial characterization of the respective proteins involved in the fungal plasma membrane and no clear evidence has emerged as to their nature (Stroobant & Scarborough, 1979; Giannini \textit{et al.}, 1988). In yeast, recent studies with membrane vesicles have demonstrated the presence of Ca\textsuperscript{2+} pumps in different intracellular membranes (Okorokov \textit{et al.}, 1993; Halachmi \textit{et al.}, 1992). Physiological studies have postulated the necessity for a Ca\textsuperscript{2+}-ATPase in fungal plasma membranes (Miller \textit{et al.}, 1990), although, so far, no such pump has been characterized in isolated fungal plasma membrane vesicles.

In this work, we present evidence for a primary Ca\textsuperscript{2+}-ATPase in \textit{Ustilago maydis} plasma membrane vesicles, which is MgATP- or MgGTP-dependent. We have also examined the effects of Ca\textsuperscript{2+}-channel blockers and erythrosin B on Ca\textsuperscript{2+} transport.

METHODS

Strain and culture. \textit{Ustilago maydis} (IMI 103761) was maintained in frozen aliquots with 9 \%(v/v) DMSO at −70 °C. Liquid cultures were inoculated with 80 mg (fresh weight) of cells and cultured for 48 h in minimal medium on a rotary shaker at 25 °C (Hargreaves & Turner, 1992).

Plasma membrane purification. Sporidia were harvested at the exponential growth phase by centrifuging at 6000 \texttimes g for 10 min (typical harvest 35 g), mixed with 50 ml homogenization buffer [50 mM HEPES to pH 7.5 with KOH, 330 mM sucrose, 5 mM EDTA, 5 mM EGTA, 0.2% BSA, 0.2% casein hydrolysate, 1 mM PMSF, 2\%(w/v) choline and 5 mM DTT] and 75 g glass beads (0.125 mm diam.). Cells were homogenized in a ‘Bead-Beater’ (Biospect Products), the homogenate filtered through nylon cloth (240 \mu m) and centrifuged at 10000 \texttimes g for 15 min. The pellet (unbroken cells, cell debris and intact mitochondria) was discarded and the supernatant centrifuged at 100000 \texttimes g for 30 min to produce a microsomal pellet, which was resuspended in 5 mM potassium phosphate buffer, pH 7.8, and 330 mM sucrose. Plasma membranes were isolated and purified using the two-phase aqueous polymer technique, as described by Larsson \textit{et al.} (1987) with the following modifications. The microsomal

\textbf{Abbreviations:} ACMA, 9-amino-6-chloro-2-methoxyacridine; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; BTP, 1,3-bis[tris(hydroxymethyl)amino]propane; CCCP, carbonyl cyanide m-chlorophenylhydrazone.
Table 1. Specific activities of markers for the microsomal and plasma membrane fractions from U. maydis obtained by two-phase partitioning

<table>
<thead>
<tr>
<th>Marker Enzyme activity</th>
<th>Microsomes</th>
<th>Plasma membranes</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>[μmol min⁻¹ (mg protein)⁻¹]</td>
<td></td>
</tr>
<tr>
<td>ATPase (pH 6.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.166 ± 0.009</td>
<td>0.426 ± 0.043</td>
</tr>
<tr>
<td>+ Vanadate</td>
<td>0.080 ± 0.007</td>
<td>0.169 ± 0.013</td>
</tr>
<tr>
<td>Latency*</td>
<td>(13.6)*</td>
<td>(4.6)*</td>
</tr>
<tr>
<td>Glucan synthetase</td>
<td>0.172 ± 10⁻³± 0.020 ± 10⁻³</td>
<td>1.643 ± 10⁻⁹± 0.045 ± 10⁻⁹</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>1.052 ± 0.002</td>
<td>0.282 ± 0.011</td>
</tr>
<tr>
<td>NADH-cytochrome c oxidoreductase</td>
<td>0.107 ± 0.001</td>
<td>0.128 ± 0.000</td>
</tr>
<tr>
<td>ATPase (pH 7.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.051 ± 0.006</td>
<td>0.168 ± 0.030</td>
</tr>
<tr>
<td>+ Nitrate</td>
<td>0.084 ± 0.000</td>
<td>0.155 ± 0.012</td>
</tr>
</tbody>
</table>

* Values given as a percentage of right-side-out vesicles.
Calcium accumulation in *Ustilago maydis*

**Fig. 1.** Net Ca\(^{2+}\) transport across *U. maydis* plasma membrane and microsomal membrane vesicles, measured in the presence of ATP. Blank values, obtained in the absence of ATP, have been subtracted. Triton X-100 (0.008 %) was added to both experiments at 20 min. △ Microsomal membranes; ◊ plasma membranes: 10 μM Ca\(^{2+}\) added to give 2.5 μM free Ca\(^{2+}\). Results are the means of three independent experiments; least significant difference between mean values = 1.06 at 95% level of confidence.

**Fig. 2.** Net Ca\(^{2+}\) transport across *U. maydis* plasma membrane vesicles, measured in the presence of ATP, GTP or ATP+erythrosin-B (15 μM). Blank values, obtained in the absence of ATP, have been subtracted. Triton X-100 (0.008 %) was added to all experiments at 20 min. □ ATP; △ GTP; ◊ erythrosin-B (15 μM): 10 μM Ca\(^{2+}\) added to give 2.5 μM free Ca\(^{2+}\). Results are the means of three independent experiments; least significant difference between mean values = 0.80 at 95% level of confidence.

former fraction. The \(^{45}\)Ca\(^{2+}\) accumulated over a 20 min period could be released from plasma membrane vesicles by treating them with Triton X-100 (0.008 %). In contrast, with microsomes, there was no release of Ca\(^{2+}\) by Triton X-100, which may be indicative of non-specific binding rather than true transport (Fig. 1). Increasing concentrations of Triton X-100 (0.008, 0.016 and 0.032 %) showed there was a concentration-dependent release of Ca\(^{2+}\) from the plasma membrane vesicles (51, 57 and 73 % of total uptake, respectively). With the addition of A23187, no consistent release was observed; at 2.5 μM, no Ca\(^{2+}\) was released from the vesicles. However, with 5, 10 and 20 μM, there was an 81, 91 and 83 % release of total uptake, respectively. Therefore, because it gave a more consistent release of Ca\(^{2+}\), it was decided to use Triton X-100 at the lowest concentration, to avoid the possibility of destroying the membrane vesicles.

In plasma membrane vesicles, when GTP was used instead of ATP, Ca\(^{2+}\) transport activity was about 25 % of the ATP-driven accumulation (Fig. 2). Addition of erythrosin-B (15 μM) to the assay mixture, in the presence of ATP, resulted in about 50 % inhibition of transport (Fig. 2). Additions of increasing amounts of erythrosin-B to the assays showed that inhibition was concentration-dependent with an \(I_{50}\) of 16.1 μM (Fig. 3).

To determine whether Ca\(^{2+}\) transport depended on the
Fig. 5. Net Ca²⁺ transport across U. maydis plasma membrane vesicles, measured in the presence of ATP+diltiazem (100 μM), nifedipine (100 μM) or verapamil (100 μM). Blank values, obtained in the absence of ATP, have been subtracted. Triton X-100 (0.008%) was added to both experiments at 20 min. ATP+diltiazem (100 μM); O, ATP+verapamil (100 μM); △, ATP+nifedipine (100 μM); 10 M Ca²⁺ added to give 2.5 μM free Ca²⁺. Results are the means of three independent experiments; least significant difference between mean values = 0.93 at 95% level of confidence.

Fig. 6. Dissipation by verapamil (0.1 mM) of the proton gradient, measured as fluorescence quenching of ACMA. The reaction (at pH 6.5 with 50 μg protein ml⁻¹) was done in the presence of ATP and started by adding 20 μl MgCl₂ (0.5 M). The reaction was finally stopped with 5 μl gramicidin (2 mg ml⁻¹).

proton gradient generated by the H⁺-ATPase, assays were done in the presence of CCCP and gramicidin. Neither of these treatments eliminated ATP-dependent Ca²⁺ transport, although it was about 20% less than in the control (Fig. 4). The addition of sodium orthovanadate (50 μM) resulted in a greater than 50% inhibition of ATP-dependent Ca²⁺ transport, indicating the presence of a P-type ATPase (Fig. 4).

None of the known Ca²⁺-channel blockers had any significant effect on Ca²⁺ transport into plasma membrane vesicles (Fig. 5). However, in our experiments, verapamil dissipated the H⁺-ATPase proton gradient across plasma membrane vesicles at the concentration used to block Ca²⁺ channels (Fig. 6). Addition of verapamil to the assay mixture, in the absence of ATP, or vesicles, had no effect on ACMA fluorescence (data not shown). Neither diltiazem nor nifedipine had any effect on the proton gradient (data not shown).

**DISCUSSION**

The increased level of Ca²⁺ transport in plasma-membrane-enriched vesicles of U. maydis with respect to the microsomes indicated that this activity was plasma-membrane-bound (Fig. 1). Accumulated Ca²⁺ was released by small concentrations of Triton X-100, which was an indication of true transport against a concentration gradient (Tada et al., 1988). With A23187 (2.5 μM), no release of Ca²⁺ occurred (data not shown). This is consistent with results from experiments with Penicillium notatum, in which Ca²⁺ was not released by A23187 from any sub-cellular fraction, including plasma membrane vesicles, although with the exception of mitochondria where a rapid release was observed (Pitt & Barnes, 1993). However, at higher concentrations of A23187 (5–20 μM), Ca²⁺ was released, but unlike with Triton X-100, this was not concentration-dependent.

Although physiological studies have pointed to the necessity for a Ca²⁺-ATPase in the fungal plasma membrane (Millet et al., 1990), previous studies using vesicles have suggested that Ca²⁺ extrusion at the plasma membrane is achieved by a H⁺/Ca²⁺ antiport (Scarborough, 1989; Giannini et al., 1988). However, our results indicated the presence of a P-type Ca²⁺-ATPase which is erythrosin-B-sensitive (I₅₀ 16±1 μM), vanadate-sensitive (I₅₀ 19±4 μM) and MgATP/GTP-dependent (Figs 2, 3 and 4), criteria described as essential characteristics of plant plasma membrane Ca²⁺-ATPases (Williams et al., 1990; Ramón et al., 1993). Furthermore, the addition of the protonophores CCCP or gramicidin to the assay medium, reduced Ca²⁺ transport only to a slight extent and verapamil had no effect at all (Figs 4 and 5). This suggests that Ca²⁺ transport was not strictly dependent on the proton gradient generated by the H⁺-ATPase.

Verapamil is widely used as a Ca²⁺-channel blocker. However, experiments with fungi, in which verapamil was used, have often given contradictory results (Frazer & Moore, 1993; Jackson & Heath, 1993). Our results indicated that verapamil dissipated the proton gradient generated by the primary proton pump (Fig. 6) which may explain some of these anomalies. This result is in agreement with the observation made by Clarkson et al. (1988) that verapamil transiently depolarized the membrane potential of root hair cells.

Thus, we can conclude that in U. maydis plasma membranes, as in plants and animals, there is a primary Ca²⁺-transporting ATPase, and that Ca²⁺ transport is not strongly dependent on the existence of a proton gradient across the plasma membrane. The operation of this Ca²⁺ pump depends on MgATP although it can use MgGTP with lesser efficiency. It is sensitive to erythrosin-B and vanadate and, thus, resembles, in most important respects, the P-type Ca²⁺-ATPases of plant plasma membranes (Evans et al., 1991; Ramón et al., 1993).
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REFERENCES


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