Ca²⁺-ATPase-driven calcium accumulation in *Ustilago maydis* plasma membrane vesicles

A. Hernández, D. T. Cooke and D. T. Clarkson

Author for correspondence: D. T. Cooke.

Department of Agricultural Sciences, University of Bristol, AFRC Institute of Arable Crops Research, Long Ashton Research Station, Bristol BS18 9AF, UK

Ca²⁺ transport has been measured across plasma membrane vesicles isolated from cells of *Ustilago maydis*. This transport was found to be ATP- (or to a lesser extent GTP) and Mg²⁺-dependent. Inconsistent release of Ca²⁺ from intact vesicles was obtained using the calcium ionophore A23187. However, Ca²⁺ was released by Triton X-100 in a concentration-dependent manner. Transport was inhibited by vanadate (> 50%) and erythrosin B (about 50%), *I*₅₀ being about 10 µM for both inhibitors. In the presence of the protonophores CCCP or gramicidin, partial inhibition of Ca²⁺ transport (about 20%) was observed, but the Ca²⁺-channel blockers, nifedipine, diltiazem and verapamil had no effect, although the latter inhibited proton transport. The results indicate that Ca²⁺ transport in *U. maydis* is regulated by a P-type ATPase with similar properties to that found in higher plants.

Keywords: *Ustilago maydis*, calcium transport, calcium-ATPase, plasma membrane

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. sarco/endoplasmic reticulum) or via Ca²⁺ channels at the plasma membrane (Berridge, 1993). However, in resting cells it is necessary to maintain low cytoplasmic Ca²⁺ concentrations in the range 0.05–1 µM, against extracellular concentrations of 1 mM. In plant cells, this is achieved by intracellular Ca²⁺ pumps located in different organelles. For example, there are P-type Ca²⁺-ATPases in the plasma membrane (Carnelli *et al.*, 1992) and in the endoplasmic reticulum (Wimmers *et al.*, 1992). Although this method of regulating intracellular Ca²⁺ 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 *et al.*, 1988). In yeast, recent studies with membrane vesicles have demonstrated the presence of Ca²⁺ pumps in different intracellular membranes (Okorokov *et al.*, 1993; Halachmi *et al.*, 1992). Physiological studies have postulated the necessity for a Ca²⁺-ATPase in fungal plasma membranes

(Miller *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 Ca²⁺-ATPase in *Ustilago maydis* plasma membrane vesicles, which is MgATP- or MgGTP-dependent. We have also examined the effects of Ca²⁺-channel blockers and erythrosin B on Ca²⁺ transport.

METHODS

Strain and culture. *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 *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 µm) and centrifuged at 10000 *g* for 15 min. The pellet (unbroken cells, cell debris and intact mitochondria) was discarded and the supernatant centrifuged at 100000 *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 *et al.* (1987) with the following modifications. The microsomal

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)methylamino]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

Microsomes contained 0.67 mg protein, the plasma membrane fraction 390.4 mg. Data are from a representative experiment $\pm$ sd.

Marker	Enzyme activity [$\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$]	
	Microsomes	Plasma membranes
ATPase (pH 6.7)		
Control	0.166 $\pm$ 0.009	0.426 $\pm$ 0.043
+ Vanadate	0.080 $\pm$ 0.007	0.169 $\pm$ 0.013
Latency*	(13.6)*	(4.6)*
Glucan synthetase	0.172 $\times 10^{-3} \pm 0.020 \times 10^{-3}$	1.643 $\times 10^{-3} \pm 0.045 \times 10^{-3}$
Cytochrome <i>c</i> oxidase	1.052 $\pm$ 0.092	0.282 $\pm$ 0.011
NADH-cytochrome <i>c</i> oxidoreductase	0.107 $\pm$ 0.001	0.128 $\pm$ 0.000
ATPase (pH 7.5)		
Control	0.051 $\pm$ 0.006	0.168 $\pm$ 0.030
+ Nitrate	0.084 $\pm$ 0.000	0.155 $\pm$ 0.012

* Values given as a percentage of right-side-out vesicles.

fraction (200–250 mg protein) was loaded on to six 20 g two-phase systems (typical load 30 mg per system) with the following final composition: 7.0% (w/w) dextran T500, 7.0% (w/w) PEG, 5 mM potassium phosphate buffer, pH 7.8, 330 mM sucrose and 3 mM KCl. After centrifuging at 3000 *g* for 3 min, the top phase was removed, diluted three times with dilution medium (DM; 10 mM MOPS/BTP, pH 7.5, 330 mM sucrose) and centrifuged at 100000 *g* for 30 min. The resulting pellet, which represented the plasma membrane fraction, was re-suspended in DM and stored at -20°C for no longer than 24 h before use.

Measurement of proton-pumping in PM vesicles. The generation of a MgATP-dependent ΔpH was assayed by monitoring the change in fluorescence emission of the fluorescent probe 9-amino-6-chloro-2-methoxyacridine (ACMA) (Molecular Probes), as described in Coupland *et al.* (1991). Vesicle suspension (about 250 μl , enough to contain at least 100 μg of protein), was added to a cuvette containing 20 mM BTP/MES (pH 6.5), 5 mM ATP, 25 mM KNO_3 , 2 μM ACMA and water to 1.980 ml. The cuvette was placed in a luminescence spectrophotometer and maintained at 30°C with constant stirring. The reaction was started by the addition of 20 μl 0.5 M MgCl_2 and the change in fluorescence emission was measured at 485 nm, with excitation at 415 nm.

Measurement of Ca^{2+} uptake. Ca^{2+} transport was measured by a membrane filtration technique (Ramón *et al.*, 1993). Plasma membrane vesicles were incubated as described above in the proton-pumping assay at pH 7.5, which is within the optimal range for the Ca^{2+} pump and for Ca^{2+} transport determinations (Evans *et al.*, 1991). When the proton gradient reached a steady-state, Ca^{2+} transport measurements were initiated by the addition of $^{45}\text{Ca}^{2+}$ (500 kBq $^{45}\text{CaCl}_2$) in 0.1–10 mM CaCl_2 to achieve 1–100 μM total Ca^{2+} in a volume of 2 ml. Normally, 1 mM CaCl_2 (20 μl) was added to give 10 μM total Ca^{2+} , calculated to be equal to a free Ca^{2+} concentration of 2.5 μM , in the presence of MgATP (5 mM). Samples of 50 μl were taken at intervals of 5 min, filtered through pre-washed cellulose nitrate filters (Whatman, 0.45 nm pore size) and washed three times

with 1 ml buffer [20 mM BTP/MES, pH 7.1, 1 mM 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA)]. Ca^{2+} transport was confirmed by its release from the vesicles with Triton X-100 (0.008% final concentration) after 20 min. Ca^{2+} release was also examined with greater concentrations of Triton X-100 (0.016 and 0.032%) and with the calcium ionophore A23187 in increasing concentrations (2.5–20 μM). The filters were dried, suspended in 3 ml scintillant and the radioactivity determined. Blanks were run in the absence of ATP. Ca^{2+} transport was also measured with GTP in place of ATP, the Ca^{2+} -ATPase inhibitor erythrosin-B (10–100 μM), the P-type ATPase inhibitor sodium orthovanadate (10–400 μM), the protonophores CCCP (2.5 μM) and gramicidin (3 μM), and the Ca^{2+} -channel blockers verapamil (100 μM), diltiazem (100 μM) and nifedipine (100 μM).

Determination of protein. Protein concentration was determined by the method of Bradford (1976) using Bio-Rad reagent and thyroglobulin as the standard.

Determination of free Ca^{2+} content. The concentration of free Ca^{2+} in solution was evaluated by using a computer program (SOLCON) developed by Dr D. S. White (University of York, York, UK) and Dr Y. E. Goldman (University of Pennsylvania, Philadelphia, PA, USA). This program uses published association constants for all components of the reaction media, adjusted to the ionic strength and temperature of the assay (Martell & Smith, 1982; Dawson *et al.*, 1986).

RESULTS

Aqueous two-phase partitioning in *U. maydis* produced preparations of highly enriched plasma membrane vesicles (at least fivefold, over microsomes), as determined from enzyme marker assays, and which were mainly (about 90%) inside-out (Table 1). Transport experiments, over a 20 min time-course, showed apparent Ca^{2+} accumulation in both plasma membrane vesicles and in microsomes (Fig. 1), although it was more than fivefold greater in the

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. Δ , Microsomal membranes; $\square$, plasma membranes: $10\text{ }\mu\text{M}$ Ca^{2+} added to give $2.5\text{ }\mu\text{M}$ 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 ($15\text{ }\mu\text{M}$). Blank values, obtained in the absence of ATP, have been subtracted. Triton X-100 (0.008%) was added to all experiments at 20 min. $\square$, ATP; Δ , GTP; $\circ$, erythrosin-B ($15\text{ }\mu\text{M}$): $10\text{ }\mu\text{M}$ Ca^{2+} added to give $2.5\text{ }\mu\text{M}$ 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 $^{45}\text{Ca}^{2+}$ 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 $2.5\text{ }\mu\text{M}$, no Ca^{2+} was released from the vesicles. However, with 5, 10 and $20\text{ }\mu\text{M}$, there was an 81, 91 and 83% release of total

Fig. 3. Effects of increasing concentrations of erythrosin-B and sodium orthovanadate on Ca^{2+} transport across *U. maydis* plasma membrane vesicles, measured in the presence of ATP. Blank values, obtained in the absence of ATP, have been subtracted. $\square$, erythrosin-B ($10\text{--}100\text{ }\mu\text{M}$); Δ , sodium orthovanadate ($25\text{--}400\text{ }\mu\text{M}$).

Fig. 4. Net Ca^{2+} transport across *U. maydis* plasma membrane vesicles, measured in the presence of ATP+CCCP ($2.5\text{ }\mu\text{M}$), gramicidin ($3\text{ }\mu\text{M}$) or sodium orthovanadate ($50\text{ }\mu\text{M}$). Blank values, obtained in the absence of ATP, have been subtracted. Triton X-100 (0.008%) was added to both experiments at 20 min. $\square$, ATP+gramicidin ($3\text{ }\mu\text{M}$); Δ , ATP+CCCP ($2.5\text{ }\mu\text{M}$); $\circ$, ATP+sodium orthovanadate ($50\text{ }\mu\text{M}$): $10\text{ }\mu\text{M}$ Ca^{2+} added to give $2.5\text{ }\mu\text{M}$ free Ca^{2+} . Results are the means of three independent experiments; least significant difference between mean values = 0.56 at 95% level of confidence.

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 ($15\text{ }\mu\text{M}$) 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 $16.1\text{ }\mu\text{M}$ (Fig. 3).

To determine whether Ca^{2+} transport depended on the

Fig. 5. Net Ca^{2+} 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. $\square$, ATP+diltiazem (100 μM); $\circ$, ATP+verapamil (100 μM); $\triangle$, ATP+nifedapine (100 μM); 10 M 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 = 0.93 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 MgCl_2 (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 Ca^{2+} 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 Ca^{2+} transport, indicating the presence of a P-type ATPase (Fig. 4).

None of the known Ca^{2+} -channel blockers had any significant effect on Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} occurred (data not shown). This is consistent with results from experiments with *Penicillium notatum*, in which Ca^{2+} 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), Ca^{2+} was released, but unlike with Triton X-100, this was not concentration-dependent.

Although physiological studies have pointed to the necessity for a Ca^{2+} -ATPase in the fungal plasma membrane (Miller *et al.*, 1990), previous studies using vesicles have suggested that Ca^{2+} extrusion at the plasma membrane is achieved by a $\text{H}^+/\text{Ca}^{2+}$ antiport (Scarborough, 1989; Giannini *et al.*, 1988). However, our results indicated the presence of a P-type Ca^{2+} -ATPase which is erythrosin-B-sensitive (I_{50} , 16.1 μM), vanadate-sensitive (I_{50} , 19.4 μM) and MgATP/GTP-dependent (Figs 2, 3 and 4), criteria described as essential characteristics of plant plasma membrane Ca^{2+} -ATPases (Williams *et al.*, 1990; Ramón *et al.*, 1993). Furthermore, the addition of the protonophores CCCP or gramicidin to the assay medium, reduced Ca^{2+} transport only to a slight extent and verapamil had no effect at all (Figs 4 and 5). This suggests that Ca^{2+} transport was not strictly dependent on the proton gradient generated by the H^+ -ATPase.

Verapamil is widely used as a Ca^{2+} -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 Ca^{2+} -transporting ATPase, and that Ca^{2+} transport is not strongly dependent on the existence of a proton gradient across the plasma membrane. The operation of this Ca^{2+} 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 Ca^{2+} -ATPases of plant plasma membranes (Evans *et al.*, 1991; Ramón *et al.*, 1993).

ACKNOWLEDGEMENTS

We wish to thank Dr D. E. Evans for helpful discussions. A. H. is the beneficiary of a grant 'Formación de Investigadores' from Gobierno Vasco, Spain.

REFERENCES

- Berridge, M. J. (1993).** Inositol trisphosphate and calcium signalling. *Nature* **361**, 315–325.
- Bradford, M. M. (1976).** A rapid and sensitive method for the quantitation of submicrogram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Carnelli, A., De Michelis, M. I. & Rasi-Caldogno, F. (1992).** Plasma membrane Ca-ATPase of radish seedlings. 1. Biochemical characterization using ITP as substrate. *Plant Physiol* **102**, 651–661.
- Clarkson, D. T., Brownlee, C. & Ayling, S. M. (1988).** Cytoplasmic calcium measurements in intact higher plant cells: results from fluorescence ratio imaging of fura-2. *J Cell Sci* **91**, 71–80.
- Coupland, D., Cooke, D. T. & James, C. S. (1991).** Effects of 4-chloro-2-methylphenoxypropionate (an auxin analogue) on plasma membrane ATPase activity in herbicide-resistant and herbicide-susceptible biotypes of *Stellaria media* L. *J Exp Bot* **42**, 1065–1071.
- Dawson, R. M. C., Elliot, W. H. & Jones, K. M. (1986).** *Data for Biochemical Research*. Oxford: Oxford Science Publications.
- Evans, D. E., Briars, S. A. & Williams, L. E. (1991).** Active calcium transport by plant cell membranes. *J Exp Bot* **42**, 285–303.
- Frazer, L. N. & Moore, D. (1993).** Antagonists and inhibitors of calcium accumulation do not impair gravity perception though they adversely affect the gravitropic responses of *Coprinus cinereus* stipes. *Mycol Res* **97**, 113–118.
- Giannini, J. A., Holt, J. S. & Briskin, D. P. (1988).** Isolation of sealed plasma membranes from *Phytophthora megasperma* f.sp. glycinea: II. Partial characterization of Ca²⁺ transport and glyceollin effects. *Arch Biochem Biophys* **266**, 644–649.
- Halachmi, D., Ghislain, M. & Eilam, Y. (1992).** An intracellular ATP-dependent calcium pump within the yeast *Schizosaccharomyces pombe*, encoded by the gene *cta3*. *Eur J Biochem* **207**, 1003–1008.
- Hargreaves, J. A. & Turner, G. (1992).** Gene transformation in plant pathogenic fungi. In *Molecular Plant Pathology – A Practical Approach*, Vol. 1, pp. 79–96. Edited by S. J. Gurr, M. J. McPherson & D. J. Bowles. Oxford and Washington D.C.: IRL Press.
- Jackson, S. L. & Heath, I. B. (1993).** Roles of calcium in hyphal tip growth. *Microbiol Rev* **57**, 367–382.
- Larsson, C., Widell, S. & Kjellbom, P. (1987).** Preparation of high purity plasma membranes. *Methods Enzymol* **148**, 558–568.
- Martell, A. E. & Smith, R. M. (1982).** *Critical Stability Constants*, Vol. 5, First Supplement. New York: Plenum Press.
- Miller, A. J., Vogg, G. & Sanders, D. (1990).** Cytosolic calcium homeostasis in fungi: dual roles of plasma membrane transport and intracellular sequestration of calcium. *Proc Natl Acad Sci USA* **87**, 9348–9352.
- Okorokov, L. A., Tanner, W. & Lehle, L. (1993).** A novel primary Ca²⁺-transport system from *Saccharomyces cerevisiae*. *Eur J Biochem* **216**, 573–577.
- Pitt, D. & Barnes, J. C. (1993).** Calcium homeostasis, signalling and protein phosphorylation during calcium-induced conidiation in *Penicillium notatum*. *J Gen Microbiol* **139**, 3053–3063.
- Ramón, A. M., Cooke, D. T. & Clarkson, D. T. (1993).** Calcium transport across plasma membrane vesicles isolated from shoots of *Stellaria media* and *Avena sativa*. *Physiol Plant* **89**, 731–740.
- Scarborough, G. A. (1989).** Isolation of everted plasma membrane vesicles from *Neurospora crassa* and measurement of transport function. *Methods Enzymol* **174**, 667–676.
- Stroobant, P. & Scarborough, G. A. (1979).** Active transport of calcium in *Neurospora* plasma membranes. *Proc Natl Acad Sci USA* **76**, 3102–3106.
- Tada, M., Kadoma, M., Inui, M. & Fujii, J. I. (1988).** Regulation of Ca²⁺-pump from cardiac sarcoplasmic reticulum. *Methods Enzymol* **157**, 109.
- Williams, L. E., Schueler, S. B. & Briskin, D. P. (1990).** Further characterization of the red beet plasma membrane Ca²⁺-ATPase using GTP as an alternative substrate. *Plant Physiol* **92**, 747–754.
- Wimmers, L. E., Ewing, N. N. & Bennett, A. B. (1992).** Higher plant Ca²⁺-ATPase: primary structure and regulation of mRNA abundance by salt. *Proc Natl Acad Sci USA* **89**, 9205–9209.

Received 6 May 1994; revised 12 July 1994; accepted 25 July 1994.