

## Characterization of a $\text{Ca}^{2+}$ -translocating ATPase from corn root microsomes

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Brauer, D., Schubert C. and Tu, S.-I. 1990. Characterization of a  $\text{Ca}^{2+}$ -translocating ATPase from corn root microsomes. - *Physiol. Plant.* 78: 335-344.

The existence of a  $\text{Ca}^{2+}$ -translocating ATPase in microsomes from maize (*Zea mays* L. cv. WF9 × Mo17) roots was evaluated using assays to follow  $\text{Ca}^{2+}$ -stimulation of ATP hydrolysis and  $\text{Ca}^{2+}$  transport by changes in the fluorescence of chlorotetracycline. ATP hydrolysis by microsomes was stimulated by the addition of  $\text{Ca}^{2+}$  and further enhanced by the Ca ionophore A23187 and bovine brain calmodulin only in the presence of  $\text{Ca}^{2+}$ . Stimulation by these agents was additive and sensitive to vanadate. These results were consistent with the presence of a  $\text{Ca}^{2+}$ -translocating ATPase in microsomal membranes. The fluorescence of chlorotetracycline in the presence of microsomes and  $\text{Ca}^{2+}$  increased upon the addition of ATP, indicating the transport of  $\text{Ca}^{2+}$ . The initial rate and extent of change in fluorescence were stimulated by calmodulin and quenched by the addition of either A23187 or EGTA, but not by protonophores. Changes in chlorotetracycline fluorescence were prevented by vanadate. Therefore, results using chlorotetracycline also indicated the presence of a  $\text{Ca}^{2+}$ -translocating ATPase. Localization experiments indicated that the majority of the  $\text{Ca}^{2+}$ -translocating ATPase was on the endoplasmic reticulum.

*Key words* – Ca-ATPase, calmodulin, Ca transport, endoplasmic reticulum, maize, roots, *Zea mays*.

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### Introduction

In recent years, a role for  $\text{Ca}^{2+}$  in the regulation of cellular processes in plants has been advanced (Marmé 1985).  $\text{Ca}^{2+}$  can behave as a second messenger, adding to the amplification of an initial stimulus. Accordingly, several enzymes from plant sources are either  $\text{Ca}^{2+}$ -activated or  $\text{Ca}^{2+}$ -dependent (Dieter and Marmé 1986, Marmé 1985). Some of these  $\text{Ca}^{2+}$ -modulated enzymes, but not all, have been found to be further activated by calmodulin (Dieter and Marmé 1986, Marmé 1985).

The concentration of cytosolic  $\text{Ca}^{2+}$  is usually maintained at levels less than  $1 \mu\text{M}$ , and rises only in response to stimuli (Kostyuk 1982, Marmé 1985). Therefore, knowledge of  $\text{Ca}^{2+}$  transport processes is important in understanding how  $\text{Ca}^{2+}$  is involved in the regulation of cellular events. Three types of  $\text{Ca}^{2+}$  transporters, Ca-ATPase,  $\text{Ca}^{2+}$ -antiporters and a  $\text{Ca}^{2+}$ -selective

channel, have been identified on membranes from eukaryotes (Blaustein and Nelson 1982, Kostyuk 1982, Schatzmann 1982). A great deal of information is available about these carriers in mammalian systems. Recent reports suggest that these 3 types of  $\text{Ca}^{2+}$  carriers probably exist on plant membranes (Graziana et al. 1988, Macklon 1984).

$\text{Ca}^{2+}$ -ATPases are believed to have important roles in regulating cytosolic  $\text{Ca}^{2+}$  levels. This type of ATPase is very similar to other E1-E2 ATPases in vanadate sensitivity, formation of a phosphorylated intermediate, activation by  $\text{Mg}^{2+}$  and the presence of one subunit of 100 to 130 kDa (De Meis and Inesi 1982, Schatzmann 1982). Characteristics peculiar to the  $\text{Ca}^{2+}$ -translocating type of E1-E2 ATPase include: 1) ATP hydrolysis is stimulated by  $\text{Ca}^{2+}$  ionophores; 2)  $K_m$  for ATP is below  $0.2 \text{ mM}$ ; and 3)  $\text{Ca}^{2+}$  transport is not eliminated by protonophores. Mammalian  $\text{Ca}^{2+}$ -ATPase may transport

Ca<sup>2+</sup> by either an electrogenic process (Gimble et al. 1982) or an exchange reaction involving protons (Villalobo and Roufogalis, 1986). Mammalian Ca<sup>2+</sup>-ATPases have been localized on the endoplasmic reticulum (De Meis and Inesi 1982) and the plasma membrane (Schatzmann, 1982). Such differences in localization have profound implications on our understanding of the transport of Ca<sup>2+</sup> in response to stimuli and the site of Ca<sup>2+</sup> reservoirs utilized to increase cytosolic concentrations.

The existence of a similar Ca<sup>2+</sup>-ATPase has been demonstrated recently in plants. In an elegant study, Bush and Sze (1986) showed that a Ca<sup>2+</sup>-ATPase exists in soybean roots and is localized on the endoplasmic reticulum. The majority of Ca<sup>2+</sup>-ATPase activity is localized on the endoplasmic reticulum in red beet storage tissue (Giannini et al. 1987a) and *Lepidium sativum* roots (Buckhout 1983). In contrast, Ca<sup>2+</sup>-ATPase activity has also been found on the plasma membrane of red beet storage tissue (Giannini et al. 1987b), and the tonoplast of apple fruit (Fukumoto and Veniz 1986). A Ca<sup>2+</sup>-activated ATP-hydrolyzing enzyme with characteristics similar to mammalian Ca<sup>2+</sup>-ATPase has been partially purified from maize leaf microsomes by two different groups (Carter and Tipton 1987, Dieter and Marmé 1981), but localization of this activity on a specific membrane system has not been addressed. An enzyme with characteristics similar to a Ca<sup>2+</sup>-pump has been identified using a highly purified plasma membrane fraction from maize leaves (Robinson et al. 1988). However, enrichment of the activity during purification was not reported. Therefore, it is difficult to determine whether this activity is localized on either the plasma membrane vesicles or a contaminating membrane of different origin.

In this study, the activity of an enzyme with characteristics of a Ca<sup>2+</sup>-ATPase was identified using both ATP hydrolysis and Ca<sup>2+</sup>-transport assays, and the majority of this activity was localized on the endoplasmic

*Abbreviations:* BTP, bis-tris-propane; Ca-ATPase, Ca<sup>2+</sup>-translocating E1-E2 type ATPase; CCCP, carbonylcyanide-*m*-chlorophenylhydrazine; CTC, chlorotetracycline;  $\Delta F_s$ , change in fluorescence at steady-state; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazine; IDPase, inosine diphosphatase;  $k_L$ , rate constant for ion leakage when the pump is not catalyzing transport;  $k_p$ , rate constant describing processes that inhibit the generation of an ionic gradient by an ion pump, Pi, inorganic phosphate.

## Materials and methods

### Isolation of membrane fractions

Microsomes were isolated from 3-day-old maize (*Zea mays* L., cv. WF9 × Mo17) roots grown on filter paper moistened with 0.1 mM CaCl<sub>2</sub> as described previously (Brauer et al. 1988). Crude microsomes were obtained

by collecting those membranes that pelleted at 90 000 g for 35 min from the supernatant after removal of mitochondria by centrifugation at 10 000 g for 12 min. Microsomes were washed by resuspending in homogenization buffer containing 0.25 M KI and centrifuging at 90 000 g for 35 min. The final microsomal pellet was dispersed in resuspension buffer (3 mM MES-BTP, pH 7.2, 10% (w/v) glycerol, 0.25 M sucrose and 2.0 mM dithiothreitol) at a protein concentration of 2 to 4 mg ml<sup>-1</sup>. Protein concentration was determined after TCA precipitation in the presence of deoxycholate by a modification of the Lowry procedure (Bensadoun and Weinstein 1976).

For purification by density centrifugation, microsomes were dispersed in the resuspension buffer at 10 to 20 mg of protein ml<sup>-1</sup>. The solution was adjusted to 5 mM EDTA by the addition of 0.5 M EDTA titrated to pH 7.0 with BTP. Density centrifugation was performed essentially as described by Hodges and Leonard (1974). Two to 3 ml of microsomes were layered over a discontinuous sucrose gradient containing 6 ml layers of 20, 25, 30, 34 and 38% (w/w) sucrose and a final 5 ml layer of 45% sucrose. The sucrose solutions contained 5 mM HEPES-BTP (pH 7.8) and 1 mM dithiothreitol. After centrifugation at 82 000 g(ave) in an SW rotor for 2.5 h, the gradient was fractionated, with the interfaces being identified by an increase in absorbance at 280 nm. Where indicated, buffers in the isolation protocol were adjusted to yield a free Mg<sup>2+</sup> concentration of 3 mM.

### Marker enzymes assays

The distribution of membranes among the fractions from the step gradient was assessed by the activities of NADH cytochrome *c* reductase (EC 1.6.99.3), cytochrome *c* oxidase (EC 1.9.3.1), vanadate-sensitive ATPase (EC 3.6.1.3), and Triton-stimulated IDPase as markers for the endoplasmic reticulum, mitochondria, plasma membrane and Golgi, respectively (Hodges and Leonard 1974, Nagahashi 1985). Cytochrome *c*-utilizing enzymes were assayed as described by Hodges and Leonard (1974), except Triton X-100 was substituted for digitonin in the oxidase assay and 25 μM antimycin A was included in the reaction medium for the reductase assay. Vanadate-sensitive ATPase activity was determined essentially as described by Brauer et al. (1988). ATP hydrolysis was assayed using 5 to 10 μl of membranes diluted to 100 μl with reaction mixture containing 17.5 mM MES-BTP (pH 6.45), 50 mM KNO<sub>3</sub>, 1.0 mM EGTA and 2.5 mM MgSO<sub>4</sub> with and without 0.2 mM vanadate. After equilibration at 18 to 22°C, the reaction was initiated by the addition of 1 μl of 0.2 M ATP titrated to pH 6.45 with BTP. Triton-stimulated IDPase activity was assayed by the procedure of Nagahashi and Nagahashi (1982). The amount of inorganic phosphate (Pi) released was determined by the malachite green assay as described previously (Tu et al. 1987).

### Proton transport assay

Proton transport catalyzed by the vanadate-sensitive ATPase was followed by changes in the absorbance of acridine orange at 492 nm as described by De Michelis et al. (1983). Typically, 200  $\mu\text{l}$  of vesicles were diluted with 2 ml of 17.5 mM MES-BTP (pH 6.45), 2.5 mM  $\text{MgSO}_4$ , 1 mM EGTA, 7.5  $\mu\text{M}$  acridine orange and 50 mM  $\text{KNO}_3$ . After the mixture had equilibrated at 18 to 22°C for 5 min, the reaction was initiated by the addition of 20  $\mu\text{l}$  of 0.2 M ATP titrated to pH 6.45 with BTP.

### ATP hydrolysis assay for $\text{Ca}^{2+}$ -ATPase

The activity of  $\text{Ca}^{2+}$ -ATPase was estimated by determining the stimulation of ATP hydrolysis by  $\text{CaCl}_2$ , A23187 and bovine calmodulin. Ten  $\mu\text{l}$  of membrane suspension were added to 88  $\mu\text{l}$  of reaction mixture and incubated for 3 min at 18 to 22°C. The reaction was initiated by the addition of 2  $\mu\text{l}$  of 10 mM ATP titrated to pH 7.5 with BTP. The reaction mixture to determine activity not attributed to the Ca-ATPase had final concentrations of 25 mM MES-BTP (pH 7.5), 50 mM  $\text{KNO}_3$ , 2 mM  $\text{MgSO}_4$  and 0.1 mM EGTA. To activate the  $\text{Ca}^{2+}$ -ATPase, the above reaction mixture also included 0.4  $\mu\text{M}$  calmodulin, 0.1  $\mu\text{g}$  A23187  $\text{ml}^{-1}$  and 0.15 mM  $\text{CaCl}_2$  to yield approximately 0.05 mM of free  $\text{Ca}^{2+}$ . The activity associated with the  $\text{Ca}^{2+}$ -ATPase was determined by difference. The approximate free  $\text{Ca}^{2+}$  concentration was calculated by a method similar to that described by Tu et al. (1989) using an association constant of  $10^{7.8}$  for Ca-EGTA. The  $\text{Ca}^{2+}$  ionophore A23187 was prepared as a 1 mg  $\text{ml}^{-1}$  stock in absolute ethanol. After a reaction time of 10 min, the amount of Pi released was determined as above. Each assay contained 5 replicates. Standard errors of the mean are presented where the range exceeds the size of the data symbol. Typically the standard errors of the mean varied between 2 and 7%. Variations on assay conditions are noted in the text, Figs and Tabs.

### Chlorotetracycline (CTC) assay

$\text{Ca}^{2+}$  transport was assayed by changes in CTC fluorescence essentially as described by Lew et al. (1986). One hundred to 200  $\mu\text{l}$  of microsomes were diluted to 2.0 ml by the addition of freshly prepared reaction buffer containing 25 mM MES-BTP (pH 7.5), 2 mM  $\text{MgSO}_4$ , 50 mM  $\text{KNO}_3$ , 0.05 mM  $\text{CaCl}_2$  and 0.02 mM CTC. After equilibration at 18 to 22°C and establishment of a baseline level of fluorescence for 5 min, transport was initiated by the addition of 4  $\mu\text{l}$  of 0.2 M ATP titrated to pH 7.5 with BTP. Fluorescence was monitored using excitation and emission wavelengths of 310 and 560 nm, respectively. After the establishment of a new steady-state level of fluorescence,  $\text{Ca}^{2+}$  transport was terminated by the addition of either 0.1  $\mu\text{g}$  A23187  $\text{ml}^{-1}$  or 5 mM EGTA.

## Results

### Development of ATP hydrolysis assay for $\text{Ca}^{2+}$ -ATPase

This work was inspired by the observation that the omission of EGTA in our assay mixture for measuring the vanadate-sensitive ATPase increased ATP hydrolysis. This enhanced level of activity could be increased further by addition of  $\text{Ca}^{2+}$ . The addition of EGTA reversed  $\text{Ca}^{2+}$  stimulation. Addition of EGTA up to 0.02 mM in the absence of added  $\text{Ca}^{2+}$  resulted in a steady decrease in ATP hydrolysis. Additional EGTA had no further effect. The  $\text{Ca}^{2+}$ -stimulated activity represented less than 10% of the total ATP hydrolysis when 2 mM ATP was used as substrate. The absolute rate of  $\text{Ca}^{2+}$ -stimulated activity remained constant when the ATP level was decreased to 0.2 mM. However, at 0.2 mM ATP, the  $\text{Ca}^{2+}$ -stimulated activity represented a greater proportion of the total ATP hydrolyzing activity. The rate of  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis decreased as the assay time increased such that after 15 min the rate of hydrolysis was less than one-half the initial rate (Fig. 1).  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis had an initial rate of  $6.7 \pm 0.7$  nmol (mg protein) $^{-1}$  min $^{-1}$  over the first 2 min. Between 15 and 20 min, the rate had declined to  $2.8 \pm 0.6$  nmol (mg protein) $^{-1}$  min $^{-1}$ . Such a decrease in  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis could have resulted from thermodynamic inhibition of the pump as a  $\text{Ca}^{2+}$  gradient was generated across the membranes. The  $\text{Ca}^{2+}$ -ionophore A23187 increased  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis.  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis over 20 min was increased from  $82 \pm 6$  to  $174 \pm 11$  nmol Pi (mg protein) $^{-1}$  by 0.1  $\mu\text{g}$  A23187  $\text{ml}^{-1}$ . In addition, in the presence of A23187, activity proceeded at a nearly constant rate for at least 20 min. During the first 3 min the rate of  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis was  $7.9 \pm 0.9$  nmol Pi (mg protein) $^{-1}$  min $^{-1}$  as compared to  $8.1 \pm 0.7$  nmol Pi (mg protein) $^{-1}$  min $^{-1}$  between 15 and 20 min. These observations were consistent with the presence of an ATPase which transported  $\text{Ca}^{2+}$  to the

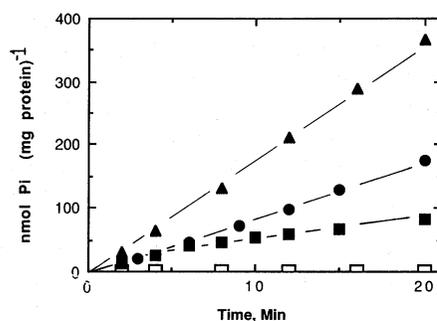


Fig. 1. Time course of  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis by microsomes as affected by the calmodulin, A23187 and vanadate. ATP hydrolysis in the presence of 0.05 mM  $\text{Ca}^{2+}$  (■) was compared to the activity in the presence of 0.05 mM  $\text{Ca}^{2+}$  plus 0.1  $\mu\text{g}$  A23187  $\text{ml}^{-1}$  (●), and in the presence of  $\text{Ca}^{2+}$ , A23187 and 0.4  $\mu\text{M}$  calmodulin (▲). Effects of 0.1 mM vanadate were assessed in the presence of 0.05 mM  $\text{Ca}^{2+}$ , 0.1  $\mu\text{g}$  A23187  $\text{ml}^{-1}$  with 0.4  $\mu\text{M}$  calmodulin (□).

intravesicular space to the point that thermodynamic inhibition began. The addition of the  $\text{Ca}^{2+}$  ionophore prevented this inhibition by dissipating the  $\text{Ca}^{2+}$  gradient.

Stimulation by  $0.1 \mu\text{g A23187 ml}^{-1}$  was dependent on the presence of  $\text{Ca}^{2+}$ . In the absence of  $\text{Ca}^{2+}$ ,  $200 \pm 10 \text{ nmol Pi (mg protein)}^{-1}$  were liberated by microsomes with a reaction time of 20 min. Addition of  $0.05 \text{ mM Ca}^{2+}$  and  $0.1 \text{ mg A23187 ml}^{-1}$  increased Pi released to  $374 \pm 15 \text{ nmol (mg protein)}^{-1} (20 \text{ min})^{-1}$ . In the presence of only A23187, Pi release was only  $206 \pm 10 \text{ nmol (mg protein)}^{-1} (20 \text{ min})^{-1}$ .

Ionophores selective for K, such as nigericin and valinomycin, did not stimulate  $\text{Ca}^{2+}$ -dependent ATP hydrolysis. When nigericin and valinomycin were substituted for A23187,  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis was  $85 \pm 8$  and  $80 \pm 6 \text{ nmol (mg protein)}^{-1} (20 \text{ min})^{-1}$ , respectively, compared to  $82 \pm 6 \text{ nmol (mg protein)}^{-1} (20 \text{ min})^{-1}$  in the absence of ionophores. Protonophores such as CCCP and FCCP also did not increase  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis.  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis over 20 min was  $88 \pm 6$  and  $85 \pm 5 \text{ nmol Pi (mg protein)}^{-1}$  in the presence of  $1 \mu\text{M CCCP}$  and  $1 \mu\text{M FCCP}$ , respectively.

The addition of  $0.4 \mu\text{M}$  calmodulin enhanced  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis by about 100% (Fig. 1). The enhancement of activity by calmodulin was prevented by the antagonists, trifluoperazine and chlorpromazine. In the presence of  $0.05 \text{ mM Ca}^{2+}$  and  $0.1 \mu\text{g A23187 ml}^{-1}$ ,  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis was increased from  $174 \pm 16$  to  $366 \pm 21 \text{ nmol Pi (mg protein)}^{-1} (20 \text{ min})^{-1}$  by  $0.4 \mu\text{M}$  calmodulin. Addition of either  $0.1 \text{ mM}$  trifluoperazine or  $0.1 \text{ mM}$  chlorpromazine in the presence of  $\text{Ca}^{2+}$ , calmodulin and A23187 reduced  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis to  $163 \pm 16$  and  $160 \pm 20 \text{ nmol Pi (mg protein)}^{-1} (20 \text{ min})^{-1}$ , respectively (data not shown). Calmodulin stimulation was dependent on the presence of  $\text{Ca}^{2+}$  (data not shown).

The  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis was abolished by the addition of  $0.1 \text{ mM}$  vanadate (Fig. 1), an inhibitor of E1-E2 type ATPases (De Meis and Inesi 1982, Schatzmann 1982). Stimulation by  $\text{Ca}^{2+}$ , A23187 and calmodulin was dependent on the presence of  $\text{Mg}^{2+}$ . In the absence of  $\text{Mg}^{2+}$ , ATP hydrolysis proceeded at  $9.9 \pm 0.5 \text{ nmol (mg protein)}^{-1} \text{ min}^{-1}$ . When  $0.4 \mu\text{M}$  calmodulin,  $0.05 \text{ mM Ca}^{2+}$  and  $0.1 \mu\text{g A23187 ml}^{-1}$  were added, the rate was virtually unchanged,  $10.3 \pm 0.6 \text{ nmol (mg protein)}^{-1} \text{ min}^{-1}$ . These results suggested that a calmodulin-stimulated  $\text{Ca}^{2+}$ -translocating ATPase was present in the maize root microsomes.

#### CTC assay

Changes in the fluorescence of CTC have been used previously to follow  $\text{Ca}^{2+}$  transport catalyzed  $\text{Ca}^{2+}$ -ATPase (Lew et al. 1986). When microsomes were incubated in the presence of  $\text{Ca}^{2+}$ , CTC and ATP, a time-dependent increase in fluorescence was observed, in-

dicating the transport of  $\text{Ca}^{2+}$  (data not shown). The rate of increase in fluorescence diminished as a function of time after ATP addition, with fluorescence achieving a steady-state value after 15 min. The fluorescence could be returned to the initial level by the addition of either the  $\text{Ca}^{2+}$ -chelator EGTA or the  $\text{Ca}^{2+}$ -ionophore A23187. The time course of ATP-induced changes in CTC fluorescence by maize membranes was very similar to that reported by Lew et al. (1986). The determination of initial rates of transport changes in either fluorescence or absorbance can be complicated because of subjective errors from drawing tangent lines through the initial portion of curves (Ho et al. 1979, Tu et al. 1987). Such errors can be eliminated by analyzing the data with respect to a mathematical model (Brauer et al. 1988, Tu et al. 1987). Data describing the generation of  $\text{Ca}^{2+}$  gradients by the pump as followed by CTC fluorescence were found to conform to the model as is evident by the linear relationships between  $\ln(1-\Delta F/\Delta F_s)$  and time after ATP addition (Fig. 2A). The slope from this plot is  $k_p$ , the rate constant describing those processes that hinder net ion transport (i.e., leakage in the direction opposite of the pump and back pressure inhibition).

Because the data concerning the buildup of gradient conformed to the model, the initial rate of ion transport

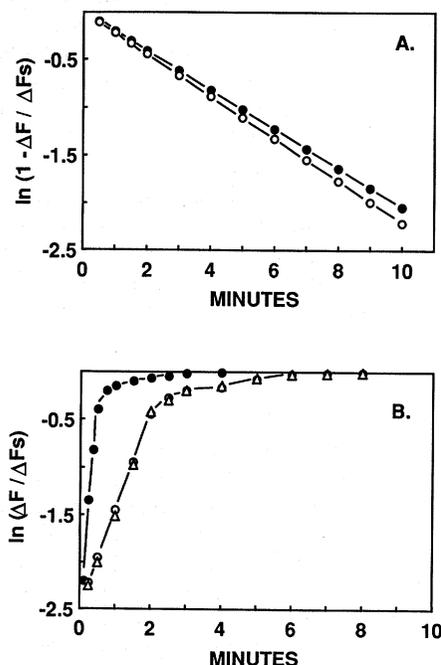


Fig. 2. Changes in chlorotetracycline fluorescence during the buildup and collapse of  $\text{Ca}^{2+}$  gradients. The data for the creation (A) and collapse of  $\text{Ca}^{2+}$  gradients (B) were transformed and fitted according to models described by Tu et al. (1987). A: the absence (○) and presence (●) of  $0.4 \mu\text{M}$  calmodulin. B: decreases in CTC fluorescence induced by the addition of  $0.1 \mu\text{g A23187 ml}^{-1}$  (●),  $0.1 \text{ mM}$  vanadate (○) or  $1.0 \text{ mM}$  EGTA (△).

Tab. 1. Effects of Ca<sup>2+</sup>, calmodulin, ionophores and vandate on ATP-dependent changes in CTC fluorescence and acridine orange absorbance to follow the transport of Ca<sup>2+</sup> and H<sup>+</sup>, respectively. Assays for both activities were conducted as described in Materials and methods. Proton transport was measured in the presence of 50 mM KNO<sub>3</sub> to completely inhibit proton transport by the tonoplast pump. Each observation is the mean ± SE of 5 determinations.

Assay conditions	Gradient generation			Gradient collapse	
	R <sub>o</sub>	k <sub>p</sub>	ΔF <sub>s</sub>	k <sub>L</sub>	ΔF <sub>s</sub>
	(mg protein) <sup>-1</sup> min <sup>-1</sup>	min <sup>-1</sup>	(mg protein) <sup>-1</sup>	min <sup>-1</sup>	(mg protein) <sup>-1</sup>
Ca <sup>2+</sup> Transport-CTC fluorescence					
a) control	4.8±0.1%	0.22	21.8%		
inhibited by 0.1 mM vanadate				1.13	21.6%
inhibited by 5 mM EGTA				1.07	21.6%
b) + 1 μM CCCP	4.7±0.1%	0.21	22.4%		
c) + 1 μM FCCP	4.7±0.1%	0.21	22.4%		
d) - 0.05 mM Ca <sup>2+</sup>	4.7±0.1%	0.21	22.4%		
e) + 0.1 μg A23187 ml <sup>-1</sup>	0.0%				
f) + 0.1 mM vanadate	0.0%				
g) +0.4 μM calmodulin	9.5±0.2%	0.20	47.5%		
inhibited ny 0.1 mM vanadate				1.11	47.2%
inhibited by 5 mM EGTA				1.08	47.3%
Nitrate-insensitive H <sup>+</sup> transport-acridine orange absorbance					
a) control	0.11±0.01A	0.41	0.26A		
b) + 1 μM CCCP	0.00				
c) + 1 μM FCCP	0.00				
d) - 0.05 mM Ca <sup>2+</sup>	0.11±0.01A	0.42	0.25A		
e) + 0.1 μg A23187 ml <sup>-1</sup>	0.00				
f) + 0.1 mM vanadate	0.02±0.01A	0.28	0.08A		
g) + 0.4 μM calmodulin	0.10±0.01A	0.41	0.25A		

by the pump, R<sub>o</sub>, can be determined by the formula (Ho et al. 1979, Tu et al. 1981):

$$R_o = -k_p \cdot \Delta F_s \quad (1)$$

There was only a slight difference in k<sub>p</sub> as determined from the slopes ln(1 - ΔF/ΔF<sub>s</sub>) vs time, averaging about 0.21 min<sup>-1</sup> over a variety of conditions (Tab. 1). Changes in R<sub>o</sub> mainly reflected changes in ΔF<sub>s</sub>. The initial rate of transport, R<sub>o</sub>, increased from 4.8 to 9.5% ΔF (mg protein)<sup>-1</sup> min<sup>-1</sup> by addition of 0.4 μM calmodulin. There was no change in CTC fluorescence when either 1 mM vanadate or 0.1 μg A23187 ml<sup>-1</sup> was added or when Ca<sup>2+</sup> was omitted from the assay mixture.

As evidenced by the linear relationships between ln(ΔF/ΔF<sub>s</sub>) and time (Fig. 2B), the initial collapse of the Ca<sup>2+</sup> gradient as followed by CTC fluorescence was found to follow first-order kinetics after the pump was inhibited or artificial leak pathways were introduced. Therefore, k<sub>L</sub>, ion leakage in the absence of an active pump, could be determined from the initial slopes in Fig. 2B. The addition of the Ca<sup>2+</sup>-ionophore A23187 induced a rapid loss of Ca<sup>2+</sup> as reflected by decreases in CTC fluorescence, with an initial first-order rate constant of about 4.8 min<sup>-1</sup>. When the Ca<sup>2+</sup> pump was stopped by addition of either vanadate or EGTA, the Ca<sup>2+</sup> gradient dissipated at a slower rate, 1.1 min<sup>-1</sup>.

The addition of CCCP, however, had little effect on ATP-dependent changes in CTC fluorescence. The lack of an effect of CCCP indicated that the changes in CTC

fluorescence could not be attributed to the transport of Ca<sup>2+</sup> via a Ca<sup>2+</sup>/proton exchanger.

Effects of Ca<sup>2+</sup>, calmodulin and ionophores on the activity of the vanadate-sensitive proton pump were assessed to further eliminate the possibility that Ca<sup>2+</sup> transport, as reflected by changes in CTC fluorescence, was linked to the action of this transporter (Tab. 1). Proton transport, as measured by changes in the acridine orange absorbance, was not affected by either 0.05 mM CaCl<sub>2</sub> or 0.4 μM calmodulin and was abolished by CCCP. Addition of 0.1 mM vanadate reduced proton transport by only 80%. Therefore, the above characteristics of proton transport were very distinct from that of CTC fluorescence.

#### Characterization of Ca-ATPase activities

The effects of various substrates and inhibitors on ATP-dependent CTC fluorescence and Ca<sup>2+</sup>-stimulated ATP hydrolysis were investigated further to provide a characterization of the Ca-ATPase. Both Ca<sup>2+</sup>-stimulated ATP hydrolysis and Ca<sup>2+</sup> transport exhibited Michaelis-Menten dependence on ATP concentration (Fig. 3). Both activities had Km's of approximately 0.05 mM. When 0.2 mM AMP was substituted for ATP, no Ca<sup>2+</sup>-stimulated Pi release or Ca<sup>2+</sup> transport was found. In the presence of 0.2 mM ADP, Ca<sup>2+</sup>-stimulated Pi release and Ca<sup>2+</sup> transport occurred at about 30% of the rates found with ATP. No change in CTC fluorescence or Ca<sup>2+</sup>-stimulated Pi formation was found when 0.2 mM

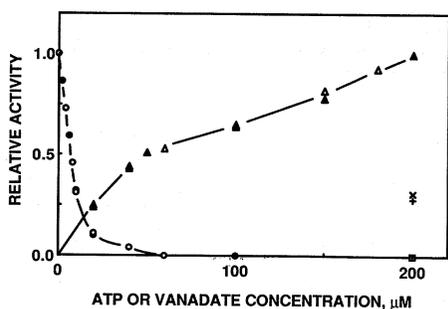


Fig. 3. Effect of ATP and vanadate concentration on rates of  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis and CTC fluorescence. The effect of ATP concentration was determined in the presence of  $0.4 \mu\text{M}$  calmodulin and  $0.05 \text{ mM}$  free  $\text{Ca}^{2+}$  ( $\Delta$ ,  $\blacktriangle$ ). The effect of vanadate was assessed in the presence of  $0.4 \mu\text{M}$  calmodulin,  $0.2 \text{ mM}$  ATP and  $0.05 \text{ mM}$   $\text{Ca}^{2+}$  ( $\circ$ ,  $\bullet$ ). Effect of substituting either AMP ( $\square$ ) or ADP ( $+$ ,  $\times$ ) for ATP was assessed at  $0.2 \text{ mM}$ .  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis was determined in the presence of  $0.1 \mu\text{g}$  A23187  $\text{ml}^{-1}$ . Data are plotted relative to the activity at  $0.2 \text{ mM}$  ATP and  $0.4 \mu\text{M}$  calmodulin, which averaged  $7.3\% \Delta\text{F} (\text{mg protein})^{-1} \text{ min}^{-1}$  and  $18.6 \text{ nmol Pi} (\text{mg protein})^{-1} \text{ min}^{-1}$  for CTC fluorescence (closed symbols) and ATP hydrolysis (open symbols).

pyrophosphate, UDP, UTP or GTP was supplied as substrate. Like other E1-E2 ATPases, the ATP hydrolysis and  $\text{Ca}^{2+}$  transport catalyzed by the  $\text{Ca}^{2+}$ -ATPase were inhibited by vanadate (Fig. 3). Both activities declined rather sharply as the vanadate concentration was increased from 1 to  $20 \mu\text{M}$ . At  $60 \mu\text{M}$  vanadate, there was essentially no activity.

In the absence of calmodulin, significant levels of both  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis and  $\text{Ca}^{2+}$  transport were found (Fig. 4). As the calmodulin concentration was increased to about  $1 \mu\text{M}$ , activity was stimulated nearly 3-fold with saturation kinetics. Both  $\text{Ca}^{2+}$  transport and ATP hydrolysis were stimulated by the presence of  $50 \text{ mM}$   $\text{KNO}_3$  in the reaction medium. The omission of  $\text{KNO}_3$  decreased ATP hydrolysis in the presence of  $0.1 \mu\text{g}$  A23187  $\text{ml}^{-1}$ ,  $0.05 \text{ mM}$   $\text{CaCl}_2$  and  $0.4 \mu\text{M}$  calmodulin by about 50%. Likewise,  $\text{Ca}^{2+}$  trans-

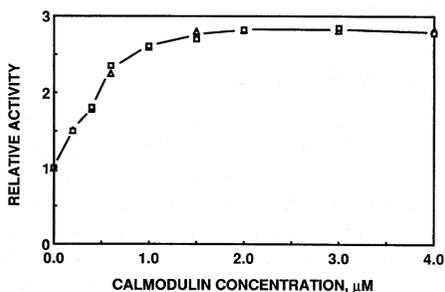


Fig. 4. Effect of calmodulin on rates of  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis and CTC fluorescence. The effect of calmodulin concentration was assessed in the presence of  $0.2 \text{ mM}$  ATP and  $0.05 \text{ mM}$   $\text{Ca}^{2+}$ . Data are plotted relative to activity in the absence of calmodulin, which averaged  $4.0\% \Delta\text{F} (\text{mg protein})^{-1} \text{ min}^{-1}$  and  $10.2 \text{ nmol Pi} (\text{mg protein})^{-1} \text{ min}^{-1}$  for CTC fluorescence ( $\Delta$ ) and ATP hydrolysis ( $\square$ ), respectively.

port in the absence of  $\text{KNO}_3$  was reduced an equal amount from 7.6 to  $3.9\% \Delta\text{F} (\text{mg protein})^{-1} \text{ ml}^{-1}$ . Substituting  $\text{KCl}$  for  $\text{KNO}_3$  did not reduce rates of ATP hydrolysis and  $\text{Ca}^{2+}$  transport. Stimulation of  $\text{Ca}^{2+}$ -ATPase by  $\text{K}^+$  has been observed previously, (De Meis and Inesi 1982).

Stimulation of the  $\text{Ca}^{2+}$ -ATPase by the  $\text{Ca}^{2+}$  ionophore A23187 and by other divalent cations was investigated using only the ATP hydrolysis assay.  $\text{Ca}^{2+}$ -stimulated ATPase activity increased as the A23187 concentration increased to  $0.1 \mu\text{g ml}^{-1}$  (Fig. 5). Between  $0.1$  and  $0.4 \mu\text{g ml}^{-1}$  activity remained relatively constant. Above  $0.4 \mu\text{g ml}^{-1}$ , the level of  $\text{Ca}^{2+}$ -stimulated activity declined. Similar results were reported in another system (Giannini et al. 1987a). ATP hydrolysis was not stimulated when  $\text{Ca}^{2+}$  was replaced by  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$  or  $\text{Zn}^{2+}$  (data not shown). Both stimulation of ATP hydrolysis and ATP-dependent changes in CTC fluorescence exhibited saturation kinetics with respect to  $\text{Ca}^{2+}$  concentration over the range of  $0.001$  to  $0.05 \text{ mM}$  (Fig. 6). Half maximal stimulation of both activities occurred at about  $5 \mu\text{M}$   $\text{Ca}^{2+}$ . Both changes in CTC fluorescence and  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis had pH optima at about 7.5 (data not shown). Changing of the pH one unit in either direction from 7.5 resulted in over half of the activity being lost.

#### Localization of $\text{Ca}^{2+}$ -ATPase

Because transport activities, like proton transport by the vanadate-sensitive ATPase, have been demonstrated to have limited stability at  $0$  to  $4^\circ\text{C}$  due to degradation of membrane lipids (Brauer et al. 1988), localization experiments utilized short centrifugation times and discontinuous sucrose gradients. The marker for mitochondria, cytochrome *c* oxidase, was enriched at the 38/45% (w/w) sucrose interface and the pellet below the 45% (w/w) sucrose step (data not shown), while the marker for endoplasmic reticulum, antimycin A-insensitive cytochrome *c* reductase, was enriched at the 20/25% (w/w) sucrose interface (Fig. 7A). Markers for Golgi and plasma membrane, Triton-stimulated IDPase and vanadate-sensitive ATPase, respectively,

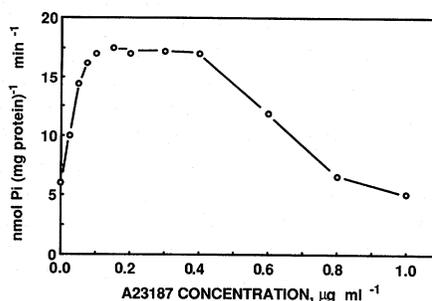


Fig. 5. Effect of A23187 concentration on rates of  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis. The effect of A23187 was assessed in the presence of  $0.2 \text{ mM}$  ATP,  $0.05 \text{ mM}$   $\text{Ca}^{2+}$  and  $0.4 \mu\text{M}$  calmodulin.

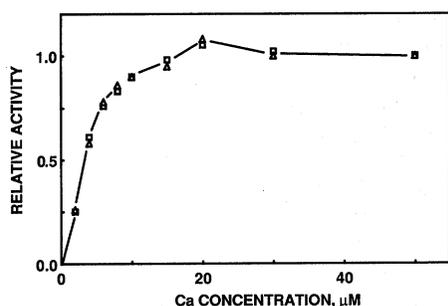


Fig. 6. Effect of  $\text{Ca}^{2+}$  concentration on rates of  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis and ATP dependent changes in CTC fluorescence, assessed in the presence of 0.2 mM ATP and 0.4  $\mu\text{M}$  calmodulin. Assays for  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis also contained 0.1  $\mu\text{g}$  A23187  $\text{ml}^{-1}$ . Data are plotted relative to activity in the presence of 0.05 mM  $\text{Ca}^{2+}$ , which averaged 7.6%  $\Delta F$  (mg protein) $^{-1}$   $\text{min}^{-1}$  and 17.4 nmol Pi (mg protein) $^{-1}$   $\text{min}^{-1}$  for the CTC fluorescence ( $\Delta$ ) and Ca-stimulated ATP hydrolysis ( $\square$ ) assays, respectively.

distributed as broad bands with the fractions highest in specific activity found at the 30/34 and 34/38% (w/w) sucrose interface, respectively (Fig. 7A). The distribution of marker enzymes was similar to that found previously for corn root (Leonard and Hotchkiss 1976). ATP hydrolysis associated with the  $\text{Ca}^{2+}$ -ATPase was found exclusively in the lighter density fractions (Fig. 7A), closely corresponding to the distribution of cytochrome *c* reductase.

To confirm that  $\text{Ca}^{2+}$ -ATPase was associated with the endoplasmic reticulum, membranes were isolated in the presence of free  $\text{Mg}^{2+}$  to maintain the ribosomes on the endoplasmic reticulum. Under such conditions, the specific activity of the endoplasmic reticulum marker, cytochrome *c* reductase, was shifted to the interfaces between 34/38 and 38/45% (w/w) sucrose and its distribution was still identical to the distribution of the  $\text{Ca}^{2+}$ -ATPase (Fig. 7B).

## Discussion

### Identification of a $\text{Ca}^{2+}$ -ATPase in maize root microsomes

The addition of  $\text{Ca}^{2+}$  was found to stimulate ATP hydrolysis (Fig. 1). The rate of  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis was found to decrease as the assay time was increased beyond 4 min. The addition of the  $\text{Ca}^{2+}$ -ionophore A23187 allowed  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis to proceed at the initial rate for at least 20 min. Ionophores that transport  $\text{K}^+$  and  $\text{H}^+$ , such as valinomycin, nigericin, CCCP and FCCP, did not mimic the effects of A23187 (Tab. 1). The easiest interpretation of such results is that  $\text{Ca}^{2+}$  was transported by an ATP-dependent process to the inside of membrane vesicles to the point that a significant  $\text{Ca}^{2+}$  gradient was produced, resulting in thermodynamic inhibition of ATP hydrolysis. The addition of A23187 provided a leak pathway so

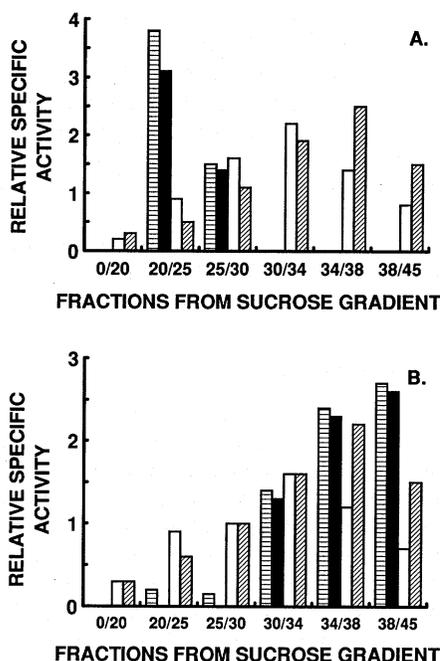


Fig. 7. Distribution of enzyme markers and  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis activities among fractions from discontinuous density sucrose gradients. Membranes were isolated as described under Materials and methods in the presence of chelators of divalent cations (A) and 3 mM  $\text{Mg}^{2+}$  (B). Activities are plotted relative to the specific activity of the microsomal fraction for cytochrome *c* reductase ( $\square$ ), Ca-stimulated ATP hydrolysis ( $\blacksquare$ ), Triton X-100 stimulated IDPase ( $\square$ ) and vana-date-sensitive ATPase ( $\text{hatched}$ ), from left to right, which averaged 8.1 A (mg protein) $^{-1}$   $\text{min}^{-1}$ , 7.6 nmol Pi (mg protein) $^{-1}$ , 25 nmol Pi (mg protein) $^{-1}$   $\text{min}^{-1}$  and 150 nmol Pi (mg protein) $^{-1}$   $\text{min}^{-1}$ , respectively.

that the  $\text{Ca}^{2+}$  gradient was not generated, thus avoiding thermodynamic inhibition.  $\text{Ca}^{2+}$ -stimulated activity was further increased by calmodulin. The results taken together indicated the presence of a calmodulin-stimulated  $\text{Ca}^{2+}$ -ATPase. Under similar assay conditions,  $\text{Ca}^{2+}$  transport was detected by changes in CTC fluorescence which was ATP-dependent,  $\text{Ca}^{2+}$ -dependent, CCCP-insensitive and A23187 sensitive. The stimulation of ATP hydrolysis by  $\text{Ca}^{2+}$  and calmodulin was sensitive to vanadate, which is a fairly specific inhibitor of E1-E2 ATPases (Schatzman 1982), indicating the involvement of one or more of this class of transport enzymes.

However,  $\text{Ca}^{2+}$ -stimulation of ATP hydrolysis and  $\text{Ca}^{2+}$  transport did not seem to involve the plasma-membrane vanadate-sensitive proton pump.  $\text{Ca}^{2+}$  stimulation of ATP hydrolysis was not reduced by CCCP and FCCP. Such results eliminated the possibility that the operation of a  $\text{Ca}^{2+}/\text{H}^+$  antiporter was dissipating a pH gradient generated by the proton pump and thus stimulating ATP hydrolysis. Results in Tab. 1 demonstrate that the characteristics of the vanadate-sensitive proton transport in maize root microsomes were quite

different from those of the  $\text{Ca}^{2+}$ -transporting activity as followed by changes in CTC fluorescence. Proton transport was eliminated by CCCP and FCCP, while  $\text{Ca}^{2+}$  transport was unaffected. Proton transport was unaffected by the presence of  $\text{Ca}^{2+}$ , suggesting that there was no coupling between the pH gradient formed by this pump and  $\text{Ca}^{2+}$  transport. These results are in sharp contrast to the results of Rasi-Caldogno et al. (1987). With radish plasma membranes, the addition of  $\text{Ca}^{2+}$  reduced proton transport, indicating the dissipation of the pH gradient by the activity of a  $\text{Ca}^{2+}/\text{H}^+$  antiporter.

#### Characteristics of the maize root $\text{Ca}^{2+}$ -ATPase

The  $\text{Ca}^{2+}$ -ATPase from maize had substantial activity in the absence of calmodulin, but calmodulin was a positive effector (Fig. 1). Similar activation of plant  $\text{Ca}^{2+}$ -ATPases by calmodulin has been reported previously for apple fruit membranes (Fukumoto and Venis 1986), spinach leaf membranes (Stusic et al. 1983) and a partially purified  $\text{Ca}^{2+}$ -ATPase from maize shoots (Carter and Tipton 1987). Calmodulin activation was the basis of the purification of a  $\text{Ca}^{2+}$ -ATPase from maize shoot tissue (Dieter and Marmé 1981). Calmodulin stimulation of the  $\text{Ca}^{2+}$ -ATPase from zucchini hypocotyls differed slightly from the maize activity. Calmodulin increased the initial rate of  $\text{Ca}^{2+}$  transport but not the steady-state levels (Lew et al. 1986), whereas both parameters were increased with maize microsomes (Tab. 1). Calmodulin modulation of the plasma membrane and endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase from red beet storage tissue was not reported by Giannini et al. (1987a,b). With maize root microsomes, both  $\text{Ca}^{2+}$ -ATP hydrolysis and  $\text{Ca}^{2+}$  transport were stimulated to a similar extent by calmodulin, indicating that activation represented enhanced enzymatic turnover rather than a change in the coupling between ATP hydrolysis and  $\text{Ca}^{2+}$  transport. In summary, it appears that calmodulin stimulation of  $\text{Ca}^{2+}$ -ATPase activity from plant tissues is relatively common. The role of this activation in cellular regulation needs further investigation to understand its *in situ* implications.

$\text{Ca}^{2+}$  transport as assayed by changes in CTC fluorescence conformed to a mathematical model which describes net transport as the differences between the pumping rate and leakage from the vesicles in the direction opposite that of the pump (Fig. 2). The model allows one to quantify the magnitude of the processes hindering the development of the  $\text{Ca}^{2+}$  gradient,  $k_p$ . Calmodulin stimulation did not alter  $k_p$  (Tab. 1), indicating that the factors inhibiting the build-up of  $\text{Ca}^{2+}$  gradient were unaffected by calmodulin. Since  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis in the absence of A23187 declined over the time period between the initiation of  $\text{Ca}^{2+}$  transport and establishment of a steady-state gradient (Figs 1 and 2), back-pressure inhibition must have contributed to  $k_p$ . Therefore, in the case of the  $\text{Ca}^{2+}$  pump,  $k_p$  was determined by both back-pressure inhibi-

tion and  $\text{Ca}^{2+}$  leakage. It was not possible to determine the relative magnitude of these 2 processes on  $k_p$ . However, in the cases of proton transport by both plasma membrane and tonoplast pumps from maize roots, the magnitude of  $k_p$  was determined primarily by the rate of proton leakage (Brauer et al. 1989, Tu et al. 1987). Values for  $k_p$  for the  $\text{Ca}^{2+}$  pump were about one-half that for the vanadate-sensitive proton pump (Tab. 1). In contrast, the values for  $\text{Ca}^{2+}$  leakage after the  $\text{Ca}^{2+}$  pump was allowed to generate a steady-state gradient and then inhibited,  $k_L$ , were higher than those reported for the proton pump. The rate constants for the dissipation of proton gradients,  $k_L$ , formed by the vanadate-sensitive ATPase averaged  $0.5 \text{ min}^{-1}$  (Brauer et al. 1988), compared to the  $1.1 \text{ min}^{-1}$  reported here for the  $\text{Ca}^{2+}$ -ATPase. The rate constant for the collapse of the  $\text{Ca}^{2+}$  gradient,  $k_L$ , was significantly greater than the rate constants of those processes hindering gradient formation during pumping,  $k_p$ ,  $1.1$  and  $0.2 \text{ min}^{-1}$ , respectively. Such results would be only possible if the leakage of  $\text{Ca}^{2+}$  decreased when the  $\text{Ca}$ -ATPase was catalyzing transport.

The maize root activity was similar to that of mammalian  $\text{Ca}$ -ATPases (De Meis and Inesis 1982, Schatzmann 1982) in its dependence on ATP (Fig. 3), stimulation by calmodulin (Fig. 4) and inhibition by vanadate (Fig. 3). The  $\text{Ca}^{2+}$ -ATPase can be distinguished from the vanadate-sensitive proton pump from maize roots on basis of a lower  $K_m$  for ATP,  $0.05 \text{ mM}$  (Fig. 3) vs  $0.5 \text{ mM}$  (Bauer et al. 1989), and a higher sensitivity to vanadate, half inhibition at less than  $10 \text{ }\mu\text{M}$  (Fig. 3) vs approximately  $50 \text{ }\mu\text{M}$  Bush and Sze 1986, Lew et al. 1986).

No general trends, however, can be seen concerning the affinity of different plant  $\text{Ca}^{2+}$ -ATPases towards ATP. Previous estimates for the  $K_m$  for ATP range from less than  $0.1 \text{ mM}$  (Lew et al. 1986) to over  $1.0 \text{ mM}$  (Bush and Sze 1986, Giannini et al. 1987a,b) with the maize root enzyme having a  $K_m$  in the lower end of this range. Not only do previous papers report differences in the affinity for ATP but also in the type of kinetics with respect to ATP. Both the maize root  $\text{Ca}^{2+}$ -ATPase (Fig. 4) and the zucchini hypocotyl (Lew et al. 1986)  $\text{Ca}^{2+}$ -ATPase exhibited Michealis-Menten kinetics with respect to ATP, whereas the carrot cell enzyme had sigmoidal kinetics (Bush and Sze 1986). The reason for these discrepancies in the kinetics for ATP among different plant  $\text{Ca}^{2+}$ -ATPases is not readily apparent. However, kinetics of mammalian  $\text{Ca}^{2+}$ -ATPases with respect to ATP can be altered by various treatments, including calmodulin (De Meis and Inesis 1982, Schatzmann 1982).

Plant  $\text{Ca}^{2+}$ -ATPases also differ widely in their activation by  $\text{Ca}^{2+}$ . The maize root enzyme had a relatively high affinity for  $\text{Ca}^{2+}$  with maximal activity occurring at  $20 \text{ }\mu\text{M}$  (Fig. 6), a value similar to that reported previously for enzyme activities from maize leaves (Carter and Tipton 1987, Robinson et al. 1988), zucchini, hypocotyl (Lew et al. 1986) and red beet storage tissue

(Giannini et al. 1987a,b). In contrast, the  $\text{Ca}^{2+}$ -ATPase activity from apple fruit tonoplast vesicles exhibited a  $K_m$  for  $\text{Ca}^{2+}$  of about  $50 \mu\text{M}$ .

On the basis of density centrifugation in the absence and presence of free  $\text{Mg}^{2+}$ , the majority of the  $\text{Ca}^{2+}$ -ATPase activity from maize root microsomes was associated with the endoplasmic reticulum (Fig. 7). Zocchi (1988) attempted to localize the ATP-dependent  $\text{Ca}^{2+}$  transport activity from maize roots by separating microsomal membranes on a two-step dextran gradient. Both fractions from the dextran gradient had ATP-dependent,  $\text{Ca}^{2+}$ -transporting activity but these activities had different sensitivity to uncouplers.  $\text{Ca}^{2+}$  transport by membranes at the higher density interface was not inhibited by  $10 \mu\text{M}$  CCCP. Both of the 2 fractions contained significant levels of markers for the various membranes, indicating a low degree of purification. The data in Fig. 7 clearly demonstrate that  $\text{Ca}^{2+}$ -ATPase activity co-purified with the endoplasmic reticulum marker, antimycin A-insensitive NADH cytochrome *c* reductase (Hodges and Leonard 1974, Nagahashi 1985, Quail 1979).

Not only do putative plant  $\text{Ca}^{2+}$ -ATPases differ widely with respect to the characteristics of enzymatic activity, as described above, but also in their subcellular localization. Plant  $\text{Ca}^{2+}$ -ATPases have been reported to reside on plasma membranes from maize leaves (Robinson et al. 1988), radish roots (Rasi-Caldogno et al. 1987), red beet storage tissue (Giannini et al. 1987b), endoplasmic reticulum from maize roots (Fig. 7) and cultured carrot cells (Bush and Sze 1986) and tonoplast vesicles from apple fruit (Fukumoto and Venis 1986). This diversity in localization may reflect differences in either the functions of the  $\text{Ca}^{2+}$ -ATPase or the sites of sequestering  $\text{Ca}^{2+}$  among different plant tissues. As knowledge of the role of  $\text{Ca}^{2+}$  and its transport in regulating cellular metabolism increases, the reason for the diversity in the characteristics of plant  $\text{Ca}^{2+}$ -ATPase may become apparent.

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