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A Ca^{2+}/H^+ Antiport System Driven by the Proton Electrochemical Gradient of a Tonoplast H^+-ATPase from Oat Roots[1]

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ABSTRACT

Two types of ATP-dependent calcium (Ca^{2+}) transport systems were detected in sealed microsomal vesicles from oat roots. Approximately 80% of the total Ca^{2+} uptake was associated with vesicles of 1.11 grams per cubic centimeter and was insensitive to vanadate or azide, but inhibited by NO_3^-. The remaining 20% was vanadate-sensitive and mostly associated with the endoplasmic reticulum, as the transport activity comigrated with an endoplasmic reticulum marker (antimycin A-insensitive NADH cytochrome *c* reductase), which was shifted from 1.11 to 1.20 grams per cubic centimeter by Mg^{2+}.

Like the tonoplast H^+-ATPase activity, vanadate-insensitive Ca^{2+} accumulation was stimulated by 20 millimolar Cl^- and inhibited by 10 micromolar 4,4′-diisothiocyano-2,2′-stilbene disulfonic acid or 50 micromolar *N,N′*-dicyclohexylcarbodiimide. This Ca^{2+} transport system had an apparent K_m for Mg-ATP of 0.24 millimolar similar to the tonoplast ATPase. The vanadate-insensitive Ca^{2+} transport was abolished by compounds that eliminated a pH gradient and Ca^{2+} dissipated a pH gradient (acid inside) generated by the tonoplast-type H^+-ATPase. These results provide compelling evidence that a pH gradient generated by the H^+-ATPase drives Ca^{2+} accumulation into right-side-out tonoplast vesicles via a Ca^{2+}/H^+ antiport. This transport system was saturable with respect to Ca^{2+} (K_m apparent = 14 micromolar). The Ca^{2+}/H^+ antiport operated independently of the H^+-ATPase since an artificially imposed pH gradient (acid inside) could also drive Ca^{2+} accumulation. Ca^{2+} transport by this system may be one major way in which vacuoles function in Ca^{2+} homeostasis in the cytoplasm of plant cells.

Although few measurements of Ca^{2+} levels have been made in plant cells (35), cytoplasmic Ca^{2+} levels are assumed to be similar to those determined for animal cells (0.1–1.0 μM) (1). The concentration of free Ca^{2+} in the vacuole of plant cells is estimated to range from 0.1 to 10 mM (23, 24). Using histochemical probes for Ca^{2+}, it has long been known that the ER lumen and the nuclear envelope contain relatively high levels of Ca^{2+} (15). In spite of the much higher levels of Ca^{2+} in the external medium (ranging from 0.1–1 mM) (10) and in the lumen of intracellular organelles, cytoplasmic Ca^{2+} levels are maintained at submicromolar concentrations. These observations, coupled with compartmental flux analyses (23), have suggested that Ca^{2+} transport systems must exist on various subcellular membranes. Such transport systems reduce cytoplasmic Ca^{2+} levels that tend to increase due to the movement of Ca^{2+} down the large electrochemical gradient.

To identify these putative Ca^{2+} transport systems, we have isolated mitochondria (19) or membrane vesicles (31) to study the properties of the Ca^{2+} pumps directly. Several ATP-dependent Ca^{2+} transport systems have already been demonstrated using this approach (3, 4, 11, 13, 14, 27, 36). With few exceptions (3, 4), the membrane identity and the specific properties of the Ca^{2+} pumps have not been established. For example, the membrane localization of a Ca^{2+}/H^+ antiport system reported by Hager and Hermsdorf (14) in corn coleoptiles and by Rasi-Caldogno *et al.* (27) in pea stems is not clear. Other studies have suggested an ATP-dependent Ca^{2+} pump was localized on the plasma membrane (11, 13, 20) without providing any supporting data.

Here we show two types of Ca^{2+} transport systems in membrane vesicles from oat roots: a vanadate-sensitive Ca^{2+} transport activity associated with the ER, and the major portion of the Ca^{2+} transport, a vanadate-insensitive or NO_3^--sensitive system, associated with the vacuole. Based on the similar properties of the H^+-pumping tonoplast ATPase and the ATP-dependent vanadate-insensitive Ca^{2+} transport, we conclude that the pH gradient established by the tonoplast H^+-ATPase is used to drive Ca^{2+} accumulation via a Ca^{2+}/H^+ antiport system. Preliminary results of this study have been presented (28).

MATERIALS AND METHODS

Plant Material. Oat seeds (*Avena sativa* L. var Lang) were germinated in the dark over an aerated solution of 0.5 mM $CaSO_4$. Roots were harvested after 4 d. Lang oats were generously provided by the Agronomy Department, University of Maryland, College Park (Dr. D. Sammons) and Southern States Cooperative, Baltimore, MD.

Preparation of Sealed Microsomal Vesicles. The procedure of Sze (30) as modified by Churchill and Sze (8) was used to isolate membrane vesicles. All procedures were conducted at 4°C. Oat roots (10–30 g) were homogenized by mortar and pestle in a medium containing 250 mM mannitol, 3 mM EGTA, 25 mM Hepes-BTP[2] (pH 7.4), 1 mM DTT, and 0.5% BSA at a medium-to-tissue ratio of 1.5 ml/g fresh weight. After filtration through two layers of cheesecloth, the debris was rehomogenized in 1 ml/g of the original tissue weight, washed in 0.5 ml/g, and filtered. The homogenate was centrifuged for 15 min at 13,000*g*. The 13,000*g* supernatant was centrifuged for 30 min at 60,000*g* (Beckman SW 28 rotor, r_{max}). The 60,000*g* pellet (crude micro-

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[2] Abbreviations: BTP, bis-tris propane or 1,3-bis(tris[hydroxymethyl]methylamine); ΔpH, pH gradient; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N′*-dicyclohexylcarbodiimide; DIDS, 4,4′-diisothiocyano-2,2′-stilbene disulfonic acid; SR, sarcoplasmic reticulum.

somal fraction) was resuspended in 250 mM mannitol, 2.5 mM Hepes-BTP (pH 7.2), and 1 mM DTT (resuspension buffer). The suspension was layered over a 10% (w/w) dextran cushion prepared in resuspension buffer. Usually, 6 ml of the microsomal suspension was layered over 10 ml of dextran and centrifuged for 2 h at 70,000*g* (SW 28.1 r_{max}). A visible band at the mannitol-dextran interface was collected and is referred to as the sealed microsomal vesicles.

Protein Concentration Determination. Protein concentration was estimated after precipitation with 10% TCA by the method of Lowry *et al.* (22), with BSA as the standard.

Calcium Transport Assays. ATP-dependent $^{45}Ca^{2+}$ uptake was measured by a filtration method (13). To initiate the reaction, sealed microsomal vesicles were added to a reaction mixture (0.5–1.0 ml) containing (final concentrations) 175 mM mannitol, 25 mM Hepes-BTP (pH 7.0), 20 mM KCl, 0.2 mM NaN_3, 40 to 100 μg/ml membrane protein, 3 mM $MgSO_4$, 10 μM $^{45}CaCl_2$ (approximately 0.25 μCi/ml) with or without 3 mM ATP. To characterize the vanadate-insensitive or NO_3^--sensitive Ca^{2+} transport system, all reaction mixtures were modified to include 100 μM vanadate. Ionophores or inhibitors dissolved in ethanol were added to reaction mixtures to give a final concentration of 0.5 to 1.0% ethanol (ethanol concentrations up to 1.0% had no effect on Ca^{2+} uptake). After a 10 min incubation (unless otherwise indicated) at 22°C, duplicate aliquots of 50 to 100 μl were filtered through Millipore filters (0.45 μm pore size). The filtration procedure involved wetting a Millipore filter with 1 ml of cold (4°C) wash solution (250 mM mannitol, 2.5 mM Hepes-BTP, 0.1 mM $CaCl_2$), filtering an aliquot of the reaction medium, and quickly rinsing with 1 ml of cold wash medium. $CaCl_2$ was included in the rinse solution to exchange any $^{45}Ca^{2+}$ bound to the vesicle surface or filter paper. The filters were dried and the radioactivity determined by liquid scintillation counting. Results are presented as nmol Ca^{2+} taken up per mg protein.

Methylamine Accumulation. ΔpH generation (acid inside) in vesicles was determined by [^{14}C]methylamine accumulation using the direct filtration procedure described by Churchill and Sze (8).

Separation of Microsomal Vesicles by Sucrose Gradients. For some experiments, resuspended crude microsomal membranes were fractionated in a linear sucrose gradient 20 to 40% (32 ml total). Two ml of resuspended microsomal vesicles were layered on top of the gradient and centrifuged for 2.5 h at 70,000*g* (SW 28 rotor, r_{max}). Eighteen fractions of 1.7 ml each were collected and tested for vanadate-sensitive or NO_3^--sensitive Ca^{2+} transport and antimycin A insensitive NADH Cyt *c* reductase activity. Sucrose density was determined using a refractometer.

Endoplasmic Reticulum Density Shifting Experiments. Vesicles enriched in RER were prepared as described by Lord *et al.* (21) with several modifications. Tissue was homogenized in 250 mM sucrose, 25 mM Hepes-BTP (pH 7.4), 2 mM $MgSO_4$, 1 mM DTT, 0.2% BSA, and 1 mM EGTA. Resuspension buffer contained 250 mM sucrose, 2.5 mM Hepes-BTP (pH 7.2), 1 mM DTT, and 2 mM $MgSO_4$. Sucrose gradient solutions contained 1 mM DTT and 2 mM $MgSO_4$. To prepare vesicles enriched in tonoplast membranes or smooth ER (light density), the tissue was homogenized in a Mg^{2+}-free medium containing 250 mM sucrose, 25 mM Hepes-BTP (pH 7.4), 3 mM EGTA, 1 mM DTT, and 0.5% BSA. All other solutions were as above without Mg^{2+}. The resuspended microsomal pellets were loaded on sucrose step gradients (29/40%) and centrifuged for 2 h at 70,000*g* (SW 28.1 rotor). The 8/29% and 29/45% interfaces were collected and assayed for vanadate-sensitive or NO_3^--sensitive Ca^{2+} transport and antimycin A insensitive NADH Cyt *c* reductase activity.

Antimycin A-Insensitive NADH Cyt *c* Reductase Activity. Antimycin A-insensitive NADH Cyt *c* reductase activity was assayed spectrophotometrically by the reduction of Cyt *c* at 550 nm at 22°C (17). A typical reaction mixture contained 50 mM K-phosphate (pH 7.5), 0.3 μM antimycin A, 30 μM Cyt *c*, and 0.1 mM NADH. The reaction was initiated by the addition of vesicles.

Artificial pH Gradient Formation. Vesicles were loaded with KCl by addition of 150 mM KCl in the homogenization, resuspension, and dextran media. The microsomal vesicles were diluted with 36 ml of resuspension buffer containing 150 mM KCl and concentrated by pelleting (90,000*g*, SW 28, r_{max}). The pellet was resuspended in buffer without KCl just prior to use and an aliquot was diluted into a reaction mixture containing 25 mM Hepes-BTP (pH 7.0), with or without 5 μM nigericin.

RESULTS

Calcium Uptake. $^{45}Ca^{2+}$ accumulation into sealed microsomal vesicles was significantly increased in the presence of ATP. This Ca^{2+} was rapidly released upon addition of A23187 (Fig. 1), an ionophore which catalyzes an electroneutral exchange of protons for Ca^{2+}.

In the absence of exogenously added ATP, approximately 0.5 nmol Ca^{2+}/mg protein was associated with the vesicles. This may represent Ca^{2+} bound to membranes and Ca^{2+} accumulated in the vesicles from an endogenous energy source as A23187 released some Ca^{2+} even in the absence of ATP (Fig. 1). Using an internal vesicle volume of 10 μl/mg protein (33), we estimated that at 10 μM $[Ca^{2+}]_{ext}$, Ca^{2+} flux into the vesicles would reach passive equilibrium when $[Ca^{2+}]_{int}$ was 0.1 nmol/mg protein. This value is close to the amount of Ca^{2+} remaining in the vesicles after addition of A23187 (Fig. 1).

In the presence of ATP, a total of 6.5 nmol Ca^{2+} was associated with 1 mg vesicle protein. If one estimates that about 6.0 nmol is diffusible Ca^{2+} (released by A23187), the accumulation ratio of $[Ca^{2+}]_i/[Ca^{2+}]_o$ would be 6.0/0.1 or about 60-fold. This may be an underestimate as Ca^{2+} uptake varied from one preparation to another and ATP could catalyze net uptake of up to 20 nmol Ca^{2+}/mg protein.

Sensitivity to Vanadate and NO_3^-. Vanadate (100 μM), an inhibitor of proton ATPases associated with the plasma membrane of higher plants (7, 32), fungi (12), and of Ca-ATPases associated with the plasma membrane (6) and SR (26) in animal cells had a small effect on Ca^{2+} accumulation (Fig. 2). Vanadate-sensitive Ca^{2+} uptake is the difference in Ca^{2+} accumulation in the absence and presence of the inhibitor. NO_3^- (10 mM), which

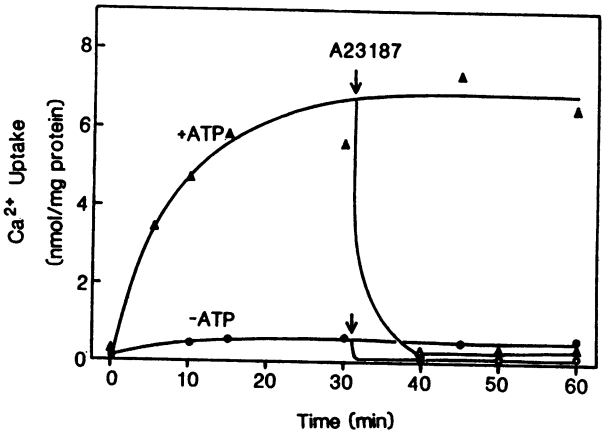

FIG. 1. Time-course of ATP-dependent Ca^{2+} accumulation in sealed microsomal vesicles from oat roots. Reaction mixtures contained 25 mM Hepes-BTP (pH 7.0), 175 mM mannitol, 20 mM KCl, 10 μM $^{45}CaCl_2$, 3 mM $MgSO_4$ with (▲) or without (●) 3 mM ATP. After 30 min incubation at 22°C, A23187 was added to give a final ionophore concentration of 5 μM and ethanol concentration of 0.5% (△, ○).

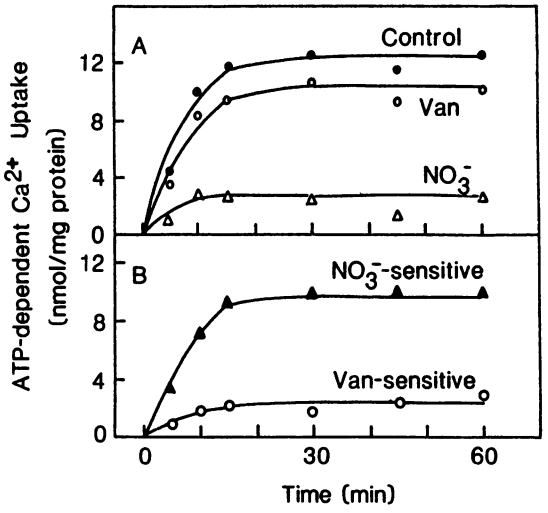

FIG. 2. Effects of vanadate or nitrate on Ca^{2+} uptake into sealed microsomal vesicles from oat roots. Reaction mixtures were as described in Figure 1. A, Total Ca^{2+} uptake in the absence (●) of inhibitors and in the presence of 10 mM BTP-NO_3 (Δ) or 100 μM Na orthovanadate (○). B, NO_3^--sensitive (▲) and vanadate-sensitive (○) Ca^{2+} uptake was calculated from the difference of uptake in the absence and presence of NO_3^- or vanadate. Results are from one representative experiment.

Table I. *Effect of Inhibitors on ATP-Dependent Ca^{2+} Uptake and Methylamine Uptake into Sealed Microsomal Vesicles of Oat Roots*

Reaction mixtures for Ca^{2+} uptake were as described in Figure 1.

Addition	ATP-Dependent Transport	
	$[^{14}C]CH_3NH_2$[a]	$^{45}Ca^{2+}$
	%	
None	100	100
Vanadate (200 μM)	112	92
DIDS (4 μM)	55	62
DCCD (50 μM)	10[b]	25
Nitrate (10 mM)	38	44
NaN_3 (0.2 mM)	80	98

[a] Data from Churchill and Sze (8). [b] H^+-pumping was determined by quinacrine fluorescence quenching (34).

inhibits a H^+-pumping ATPase associated with the tonoplast in oat roots (8), inhibited Ca^{2+} accumulation approximately 80% (Fig. 2). Since the magnitude and density of the NO_3^--sensitive activity was similar to that of the vanadate-insensitive Ca^{2+} transport (see following sections), we concluded these two properties are characteristic of one Ca^{2+} transport system. A second type of Ca^{2+} pump in these microsomal vesicles was characterized as vanadate-sensitive and NO_3^--insensitive.

Azide (0.2 mM) did not affect Ca^{2+} transport (Table I) suggesting the vesicle preparation was not contaminated by mitochondria which can display ATP-dependent Ca^{2+} transport (11, 19). To eliminate the possibility of Ca^{2+} transport from mitochondrial contamination, azide was routinely added to the reaction mixture.

Localization of the Two Calcium Transport Systems. Both the vanadate-insensitive (or NO_3^--sensitive) and vanadate-sensitive Ca^{2+} pumps equilibrated at similar sucrose densities corresponding to 1.11 g/cm³ (Fig. 3). These fractions were enriched in ER as measured by antimycin A-insensitive NADH Cyt *c* reductase activity (not shown).

To determine whether one of the Ca^{2+} transport systems was associated with the ER, Mg^{2+} was included in the isolation media to stabilize ribosomal attachment to the ER and redistribute the

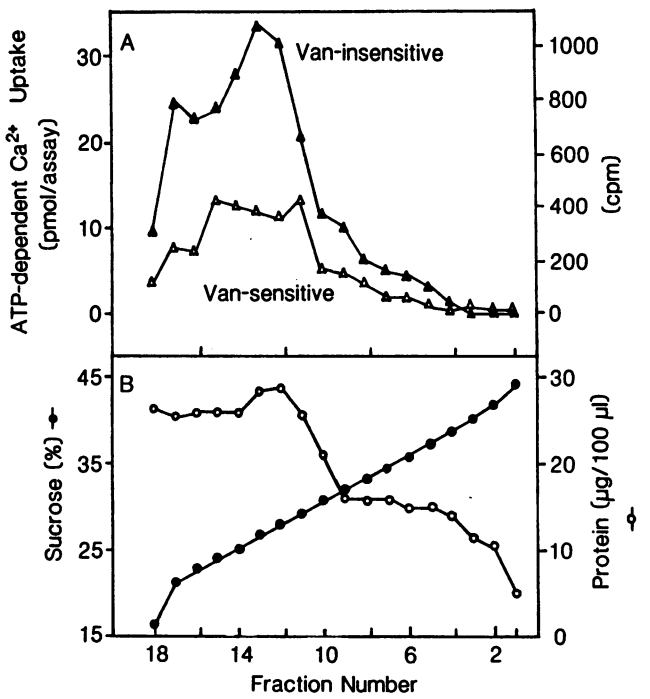

FIG. 3. A, Distribution of the vanadate-sensitive (Δ) and vanadate-insensitive (▲) Ca^{2+} transport system in vesicles separated with a linear sucrose gradient (20–40%). Net ATP-dependent Ca^{2+} uptake at 20 min was measured in the absence or presence of 100 μM Na orthovanadate. Each assay consisted of 60 μl vesicles. B, Distribution of protein (○) on a linear sucrose density gradient (●).

Table II. *Effect of Mg^{2+} on the Relative Density of Vanadate-Sensitive or Nitrate-Sensitive Ca^{2+} Transport in Vesicles from Oat Roots*

Reaction mixtures were as described in "Materials and Methods."

	Type of Transport	ATP-Dependent Ca^{2+} Uptake		
		Total activity (L + H)	8/29% (Light)	29/45% (Heavy)
		nmol	*nmol/fraction (% total)*	
A.	$-Mg^{2+}$			
	NO_3^--sensitive	0.42	0.41 (98)	0.01 (2)
	Vanadate-sensitive	0.23	0.23 (100)	0 (0)
	ER marker (rel. distribution)			
	Ant. A-insensitive NADH Cyt *c* reductase		62%	38%
B.	$+Mg^{2+}$			
	NO_3^--sensitive	0.27	0.24 (91)	0.02 (9)
	Vanadate-sensitive	0.24	0.09 (38)	0.15 (62)
	ER marker (rel. distribution)			
	Ant. A-insensitive NADH Cyt *c* reductase		39%	61%

ER to a heavier density. Under these conditions the RER was shifted to a density of 1.20 g/cm³. Based on this information, microsomal vesicles were separated with discontinuous (29 and 45%) sucrose gradients made either with or without Mg^{2+}. In the absence of Mg^{2+}, both the vanadate-sensitive and NO_3^--sensitive Ca^{2+} transport activities were localized in the light (8/29%) fraction and little Ca^{2+} uptake was seen in the heavy (29/45%) fraction (Table II). When Mg^{2+} was included in the preparation,

the NO_3^--sensitive portion remained in the lighter fraction while 60% of the vanadate-sensitive Ca^{2+} transport shifted to the heavier fraction. The ER marker (antimycin A-insensitive NADH Cyt *c* reductase activity) shifted from the 8/29% interface (no Mg^{2+}) to the 29/45% interface when Mg^{2+} was added (Table II). These results suggest that most of the vanadate-sensitive Ca^{2+} transport system is localized on the ER, while the vanadate-insensitive (or NO_3^--sensitive) Ca^{2+} transport system is associated with low-density smooth membranes.

Properties of the Vanadate-Insensitive Calcium Transport. We tested the possibility that the proton-motive force from the tonoplast-type H^+-ATPase (8, 9) was used to drive Ca^{2+} accumulation via a Ca^{2+}/H^+ antiport mechanism. If so, the vanadate-insensitive Ca^{2+} uptake would have the same properties as the tonoplast-type H^+-ATPase (34). Furthermore, a ΔpH (acid inside) should be collapsed by Ca^{2+} entry. The following results support this model.

Dependence of Calcium Transport on a pH Gradient. Nigericin, an ionophore which exchanges K^+ for H^+ and thus dissipates pH gradients in low density vesicles from oat roots (8), eliminated active Ca^{2+} transport (Table III). Similar results were seen with protonophores such as CCCP and gramicidin which equilibrate H^+ across the membrane (Table III). CCCP plus valinomycin in the presence of K^+ completely eliminated active Ca^{2+} transport, and dissipated the H^+-electrochemical gradient (Table III). $(NH_4)_2SO_4$ or NH_4Cl at 10 mM act as uncouplers and also inhibited Ca^{2+} transport (Table III). These results suggest that Ca^{2+} accumulation is driven by a pH gradient.

When nigericin was added after a Ca^{2+} gradient was established, Ca^{2+} loss from the vesicles was fairly rapid ($T_{1/2} \sim 10$ min) (Fig. 4), suggesting that a steady state Ca^{2+} gradient is maintained by continuous Ca^{2+} accumulation dependent on a ΔpH. The rate of Ca^{2+} accumulation at steady state would be equivalent to the rate of Ca^{2+} loss seen when the system is uncoupled (Fig. 4).

Chloride Requirement. The vanadate-insensitive Ca^{2+} transport was stimulated 15-fold by 20 mM Cl^- (Table IV). Cations had little or no effect as both KCl and BTP-Cl stimulated Ca^{2+} accumulation (data not shown). These results are consistent with those of Churchill and Sze (8, 9) where Cl^- stimulated ΔpH formation and dissipated the electrical potential. Oxalate (30 mM) did not alter Ca^{2+} accumulation in the vesicles (data not shown). Chloride stimulation of the vanadate-sensitive Ca^{2+} transport may also reflect a requirement to dissipate the electrical potential.

Inhibitor Sensitivities. DCCD, a proton channel blocker, inhibited Ca^{2+} transport in the same concentration range as its inhibition of the tonoplast H^+-ATPase (34). DCCD (50 μM) inhibited Ca^{2+} transport 75% (Table I). DIDS inhibited the vanadate-insensitive Ca^{2+} transport by 40% at 2 to 4 μM (Fig. 5A), similar to its effect on both H^+-pumping and tonoplast ATPase activity (9) (Table I). At 4 μM DIDS, the vanadate-sensitive Ca^{2+} transport was not affected (Fig. 5B).

Mg-ATP Concentration Dependence. Calcium uptake was dependent on Mg-ATP concentration (Fig. 6). Analysis of a Hanes-Woolf plot yielded an apparent K_m for Mg-ATP of 0.24 mM.

pH Dependence. The vanadate-insensitive (or the NO_3^--sensitive) Ca^{2+} uptake showed a broad pH dependence with optimal activity at pH 6 to 7 and at pH 8.0 (Fig. 7). These results have been observed in four out of five experiments.

Effect of Calcium on ΔpH. The ATP-dependent pH gradient (acid inside), measured as [^{14}C]methylamine accumulation, was progressively reduced with increasing Ca^{2+} concentrations (Fig. 8). Since Ca^{2+} (up to 1 mM) did not inhibit the tonoplast-type ATPase activity (data not shown), the results suggest Ca^{2+} was taken up in exchange for H^+ loss. One can examine the effect of Ca^{2+} concentration on H^+ loss by assuming the initial rate of methylamine loss from the vesicles reflected H^+ efflux. Initial rate of methylamine loss as a function of Ca^{2+} concentration showed apparent saturation kinetics, supporting the idea that Ca^{2+} uptake and H^+ loss occur via a Ca^{2+}/H^+ exchange system.

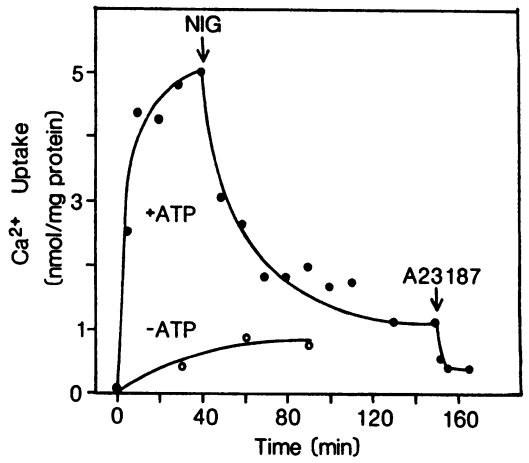

FIG. 4. Decrease of Ca^{2+} gradient by nigericin in sealed microsomal vesicles of oat roots. Ca^{2+} uptake into vesicles in the presence (●) or absence (○) of ATP was determined in a 1 ml reaction mixture as described in Figure 1 plus 100 μM Na orthovanadate. After a 40 min incubation at 22°C, 4 μl of 1 mM nigericin was added to give a final concentration of 5 μM. Aliquots of 50 μl were filtered and the amount of $^{45}Ca^{2+}$ associated with the vesicles was measured. One experiment representative of two.

Table III. *Effect of Ionophores and Uncouplers on ATP-Dependent Calcium Uptake and Methylamine Uptake into Sealed Microsomal Vesicles of Oat Roots*

Reaction mixtures for calcium uptake were as described in Figure 1 plus 100 μM Na vanadate.

Addition	ATP-Dependent Transport	
	[^{14}C]CH_3NH_2[a]	$^{45}Ca^{2+}$
	%	
None	100	100
NH_4Cl (10 mM)	10	19
$(NH_4)_2SO_4$ (10 mM)	ND[b]	23
Nigericin (5 μM)	13	18
CCCP (5 μM)	14	20
Gramicidin (5 μM)	32	33
CCCP + valinomycin (5 μM)	0	0

[a] Data from Churchill and Sze (8). [b] Not determined.

Table IV. *Effect of 20 mM Chloride on ATP-Dependent Calcium Uptake into Sealed Microsomal Vesicles of Oat Roots*

Reaction mixtures were as described in Figure 1 with or without KCl. Chloride stimulation (ΔCl^-) is calculated as the difference in uptake in the presence and absence of Cl^-.

	Type of Transport	ATP-Dependent Ca^{2+} Uptake		
		$-Cl^-$	$+Cl^-$	ΔCl^-
		nmol/mg protein·10 min		
A.	Total	0.75	11.6	10.85
B.	Vanadate-insensitive	0.63	8.3	7.62
	NO_3^--sensitive	0.75	8.5	7.75
C.	Vanadate-sensitive	0.09	3.3	3.21
	NO_3^--insensitive	0	3.5	3.50

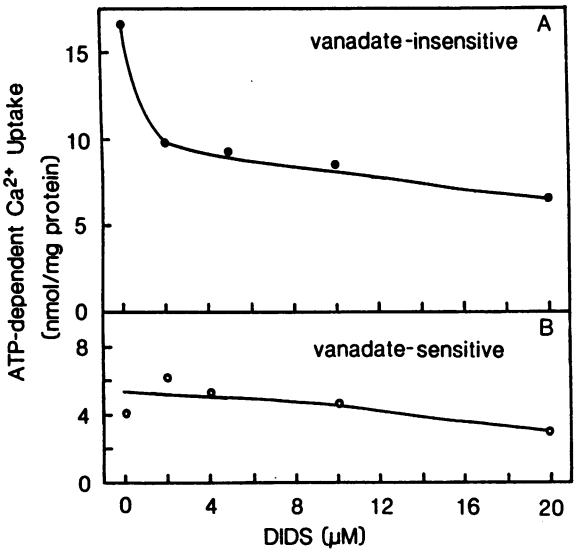

FIG. 5. Differential inhibition by DIDS of the vanadate-insensitive and vanadate-sensitive Ca^{2+} transport systems. Reaction mixtures were as in Figure 1 except 100 μM vanadate was added when appropriate. A, DIDS inhibition of the vanadate-insensitive Ca^{2+} transport system; B, resistance to DIDS of the vanadate-sensitive Ca^{2+} pump. Results are an average of three experiments.

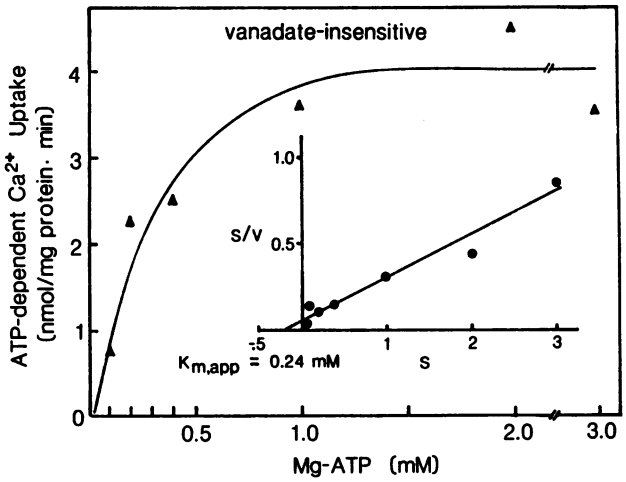

FIG. 6. Mg-ATP concentration dependence of the vanadate-insensitive Ca^{2+} uptake into sealed microsomal vesicles from oat roots. Hanes-Woolf plot showed a K_m apparent of 0.24 mM. NO_3^--sensitive Ca^{2+} transport activity also showed saturation kinetics as a function of Mg-ATP concentration with a K_m apparent of 0.13 to 0.25 mM (not shown). Results are the average of three experiments.

Calcium Concentration Dependence. To determinine the affinity of the Ca^{2+}/H^+ antiport for Ca^{2+}, initial rates of Ca^{2+} uptake (at 5 min) were measured over a range of Ca^{2+} concentrations from 10 to 200 μM. An apparent K_m for Ca^{2+} of 14 μM was determined by a linear regression analysis of a Hanes-Woolf plot (Fig. 9). These results show the Ca^{2+}/H^+ antiport is mediated by a saturable system. Saturation was not due to a limiting ΔpH as Ca^{2+} concentrations up to 1 mM did not completely dissipate ΔpH (Fig. 8).

Artificial pH Gradient Driven Calcium Uptake. An artificial pH gradient ([^{14}C]methylamine accumulation) was generated by diluting K^+-loaded vesicles into a K^+-free medium with nigericin (Fig. 10). In the presence of Ca^{2+}, nigericin caused accumulation of Ca^{2+} (Fig. 10) which could be released with A23187. In the absence of nigericin, neither methylamine nor Ca^{2+} were accumulated suggesting that nigericin exchanged K^+ for H^+ to generate a pH gradient which can drive Ca^{2+} accumulation.

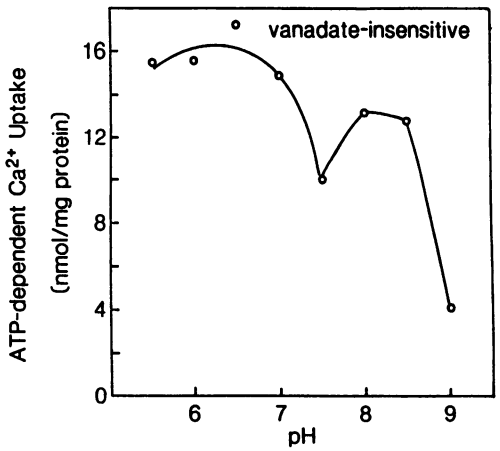

FIG. 7. pH dependence of the vanadate-insensitive ATP-dependent Ca^{2+} transport in sealed microsomal vesicles from oat roots. Reaction mixtures were as described in Figure 4. The pH was adjusted with 25 mM Hepes-BTP. One experiment representative of four. NO_3^--sensitive Ca^{2+} transport showed similar pH dependence.

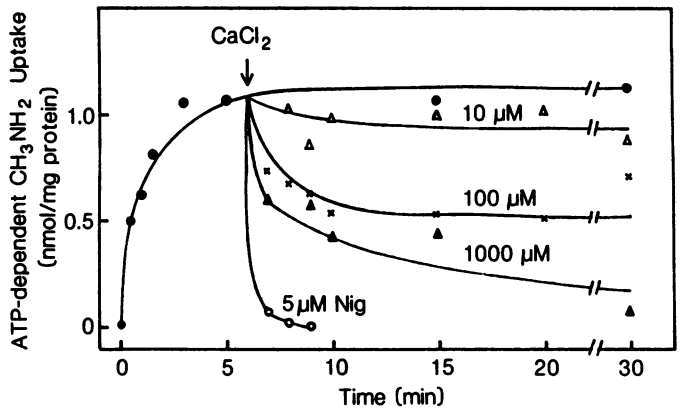

FIG. 8. Dissipation of a pH gradient by Ca^{2+} in sealed microsomal vesicles from oat roots. ATP was used to generate a pH gradient (acid inside), as measured by [^{14}C]methylamine accumulation (O). The 1.0 ml reaction mixture contained 25 mM Hepes-BTP (pH 7.0), 175 mM mannitol, 20 mM BTP-Cl, 3 mM $MgSO_4$, 0.2 mM NaN_3, 20 μM [^{14}C]methylamine (1–1.5 μCi/ml), with or without 3 mM ATP. At 6 min, 10 μl of nonradiolabeled Ca^{2+} solutions were added to give final concentrations of 10 (Δ), 100 (×), or 1000 μM (▲). Aliquots of 100 μl were removed and filtered. Nigericin (5 μM) decreased methylamine associated with the vesicles to passive levels (O).

DISCUSSION

Ca^{2+}/H^+ Antiport is Dependent on a ΔpH Generated by the Tonoplast-type H^+-ATPase. We have shown that a ΔpH generated by the tonoplast-type H^+-ATPase drives Ca^{2+} accumulation by a Ca^{2+}/H^+ antiport as summarized below (see model on Fig. 11). (a) When the pH gradient, established either with ATP or artificially, was dissipated (ionophores, uncouplers), Ca^{2+} transport was abolished (Table III; Fig. 10). (b) An artificially imposed pH gradient could drive Ca^{2+} accumulation (Fig. 10). (c) A pH gradient (acid inside) generated by the tonoplast H^+-ATPase could be dissipated by Ca^{2+} (Fig. 8). (d) Requirements for ATP-dependent Ca^{2+} transport mimic those for H^+ pumping (8) or ATPase activity (34) in terms of ATP concentration dependency (Fig. 6), and pH optimum (Fig. 7). (e) Both Ca^{2+} uptake and H^+-

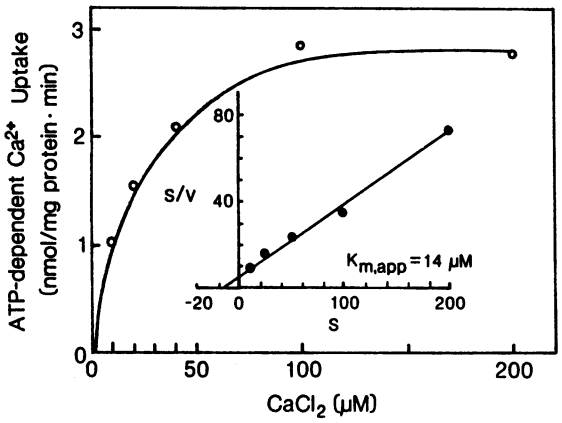

FIG. 9. Effect of Ca^{2+} concentration on the vanadate-insensitive Ca^{2+} uptake into sealed microsomal vesicles from oat roots. Reaction mixtures were as described in Figure 4 with $CaCl_2$ concentrations varying from 10 to 200 μM. Uptake at 5 min reflected initial rates of transport. Hanes-Woolf plot showed a K_m apparent for Ca^{2+} of 14 μM. Data represent the average of three experiments.

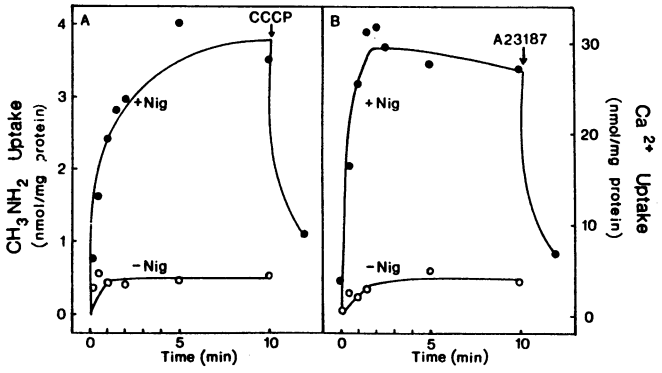

FIG. 10. Artificially generated pH gradient and its effect on Ca^{2+} uptake in sealed microsomal vesicles from oat roots. A, pH gradient generation (acid inside) as measured by [^{14}C]methylamine uptake. Vesicles preloaded with 150 mM KCl were diluted into a KCl-free medium containing 25 mM Hepes-BTP at pH 7.0 and 20 μM [^{14}C]methylamine. Nigericin (5 μM) in ethanol (●) or ethanol alone (○) was added to give a final concentration of 0.5%. B, Δ pH-dependent $^{45}Ca^{2+}$ uptake into vesicles. Vesicles preloaded with 150 mM KCl were diluted into a KCl-free medium with 25 mM Hepes-BTP (pH 7.0), 10 μM $^{45}CaCl_2$, with (●) or without (○) nigericin (5 μM).

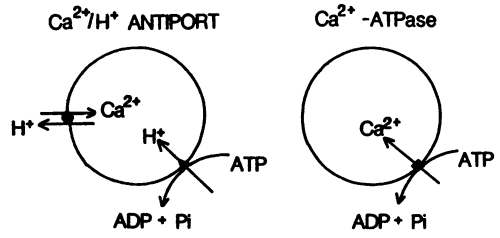

FIG. 11. Working model of two types of ATP-dependent Ca^{2+} transport systems. Ca^{2+}/H^+ antiport dependent upon a pH gradient from a H^+-pumping ATPase (left) and a Ca^{2+}-pumping ATPase (right). Our results suggest the Ca^{2+}/H^+ antiport is located on the tonoplast and a Ca^{2+}-ATPase is associated with the ER. Assuming that ATP is accessible only to the cytoplasmic face, Ca^{2+} accumulation would occur in right-side-out vesicles of the tonoplast and ER (32).

ATPase activity were insensitive to mitochondrial (azide) and plasma membrane H^+-ATPase (vanadate) inhibitors (Table I). Furthermore, the vanadate-insensitive Ca^{2+} transport was affected by all conditions which modulated the H^+-ATPase directly (stimulation by Cl^-, inhibition with DIDS, NO_3^- and DCCD) (Tables I and IV). (f) Finally, the density of vesicles showing Ca^{2+} accumulation (1.09–1.13 g/cm^3) and tonoplast H^+-ATPase activity (1.10–1.13 g/cm^3) were similar (Fig. 3; 7, 8). From the results of (c) to (f), we conclude that the ΔpH-dependent Ca^{2+} transport system is of tonoplast origin. The possibility that this Ca^{2+} transport activity may also be localized on the SER and Golgi cannot be ruled out at present. Association of the vanadate-insensitive, NO_3^--sensitive Ca^{2+} transport to vesicles prepared from intact vacuoles provides direct evidence that the Ca^{2+}/H^+ exchange system is localized on the tonoplast (5).

The Ca^{2+}/H^+ antiport of oat roots described here may be similar to exchange systems characterized in vesicles from other plants and organisms. Similar transport systems have been identified in *Escherichia coli* plasma membrane (2), *Neurospora* plasma membrane (29), and yeast vacuole (25) preparations. In higher plants, Hager and Hermsdorf (14) gave indirect evidence for a Ca^{2+}/H^+ antiport system based on the ability of Ca^{2+} to dissipate a DCCD-sensitive pH gradient. They suggested the antiport was localized on the ER and/or Golgi. Rasi-Caldogno *et al.* (27) identified a FCCP-sensitive Ca^{2+} transport activity in a low-density, nonmitochondrial fraction from pea internodes but did not identify the membrane or the source of the ΔpH. In light of our results, it is possible that the Ca^{2+}/H^+ antiport systems seen in pea stems (27), corn coleoptiles (14), or corn roots (36) originated in large part from the tonoplast and were dependent on the tonoplast H^+-ATPase.

Vacuole and ER Sequester Cytoplasmic Ca^{2+} by Two Types of Ca^{2+} Pumps. According to our working model (32), ATP hydrolysis would be coupled to H^+ pumping into right-side-out tonoplast vesicles (Fig. 11). If so, our results suggest that vacuoles accumulate high concentrations of Ca^{2+} using a Ca^{2+}/H^+ antiport mechanism.

ER vesicles showing ATP-dependent Ca^{2+} accumulation are postulated to be oriented right-side-out (Fig. 11) (32). Based on its sensitivity to vanadate, Ca^{2+} may be accumulated in the ER lumen via a primary Ca^{2+}-pumping ATPase, perhaps similar to the SR Ca^{2+}-pumping ATPase (18). This ER pump could be similar to the one characterized from *Lepidium* roots (3, 4) although vanadate sensitivity was not determined in that system.

There seems to be little Ca^{2+} transport associated with the plasma membrane in our oat root preparations for the following reasons: (a) Ca^{2+} transport was enriched in vesicles of lighter density (1.09–1.13 g/cm^3) than plasma membranes (Fig. 3). The activity of the plasma membrane (vanadate-sensitive) H^+-ATPase distributes around 1.13 to 1.16 g/cm^3 (7), where little Ca^{2+} uptake was detected. (b) It has been difficult to prepare (in quantity) sealed plasma membrane vesicles from oat roots (capable of holding a pH gradient) for reasons that are not yet clear. Obtaining plasma membrane vesicles that can maintain ATP-dependent Ca^{2+} or other ion gradients may depend on the age or type of tissue or cell, vesicle orientation as well as the preparation and assay procedures (32). Despite our results, it is possible that a small part of the vanadate-sensitive Ca^{2+} transport (Fig. 3) originated from the plasma membrane. This Ca^{2+} transport could be driven in part by a Ca^{2+}-pumping ATPase and in part by a Ca^{2+}/H^+ antiport system which is dependent on a vanadate-sensitive H^+ pump, similar to the plasma membrane Ca^{2+}/H^+ antiport system of *Neurospora* (29).

The relative importance of the Ca^{2+} pumps has yet to be determined. In oat roots, 75 to 80% of the Ca^{2+}-pumping activity in microsomal vesicles originates from the Ca^{2+}/H^+ antiport suggesting that the vacuolar Ca^{2+} transport system may be im-

portant in removing excess Ca^{2+} from the cytoplasm. Cytoplasmic Ca^{2+} concentrations may increase from physical as well as chemical stimuli (16), causing Ca^{2+} to diffuse down its electrochemical gradient into the cytoplasm from outside the cell or from the vacuole, ER lumen or mitochondria. Active uptake mechanisms such as the vacuolar and ER Ca^{2+} pumps identified in this paper might operate to return cytosolic Ca^{2+} concentrations to unstimulated levels.

Artificial ΔpH-Driven Calcium Accumulation—Approach to Characterize the Ca^{2+}/H^+ Antiport System. The vanadate-insensitive Ca^{2+}/H^+ antiport system represents a secondary active transport system. Many of the initial characterizations shown here reflect the activity of the primary pump (the tonoplast H^+-ATPase), rather than the Ca^{2+}/H^+ antiport. Ca^{2+} accumulation using an artificially imposed pH gradient bypassed the ATPase (Fig. 10). This approach will allow us to characterize the Ca^{2+}/H^+ antiport system directly in terms of stoichiometry, Ca^{2+} affinity, substrate specificity and inhibitor sensitivity. These studies will help us determine the mechanism of Ca^{2+} transport as well as understand the physiological role for this Ca^{2+} transport system in regulating cytoplasmic Ca^{2+} levels.

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