Plant Physiol. (1991) 97, 1535–1544
0032-0889/91/97/1535/10/$01.00/0

Received for publication June 3, 1991
Accepted July 29, 1991

Calcium-Pumping ATPases in Vesicles from Carrot Cells[1]

Stimulation by Calmodulin or Phosphatidylserine, and Formation of a 120 Kilodalton Phosphoenzyme

Wen-Ling Hsieh, Wayne S. Pierce[2], and Heven Sze*

Department of Botany, University of Maryland, College Park, Maryland 20742

ABSTRACT

Ca^{2+}-ATPases keep cytoplasmic [Ca^{2+}] low by pumping Ca^{2+} into intracellular compartments or out of the cell. The transport properties of Ca^{2+}-pumping ATPases from carrot (*Daucus carota* cv Danvers) tissue culture cells were studied. ATP-dependent Ca^{2+} transport in vesicles that comigrated with an endoplasmic reticulum marker, was stimulated three- to fourfold by calmodulin. Cyclopiazonic acid (a specific inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase) partially inhibited oxalate-stimulated Ca^{2+} transport activity; however, it had no effect on calmodulin-stimulated Ca^{2+} uptake driven by ATP or GTP. The results would suggest the presence of two types of Ca^{2+}-ATPases, an endoplasmic reticulum- and a plasma membrane-type. Interestingly, incubation of membranes with [gamma^{32}P]ATP resulted in the formation of a single acyl [^{32}P]phosphoprotein of 120 kilodaltons. Formation of this phosphoprotein was dependent on Ca^{2+}, but independent of Mg^{2+}. Its enhancement by La^{3+} is characteristic of a phosphorylated enzyme intermediate of a plasma membrane-type Ca-ATPase. Calmodulin stimulated Ca^{2+} transport was decreased by W-7 (a calmodulin antagonist), ML-7 (myosin light chain kinase inhibitor) or thyroxine. Acidic phospholipids, like phosphatidylserine, stimulated Ca^{2+} transport, similar to their effect on the erythrocyte plasma membrane Ca^{2+}-ATPase. These results would indicate that the calmodulin-stimulated Ca^{2+} transport originated in large part from a plasma membrane-type Ca^{2+} pump of 120 kilodaltons. The possibility of calmodulin-stimulated Ca^{2+}-ATPases on endomembranes, such as the endoplasmic reticulum and secretory vesicles, as well as the plasma membrane is suggested.

Many stimuli-induced responses in plants are coupled to changes in cytoplasmic calcium levels (17). The role of Ca in signaling necessitates regulation of the cytosolic ion activity, accomplished in part by various Ca channels and transporters. Under most growth conditions, there is a steep electrochemical gradient favoring Ca^{2+} movement from the external medium and from organellar compartments (*e.g.*, vacuole) into the cytosol. Signal-induced passive Ca^{2+} fluxes are mediated by opening of Ca^{2+} channels on the plasma membrane and organellar membranes (28). When cytoplasmic [Ca^{2+}] increases, active Ca^{2+}-transport systems work to restore the resting cytoplasmic Ca^{2+} to submicromolar levels.

Several types of active Ca^{2+} transport systems have been documented in plant cells. The primary Ca^{2+}-pumping ATPases that couple ATP hydrolysis directly to Ca^{2+} transport have been found in the ER (2, 5, 6, 14) and plasma membrane (4, 15, 19, 25, 26, 32). Calcium also accumulates in the vacuole, via a Ca^{2+}/H^+ exchange system. H^+-ATPase and H^+-PPase generate a H^+ electrochemical gradient that can drive Ca^{2+} uptake mediated by the Ca^{2+}/H^+ antiporter (6, 11, 29). Since the affinity for Ca^{2+} of the Ca^{2+}-pumping ATPases is relatively high ($K_{m[Ca]}$ about 1 μM), these pumps are thought to play an important role in reducing Ca^{2+} concentration to submicromolar levels. Both the plasma membrane Ca-ATPase and the ER Ca-ATPase are vanadate-sensitive, suggesting the enzymes form a phosphorylated intermediate or are P-type enzymes. However, because these two types of Ca pumps share similar properties, they are difficult to differentiate based on their biochemical activities.

In animal cells, the two types of Ca-pumping ATPases which are P-type (phosphorylated intermediate) or E_1E_2-type transport ATPases, can be separated by a few key features (27). The plasma membrane Ca-ATPase, extensively studied using the human erythrocyte, has a molecular mass of 130 to 140 kD and is directly stimulated by CaM[3] (8). In contrast, the sarcoplasmic reticulum Ca-ATPases, in which the slow-type is similar to the endoplasmic reticulum Ca-ATPase, has a mol mass of 100–118 kD. This enzyme differs from the plasma membrane-type by its sensitivity to cyclopiazonic acid (30). The SR/ER type Ca-ATPase is stimulated indirectly by calmodulin-dependent kinase via phosphorylation of a regulatory protein termed phospholamban (7, 31). It is not clear at this time whether the Ca-ATPases detected in plants are strictly analogous to those from animal cells.

[1] Supported by Department of Energy Grant DE FG05–86ER13461 to H.S. and Maryland Agricultural Experiment Station, Contribution No. 8358, scientific article no. A-6189.

[2] Permanent address: Department of Biology, California State University, Stanislaus, Turlock, CA 95380.

[3] Abbreviations: CaM, calmodulin; A23187, Ca ionophore, $C_{29}H_{37}N_3O_6$; CPA, cyclopiazonic acid; E_1E_2-ATPase, P-type ATPase with two enzyme conformational states; ML-7, 1-(5-iodonaphthalene-1-sulfonyl)-1 H-hexahydro-1,4-diazepine HCl; MLC kinase, myosin light chain kinase; P-type ATPase, phosphorylated type of ion motive ATPase; PI, phosphatidylinositol; PS, phosphatidylserine; SR, sarcoplasmic reticulum; W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide HCl.

Studies to identify CaATPases from plant cells are still at an early stage and results suggest there may be variations among different plants and tissues (4, 11). For example, Briars and Evans (3, 11) identified a plasma membrane Ca-ATPase of 140 kD in corn microsomes by formation of a phosphoprotein. However, in red beets, both the ER and the plasma membrane Ca-ATPase were determined to be about 100 kD (32).

Sensitivity to calmodulin also varies from one study to another (4, 11). For example, calmodulin stimulated Ca^{2+} uptake by 100 to 300% in vesicles from corn coleoptiles (2, 10, 11), and by 30% in vesicles from red beets (32). In contrast, little or no calmodulin stimulation (0–18%) was found in several species such as pepper grass, pea, day flower and radish (5, 11, 15, 25). This discrepancy is not yet understood .

Our laboratory previously reported an ATP dependent Ca^{2+} transport activity in ER-enriched vesicles isolated from cultured carrot cells (6). Transport was inhibited by vanadate and Ca dependent kinetics showed apparent K_m[Ca] of about 1 μM. However, it was not clear whether the activity arose from an endoplasmic reticulum-type or plasma membrane-type Ca pump or both. Here we show evidence for both types of Ca pumps. Calmodulin or phosphatidylserine stimulated Ca^{2+} transport characteristic of a plasma membrane-type Ca pump. However, inhibition by cyclopiazonic acid, a specific inhibitor of the sarcoplasmic/endoplasmic reticulum, suggested that part of the Ca transport activity originated from an ER-type Ca-ATPase. Preliminary results of a part of this study were presented previously (24).

MATERIALS AND METHODS

Plant Material

Suspension cell cultures of carrot (*Daucus carota,* Danvers) were subcultured weekly into Murashige and Skoog medium (6) supplemented with 1 mg/L dichlorophenoxyacetic acid and 0.1 mg/L 6-benzyladenine. Experiments were routinely performed on the cells 5 d after subculturing.

Preparation of Membrane Vesicles

Vesicles were prepared by the method of Bush and Sze (6) with some modifications. All isolation procedures were conducted at 4°C. Cells were collected on filter paper using a Buchner funnel and washed with about 3 volumes of cold deionized water. Three flasks were used with a typical yield of 13 to 14 g. Cells were homogenized by mortar and pestle in a medium containing 250 mM sucrose, 25 mM Hepes-BTP (pH 7.4), 3 mM EGTA, 1 mM DTT, 1 mM PMSF and 0.5% BSA using a grinding medium to tissue ratio of 1 mL per g (4 min), 0.5 mL/g (3 min), and 0.5 mL/g (3 min) for each of the three grinds, respectively. After filtration through 6 layers of cheesecloth, the homogenate was centrifuged at 1000*g* for 10 min, and 7500*g* for 20 min to remove cellular debris and mitochondria. The supernatant was layered on a sucrose step gradient (initially of 8 mL 35%, 6 mL 26%, and 6 mL 15%, and later modified to 6 mL 32%, 5 mL 22%, and 5 mL 15%) and centrifuged at 70,000*g* for 2 h. Gradient solutions contained 2.5 mM Hepes-BTP pH 7.2, 0.5 mM DTT and 1 mM PMSF. After centrifugation, the 26/35% or 22/32% interface was collected, and frozen at -70°C (for subsequent transport studies).

To further purify the membranes, the 26/35% interface was diluted four-to fivefold and layered on a continuous (20–45%) sucrose gradient (without DTT which interferes with Cyt *c* reductase activity). After centrifugation for 3 h at 100,000*g* (23,000 rpm, SW 28), fractions of 1.2 mL were collected and immediately frozen at -70°C for subsequent use.

Ca^{2+} Transport

ATP-dependent $^{45}Ca^{2+}$ uptake into membrane vesicles was measured by the filtration method (6, 29) at 22°C. Transport was initiated by adding 50 to 100 μL of vesicles (35–85 μg protein) to a reaction mixture (final vol. 0.5 mL) containing 200 mM sucrose, 25 mM Hepes-BTP pH 7.0, 0.1 mM NaN_3, 10 μM $CaCl_2$ (0.5 μCi ^{45}Ca/mL), 10 mM KCl, 3 mM $MgSO_4$, 3 mM ATP, with or without 10 mM K-oxalate. Duplicate 0.2 mL aliquots were filtered onto 0.45 μm Millipore filters premoistened and washed with 2 mL of cold rinse solution (250 mM sucrose, 2.5 mM Hepes-BTP pH 7.0 and 0.2 mM $CaCl_2$). The radioactivity associated with the filters was determined by liquid scintillation counting. Active transport was defined as the difference between uptake in the presence and absence of MgATP.

To study the effects of phospholipids, precautions to prevent oxidation were taken. Phospholipids in $CHCl_3$ were dried by N_2, dissolved in ether and vaporized under N_2. After drying for 20 min in vacuum, phospholipids were sonicated for 15 min (5 min intervals) at 4°C with 20 mM Hepes-BTP buffer at pH 7.0, and used immediately.

Enzyme Assays and Protein Determination

Ca^{2+}-ATPase activity was determined in a mixture containing (final concentrations) 25 mM Hepes-BTP pH 7.0, 3 mM ammonium molybdate, 0.5 mM NaN_3, 20 mM KNO_3, 3 mM ATP-BTP pH 7.0, 3 mM $MgSO_4$, 2 μg/mL A23187, and 0.5 mM EGTA or 20 μM $CaCl_2$. Reactions were initiated by addition of ATP and incubated at 35°C for 30 min. Inorganic phosphate (Pi) was determined by the Fiske and SubbaRow method (13). Antimycin A-insensitive NADH Cyt *c* reductase activity was assayed (6) by monitoring the reduction of cytochrome *c* spectrophotometrically at 550 nm.

Protein was determined according to Bradford (1) after precipitation with 10% TCA by using BSA as standard.

Formation of Acyl [^{32}P]Phosphoprotein

The formation of [^{32}P]phosphorylated proteins in vesicles collected from the 22/32% sucrose interface was performed using published methods (3, 23). Vesicles were diluted 10 fold with suspension buffer (250 mM sucrose, 25 mM Hepes-BTP pH 7.0) and centrifuged for 1 h at 185,000*g* (45,000 rpm, Ty65) to concentrate the membranes. The pellet (0.8–1 mg) was resuspended in 1 to 1.5 mL suspension buffer. The reaction mixture (0.4 mL) contained 70 to 150 μg membrane protein, 8 nM or 100 nM [gamma-^{32}P]ATP (4–10 μCi, Amersham), 50 μM $CaCl_2$, 100 mM KCl, 25 mM Hepes-BTP pH

6.0, with or without 12 μM $MgSO_4$. In a control mixture, 500 μM EGTA was added instead of Ca. Various inhibitors were added to a final concentration of 50 μM $LaCl_3$, 100 nmol CPA/mg protein or 100 μM Na vanadate. [^{32}P]ATP was added to start the reaction. After 15 s at 0°C, the reaction was terminated by the addition of 1 mL of ice cold 10% TCA, 1 mM ATP, and 5 mM NaH_2PO_4. The effect of hydroxylamine was tested by washing the acid-precipitated proteins in 50 mM hydroxylamine made in 100 mM 2(N-morpholino)ethanesulphonic acid (MES) brought to pH 6.0 with KOH. Control proteins were washed in MES/KOH buffer alone. The pelleted proteins were then washed with ice cold stop solution and in water.

The pellets were suspended in SDS-PAGE sample buffer containing 2% SDS, 10% glycerol, 5% mercaptoethanol, 100 mM sodium phosphate buffer pH 6.0, 0.001% bromophenol blue or pyronin Y, 8 M urea and 5 mM PMSF. After incubation on ice for 30 min, proteins were separated with a 5% acrylamide gel (20) modified to pH 6.0 with Na phosphate buffer. The running buffer and the electrode buffer were modified to pH 6. Polypeptides and molecular mass standards run in adjacent lanes were stained with Coomassie blue (22). Gels were dried using a Biorad 583 gel drier and exposed for 3 hr or overnight with Kodak XAR-5 film at -70°C. The intensity of the bands were quantified using a Biorad video-300A gel densitometer. Apparent molecular mass was estimated by comparison of autoradiographs with stained standards (Biorad high and low molecular mass standards). In some cases, the bands corresponding to the [^{32}P]phosphorylated proteins were excised and the radioactivity was determined by scintillation spectroscopy (Beckman LS 3801).

Chemicals

$^{45}CaCl_2$ was obtained from New England Nuclear. ATP, obtained from Boehringer Mannheim as the disodium salt, was converted to ATP-BTP using Dowex 50W ion exchange resin and titrating with BTP to pH 7. Gamma-labeled [^{32}P] ATP (3 Ci/mmol) was obtained from Amersham as the triethylammonium salt. Calmodulin (bovine brain type) was obtained from Sigma. All other chemicals were reagent grade.

RESULTS

Our primary goals were to characterize the properties of Ca transport in vesicles as a means to identify the types of Ca-pumping ATPases in carrot cells, and to begin studies to understand the regulation of Ca pumps. Since regulatory proteins might be peripheral to the membrane, we have chosen to use isolation methods that would be least disruptive in order to maintain native conditions. For these reasons, our approach to distinguish between the endoplasmic reticulum-type and plasma membrane-type CaATPase initially involved characterization of the Ca transport properties in native membrane vesicles.

Calmodulin-Stimulated Ca^{2+} Transport Comigrated with an ER Marker

Oxalate Stimulation

In the presence of oxalate, the rate of Ca^{2+} uptake into the vesicles was linear for 40 min or more, and the total Ca^{2+} taken up was enhanced by 10 to 20-fold (Fig. 1b). In contrast, in the absence of oxalate, the initial rate of Ca^{2+} uptake was linear for less than 5 min (Fig. 1a). Oxalate stimulation is thought to be caused by formation of a Ca-oxalate precipitate inside the vesicles (6), thus decreasing the magnitude of the Ca^{2+} chemical gradient across the membrane. This interpretation implies that an oxalate transporter or channel is present on the membrane. A23187, a Ca^{2+} ionophore, was unable to dissipate the Ca^{2+} gradient rapidly (Fig. 1b), probably due to the formation of Ca-oxalate precipitate inside the vesicles. Other anions were less effective than oxalate. Potassium phosphate (10 mM) slightly stimulated net Ca^{2+} uptake (2.7-fold) while sulfate had no effect (0.85-fold).

Calmodulin Stimulation

Calmodulin stimulated Ca^{2+} transport by 2–4 fold (Fig. 2), and about 10 μg/mL (or 0.6 μM) calmodulin was required to

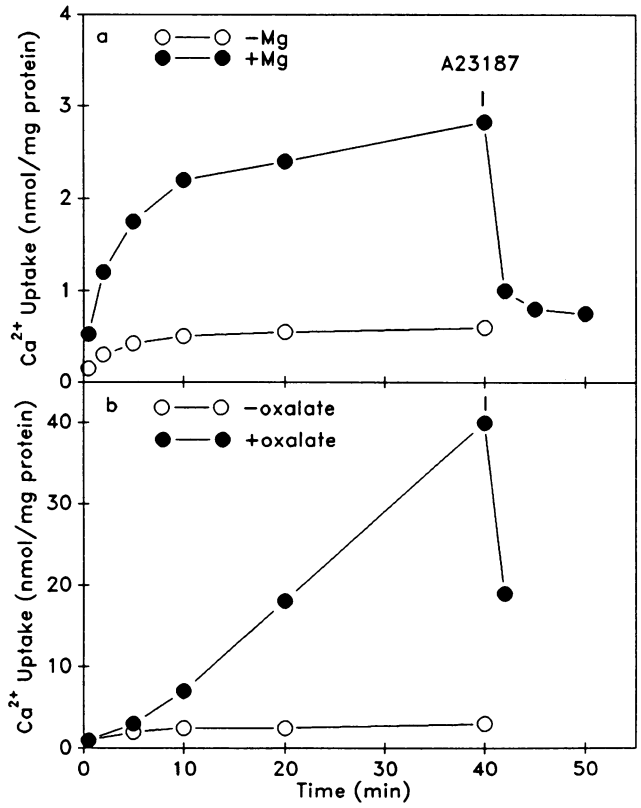


Figure 1. Oxalate stimulation of ATP-dependent Ca^{2+} uptake into microsomal vesicles (26/35% sucrose). Reaction mixtures in (a) contained 200 mM sucrose, 25 mM Hepes-BTP pH 7.0, 20 mM KCl, 0.2 mM NaN_3, 3 mM ATP, 10 μM $CaCl_2$ with (●) or without 3 mM $MgSO_4$(o). A23187 (in ethanol) was added at 40 min to a final concentration of 2.5 μg/mL. MgATP-dependent Ca^{2+} uptake in (b) was measured as described in the absence (o) or presence (●) of 10 mM K_2-oxalate.

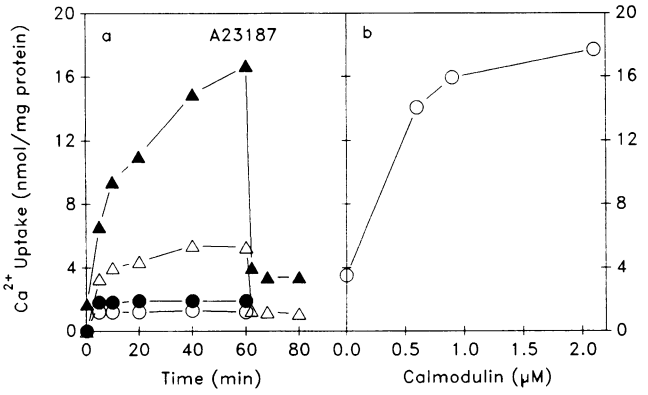


Figure 2. Calmodulin stimulated Ca^{2+} uptake in vesicles (22/32% sucrose interface). (a) Reaction mixtures are as described in Figure 1, with (△, ▲) or without (o,o) 3 mM $MgSO_4$ and with (▲, ●) or without (△, o) 0.3 μM (5 μg/mL) calmodulin. Final concentration of A23187 was 2.5 μg/mL. (b) Effect of calmodulin concentration on Ca^{2+} uptake (nmol/mg) at 30 min.

give maximal stimulation (Fig. 2b). Calmodulin decreased the affinity for ATP as the apparent K_m increased from 0.30 (-CaM) to 0.67 mM (+CaM) (not shown). The V_{max} was enhanced from 1.01 (-CaM) to 4.97 nmol/mg protein.min (+CaM).

Calmodulin also stimulated Ca^{2+}-dependent ATPase activity by threefold. In the presence of EGTA, ATP hydrolysis was 2.72 μmol/mg.h. Ca^{2+} (20 μM) consistently stimulated ATP hydrolysis to 3.49 μmol/mg.h. Calmodulin further stimulated ATP hydrolysis to 4.77 μmol/mg.h. The specific activity of Ca-ATPase (12 nmol/mg.min) was relatively low in membrane fractions suggesting that the Ca pump made up a minor component of the total membrane protein. Since the basal (Ca independent) Mg-ATPase activity represented about 80% of the total activity (+Ca), we have used ATP-dependent Ca transport activity, a more specific assay, to characterize the Ca pump in all subsequent studies.

Ca Transport Comigrate with an ER Fraction

Bush and Sze (6) had shown that Ca^{2+} transport in vesicles isolated from carrot cells was associated with ER by a Mg-shifting protocol, where the oxalate-stimulated Ca^{2+} transport activity comigrated with the antimycin A-insensitive NADH cytochrome *c* reductase activity (6). Here we show that most of the calmodulin-stimulated Ca^{2+} transport also comigrated with this enzyme activity on sucrose gradients to a density of 1.12 g/mL. However, a plot of the specific activity revealed two peaks of calmodulin-stimulated Ca^{2+} transport (Fig. 3a): one at 1.10 to 1.12 g/mL (25–28% sucrose) comigrated with the activities of Cyt *c* reductase and oxalate-stimulated Ca^{2+} transport; and another peak of calmodulin-stimulated Ca^{2+} transport at 1.16 to 1.18 g/mL (37–41% sucrose) was not accompanied by Cyt *c* reductase activity. It is possible that the activity at 1.16 to 1.18 g/mL is due to the plasma membrane Ca^{2+}-ATPase, although this fraction represented a minor component (5%) of the total Ca transport activity. Furthermore, the putative plasma membrane Ca^{2+}-ATPase was stimulated slightly by oxalate (two- to fivefold), whereas the ER-associated Ca^{2+}-ATPase was stimulated 10- to 15-fold (Fig. 3b).

In subsequent experiments, we routinely prepared vesicles by separating the 7000*g* supernatant on a discontinous sucrose gradient and collecting vesicles at the 22/32% sucrose interface. For simplicity, this fraction was referred to as the ER-enriched vesicles.

Inhibition by Cyclopiazonic Acid

CPA, an indole tetramic acid (Fig. 4), has been reported as a specific inhibitor of the SR Ca^{2+}-ATPase (both slow and fast-twitch muscles) (30). CPA is thought to stabilize the Ca-ATPase in an enzymatically inactive conformation. Because the inhibition of ATPase activity by CPA was particularly pronounced at low ATP concentration (30), we reduced ATP in the reaction mixture to 0.6 mM. CPA inhibited Ca^{2+} transport in the presence of oxalate by 30–57% at 100 nmol/mg in ER-enriched vesicles (Fig. 4). At 100 nmol/mg, CPA inhibited the SR Ca^{2+}-ATPase by 59 to 89%, however, it had no effect on the plasma membrane-type Ca^{2+}-ATPase (erythrocyte) even at 1000 nmol of CPA/mg protein (30). These results further confirm that part of the Ca^{2+} transport in vesicles from carrot cells was catalyzed by an ER-type Ca^{2+}-ATPase.

It is possible the membrane preparation contained a mix-

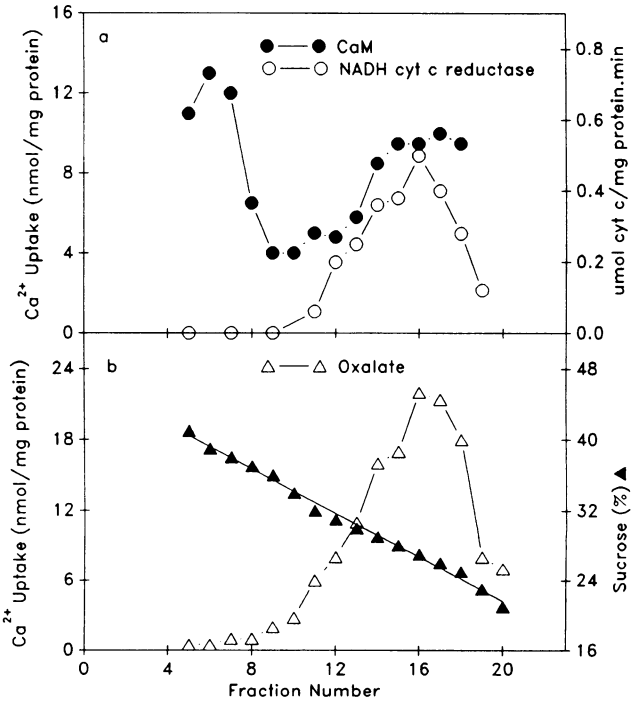
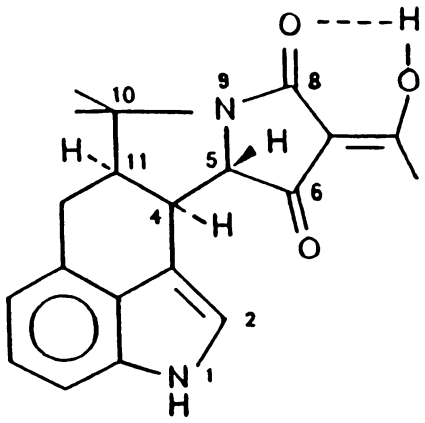


Figure 3. Calmodulin- and oxalate-stimulated Ca^{2+} uptake comigrates with the ER marker on a continuous sucrose gradient. Vesicles from a 26/35% interface were diluted and separated with a linear 20–45% sucrose gradient. Transport period was 30 min. Calmodulin or K_2-oxalate were present at 5 μg/mL or 10 mM, respectively. (a) Calmodulin (CaM) stimulated Ca^{2+} transport and antimycin A-insensitive NADH Cyt *c* reductase. (b) Oxalate-stimulated Ca^{2+} transport.



The structure of cyclopiazonic acid.

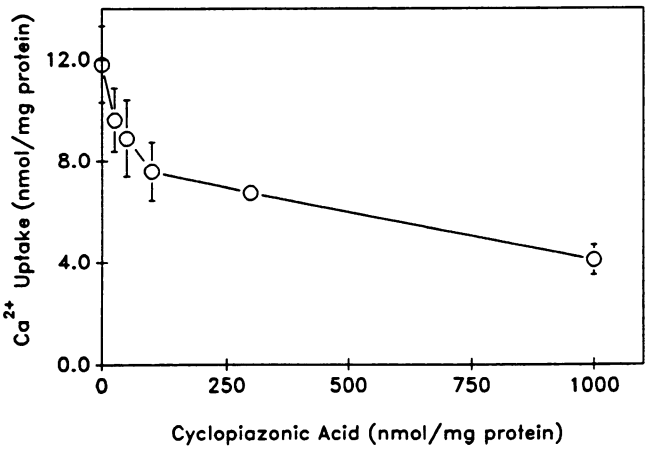


Figure 4. Effect of cyclopiazonic acid (CPA) concentration on Ca^{2+} uptake into vesicles (22/32%) from carrot cells. Reaction mixtures contained 25 mM Hepes-BTP pH 7.0, 250 mM sucrose, 10 mM KCl, 0.1 mM NaN_3, 10 mM oxalate, 5 mM KNO_3, 10 μM $^{45}CaCl_2$, 3 mM $MgSO_4$, and 0.6 mM ATP. Net ATP-dependent Ca^{2+} uptake for 20 min was measured. Data represent an average of three experiments. Error bars indicate ± SE.

ture of Ca-ATPases, due to the presence of CPA-insensitive activity. Interestingly, there was little or no reduction of the calmodulin-stimulated Ca transport by 100 nmol/mg CPA (Table I), suggesting that most of the calmodulin-dependent activity was insensitive to CPA. Furthermore, GTP, an alternative substrate for the plant plasma membrane CaATPase (32), supported Ca transport that was stimulated by calmodulin, but insensitive to CPA (Table I). To distinguish the two types of Ca pumps, we determined the mol mass of Ca-pumping ATPases by formation of Ca-dependent phosphoenzyme.

Formation of an Acyl Phosphoprotein

Incubation of membranes with $\gamma[^{32}P]$ATP for 15 s resulted in the formation of a single phosphoprotein with an apparent molecular mass of 120 kDa. Formation of the phosphoenzyme was dependent on Ca^{2+} but not on Mg^{2+} (Fig. 5). Our results are consistent with the proposed reaction cycle of E_1E_2-

Table I. *Effect of CPA on CaM-Stimulated Ca Uptake into Vesicles from Carrot Cells*

Net Mg-dependent Ca^{2+} uptake (20 min) was determined in reaction mixtures as described in Figure 2, except NTP and CaM concentrations were 0.6 mM and 1 μM, respectively.

Substrate	CPA Concn.	Net Ca^{2+} Uptake		
		−CaM	+CaM	CaM-stimulated
	nmol/mg		*nmol/mg*	%
ATP	0	1.57	5.64	4.07 100
	100	1.61	5.21	3.60 88
	200	1.43	4.85	3.42 84
GTP	0	1.08	4.80	3.72 100
	100	1.02	4.73	3.71 100
	200	1.02	4.56	3.55 95

type Ca^{2+}-ATPases (Fig. 6 taken from ref. 9), where Mg facilitates dephosphorylation. In addition, hydroxylamine decreased the amount of phosphoprotein (Fig. 5, lane 6) relative to a buffer-treated control (Fig. 5, lane 5). Little or no $[^{32}P]$ phosphoprotein was detected when excess (1 mM) unlabeled ATP was added (data not shown). The rapid turnover and the sensitivity to hydroxylamine indicate that this phosphoenzyme was formed from an acyl-phosphate linkage characteristic of E_1E_2-type transport ATPases (3, 8, 27). The phosphoenzyme was identified as a Ca^{2+}-ATPase based on the requirement for Ca^{2+} and the enhancement by La^{3+}.

Formation of this Ca^{2+}-dependent phosphoenzyme was sensitive to La and vanadate. Lanthanum increased the phosphorylation by 34% as estimated by densitometric spectroscopy (Fig. 6). La^{3+} is thought to block the conversion of E_1P

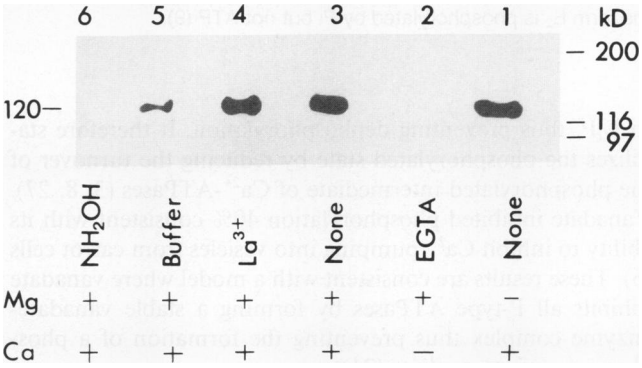


Figure 5. Calcium-dependent formation of an acyl $[^{32}P]$phosphoprotein in membranes from carrot cells. [gamma-^{32}P] ATP (4–10 μCi) 8 nM was added to a reaction mixture (0.4 mL) containing 70–150 μg membrane protein, 25 mM Hepes-BTP pH 6.0, and 100 mM KCl. Other additions are as indicated: 50 μM $CaCl_2$, 500 μM EGTA, 12 μM $MgSO_4$ and 50 μM $LaCl_3$. After incubation on ice for 15 s, the reaction was terminated by a solution containing 10% TCA, 1 mM ATP, and 5 mM NaH_2PO_4 (1 mL). In lane 5 or 6, the pelleted vesicles were washed with either buffer alone or with 50 mM hydroxylamine in 100 mM Mes/KOH pH 6.0 prior to SDS-PAGE (5%) at pH 6.0. The gel was dried and exposed for 3 hr with X-ray film at -70°C. Results are from one representative experiment of five.

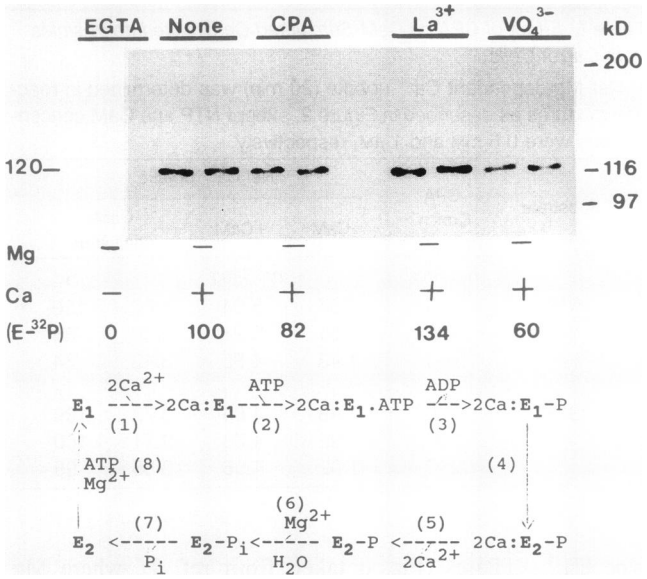


Figure 6. (Top panel) Effect of lanthanum, vanadate and CPA on formation of a phosphoprotein in membranes from carrot cells. Mg^{2+} was absent from the duplicate reaction mixtures which were similar to that described in Fig 5. The reaction was started by the addition of 100 nM [gamma-^{32}P]-ATP (4 μCi). Other additions are as indicated: 100 nmol CPA/mg protein, or 50 μM $LaCl_3$ or 100 μM vanadate. The relative amount of the phosphoprotein was estimated with a densitometer, and the control was set to 100. Results are from one representative experiment of three. (Bottom panel) The reaction sequence shows the steps involved in the process of ATP hydrolysis and Ca^{2+} transport. This sequence includes two distinct functional states of the enzyme, E_1 and E_2. The Ca^{2+} binding site in the E_1 form faces the outer surface of the vesicles and has an apparent Km for Ca^{2+} in the range of 0.2–2 μM at pH 7.0 (high affinity). In the E_2 form the Ca^{2+}-binding site faces the inner surface of the vesicles and has an apparent Km for Ca^{2+} in the range of 1–3 mM at pH 7.0 (low affinity). The E_1 form is phosphorylated by ATP but not by Pi, while the form E_2 is phosphorylated by Pi but not ATP (9).

to E_2P, thus preventing dephosphorylation. It therefore stabilizes the phosphorylated state by reducing the turnover of the phosphorylated intermediate of Ca^{2+}-ATPases (3, 8, 27). Vanadate inhibited phosphorylation 40% consistent with its ability to inhibit Ca^{2+} pumping into vesicles from carrot cells (6). These results are consistent with a model where vanadate inhibits all P-type ATPases by forming a stable vanadate-enzyme complex thus preventing the formation of a phosphoenzyme intermediate (21).

Increase of phosphoenzyme formation by La is characteristic of the plasma membrane-type CaATPase (8, 27). Cyclopiazonic acid had little effect (12–15%) on phosphorylation, similar to its effect on the calmodulin stimulated Ca transport (Table I). Furthermore, [^{32}P]GTP also labeled a single phosphoprotein of 120 kD (not shown). Taken together, these results suggested that the 120 kD phosphorylated polypeptide represented a phosphoenzyme intermediate of a plasma membrane-type Ca^{2+}-ATPase. However, the possibility of an ER-type CaATPase with the same molecular mass cannot be eliminated.

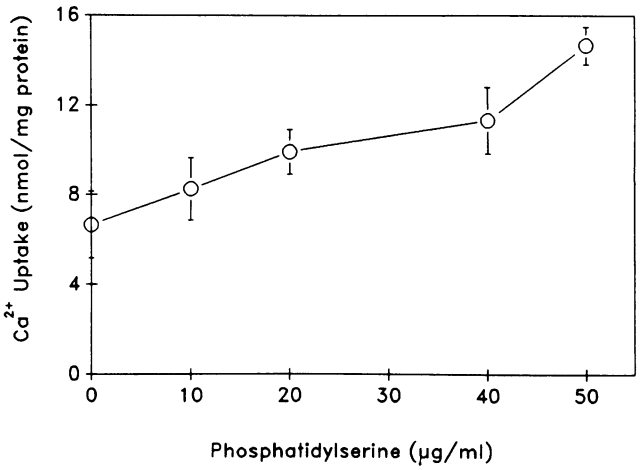


Figure 7. Phosphatidylserine (PS) stimulation of Ca^{2+} uptake into vesicles (22/32%) from carrot cells. The reaction mixture for Ca^{2+} transport contained 25 mM Hepes-BTP pH 7.0, 250 mM sucrose, 10 mM KCl, 0.1 mM NaN_3, 10 μM $^{45}CaCl_2$, 3 mM $MgSO_4$ and 3 mM ATP. Transport period was 20 min. Results are an average of four experiments. Error bars indicates ± SE.

Regulation of Ca^{2+} Transport Activity

Phosphatidylserine Stimulation

PS (40 μg/mL) stimulated Ca^{2+} transport by 1.7- to twofold and the stimulation was concentration dependent (Fig. 7). However, we found no diacylglycerol effect on Ca^{2+} uptake in our system. Phosphatidylinositol (PI) also stimulated Ca^{2+} uptake by 2.5-fold, but there was no significant effect by phosphatidylcholine or by phosphatidylethanolamine (Table II). PS stimulated the maximal transport velocity by about 2 fold (not shown). The V_{max} for ATP increased from 1.01 to 2.02 nmol/mg protein·min.

Calmodulin-Stimulated Ca Transport is Reduced by Salt Washes

To remove endogenous calmodulin, we washed vesicles with either buffer, EGTA, KCl or KI (Table III), which are

Table II. *Effect of Phospholipids on Ca^{2+} Uptake into Interface (22/32%) Vesicles from Carrot Cells*

All phospholipids were pretreated as described in "Materials and Methods" and were present at 40 μg/mL. Reaction mixtures for Ca^{2+} transport were as described in Figure 7. Transport period was 20 min at 22°C. Data are from one representative experiment of two.

Phospholipids	Relative Ca^{2+} Uptake
	%
None	100[a]
PS	218
Phosphatidylcholine	129
Phosphatidylinositol	256
Phosphatidylethanolamine	89

[a] 6.28 nmol Ca^{2+}/mg protein·20 min.

Table III. *Effect of Washing Microsomal Vesicles with EGTA or KCl on Ca^{2+} Transport*

Vesicles (125 μg protein in 0.5 mL) collected from the 22/32% sucrose interface were incubated for 30 min on ice in either 15 mL of 25 mM Hepes-BTP (pH 7.0), 3 mM EGTA, 0.2 M KI, or 0.5 M KCl and then pelleted at 70,000k *g* for 1 h. The pellets were resuspended in 0.5 mL suspension buffer, and 0.1 mL was used for each 0.5 mL transport reaction. Reaction mixtures contained 25 mM Hepes-BTP (pH 7.0), 250 mM sucrose, 10 mM KCl, 0.1 mM NaN_3, 10 mM oxalate, 10 μM $^{45}CaCl_2$, 3 mM $MgSO_4$, and 3 mM ATP. After 30 min at 22°C, 0.2 mL was filtered per assay. CaM concentration when added was 0.3 μM. Data represent an average of eight experiments.

Treatment	Ca^{2+} Transport Activity		Stimulation
	−CaM	+CaM	
	pmol/assay		*-fold*
None	65.6 ± 7.0	256.4 ± 53	3.9
Buffer	25.6 ± 1.1	134.1 ± 14	5.2
EGTA (3 mM)	10.1 ± 2.4	61.2 ± 21	6.0
KCl (0.5 M)	4.7 ± 2.1	49.3 ± 11	10.5
KI (0.2 M)	4.0 ± 2.6	32.1 ± 3	7.9

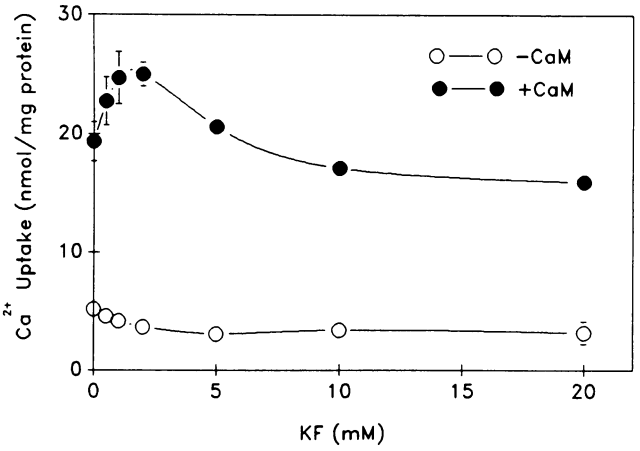


Figure 8. Effect of KF concentration on Ca^{2+} uptake into interface (22/32%) vesicles from carrot cells. Net ATP-dependent Ca^{2+} uptake at 30 min was measured in the absence (o) or presence (●) of 1 μM calmodulin. The reaction mixture for Ca^{2+} transport measurement was as described in Fig. 7. in the absence of oxalate. Data represent an average of three experiments. Error bars indicate ± SE.

commonly used to remove peripheral proteins including calmodulin. Net Ca^{2+} uptake was decreased by washing the vesicles in buffer alone. However, KCl (0.5 M) or KI (0.2 M) reduced net Ca^{2+} uptake by 64 to 85% relative to the buffer-washed vesicles.

The results suggest that factors solubilized by these treatments were required for optimal Ca transport activity. Washing with KCl or KI was more effective in removing endogenous CaM than EGTA. KCl wash increased CaM stimulation from five- to 10-fold relative to the buffer control. In contrast, after EGTA wash calmodulin stimulated net Ca transport by sixfold. Apparently, factors other than calmodulin were important as the addition of exogenous calmodulin only partially restored transport activity. Although we do not understand the reasons for this loss in activity, the results suggest that washing membranes with 0.2 M KI or KCl during isolation (4, 32) could alter and reduce the intrinsic activities.

We tested whether some cytosolic factor was required for maximal Ca^{2+} transport. After centrifugation at 185,000*g* (45,000 rpm, TY 65), transport in the pelleted vesicles was reduced about three- to fourfold relative to the control vesicles; however, adding back the supernatant did not restore activity (not shown). Perhaps Ca transport activity was labile, due to hydrolytic enzymes (proteases or lipases) or to an increase in vesicle permeability.

To further understand the interaction of calmodulin with CaATPase, we tested the effects of phosphatase inhibitors, calmodulin antagonists and protein kinase inhibitors on Ca^{2+} uptake.

Fluoride Stimulated Ca^{2+} Uptake

Interestingly, KF at 1 mM stimulated Ca^{2+} transport activity in the presence of calmodulin (Fig. 8). Other anions, like Br or I were less effective than F^- (Table IV). Fluoride inhibition of a phosphatase might enhance calmodulin stimulation of Ca^{2+} transport. Alternatively, KF could act as oxalate and Ca^{2+} transport activity was stimulated due to the formation of a CaF_2 precipitate, while $CaBr_2$ and CaI_2 are very soluble in aqueous solutions.

Fluoride had only a slight inhibitory effect on Ca^{2+} uptake. In the absence of calmodulin, Ca^{2+} uptake was inhibited by 40% at [KF] higher than 5 mM (Fig. 8). In the presence of calmodulin, a partial inhibition of about 20% on Ca^{2+} transport by KF (>2 mM) was detected. Results suggest that high concentrations of KF inhibited uptake consistent with its ability to inhibit phosphatases. However, only 20 to 40% inhibition was observed in the presence of 20 mM KF (Fig. 8), indicating that this Ca^{2+}-ATPase was not a highly F^--sensitive ATPase.

No effect on Ca^{2+} transport by molybdate and alpha-naphthyl phosphate was found, and slight inhibition by ATP-gamma-S was detected in the presence of calmodulin.

Calmodulin Antagonists & Protein Kinase Inhibitors

Calmodulin antagonists, like W-7, inhibit CaM-dependent enzyme activities. Ca binding to CaM induces a conforma-

Table IV. *Effect of F^-, Br^-, and I^- on CaM-Stimulated Ca^{2+} Uptake into Interface (22/32%) Vesicles from Carrot Cells*

Conditions for Ca^{2+} uptake assay were as described in Figure 7. CaM when added was 1 μM. Net uptake was at 30 min. Data are from one representative experiment of two.

Addition	Ca^{2+} Transport Activity	
	−CaM	+CaM
	nmol/mg protein	
None	6.85	21.20
KF (1 mM)	5.57	28.61
KBr (1 mM)	6.64	17.58
KI (1 mM)	6.72	21.96

tional change that exposes hydrophobic groups which then binds to and activates the enzyme. W-7 prevents this by binding to the CaM-Ca complex through hydrophobic and electrostatic interactions (18).

W-7 inhibited Ca^{2+} uptake by 95% at 100 μM in the presence of calmodulin. However, in the absence of exogenous calmodulin, transport was also inhibited by 100 μM W-7 to a similar level (Fig. 9). These results might be caused by residual endogenous calmodulin present in the membrane preparations. Similar results were obtained with ML-7, a W-7 derivative which inhibited Ca^{2+} transport activity by 70% at 100 μM in the presence and absence of calmodulin (Fig. 10a). The concentration of W-7 required to inhibit CaM-dependent Ca transport by 50% was 38 μM. Our findings are very similar to W-7 inhibition of calmodulin-dependent enzymes, like MLC kinase (I_{50} = 34 μM) (18).

Interestingly, L-Thyroxine inhibited Ca^{2+} uptake about 60% in the presence of calmodulin but had little effect on transport in the absence of calmodulin (Fig. 10b). Assuming that thyroxine-insensitive activity was not dependent on calmodulin, we estimate that the concentration required for 50% inhibition of transport activity was 10 μM. Our result is strikingly similar to an I_{50} of 7 to 9 μM reported for the MLC kinase (16).

L-Thyroxine, unlike W-7 and ML-7, is a unique type of MLC kinase inhibitor. It inhibits calmodulin-induced activation of MLC-kinase by direct binding at or near the calmodulin-binding domain of MLC-kinase, and is not competitive with ATP (16). W-7 and ML-7 inhibit MLC kinase in a competitive fashion with ATP, suggesting they bind at or near the ATP-binding site of the enzyme (18). This difference could account in part for the inhibition of transport in the absence of exogenous CaM seen with W-7 and ML-7, but not with thyroxine.

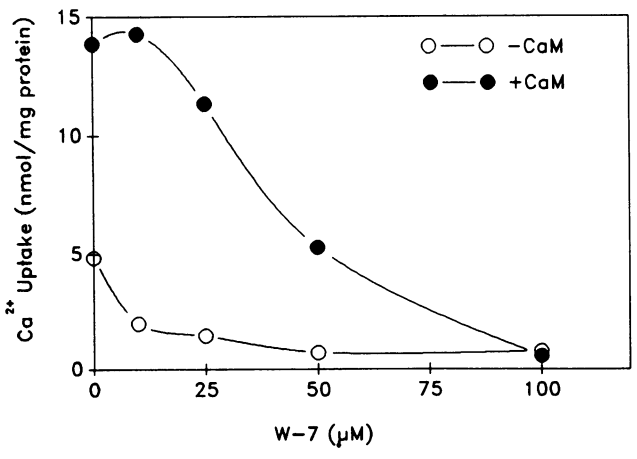


Figure 9. Effect of W-7 concentration on Ca^{2+}-uptake into interface (22/32%) vesicles from carrot cells. Net ATP-dependent Ca^{2+} uptake at 30 min was measured in the absence (o) or presence (●) of 1 μM calmodulin. Conditions for the Ca^{2+} uptake assay were as described in Fig. 7. Results are from one representative experiment of three.

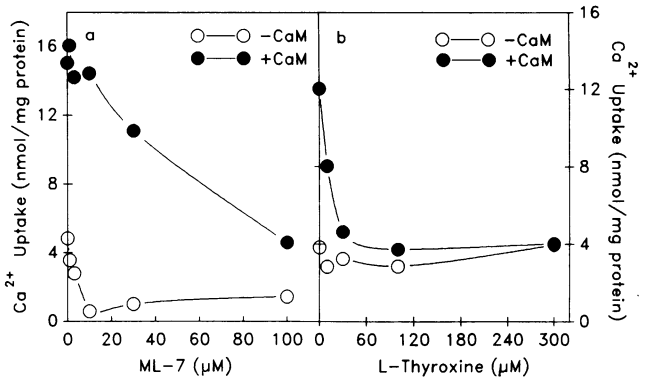


Figure 10. Effects of protein kinase inhibitors (a) ML-7 and (b) L-thyroxine on Ca^{2+}-uptake into interface (22/32%) vesicles from carrot cells. Vesicles were preincubated with inhibitors in a reaction mixture as described in Fig. 7 (minus ATP) at 22°C for 15 min and 3 mM ATP was added to start the reaction. Net ATP-dependent Ca^{2+} uptake at 30 min was measured in the absence (o) or presence (●) of 1 μM calmodulin. Data are from one representative experiment of two.

DISCUSSION

Our results taken together would indicate that there are at least two types of Ca-pumping ATPases in ER-enriched vesicles from carrot cells. They could be partially distinguished by their transport activities and their sensitivity to cyclopiazonic acid, a specific inhibitor of the SR/ER type Ca-ATPase. One CaATPase, the ER-type, was stimulated by oxalate, inhibited by CPA, and shows little or no stimulation by calmodulin. These conclusions were drawn from the results which showed oxalate stimulated Ca transport was particularly sensitive to CPA inhibition while calmodulin-stimulated Ca transport was not (Fig. 4, Table I). The second type of Ca transport activity was CPA-insensitive activity and stimulated by calmodulin. This activity is tentatively refered as the plasma membrane-type Ca pump.

The mol mass of a Ca-ATPase was near 120 kD as determined from the formation of phosphoprotein under conditions specific for phosphorylation of P-type Ca-ATPases. Even under conditions preventing proteolysis, only one major phosphoenzyme band was detected. Our results suggested that the mol mass of 120 kD in carrot cells represented the plasma membrane-type CaATPase. It is possible the molecular mass of Ca-pumping ATPases from plants differs slightly from tissue to tissue. In red beets, both the ER-type and the plasmamembrane-type CaATPase had a mol mass of about 100 kD (4, 14, 32). However, in corn microsomes, the calmodulin-stimulated CaATPase has a molecular mass of 140 kD (3, 11), and its identity as a plasma membrane-type Ca pump was confirmed by its reactivity with antibodies to the erythrocyte Ca pump (11).

In animal cells, calmodulin stimulated Ca pumping is generally thought to be a property of the plasma membrane-type CaATPase (8, 27). Direct binding of calmodulin to a domain of the 130 to 140 kD ATPase induces a conformational change that results in an activated state of the pump (8, 12). Phosphatidylserine, an acidic phospholipid, stimulated the

plasma membrane Ca pump, like calmodulin (23). In contrast, the SR/ER type Ca ATPase of 100 to 118 kD do not contain a calmodulin binding domain, but is stimulated indirectly by a calmodulin-dependent kinase which phosphorylates an acidic protein, phospholamban. The SR Ca transport can also be activated by phosphorylation of phospholamban with either a cAMP-dependent kinase or protein kinase c (31). Unphosphorylated phospholamban interacts with the inactive form of the CaATPase. However, after phosphorylation via protein kinases, phospholamban is released and the pump becomes activated. It is unclear whether the plant ER-type Ca ATPase is stimulated by calmodulin via a similar mechanism or not.

Several observations suggest that the calmodulin-stimulated Ca transport seen in carrot cells originated from a plasma membrane-type Ca pump of 120 kD. First, the calmodulin-stimulated activity supported by either ATP or GTP was not sensitive to cyclopiazonic acid (Table I). Second, the stimulation of Ca transport by acidic phospholipids, such as PS and PI (Table II, Fig. 7) is analogous to their effect on the erythrocyte Ca pump. Third, the inhibition of calmodulin-stimulated Ca transport by thyroxine, W-7 or ML-7 (Figs. 9, 10) were similar to their effects on the MLC kinase, a calmodulin-dependent enzyme. The simplest interpretation of these results is that calmodulin stimulated the Ca ATPase through direct binding to the enzyme. Finally, removal of peripheral proteins by salt washes did not eliminate calmodulin stimulation. Taken together, these results would support a model of a direct interaction between calmodulin and the Ca-ATPase, similar to the plasma membrane-type Ca pump.

It is interesting and important to note that calmodulin-stimulated Ca transport in plants have been detected in ER-enriched vesicles or the microsomal fraction. Although a 140 kD CaATPase was detected in corn microsomes (3, 11), the major part of the calmodulin stimulated transport was associated with the ER fraction from corn (2) similar to results reported here for carrot cells. At this point, we cannot eliminate the possibility that plasma membrane vesicles from carrot cells migrate closely with the ER fraction. However, together with results from corn, the possibility of the plasma membrane-type Ca pump on endomembranes, such as secretory vesicles (destined for the plasma membrane) as well as on the plasma membrane need to be considered. The full role and significance of a calmodulin-stimulated CaATPase in plant cell growth and development is still unclear. It is also not known whether Ca pumps in plants are regulated by phosphorylation by protein kinases. Stimulation of the calmodulin sensitive Ca uptake by KF (a phosphatase inhibitor) but not of the calmodulin-independent Ca transport is provocative. Future studies of the solubilized and purified Ca-ATPases as well as molecular studies will help distinguish the different types of Ca pumps as well as their potentially different modes of regulation.

ACKNOWLEDGMENTS

We thank Dr. Paul Bottino for the carrot cells and use of his tissue culture facilities, Drs. Ernesto Carafoli, Larry Jones, J. Penniston and John M. Ward for stimulating discussions.

LITERATURE CITED

1. **Bradford MM** (1979) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254
2. **Brauer D, Schubert C, Tsu SI** (1990) Characterization of a Ca^{2+}-translocating ATPase from corn root microsomes. Physiol Plant **78:** 335–344
3. **Briars SA, Evans DE** (1989) The calmodulin-stimulated ATPase of maize coleoptiles form a phosphorylated intermediate. Biochem Biophys Res Commun **159:** 185–191
4. **Briskin DP** (1990) Ca-translocating ATPase of the plasma membrane. Plant Physiol **94:** 397–400
5. **Buckhout TJ** (1984) Characterization of Ca^{2+} transport in purified endoplasmic reticulum membrane vesicles from *Lepidium sativum* L. roots. Plant Physiol **76:** 962–967
6. **Bush DR, Sze H** (1986) Calcium transport in tonoplast and endoplasmic reticulum vesicles isolated from cultured carrot cells. Plant Physiol **80:** 549–555
7. **Carafoli E** (1987) Intracellular calcium homeostasis. Annu Rev Biochem **56:** 395–433
8. **Carafoli E** (1991) Calcium pump of the plasma membrane. Physiol Rev **71:** 129–153
9. **de Meis L** (1988) Approaches to studying the mechanisms of ATP synthesis in sarcoplasmic reticulum. Methods Enzymol **157:** 190–206
10. **Dieter P, Marme D** (1980) Calmodulin activation of plant microsomal Ca^{2+} uptake. Proc Natl Acad Sci USA **77:** 7311–7314
11. **Evans DE, Briars SA, Williams LE** (1991) Active calcium transport by plant cell membranes. J Exp Bot **42:** 236–303
12. **Enyedi A, Vorherr T, James P, McCormick DJ, Filoteo AG, Carafoli E, Penniston JT** (1989) The calmodulin binding domain of the plasma membrane Ca^{2+} pump interacts both with calmodulin and with another part of the pump. J Biol Chem **264:** 12313–12321
13. **Fiske CH, SubbaRow Y** (1925) The colorimetric determination of phosphorus. J Biol Chem **66:** 375–400
14. **Giannini JL, Ruiz-Cristin J, Briskin DP** (1987) Calcium transport in sealed vesicles from red beet (*Beta vulgaris* L.) storage tissue. I. Characterization of a Ca^{2+}-pumping ATPase associated with the endoplasmic reticulum. Plant Physiol **85:** 1129–1136
15. **Graf P, Weiler EW** (1989) ATP-driven Ca^{2+} transport in sealed plasma membrane vesicles prepared by aqueous two-phase partitioning from leaves of *Commelina communis*. Physiol Plant **75:** 469–478
16. **Hagiwara M, Mamiya S, Ochiai M, Hidaka H** (1988) Thyroid hormones inhibit the Ca^{2+} calmodulin-induced activation of myosin light chain kinase. Biochem Biophys Res Commun **152:** 270–276
17. **Hepler PK, Wayne RO** (1985) Calcium and plant development. Annu Rev Plant Physiol **36:** 397–439
18. **Hidaka H, Hagiwara M** (1987) Biopharmacological regulation of protein phosphorylation. Prog Clin Biochem Med **5:** 48–65
19. **Kasai M, Muto S** (1990) Ca^{2+} pump and Ca^{2+}/H^{+} antiporter in plasma membrane vesicles isolated by aqueous two-phase partitioning from corn leaves. J Membr Biol **114:** 133–142
20. **Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685
21. **Macara GI** (1980) Vanadium—an element in search of a role. Trends Biochem Sci **5:** 92–94
22. **Neuhoff V, Arold N, Taube D, Ehrhardt W** (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with a clear background at nanogram sensitivity using Coomassie brilliant blue G-250 and R250. Electrophoresis **9:** 255–262

23. **Niggli V, Adunyah ES, Carafoli E** (1981) Acidic phospholipids, unsaturated fatty acids, and limited proteolysis mimic the effect of calmodulin on the purified erythrocyte Ca^{2+}-ATPase. J Biol Chem **256:** 8588–8592
24. **Pierce WS, Sze H** (1987) Calmodulin stimulation of calcium transport in carrot microsomal vesicles (abstract No. 321). Plant Physiol **83:** S-53
25. **Rasi-Caldogno F, Pugliarello MC, Olivari C, De Michelis MI** (1989) Identification and characterization of the Ca^{2+}-ATPase which drives active transport of Ca^{2+} at the plasma membrane of radish seedlings. Plant Physiol **90:** 1429–1434
26. **Robinson C, Larsson C, Buckhout TJ** (1988) Identification of a calmodulin-stimulated (Ca^{2+}+Mg^{2+})-ATPase in a plasma membrane fraction isolated from maize (*Zea mays*) leaves. Physiol Plant **72:** 177–184
27. **Schatzmann HJ** (1989) The calcium pump of the surface membrane and the sarcoplasmic reticulum. Annu Rev Physiol **51:** 473–485
28. **Schroeder JI, Thuleau P** (1991) Ca^{2+} channels in higher plant cells. Plant Cell **3:** 555–559
29. **Schumaker KS, Sze H** (1986) Calcium transport into the vacuole of oat roots. Characterization of Ca^{2+}/H^{+} exchange activity. J Biol Chem **261:** 3944–3946
30. **Seidler NW, Jona I, Vegh M, Martonosi A** (1989) Cyclopiazonic acid is a specific inhibitor of the Ca^{2+}-ATPase of sarcoplasmic reticulum. J Biol Chem **264:** 17816–17823
31. **Tada M, Kadoma M, Inui M, Fujii JI** (1988) Regulation of Ca^{2+} pump from cardial sarcoplasmic reticulum. Methods Enzymol **157:** 107–154
32. **Williams LE, Schueler SB, Briskin DP** (1990) Further characterization of the red beet plasma membrane Ca^{2+}-ATPase using GTP as an alternative substrate. Plant Physiol **92:** 747–754