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A Plasma Membrane-Type Ca^{2+}-ATPase of 120 Kilodaltons on the Endoplasmic Reticulum from Carrot (*Daucus carota*) Cells[1]

Properties of the Phosphorylated Intermediate

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Cytosolic Ca^{2+} levels are regulated in part by Ca^{2+}-pumping ATPases that export Ca^{2+} from the cytoplasm; however, the types and properties of Ca^{2+} pumps in plants are not well understood. We have characterized the kinetic properties of a 120-kD phosphoenzyme (PE) intermediate formed during the reaction cycle of a Ca^{2+}-ATPase from suspension-cultured carrot (*Daucus carota*) cells. Only one Ca^{2+}-dependent phosphoprotein was formed when carrot membrane vesicles were incubated with [γ-^{32}P]ATP (W.L. Hsieh, W.S. Pierce, and H. Sze [1991] Plant Physiol 97: 1535–1544). Formation of this 120-kD phosphoprotein was inhibited by vanadate, enhanced by La^{3+}, and decreased by hydroxylamine, confirming its identification as an intermediate of a phosphorylated-type Ca^{2+}-translocating ATPase. The 120-kD Ca^{2+}-ATPase was most abundant in endoplasmic reticulum-enriched fractions, in which the Ca^{2+}-ATPase was estimated to be 0.1% of membrane protein. Direct quantitation of Ca^{2+}-dependent phosphoprotein was used to examine the kinetics of PE formation. PE formation exhibited a K_m for Ca^{2+} of 1 to 2 μM and a K_m for ATP of 67 nM. Relative affinities of substrates, determined by competition experiments, were 0.075 μM for ATP, 1 μM for ADP, 100 μM for ITP, and 250 μM for GTP. Thapsigargin and cyclopiazonic acid, specific inhibitors of animal sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, had no effect on PE formation; erythrosin B inhibited with 50% inhibition at <0.1 μM. Calmodulin (1 μM) stimulated PE formation by 25%. The results indicate that the carrot 120-kD Ca^{2+}-ATPase is similar but not identical to animal plasma membrane-type Ca^{2+}-ATPase and yet is located on endomembranes, such as the endoplasmic reticulum. This type of Ca^{2+} pump may reside on the cortical endoplasmic reticulum, which is thought to play a major role in anchoring the cytoskeleton and in facilitating secretion.

Ca^{2+} transport proteins are the dominant regulators of the signaling function of Ca^{2+}. Cytoplasmic Ca^{2+} increases when Ca^{2+} is imported passively through Ca^{2+} channels and decreases when Ca^{2+} is exported actively. In plant cells, at least two types of transporters export cytosolic Ca^{2+} across the PM or into organellar lumen: Ca^{2+}-ATPases, located on the ER and the PM, and a Ca^{2+}/H^+ antiporter, located on the vacuolar membrane (Schumaker and Sze, 1986; Evans et al., 1991). Because the affinity for Ca^{2+} of the Ca^{2+}-ATPases (K_m = approximately 1 μM) is higher than that of the antiporter (K_m = 10–20 μM), the Ca^{2+}-ATPases are thought to play the main role in fine tuning intracellular Ca^{2+}.

Although several Ca^{2+}-ATPases from plant cells have been reported (Briskin, 1990; Evans et al., 1991), the differences between Ca^{2+}-ATPases of the PM and of the ER have not been well characterized. Furthermore, discrepancies in sensitivity to calmodulin and the molecular masses among Ca^{2+}-ATPases in the same membrane would suggest the possibility of variations among species or tissues. For example, exogenous calmodulin stimulated Ca^{2+} uptake in maize and spinach leaf PM vesicles (Malatialy et al., 1988; Robinson et al., 1988) but had little or no effect on Ca^{2+} uptake into PM vesicles from *Lepidium sativum*, corn roots, *Commelina communis* leaf, and radish seedling (Buckhout, 1984; Rasi-Caldogno et al., 1989; Graf and Weiler, 1990; Evans et al., 1991). Also, the molecular mass of a putative PM Ca^{2+}-ATPase was estimated as 140 kD in corn (Briars et al., 1988), whereas both the ER and PM Ca^{2+} pumps were about 100 kD in red beets (Briskin, 1990).

The concept that there are some distinctly different characteristics between the PM and the ER Ca^{2+}-ATPases is derived from the model that has emerged for animal cells. The animal PM Ca^{2+}-ATPase has been extensively characterized, mainly in the erythrocyte (Schatzmann, 1982; Carafoli, 1991). This Ca^{2+}-ATPase has a molecular mass of 134 to 140 kD and can directly bind to and be activated by calmodulin and acidic phospholipids (Carafoli, 1991). An animal SER-type Ca^{2+}-ATPase, highly enriched in sarcoplasmic reticulum (Schatzmann, 1989), has a molecular mass of 100 to 120 kD, is not directly activated by calmodulin, and is sensitive to specific inhibitors, thapsigargin (Sagara and Inesi, 1991) and

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Abbreviations: BiP, tobacco-binding protein; BTP, bis-Tris-propane or 1,3-bis[tris(hydroxymethyl)methylamino]propane; CPA, cyclopiazonic acid; PE, phosphoenzyme; PM, plasma membrane; SER, sarcoplasmic/endoplasmic reticulum.

CPA (Goeger and Riley, 1989; Seidler et al., 1989). The presence of two types of Ca^{2+}-ATPases makes possible differential regulation of Ca^{2+} transport at the PM and ER sites. Whether the animal model will apply to plant systems is not yet clear.

We have previously characterized ATP-dependent Ca^{2+}-pumping activity in ER-enriched membrane fractions of carrot (*Daucus carota*) cells (Bush and Sze, 1986); however, the molecular mass and specific biochemical properties of the Ca^{2+} pump(s) involved were not clear. Ca^{2+} uptake was stimulated 3- to 4-fold by calmodulin but was also inhibited partially by CPA (Hsieh et al., 1991). In this study, we demonstrate that a Ca^{2+}-dependent phosphoprotein of 120 kD has kinetic properties characteristic of a catalytic intermediate of a Ca^{2+}-pumping ATPase. The 120-kD Ca^{2+}-ATPase from carrot cells resembles the PM-type Ca^{2+} pump from animal cells and yet is associated with the ER. Preliminary results of this work have been presented (Chen et al., 1992).

MATERIALS AND METHODS

Plant Material

Suspension-cell cultures of carrot (*Daucus carota*, Danvers) were subcultured weekly into Murashige-Skoog medium supplemented with 1 mg L^{-1} of 2,4-D and 0.1 mg L^{-1} of 6-BA. Membrane vesicles were isolated from the cells 5 d after subculturing.

Preparation of Membrane Vesicles

Vesicles were isolated as described by Hsieh et al. (1991) with some modifications. All isolation procedures were conducted at 4°C. Cells were filtered and washed with about 3 volumes of cold deionized water. Eight to 10 flasks (250 mL) were used with a typical yield of 80 to 100 g fresh weight. Cells were homogenized with a mortar and pestle in a grinding medium (50–60 mL $grind^{-1}$) containing 250 mM Suc, 25 mM Hepes-BTP (pH 7.0), 3 mM EGTA, 1 mM DTT, and 1 mM PMSF for three 5-min grinds. After filtration through six to eight 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 Suc step gradient (6 mL of 32%, 5 mL of 22%, and 5 mL of 15%) and centrifuged at 70,000*g* for 2 h. Gradient solutions contained 2.5 mM Hepes-BTP (pH 7.0), 1 mM DTT, and 1 mM PMSF. After centrifugation, the 22/32% interface or all interfaces including the pellet were collected and stored at −80°C. For simplicity, the 22/32% fraction is also referred to as ER vesicles. The protein concentration, after precipitation with 10% TCA, was determined by a modified Lowry method (Bensadoun and Weinstein, 1976) with BSA as the standard.

Formation of Acyl Phosphoprotein with [γ-^{32}P]ATP

[^{32}P]Phosphoproteins were formed using published methods (Inesi et al., 1988; Hsieh et al., 1991) with some modifications. To concentrate the membranes, usually 9 mL of vesicles (0.2–0.4 mg of protein mL^{-1}) were diluted 8- to 10-fold with suspension buffer (25 mM Hepes-BTP [pH 7.0], 1 mM PMSF, and 1 mM DTT) and centrifuged for 1 h at 70,000*g*. The pellet was suspended in the same buffer to about 1 mg of protein mL^{-1}.

The phosphorylation reaction mixture (0.5 mL) contained 25 mM Hepes-BTP (pH 7.0), 50 μM $CaCl_2$ or 0.5 mM EGTA, 100 mM KCl, various amounts of [γ-^{32}P]ATP (as indicated in each figure legend), and about 50 to 100 μg of membrane protein. Inhibitors, as indicated in the figure and table legends, were preincubated with membrane in reaction mixtures for 20 min at 25°C before assay. [γ-^{32}P]ATP was added to chilled reaction mixtures at 0°C to start the reaction. When the substrate specificity of the enzyme was being tested, various concentrations of different nucleotides were mixed with 1 nM [γ-^{32}P]ATP (1 μCi). After incubation on ice for various periods, the reaction was quenched by adding 0.7 mL of ice-cold 12% TCA, 1 mM ATP, and 50 mM NaH_2PO_4. The TCA precipitate was incubated on ice for at least 15 min, followed by a centrifugation at 14,000 rpm (Eppendorf 5415 C) for 20 min. The TCA pellet was resuspended again with the same TCA solution and separated by centrifugation. Pellets were washed with water and then suspended in 50 μL of SDS-PAGE sample buffer containing 2% SDS, 10% glycerol, 5% mercaptoethanol, 100 mM sodium phosphate buffer (pH 6.0), 8 M urea, and 5 mM PMSF. The suspensions were subjected to either scintillation counting for quantitative assays, SDS-PAGE, or both.

The effect of hydroxylamine was tested by suspending the first TCA pellet in 1 mL of 100 mM Mes brought to pH 6.0 with KOH in the presence or absence of 50 to 100 mM hydroxylamine. After incubation for 15 min on ice, the proteins were precipitated again with 0.2 mL of 50% TCA (final concentration 8%) and separated by centrifugation. Pellets were rinsed in water, suspended in sample buffer, and counted.

SDS-PAGE and Autoradiography

To detect [^{32}P]phosphoproteins, proteins were separated in a 5% acrylamide gel modified to pH 6.0 with sodium phosphate buffer (Weber and Osborn, 1969). The running buffer and the electrode buffer were modified to pH 6.0. One microliter of 0.01% bromophenol blue was added to each sample before loading. Polypeptides and molecular mass standards run in adjacent lanes were stained with Coomassie blue. Gels were dried using a Bio-Rad 583 drier and exposed overnight or 3 d with Kodak XAR-5 film at −80°C. Apparent molecular mass was estimated by comparison of autoradiographs with stained standards (Bio-Rad, Richmond, CA).

Immunoblotting

For immunostaining, membrane proteins were precipitated with 10% TCA and washed with 100% acetone. After separation on standard Laemmli SDS-PAGE (pH 8.8), the gels were blotted to Immobilon P (Millipore) in 25 mM Tris, 192 mM Gly, and 15% methanol at 4°C. The membrane was blocked in 1% BSA, 5% nonfat milk, and 1 M Gly in PBS (5 mM NaH_2PO_4, 150 mM NaCl [pH 7.4]), washed in 0.05% Tween 20 in PBS, and incubated with rabbit antisera against BiP (Hofte and Chrispeels, 1992). The membrane was washed, incubated with goat anti-rabbit immunoglobulin G

conjugated to alkaline phosphatase, and developed with 5-bromo-4-chloro-3-indoyl phosphate and nitro blue tetrazolium (Blake et al., 1984).

Determination of Free [Ca^{2+}]

To determine the effect of Ca^{2+} concentration on the formation of PE, Ca^{2+} levels were adjusted by the addition of EGTA (0–10, 30, 100, 1000 μM) or $CaCl_2$ (0–100 μM) to reaction mixtures. Free [Ca^{2+}] at micromolar levels was determined by titration of reaction mixtures with EGTA. The titration was monitored with a Ca^{2+}-selective ion electrode (Orion model 93–20; Orion Research Inc., Boston, MA). The free [Ca^{2+}] is equal to the added EGTA at the end point of titration. For free [Ca^{2+}] less than 1 μM, Ca^{2+} concentration was estimated using the fluorescent indicator quin2 (Tsien et al., 1982). The fluorescence (excitation 339 nm, emission 492 nm) of 20 μM quin2 in reaction mixtures containing membrane at 0°C was determined. The free [Ca^{2+}] of reaction mixtures (200 μL) containing membrane vesicles (30 μg of protein) without added EGTA or $CaCl_2$ was determined to be 2.5 μM.

Ca^{2+} Transport

ATP-dependent Ca^{2+} uptake into membrane vesicles was measured by the filtration method (Bush and Sze, 1986). Transport was initiated by adding 60 to 150 μL of vesicles (approximately 20–50 μg of protein) to a reaction mixture (0.2–0.5 mL) containing 250 mM Suc, 25 mM Hepes-BTP (pH 7.0), 10 mM KCl, 0.1 mM NaN_3, 5 mM KNO_3, 10 μM $^{45}CaCl_2$ (1 μCi mL^{-1}), and 3 mM ATP with or without 3 mM $MgSO_4$. Azide and nitrate were included to minimize activity from the mitochondrial or vacuolar H^+-ATPases, respectively. After the desired transport period at 20°C, 0.1-mL aliquots were filtered onto 0.45-μm Millipore filters premoistened with a rinse solution (250 mM Suc, 2.5 mM Hepes-BTP [pH 7.0], and 1 mM $CaCl_2$). The vesicles on the filters were washed with 4 mL of cold rinse solution. The $^{45}Ca^{2+}$ radioactivity associated with the filters was determined by liquid scintillation counting. Active transport is defined as the difference between activity in the presence and absence of $MgSO_4$ and expressed as nmol of Ca^{2+} mg^{-1} of protein.

Chemicals

γ-Labeled [^{32}P]ATP (3000 or 4000 Ci $mmol^{-1}$) was obtained from Amersham (Arlington Heights, IL) or New England Nuclear (Boston, MA) as the triethylammonium salt. ATP, AMP, ADP, GTP, and ITP were obtained from Boehringer-Mannheim as the sodium salt. Calmodulin (bovine brain type), CPA, and erythrosin B were obtained from Sigma. Thapsigargin was purchased from LC Service Co. (Woburn, MA) or Sigma. All other chemicals were reagent grade.

RESULTS

Formation of a 120-kD [^{32}P]Phosphoprotein Could Be Directly Quantitated

Because incubation of carrot microsomal membranes with [γ-^{32}P]ATP resulted in the formation of a single [^{32}P]phosphoprotein (Hsieh et al., 1991), we tested whether formation of the [^{32}P]PE could be quantitated by direct counting of the TCA-precipitated proteins. To compare the results of direct counting of total TCA-precipitated proteins with those of SDS-PAGE analysis of phosphoproteins, vesicles from the 22/32% Suc interface were incubated with [γ-^{32}P]ATP, and the reactions were stopped by TCA. Half of the washed TCA pellet was analyzed by SDS-PAGE and autoradiography (Fig. 1), and the radioactivity in the remaining half was quantitated by scintillation spectroscopy (Tables I and II represent data from two separate experiments).

A major phosphoprotein of about 120 kD was formed in the presence of Ca^{2+} (Fig 1, lanes 7 and 8) but not in the absence of Ca^{2+} (lanes 1–4). Formation of the Ca^{2+}-dependent phosphoprotein was inhibited by vanadate (Fig. 1A, lanes

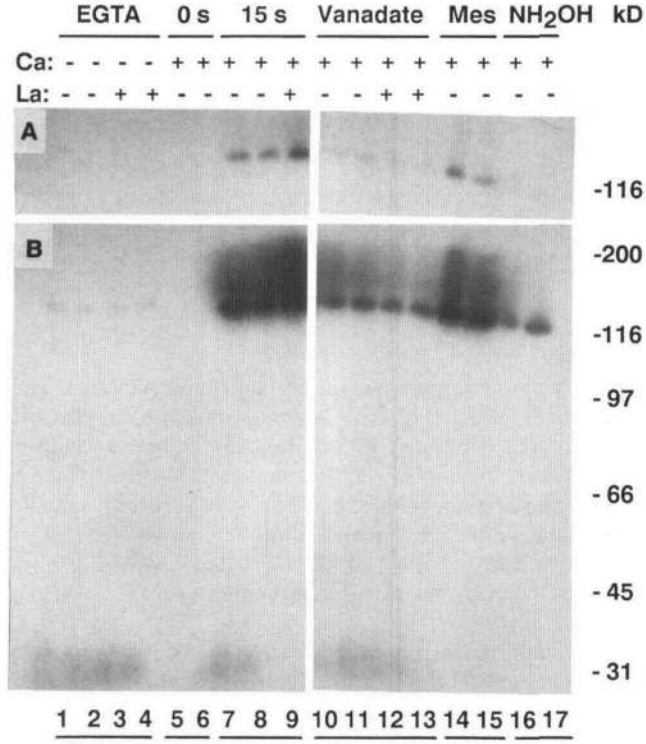

Figure 1. Formation of [^{32}P]phosphoprotein is associated with a 120-kD protein. [γ-^{32}P]ATP (1 nM; 1 μCi $assay^{-1}$) was added to start the reaction. Reaction mixtures (0.5 mL) contained 25 mM Hepes-BTP (pH 7.0), 100 mM KCl, 50 μM $CaCl_2$ or 0.5 mM EGTA, and about 100 μg of ER vesicle protein. When present, the vanadate or La concentration was 200 or 50 μM, respectively. Except for a control reaction stopped at 0 s (lanes 5 and 6), all reactions were stopped after 15 s at 0°C with TCA. In two reactions (lanes 14–17), the pellets were resuspended in 1 mL of buffer alone (Mes) or 50 mM hydroxylamine in 100 mM Mes/KOH (pH 6) and kept on ice for 15 min. Pellets were precipitated again with TCA, rinsed with water, and then resuspended in 50 μL of sample buffer without the dye. Half of the suspension (25 μL) was used for determination of radioactivity (Tables I and II), and the remaining half was analyzed by SDS-PAGE at pH 6 in duplicate lanes, followed by autoradiography for either 20 h (A) or 5 d (B). The autoradiograph in B shows the absence of any phosphoproteins smaller than 120 kD. Data are from one experiment representative of three to six experiments.

Table I. *Formation of Ca^{2+}-dependent phosphoprotein in membrane vesicles from carrot cells*

Enhancement by La and inhibition by vanadate. Assay conditions were as described in legend to Figure 1. [γ-^{32}P]ATP (1 nM) was added to start the reaction (0.5 mL). Reaction mixtures contained 25 mM Hepes-BTP (pH 7.4), 100 mM KCl, 50 μM $CaCl_2$ or 0.5 mM EGTA, and about 100 μg of ER vesicle protein. When present, vanadate or La concentration was 200 or 50 μM, respectively. Except for a control reaction stopped at 0 s, all other reactions were stopped after 15 s at 0°C with TCA. Pellets were precipitated again with TCA, rinsed with water, and then resuspended in 50 μL of sample buffer. Half was used for determination of radioactivity. Data are from one experiment representative of three to six experiments each with duplicates.

Treatments	[^{32}P]Phosphoprotein Formation			
	Total radioactivity	Ca^{2+}-dependent		
		Radioactivity	Specific activity	Relative activity
	cpm	*cpm*	*fmol mg^{-1}*	%
EGTA	5,695			
EGTA + La^{3+}	5,268			
Ca^{2+} (0 s)	412			
Ca^{2+} (15 s)	41,939	36,457	50.3	100
Ca^{2+} + La^{3+}	105,681	100,199	138.2	275
Ca^{2+} + VO_4^{3-}	28,036	22,554	31.1	62
Ca^{2+} + La^{3+} + VO_4^{3-}	27,959	22,477	31.0	62

10–13), an inhibitor of phosphorylated-type ATPases, and enhanced by La^{3+} (Fig. 1A, lane 9), an inhibitor of Ca^{2+} transport. The denatured PE was sensitive to hydroxylamine (Fig. 1, lanes 16 and 17), indicating that the phosphate is linked by an acyl phosphate bond to a carboxyl group, usually of an Asp residue (Schatzmann, 1989). Taken together, these results confirm that the 120-kD, Ca^{2+}-dependent phosphoprotein is the phosphorylated intermediate of a Ca^{2+}-ATPase (Hsieh et al., 1991).

The radioactivity associated with the TCA-precipitable membrane pellet (Tables I and II) correlated with the amount of the 120-kD [^{32}P]phosphoprotein visualized by autoradiography (Fig. 1). Ca^{2+} stimulated the levels of phosphoprotein by 5- to 8-fold over the EGTA control. Ca^{2+}-dependent formation of [^{32}P]phosphoprotein was enhanced approximately 2-fold by La^{3+} (Table I). Vanadate inhibited phosphoprotein formation by 40 or 78% in the absence or presence of La^{3+}, respectively. Furthermore, phosphoprotein levels were decreased 63% in vesicles treated with hydroxylamine (Table II). These results indicate that the formation of the phosphorylated intermediate of a Ca^{2+}-ATPase can be directly quantitated by the measurement of Ca^{2+}-dependent [^{32}P]phosphoprotein in carrot microsomal membranes.

A 120-kD Ca^{2+}-ATPase Was Associated with a Vesicle Fraction Enriched in ER

Surprisingly, only one Ca^{2+}-dependent [^{32}P]phosphoprotein was formed in all of the membrane fractions separated by a step Suc gradient (Fig. 2A). Most of the activity (65% of the total Ca^{2+}-dependent PE) was associated with vesicles derived from the 22/32% Suc interface. This fraction was previously shown to have the highest Ca^{2+}-transport activity and greatest enrichment in ER vesicles of the fractions, as determined by antimycin A-insensitive NADH-dependent Cyt *c* reductase activity (Bush and Sze, 1986). This parallel with Ca^{2+}-transport activity further supports the identification of this 120-kD phosphoprotein as an intermediate of a Ca^{2+}-translocating ATPase. The association of Ca^{2+}-dependent phosphoprotein with ER membranes was further confirmed by its parallel distribution on Suc step gradients with BiP (binding protein), an ER lumen protein. Western blotting of the membrane fractions with antibody to BiP showed an enrichment of a 78-kD polypeptide in the 22/32% Suc interface (Fig. 2B).

Kinetics of PE Formation

The formation of PE increased with time for about 30 s at 0°C and reached a steady-state level by 60 to 120 s (as shown later in Fig. 5). In subsequent experiments, the rate of phosphoprotein formation was determined after 15 to 20 s of incubation of membranes with [γ-^{32}P]ATP; whereas steady-state levels of phosphoprotein were measured at 2 min. This time course is similar to that observed for PE formation of the erythrocyte Ca^{2+}-ATPase (Schatzmann, 1982).

The affinity for Ca^{2+} was determined by measuring the effect of Ca^{2+} concentration on the steady-state level of phosphoprotein formation. The K_m for Ca^{2+} determined in four experiments was 1 to 2 μM (Fig. 3). This value is very close to the K_{mCa} of 0.7 to 2 μM previously determined for ATP-dependent Ca^{2+} pumping into vesicles from carrot cells (Bush and Sze, 1986). This further confirms the parallel

Table II. *Sensitivity of phosphoprotein to hydroxylamine*

Assay conditions were similar to those described in Table I and Figure 1 except reaction mixtures contained 5 nM ATP (1.8 μCi assay^{-1}). After 15 s, the reactions were quenched with TCA. The pellets were first suspended in 1 mL of Mes-KOH at pH 6.0 and separated by centrifugation. The pellets were then suspended in 1 mL of buffer alone or 100 mM NH_2OH in 100 mM Mes-KOH at pH 6.0 and incubated on ice for 15 min. Proteins were pelleted with TCA (final concentration 10%), washed, and suspended in sample buffer for counting. Results are from one experiment representative of two.

Treatments	[^{32}P]Phosphoprotein Formation			
	Total counts	Ca^{2+}-dependent		
		Counts	Specific activity	
	cpm	*cpm*	*fmol mg^{-1}*	%
EGTA + La^{3+}	9,100			
Ca^{2+}	43,149	34,656	210	100
Ca^{2+} + La^{3+}	97,758	88,658	540	256
EGTA + Mes-KOH	6,273			
Ca^{2+} + Mes-KOH	34,803	28,530	170	100
Ca^{2+} + NH_2OH/Mes-KOH	17,010	10,737	65	38
EGTA + La^{3+} + NH_2OH	4,013			
Ca^{2+} + La^{3+} + Mes-KOH	96,703	92,690	560	100
Ca^{2+} + La^{3+} + NH_2OH/Mes-KOH	37,900	33,887	210	37

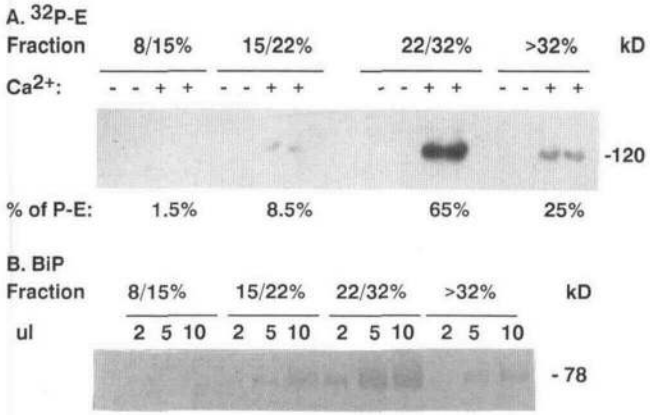

Figure 2. Ca^{2+}-dependent 120-kD phosphoprotein is associated with a membrane fraction enriched in ER. A, Ca^{2+}-dependent [^{32}P] phosphoprotein is enriched in the 22/32% Suc interface. Membranes were collected from all interfaces of the step Suc gradient, diluted, and then pelleted (100,000*g* for 1 h). All membrane fractions were resuspended in 0.8 mL, and an equal volume fraction was used for the assays. [γ-^{32}P]ATP (1 nM, 1 μCi assay^{-1}) was added to start the reaction containing 25 mM Hepes-BTP (pH 7.4), 100 mM KCl, 50 μM $CaCl_2$ or 0.5 mM EGTA, and 100 μL of vesicles. After incubation for 1 min on ice, proteins were precipitated with TCA twice, rinsed, and resuspended in 50 μL of sample buffer (minus dye). Half was used for determination of radioactivity, and the remaining half was analyzed by SDS-PAGE at pH 6 in duplicate lanes, followed by autoradiography. The result is from one experiment representative of four. B, BiP detected by immunostaining is also enriched in the 22/32% Suc interface. Equal volume fractions of 2, 5, or 10 μL of membranes in sample buffer (100 μL total) were separated by SDS-PAGE at pH 8.8, transferred, and immunoblotted with rabbit anti-BiP. On average, the relative protein recovered in fractions 8/15, 15/22, 22/32, and >32% pellet were 21, 20, 31, and 28% of total microsomal protein, respectively.

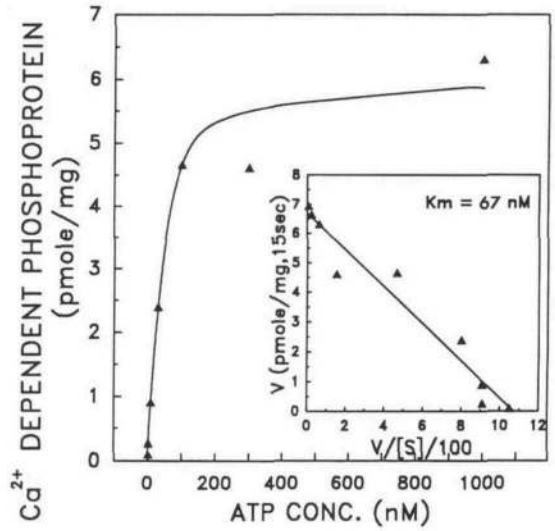

Figure 4. Dependence of phosphoprotein formation on a high-affinity ATP-binding site. Reaction mixtures (0.5 mL) contained 25 mM Hepes-BTP (pH 7.4), 100 mM KCl, 50 μM $LaCl_3$, 50 μM Ca^{2+} or 0.5 mM EGTA, and about 100 μg of vesicle protein. Various concentrations of nonlabeled ATP mixed with 1 to 10 μCi of [γ-^{32}P]ATP was added to start the reactions that were incubated for 15 s on ice. Inset, Eadie-Hofstee plot of the same data shows a K_{mATP} = 67 nM. Data are from one experiment representative of two to six experiments. The average K_{mATP} detected in the absence of La^{3+} was similar at 60 nM.

between Ca^{2+} transport and Ca^{2+}-dependent PE formation in these membranes.

The apparent K_m for ATP was determined by measuring the rate of phosphoprotein formation at 15 s. The K_{mATP} measured either in the absence (not shown) or presence of La^{3+} was about 67 nM (Fig. 4). In the presence of La^{3+}, the

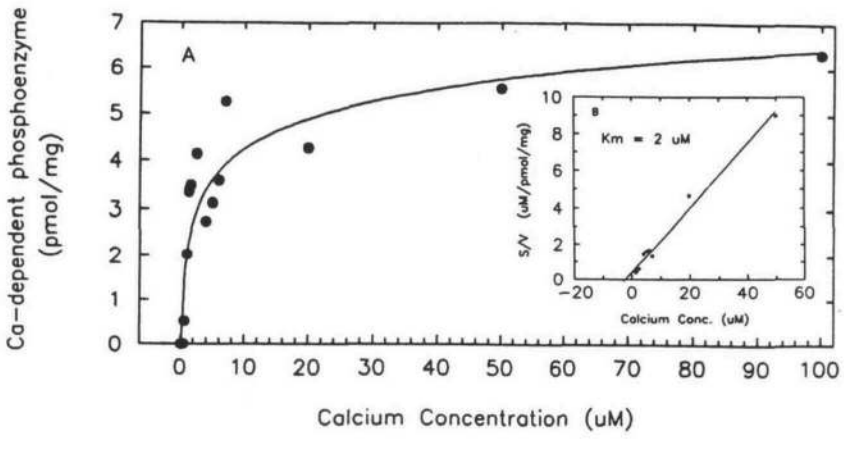

Figure 3. Dependence of steady-state PE formation on free Ca^{2+} concentration. Reaction mixtures (0.2 mL) contained 25 mM Hepes-BTP (pH 7), 100 mM KCl, 200 nM ATP (2.5 μCi of [γ-^{32}P]ATP), and 29 μg of protein (ER vesicles pelleted) with additions of EGTA or $CaCl_2$ to give the corresponding free Ca^{2+} level. Free [Ca^{2+}] was determined in complete reaction mixtures containing ER vesicles with quin2 for [Ca^{2+}] concentrations <1 μM and with EGTA titration as monitored with a Ca^{2+}-selective electrode for [Ca^{2+}] concentrations >1 μM. Background (1 mM EGTA instead of Ca^{2+}) was subtracted to determine the Ca^{2+}-dependent PE level. Vesicles were incubated with Ca^{2+}-/EGTA-containing reaction mixtures for 20 min at room temperature before assay. Reactions were stopped after 2 min at 0°C. Inset, Hanes-Woolf plot of the same data; K_{mCa} = 2 μM. Results are from one experiment representative of four.

V_{max} was 6.9 pmol mg^{-1} 15 s^{-1} as determined by the Eadie-Hofstee plot.

La^{3+} Enhanced [^{32}P]PE Formation by Inhibiting Dephosphorylation

To gain insight regarding the stimulation of phosphoprotein levels by La^{+3} (Fig. 1, Table II), we tested the effect of La^{3+} on dephosphorylation. In the absence of La^{3+}, the rate of PE formation was linear for about 10 to 15 s at 0°C and reached a steady-state level by 60 to 120 s (Fig. 5). When excess nonradiolabeled ATP was added at 60 s, the steady-state level of [^{32}P]phosphoprotein was significantly reduced within 1 min to levels below that detected in the presence of 0.5 mM EGTA (Fig. 5, left).

In contrast, excess nonradioactive ATP had little or no effect on the steady-state PE level in the presence of La^{3+} (Fig. 5, right), indicating that dephosphorylation was blocked. These results are similar to those of the erythrocyte PM Ca^{2+}-ATPase. La^{3+}, an inhibitor of Ca^{2+}-pumping activity in PM vesicles in animal cells (Carafoli, 1992), is thought to inhibit a step in the reaction cycle that blocks the dephosphorylation of PE intermediate (Schatzmann, 1982). Blockage of dephosphorylation results in an increase of the net equilibrium level of PE and a reduction in the overall turnover of the Ca^{2+}-pumping enzyme, which then results in a reduction of Ca^{2+} transport. An La^{3+}-induced increase in steady-state levels of PE is diagnostic for PM-type Ca^{2+} pumps because it is not observed for animal SER-type Ca^{2+}-ATPases (Carafoli, 1992).

La^{3+} apparently increased the rate of PE formation by about 2-fold (Fig. 5). At 200 nM ATP, the initial rates in the absence and presence of La^{3+} were 0.23 ± 0.06 and 0.44 ± 0.06 pmol mg^{-1} s^{-1} (n = 2), respectively. However, the steady-state level of PE formation was enhanced by 47%. La^{3+} increased the level of Ca^{2+}-dependent PE formed at 3.5 min from 6.20 ± 1.13 to 9.15 ± 0.07 pmol mg^{-1} (n = 2).

Added Mg^{2+} was not required for the formation of Ca^{2+}-dependent PE, but its presence resulted in the reduction of the steady-state level of PE (Fig. 6). A specific reduction of

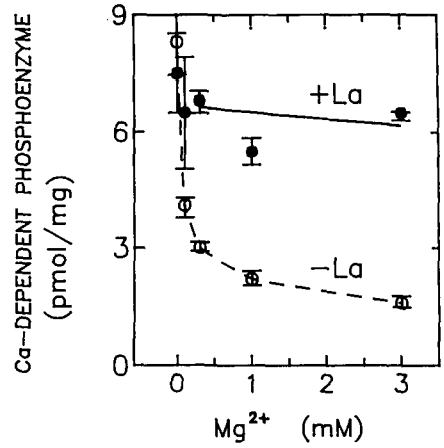

Figure 6. Effect of Mg^{2+} on the steady-state level of PE. PE level at 2 min (0°C) was determined for reaction mixtures containing 25 mM Hepes-BTP (pH 7), 100 mM KCl, 50 μM $CaCl_2$ or 500 μM EGTA, 200 nM ATP, 0 to 3 mM $MgCl_2$, and 30 μg of protein with or without 50 μM $LaCl_3$. Data are means ± SE for one representative experiment.

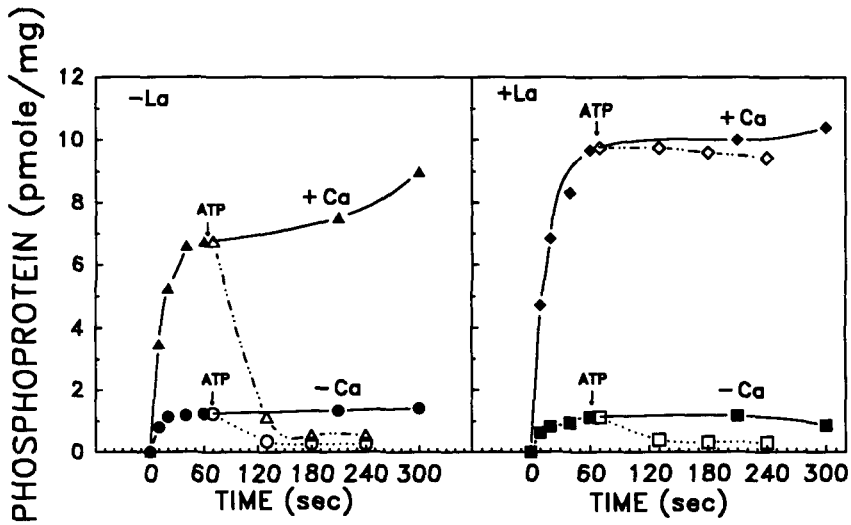

Figure 5. Effect of La^{3+} on the rate of phosphorylation and dephosphorylation. [^{32}P]ATP (200 nM, 25 μCi) was added to a 5-mL reaction mixture containing 25 mM Hepes-BTP (pH 7.4), 100 mM KCl, 50 μM $CaCl_2$ (+Ca: ▲, ◆) or 0.5 mM EGTA (−Ca: ●, ■), with (right panel) or without (left panel) 50 μM La, and about 1 mg of vesicle protein (ER vesicles, not pelleted). After incubation on ice for various periods indicated, a 0.5-mL aliquot (2.5 μCi) was removed and mixed immediately with 0.7 mL of 12% TCA, 1 mM ATP, and 50 mM Na_2HPO_4 to stop the reaction. After 60 s of incubation, aliquots of 0.5 mL each were pipetted into tubes containing 17 μL of 30 mM nonradioactive ATP to give a final concentration of 1 mM. After incubation with ATP for 60 to 180 s, the reaction was quenched with 0.7 mL of TCA (+Ca: △, ◇; −Ca: ○, □). The radioactivity of the ^{32}P-labeled protein was quantitated. Results are from one experiment representative of two to four experiments.

120-kD PE by millimolar levels of Mg^{2+} was confirmed by SDS-PAGE analysis of [^{32}P]phosphoproteins (data not shown). La^{3+} (50 μM) prevented this reduction of Ca^{2+}-dependent PE level (Fig. 6). This result indicates that Mg^{2+} enhanced the dephosphorylation of the PE, a step that is blocked by La^{3+}. In the erythrocyte Ca^{2+}-ATPase, Mg^{2+} enhances not only the dephosphorylation of the PE but also enhances the rate of other steps in the reaction cycle, leading to a net increase in steady-state PE level in the presence of Mg^{2+} (Adamo et al., 1990). The difference in the response of the carrot Ca^{2+}-ATPase PE to Mg^{2+} may indicate some different features of the reaction cycle of plant Ca^{2+}-ATPases.

Relative Abundance of the 120-kD Ca^{2+}-ATPase

Assuming that most of the Ca^{2+}-ATPase was phosphorylated in the presence of La^{3+}, we estimated the relative abundance of the Ca^{2+} pump on the membrane. The maximum rate of PE formation was reached by 200 nM ATP (Fig. 4), and the steady-state level of Ca^{2+}-dependent PE at that concentration was about 9 pmol mg^{-1} of protein when La^{3+} was present (Fig. 5). Assuming that there is only one acyl-linked phosphate per enzyme, there would be about 9 pmol of Ca^{2+}-ATPase mg^{-1} of protein. With a molecular mass of 120,000 D, the Ca^{2+} pump would, therefore, represent at least 0.1% of the total membrane protein.

Because this is the first estimate for the relative abundance of a Ca^{2+}-ATPase from plant membranes, it is unclear how this value compares with other plant tissues. The amount of Ca^{2+}-ATPase in microsomal membranes from suspension-cultured carrot cells is comparable with that of erythrocyte PM in which the Ca^{2+} pump represents 0.01 to 0.1% of the total membrane protein (Carafoli, 1992).

Substrate Specificity for Formation of [^{32}P]Phosphoprotein

To determine whether other nucleotides could act as alternate substrates, we tested the effect of different nucleotides on the formation of [^{32}P]phosphoprotein by 1 nM [γ-^{32}P]ATP (Fig. 7A). Nucleotides that bind to the catalytic site will compete with [^{32}P]ATP and the relative affinity (K_D) of the enzyme for the substrates can be determined from the concentration required to decrease PE formation by 50%. The estimated K_D values were as follows:

$$\text{ATP} < \text{ADP} < \text{ITP} < \text{GTP}$$

$$0.075\ \mu\text{M} < 1\ \mu\text{M} < 100\ \mu\text{M} < 250\ \mu\text{M}$$

The enzyme has the highest affinity for ATP, with a K_m of 75 nM, which is close to the value of 67 nM estimated independently in Figure 4. ADP or AMP decreased PE formation probably by binding to the ATP-binding site and acting as a competitive inhibitor rather than a substrate. ADP can also promote the phosphorylation back reaction (Schatzmann, 1982).

The ability of GTP to act as an alternate substrate (Williams et al., 1990) was further demonstrated by formation of a Ca^{2+}-dependent phosphoprotein of 120 kD with [γ-^{32}P]GTP (Fig. 7B). The low affinity of the enzyme for GTP (K_m = 0.25 mM) probably resulted in formation of subdetectable levels

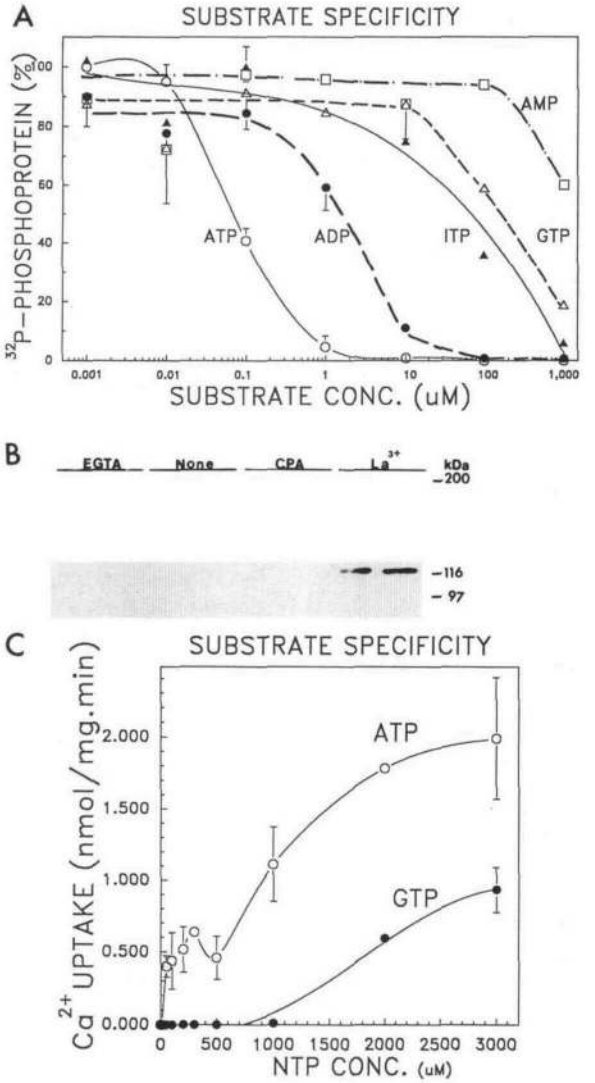

Figure 7. Effect of different nucleotide substrates on Ca^{2+}-dependent PE formation and on Ca^{2+} transport. A, Substrate specificity of the Ca^{2+}-dependent phosphoprotein. Reaction mixtures (0.5 mL) contained 25 mM Hepes-BTP (pH 7.4), 100 mM KCl, 50 μM Ca^{2+} or 0.5 mM EGTA, and about 100 μg of vesicle protein. Different concentrations of nonlabeled nucleotide plus 1 nM [γ-^{32}P]ATP (about 1 μCi) was added to start the reactions, which were stopped by TCA after 15 s on ice. The activity in the presence of 1 nM ATP was set to 100% (32 fmol mg^{-1} of protein). Data are from an average of three experiments. B, GTP-dependent [^{32}P]phosphoprotein formation. The reaction mixture contained about 100 μg of membrane protein, 25 mM Hepes-BTP (pH 6), and 100 mM KCl. Except for the control (0.5 mM EGTA), all other reactions contained 50 μM $CaCl_2$. Other additions are as indicated: None, 100 nmol of CPA mg^{-1} of protein or 50 μM $LaCl_3$. The assay was started with 100 nM [γ-^{32}P]-GTP (4 μCi $assay^{-1}$) and stopped after 15 s with 10% TCA containing 1 mM GTP and 50 mM $NaHPO_4$. After the samples were washed, they were separated by SDS-PAGE at pH 6.0, and the gel was exposed to x-ray film. C, Ca^{2+} uptake into ER membrane vesicles from carrot cells driven by Mg-ATP or Mg-GTP. $^{45}Ca^{2+}$ uptake was measured in reaction mixtures as described in "Materials and Methods" except the concentrations of $MgSO_4$, ATP, or GTP were varied from 50 μM to 3 mM. Transport period was 5 min. Results are the average of three experiments, each with duplicates or triplicates. The two K_{mATP} values determined using a Hanes-Woolf plot were 10 μM and 1.5 mM.

of the PE unless La^{3+} was present (Fig. 7B). Previously, we showed that 3 mM GTP was effective in driving calmodulin-stimulated Ca^{2+} uptake into membrane vesicles from carrots (Hsieh et al., 1991). The competition study (Fig. 7A) also confirms that ITP can serve as an alternate substrate of plant Ca^{2+}-ATPase at millimolar concentrations (Carnelli et al., 1992).

Affinity for ATP or Alternate Substrate GTP of Ca^{2+} Transport

The Mg-ATP concentration dependence of Ca^{2+} uptake was complex. The initial rate of Ca^{2+} pumping showed biphasic dependence on ATP (Fig. 7C). Assuming the model of two ATP sites with different affinities (Schatzmann, 1989), the K_ms for ATP of the high-affinity and low-affinity sites were estimated to be 10 μM and 1.5 mM, respectively. These values are about 1 order of magnitude higher than the K_m of 2 μM and 0.18 mM for the PM Ca^{2+}-ATPase of red blood cells (Carafoli, 1992). The K_m for ATP determined for Ca^{2+} transport (Fig. 7C) is much higher than that determined for 120-kD PE formation (Fig. 4). This difference may be because transport requires a complete turnover of the enzyme, whereas PE formation measures only a partial reaction. ATP may have multiple roles in the reaction cycle, as it does for both the PM- and SER-type animal Ca-ATPases (Schatzmann, 1989).

The V_{max} of Ca^{2+} transport was lower with GTP as substrate than with ATP. The kinetics of GTP activation was characterized by one rather than by two phases perhaps due to the insensitivity of the assay when activity is low. The K_m for GTP of Ca^{2+} transport was more than 1 mM, similar to the low-affinity site for ATP (Fig. 7C).

Thapsigargin or CPA Had Little or No Effect on PE Formation

Although CPA completely inhibits animal SER Ca^{2+}-ATPases at 8 nmol mg^{-1} of protein at low ATP concentrations (0.5–2 μM ATP; Seidler et al., 1989) and inhibits PE formation (Goeger and Riley, 1989), it had no effect on the formation of the carrot Ca^{2+}-dependent PE up to concentrations of 300 nmol mg^{-1} of protein (Fig. 8A). Erythrosin B, an inhibitor of ATPases in general, inhibited Ca^{2+}-dependent phosphoprotein formation completely at 1 μM (50% inhibition at <0.1 μM) (Fig. 8C). This is consistent with the high sensitivities of plant Ca^{2+}-ATPases to this inhibitor (Briskin, 1990).

Thapsigargin inhibits animal SER Ca^{2+}-ATPases completely and specifically at concentrations equal to a ratio of 1:1 thapsigargin:Ca^{2+}-ATPase enzyme (Sagara and Inesi, 1991). We found little or no effect on the rate of Ca^{2+}-dependent PE by thapsigargin over a concentration range including 9 pmol of thapsigargin mg^{-1} of membrane protein, an estimated 1:1 inhibitor:pump ratio, up to concentrations of 1000 pmol mg^{-1} of protein (Fig. 8B). Inhibition at the highest concentration (10,000 pmol mg^{-1}) may be due to nonspecific inhibition, because excessive concentrations can also inhibit animal PM Ca^{2+}-ATPases (Y. Sagara, personal communication).

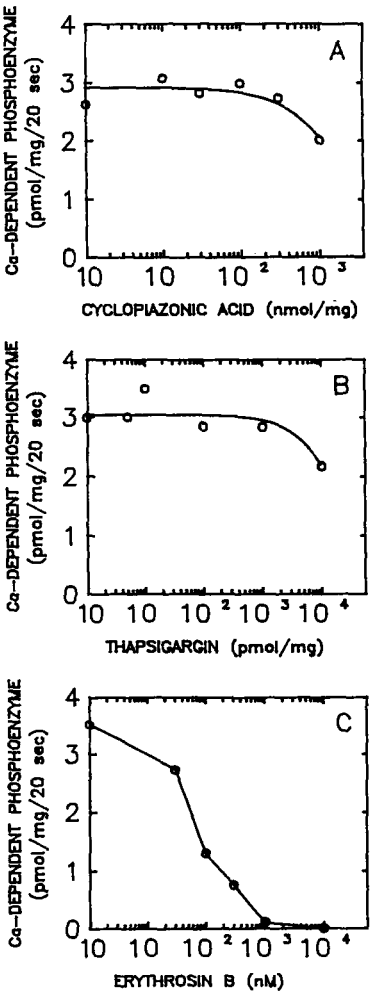

Figure 8. Effect of CPA (A), thapsigargin (B), and erythrosin B (C) on the initial rate of PE formation. PE level was determined after 20 s of incubation with [γ-^{32}P]ATP at 0°C. Reaction mixtures contained 25 mM Hepes-BTP (pH 7), 100 mM KCl, 50 or 150 μM $CaCl_2$, 200 nM ATP, and 30 μg of protein (ER vesicles). For erythrosin treatments, vesicles were preincubated for 20 min at 24°C in reaction mixtures with inhibitor. For CPA and thapsigargin treatments, vesicles (100 μg 100 μL^{-1}) were preincubated with inhibitor at 24°C for 20 min before addition to reaction mixtures. EGTA (500 μM) was added to the vesicle/thapsigargin mixtures for the preincubation treatment; a higher $CaCl_2$ addition (150 μM) was made to +Ca^{2+} reaction mixtures to compensate for EGTA carryover. This type of preincubation is necessary for maximal thapsigargin inhibition of SER Ca^{2+}-ATPases (Sagara and Inesi, 1991).

Calmodulin-Stimulated [^{32}P]PE Formation

The steady-state level of phosphoprotein was enhanced 21 to 34% with exogenous calmodulin (1 μM, Fig. 9), when vesicles were washed with either 3 mM EGTA or resuspension buffer. These washing treatments were introduced to remove membrane-associated endogenous calmodulin. The small stimulatory effect of calmodulin on PE formation was consistently observed if the ATP concentration was at 200 nM but not at 1 nM ATP (not shown). Furthermore, the stimula-

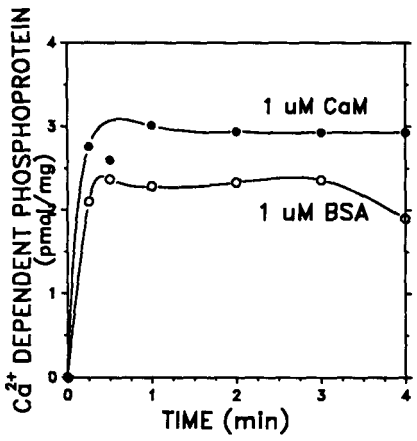

Figure 9. Stimulation of PE formation by calmodulin. Vesicles were diluted 6-fold either in suspension buffer or in 3 mM EGTA made in 25 mM Hepes-BTP (pH 7.0), 250 mM Suc, 1 mM PMSF, and 1 mM DTT. The pelleted membranes were then suspended in suspension buffer and repelleted. Ca-dependent phosphoprotein formation (+50 μM La) was then determined in the presence of 1 μM BSA or 1 μM calmodulin. Reaction mixtures containing 200 nM ATP were as described for Figure 5. Results are from one experiment representative of four.

tion was seen in either the presence or absence of La (data not shown).

It is interesting that, for the erythrocyte Ca^{2+}-pump, a calmodulin-induced elevation in steady-state PE level was also relatively small (1.4 fold) compared to a 3-fold stimulation of ATPase activity observed under the same conditions (Schatzmann, 1982). Because PE formation reflects only the partial reaction, calmodulin stimulation of PE in carrot membranes (Fig. 9) is not necessarily similar to the observed 3- to 5- fold calmodulin-stimulation of Ca^{2+} transport (Hsieh et al., 1991). Taken together, a stimulation by calmodulin of PE level again indicates that the carrot 120-kD ATPase is more similar to the PM- than to the SER-type animal Ca^{2+}-ATPases.

DISCUSSION

This study has demonstrated the kinetic properties and inhibitor sensitivities of a Ca^{2+}-dependent PE intermediate formed during the reaction cycle of a 120-kD Ca^{2+}-pumping ATPase from plants. We took advantage of a previous observation that only a single phosphoprotein was formed when carrot membranes were incubated with [γ-^{32}P]ATP (Hsieh et al., 1991). The following results support the idea that a Ca^{2+}-dependent phosphoprotein from carrot membranes has all of the characteristics of a catalytic intermediate corresponding to a Ca^{2+}-pumping ATPase of 120 kD: (a) the phosphoprotein formed is dependent on micromolar levels of Ca^{2+} (Fig. 3) but not on Mg (Hsieh et al., 1991); (b) the phosphoprotein is rapidly formed, with the maximum phosphorylation seen in 60 s at 0°C (Fig. 5); (c) it turns over rapidly because the addition of excess unlabeled ATP results in complete loss of label from the protein in less than 60 s at 0°C (Fig. 5); (d) its sensitivity to hydroxylamine (Fig. 1) suggests that an acyl phosphate bond was formed rather than a hydroxyl-phosphate characteristic of protein kinase activity; (e) the Ca^{2+}-dependent phosphoprotein was distributed on membranes in parallel with Ca^{2+}-pumping activity (Fig. 2) (Bush and Sze, 1986; Hsieh et al., 1991); (f) La^{3+} enhances the steady-state levels of PE formed by inhibiting dephosphorylation (Fig. 5); and (g) substrate specificity required for PE formation is consistent with that of Ca^{2+} pumping (Figs. 7, A–C).

The properties of the 120-kD Ca^{2+}-ATPase enriched in carrot membranes are similar but not strictly identical with the PM-type ATPase from animal tissues (Table III). The carrot Ca^{2+}-ATPase resembled the PM-type pump in the following ways: (a) both Ca^{2+} transport (Hsieh et al., 1991) and PE formation (Fig. 9) are calmodulin stimulated (Schatzmann, 1982; Carafoli, 1992). This stimulation is due to direct binding of the pump with calmodulin, because a 120-kD Ca^{2+}-ATPase could be partially purified by calmodulin-affinity chromatography (D.M. Ratterman and H. Sze, unpublished data), a result similar to one in a recent study by Askerlund and Evans (1992); (b) Formation of the PE was stimulated by La^{3+} (Fig. 5), which is characteristic of a phosphorylated intermediate of PM Ca^{2+}-ATPases (Carafoli, 1991); (c) Unlike the SER Ca^{2+}-ATPase, formation of Ca-dependent PE is not dependent on Mg (Fig. 6) (Schatzmann, 1989); and most important (d) specific SER Ca^{2+}-ATPase inhibitors, CPA and thapsigargin (Fig. 8), have little or no effect on Ca^{2+} pumping or PE formation in carrot membranes. However, neither antibodies from animal PM or SER Ca^{2+}-ATPases reacted with the 120-kD Ca^{2+} pump of carrots (not shown). Also, the molecular mass of the Ca^{2+} pump from carrot is less than the 135 to 140 kD of most PM-type Ca^{2+} pumps. Because there is no evidence of proteolytic breakdown (Fig. 1B), we conclude that the native size of a plant form of PM-like Ca-ATPase is about 120 kD.

Perhaps the most provocative finding was that the Ca^{2+}-dependent PE, like the calmodulin-stimulated Ca^{2+} pumping (Bush and Sze, 1986; Hsieh et al., 1991), comigrates with an ER marker. Bush and Sze (1986) showed that Ca^{2+}-pumping ATPase could be shifted by Mg in parallel with NADH Cyt *c* reductase activity, an ER marker. Subsequently, Hsieh et al. (1991) demonstrated that this Ca^{2+}-ATPase was stimulated

Table III. *Comparison of a carrot 120-kD Ca^{2+}-ATPase with the animal PM and the SER Ca^{2+}-ATPases*

Property	Ca^{2+}-ATPase		
	Carrot[a]	Animal PM[b]	Animal SER[b]
Molecular mass (kD)	120	134	110–115
Stimulation by Calmodulin	Yes, direct	Yes, direct	Indirect
La on PE level	Increase	Increase	No effect
PE requires Mg	No	No	Yes
Inhibitors			
Vanadate (200 μM)	Yes	Yes	Yes
Erythrosin B (1 μM)	Yes	Yes	Yes
Thapsigargin (1:1 M ratio)	No	No	Yes
CPA (100 nmol mg^{-1})	No	No	Yes

[a] Taken from Bush and Sze, 1986; Hsieh et al., 1991; and this study. [b] Taken from Schatzmann, 1989; Seidler et al., 1989; Sagara and Inesi, 1991; Carafoli, 1992.

by calmodulin and suggested the possibility of calmodulin-stimulated Ca^{2+} transport ATPase being on endomembranes, such as the ER. Comigration of the 120-kD PE with membranes enriched in Ca^{2+} transport as well as with the ER marker BiP (Fig. 2) extends and confirms this idea. Recent findings by others also support the conclusion that a calmodulin-stimulated Ca^{2+} pump is associated with the ER in actively growing or secreting tissues, such as in corn roots (Brauer et al., 1989), cauliflower (Askerlund and Evans, 1992), and barley aleurone (Gilroy and Jones, 1993). Although the notion of a calmodulin-stimulated Ca^{2+}-ATPase on the ER is not consistent with the membrane distribution of the PM and SER Ca^{2+} pumps from animal tissues, distinct Ca^{2+} pumps may have evolved in plants to provide the conditions required for their growth and development.

One possibility is that the calmodulin-stimulated Ca^{2+} pump in carrot suspension-cultured cells is associated with the cortical ER (Hepler et al., 1990). The cortical ER consists of an extensive interconnected network of tubules and flat cisternae that are situated immediately adjacent to the PM. Although the specific function of the cortical ER is not known, it arises at cytokinesis and is thought to play a key role in anchoring the cytoskeleton and facilitating secretion. Suspension-cultured cells, as in carrot, are actively dividing and secreting new cell-wall materials. The presence of Ca^{2+} pumps on cortical ER in many plant cells would be consistent with the idea that the cortical ER participates in signal transduction and in regulation of cytoplasmic [Ca]. Cytochemical studies to localize Ca^{2+} pumps in plant cells will provide insight into this question.

Many questions arise. For example, where are the other PM-type ATPases that form PE intermediates in carrot membranes, and where is a putative ER-type Ca^{2+} pump previously shown to be partially CPA sensitive (Hsieh et al., 1991)? The major PM H^+ pump of 100 kD was not detected in our membranes, probably because, unlike the PM-type Ca^{2+}-ATPase, the PE formation of H^+-ATPase requires Mg (Serrano, 1990). Similarly, the absence of added Mg under our assay conditions would have prevented the formation of a Mg-dependent PE from SER-type Ca^{2+}-ATPases and phosphorylation by Mg-dependent protein kinases. In preliminary experiments, we have observed formation of multiple phosphoproteins when Mg is included in the phosphorylation reaction mixture. Thus, a relatively abundant 120-kD Ca^{2+}-ATPase associated mainly with the ER of carrot cells may be selectively detected with the conditions described here.

It is not clear whether the PM-type Ca^{2+}-ATPase from carrots is related to or distinct from the ER-type Ca^{2+}-ATPase of tomato recently characterized by molecular cloning (Wimmers et al., 1992). The deduced amino acid sequence from tomato specified a protein of 1048 amino acids with a mol mass of 116 kD. The protein structure is more related to animal SER Ca^{2+}-ATPases than to any other P-type ATPases; however, it is not known whether this Ca^{2+} pump is sensitive to thapsigargin. Apparently, there is no clear calmodulin-binding domain in the protein (Wimmers et al., 1992), suggesting that this ER-type Ca^{2+}-ATPase is distinct from the 120-kD Ca^{2+} pump in carrots. However, the carrot 120-kD Ca^{2+} pump appears to be related to a calmodulin-stimulated 115-kD Ca-ATPase recently reconstituted and purified from cauliflower (Askerlund and Evans, 1992). The diversity of Ca^{2+} pumps observed in different plant tissues probably reflects the presence of a family of Ca^{2+}-ATPases (Carafoli, 1991) of which expression and activity are differentially regulated depending on the species, tissue or cell type, subcellular membrane, and developmental stage. Future studies to characterize Ca^{2+} pumps from carrots and other plants at the molecular level will be a step toward understanding the types of Ca^{2+} pumps in plants as well as their regulation and physiological functions.

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