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Investigation of the Calcium-Transporting ATPases at the Endoplasmic Reticulum and Plasma Membrane of Red Beet (*Beta vulgaris*)[1]

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Calcium-transporting ATPases were compared in endoplasmic reticulum (ER)- and plasma membrane-enriched fractions of red beet (*Beta vulgaris* L.) storage tissue by measuring ^{45}Ca uptake and calcium-dependent phosphoenzyme formation. The plasma membrane fraction was prepared by aqueous two-phase partitioning of a microsomal fraction and collecting the upper phase. The ER-enriched fraction was obtained by submitting a sucrose-gradient ER-enriched fraction to aqueous two-phase partitioning and collecting the lower phase; this reduced contaminating plasma membrane, which partitioned into the upper phase. The ATP-dependent calcium uptake observed in both fractions was released by the calcium ionophore A23187. Calcium uptake showed saturation kinetics for calcium with K_m values of 0.92 mmol m^{-3} for the ER fraction and 1.24 mmol m^{-3} for the plasma membrane fraction. Uptake into both fractions was inhibited by vanadate and erythrosin B, although the plasma membrane system was slightly more sensitive to both inhibitors. Cyclopiazonic acid and thapsigargin, at low concentrations, had no marked effect on uptake. The plasma membrane system was less substrate-specific for ATP than the ER system, since it was able to use GTP and ITP to drive calcium transport at up to 50% of the level obtained with ATP. Following phosphorylation with [γ-^{32}P]ATP, two high molecular mass, calcium-dependent phosphoproteins (119 and 124 kD) and a low molecular mass, calcium-independent phosphoprotein (17 kD) were observed in the plasma membrane fraction. The ER fraction showed one high molecular mass phosphoprotein (119 kD) in the presence of calcium and two low molecular mass phosphoproteins (17 and 20 kD) that showed no calcium dependence. The low molecular mass phosphoproteins were insensitive to hydroxylamine, but they did show turnover. The identity of these proteins is unknown, but they do not have the properties of phosphorylated intermediates of calcium-ATPases. In contrast, the high molecular mass phosphoproteins displayed properties consistent with their representing phosphorylated intermediates of E_1E_2-type ATPases; they were hydroxylamine-sensitive, showed rapid turnover, and were inhibited by vanadate. Because they showed calcium-dependent phosphorylation and were sensitive to erythrosin B, the 119- and 124-kD phosphoproteins may be phosphorylated intermediates of the ER and plasma membrane calcium ATPases. These phosphoproteins were characterized further with respect to inhibitor sensitivity, responses to ions, and substrate specificity.

Calcium transport into partially purified membrane vesicles has been described for a number of plant tissues (for review, see Evans et al., 1991). Generally, the plasma membrane and ER of plant cells have been shown to possess primary calcium-transporting ATPases that use ATP hydrolysis to drive the direct transport of calcium ions. These enzymes are P-type (E_1E_2-type) ATPases, which form phosphorylated intermediates during their catalytic cycles (Briars and Evans, 1989; Hsieh et al., 1991). In contrast, calcium transport into the plant cell vacuole is mediated by a tonoplast Ca^{2+}/H^+ antiport (Blumwald and Poole, 1986), the exchange of calcium ions for protons being driven by the proton motive force created by the tonoplast H^+-ATPase (Schumaker and Sze, 1985), and the tonoplast H^+-PPase (Chanson, 1991).

In most reports characterizing calcium transport at particular cell membranes, the membrane fractions were isolated by discontinuous Suc gradients (Evans et al., 1991); this includes previous studies of the transporters at the ER and plasma membrane of red beet (*Beta vulgaris*) (Giannini et al., 1987a, 1987b; Williams et al., 1990). However, a comparison of the properties of the calcium transporters at particular cell membranes is complicated because these fractions show some degree of cross-contamination (Giannini et al., 1987a). This is especially important when comparing the ER and plasma membrane transporters, which operate by similar mechanisms.

The formation of the phosphorylated intermediates of calcium pumps is dependent on the presence of calcium (Briars and Evans, 1989; Hsieh et al., 1991). However, although previous phosphorylation studies in red beet were conducted in the presence of calcium (Giannini et al., 1987a; Williams et al., 1990), the calcium-dependence of the phosphoenzymes observed in the Suc-gradient-enriched ER fractions (Giannini et al., 1987a) and plasma membrane fractions (Williams et al., 1990) was not established, since the phosphoenzymes were not characterized in detail. In addition, assigning a

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Abbreviations: BTP, bis-tris propane or 1,3-bis (tris[hydroxymethyl]-methylamine)propane; CPA, cyclopiazonic acid; EB, erythrosin B; GER, gradient ER fraction; GPM, gradient plasma membrane fraction; I_{50}, concentration causing 50% inhibition; LDS, lithium dodecyl sulfate; PER, phased endoplasmic reticulum fraction; PPM, phased plasma membrane fraction; SR, sarcoplasmic reticulum; TEMED, *NNNN*-tetramethylethylenediamine; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

precise membrane location to these phosphoproteins is difficult because of possible cross-contamination of the fractions used. For example, the 96-kD phosphoenzyme formed when a Suc-gradient, ER-enriched fraction from red beet was incubated with [γ-^{32}P]ATP (Giannini et al., 1987a) may not necessarily be the phosphorylated intermediate of an ER enzyme, but could instead be located on contaminating plasma membrane vesicles. The present study was carried out to establish whether there are any discriminatory features in the characteristics of the primary calcium pumps at the plasma membrane and ER by comparing calcium uptake and the formation of phosphorylated intermediates. A phase-partitioned plasma membrane fraction from red beet, which shows negligible contamination by ER, is compared with a Suc-gradient ER fraction that has been subjected to phase partitioning to remove much of the contaminating plasma membrane. Previous studies of these transporters in red beet used ^{45}Ca and chlorotetracycline fluorescence, respectively, to study the characteristics of the plasma membrane and ER systems (Giannini et al., 1987a, 1987b). For a more direct comparison, this study uses ^{45}Ca for both systems.

MATERIALS AND METHODS

Plant Material

Red beet (*Beta vulgaris* L.) roots were either obtained commercially or harvested from the field. The tops of the plants were removed, and the roots were stored in moist vermiculite at 4°C until use. All root tissue was stored for at least 10 d to ensure uniformity in membrane isolation.

Membrane Isolation

Microsomal Fraction

Microsomal vesicles were isolated from red beet storage roots according to a modification of the method of Giannini et al. (1987a). The homogenization medium contained 250 mol m^{-3} Suc, 2 mol m^{-3} EDTA, 5 mol m^{-3} $MgSO_4$, 0.5% (w/v) PVP (40,000 mol wt), 0.2 mol m^{-3} PMSF, 15 mol m^{-3} β-mercaptoethanol, 4 mol m^{-3} DTT, 10% (v/v) glycerol, and 70 mol m^{-3} Tris-HCl (pH 8.0). DTT, PMSF, and β-mercaptoethanol were added to the medium just prior to use. BSA (0.5%, w/v) was included in the homogenization medium when measurements of NADH-Cyt *c* reductase were performed in order to maintain enzyme activity. The beet tissue was peeled, cut into small pieces, and added to the ice-cold homogenization medium in a 1:1 (v/v) ratio. Following vacuum infiltration on ice for at least 10 min, the tissue was homogenized in a chilled Waring blender, using 20-s high-speed bursts. The homogenate was filtered twice though four layers of muslin and then centrifuged at 13,000*g* for 15 min at 4°C in a Sorvall SS34 rotor. The supernatant was then centrifuged at 80,000*g* for 45 min at 4°C in a 10 × 100 cm^3 Prepspin rotor, and the resulting microsomal pellet was resuspended in buffer containing 250 mol m^{-3} Suc, 10% (v/v) glycerol, 1 mol m^{-3} DTT (added fresh), and 2 mol m^{-3} BTP-Mes (pH 7.0). The microsomal fraction was frozen in liquid nitrogen and stored at −70°C until use.

Suc Density Gradient Centrifugation

Microsomal fractions were suspended on top of a discontinuous Suc gradient containing 14 cm^3 of 22% (w/w) Suc layered over 10 cm^3 of 26% (w/w) Suc layered over 7 cm^3 of 38% (w/w) Suc; all Suc solutions contained 3 mol m^{-3} $MgCl_2$ and 1 mol m^{-3} DTT and were buffered to pH 7.2 with 2 mol m^{-3} Tris-Mes. The gradient was centrifuged at 80,000*g* for 2 h at 4°C in a Sorvall AH629 swing-out rotor. The membranes at the top of the gradient, at the 22/26% Suc interface, and at the 26/38% interface were collected, diluted at least 4-fold in suspension buffer, and centrifuged at 100,000*g* for 45 min at 4°C in a Sorvall A841 rotor. The resulting pellets were resuspended in suspension buffer, frozen in liquid nitrogen, and stored at −70°C until use.

Magnesium Shift

The effect of magnesium on the separation of the different membrane types on a discontinuous Suc density gradient was studied. Homogenization of the red beet tissue was performed in the presence of 0, 2, and 5 mol m^{-3} $MgSO_4$ and the microsomal fractions produced were centrifuged on 22/26/38% discontinuous Suc gradients containing, respectively, 0, 0, and 3 mol m^{-3} $MgCl_2$ throughout. In each case, the same gradient fractions were removed and subjected to marker enzyme analysis.

Aqueous Two-Phase Partitioning of the Microsomal Fraction

Phase-partitioned plasma membrane was prepared according to a modification of the method described by Williams et al. (1992). Red beet microsomal protein (33–35 mg) was suspended in 1.6 cm^3 of phase suspension medium containing 250 mol m^{-3} sorbitol, 5 mol m^{-3} potassium phosphate buffer (pH 7.0), and 1 mol m^{-3} DTT. This was added to a phase partition mixture to form a 32-g phase system with a final composition of 6.5% (w/w) Dextran T500, 6.5% (w/w) PEG (mol wt 3350), 250 mol m^{-3} sorbitol, 3 mol m^{-3} KCl, 5 mol m^{-3} potassium phosphate (pH 7.8), and 1 mol m^{-3} DTT. The tubes were mixed thoroughly by repeated inversion for 20 s. The two phases were separated by centrifugation at 2500*g* for 5 min in a Sorvall HB4 rotor at 4°C. The upper and lower phases were separated, fresh upper and lower phases were added, and the partitioning was repeated. Following a total of three partitions, the resulting upper and lower phases were diluted about 10-fold in phase-diluting medium containing 250 mol m^{-3} sorbitol, 25 mol m^{-3} K_2SO_4, 20 mol m^{-3} BTP-Mes (pH 7.0), and 1 mol m^{-3} DTT. The membranes were collected by centrifugation for 40 min in a Sorvall A841 rotor at 100,000*g* at 4°C. The pellets were resuspended in suspension buffer containing 250 mol m^{-3} Suc, 10% (v/v) glycerol, 1 mol m^{-3} DTT (added fresh), and 2 mol m^{-3} BTP-Mes (pH 7.0). The upper phase, referred to as the PPM, and the lower phase were immediately frozen in liquid nitrogen and stored at −70°C until use.

Aqueous Two-Phase Partitioning of the Suc Gradient ER-Enriched Membrane Fraction

Phase partitioning of the ER-enriched membrane fraction, collected from the 22/26% Suc interface, was performed

according to the method described above with minor modifications. Typically, membrane protein prepared from six discontinuous Suc density gradients (3–5 mg) was suspended in 0.4 cm^3 of phase-suspension buffer and was partitioned on an 8-g phase system. Following three partitions, the upper and lower phases were diluted, centrifuged, and stored as described above. The lower phase is referred to as the PER.

Marker Enzyme Analysis

ATPase activity and K^+-stimulated pyrophosphatase activity were determined by measuring Pi release according to the method of Ohnishi et al. (1975) with slight modifications (Williams et al., 1990). Antimycin A-insensitive NADH-Cyt *c* reductase and Cyt *c* oxidase determinations were carried out according to Hodges and Leonard (1974). Marker enzyme analysis was carried out on at least three different membrane preparations and the assays were conducted in triplicate. The results shown are from representative experiments.

Uptake of Radiolabeled Calcium

Uptake of radiolabeled calcium into microsomal and Suc gradient fractions was determined at 22°C in a 0.2-cm^3 reaction volume containing 250 mol m^{-3} Suc, 25 mol m^{-3} BTP-Mes (pH 7.25), 3.75 mol m^{-3} $MgSO_4$, 3.75 mol m^{-3} ATP-BTP (pH 7.25), 100 mol m^{-3} KCl, 15 mmol m^{-3} $CaCl_2$ (containing 2–3 μCi cm^{-3} ^{45}Ca), and 0.4 mol m^{-3} NaN_3 (transport buffer). All solutions were made up in distilled deionized water. Uptake was initiated by the addition of membrane protein. For uptake into PPM and PER, KNO_3 replaced KCl in the medium and 1 mmol m^{-3} nigericin was included in the assay. After 5 min, 0.19 cm^3 of the reaction mixture was removed and filtered by vacuum filtration through presoaked 0.45-μm Whatman cellulose nitrate filters. The filters were washed with 4 cm^3 of ice-cold washing buffer (transport buffer without ATP-BTP and ^{45}Ca but with 30 mmol m^{-3} unlabeled $CaCl_2$), and radioactivity associated with the filters was determined by liquid scintillation spectroscopy. For time-course experiments, the reaction volume was 1 cm^3, and aliquots of 0.15 cm^3 were taken at various time intervals and treated as above. The calcium ionophore A23187 (4.5 mmol m^{-3}) was added at the end of the incubation period. To determine the substrate specificity of calcium uptake, the substrates were supplied as the BTP salt at a concentration of 3.75 mol m^{-3}, except for PPi, which was supplied at 0.2 mol m^{-3}. The effect of CPA on calcium transport was measured in the presence of 0.6 mol m^{-3} ATP. The dependence of calcium uptake on calcium concentration was determined by keeping the magnesium and ATP concentrations at 3.75 mol m^{-3} and varying the free calcium concentration using calcium/EGTA buffers.

Results of the time-course experiments are the means of uptake values obtained for at least two different membrane preparations. For uptake experiments conducted at specific time points, the values given are the means of three to four determinations from representative experiments, and within the experiment SE values were less than 10% of the means.

Phosphorylation and Dodecyl Sulfate Gel Electrophoresis

Phosphorylation reactions were carried out according to the method of Briars and Evans (1989) at ice temperature in a 500-μL volume containing 20 mol m^{-3} Tris-Mes (pH 7.4), 12.5 mmol m^{-3} $MgCl_2$, 37 kBq of [γ-^{32}P]ATP (0.66 μmol m^{-3} final concentration), or 370 kBq of [γ-^{32}P]GTP (3.4 μmol m^{-3} final concentration), 50 mmol m^{-3} $CaCl_2$ (plus calcium conditions), and, typically, 40 to 80 μg of membrane protein. For minus-calcium conditions, 0.5 mol m^{-3} EGTA was included in the assay. All solutions were made in distilled, deionized water. The reaction was initiated by the addition of [γ-^{32}P]-ATP or [γ-^{32}P]GTP in a 10-mm^3 volume. The reaction was stopped after 15 s by the addition of 0.5 cm^3 of ice-cold stop solution containing 10% (w/v) TCA, 0.5 mol m^{-3} ATP, and 0.005% (v/v) phosphoric acid (final concentrations). For ATP and GTP chases, the assay was carried out as above except that after 15 s of incubation, 25 mm^3 of 100 mol m^{-3} unlabeled ATP or GTP (final concentration 4.76 mol m^{-3}) was added to the assay. After mixing and further incubation for 45 s, the reaction was stopped as above with TCA. After 15 min of incubation on ice, the TCA-precipitated protein was pelleted by centrifugation at 13,000*g* (10,400 rpm) for 5 min at 4°C in a Sorvall SS34 rotor. Hydroxylamine treatment was achieved by treating the TCA-precipitated protein with 250 mol m^{-3} hydroxylamine in 50 mol m^{-3} Mes-Tris (pH 5.2) for 30 min on ice. For the controls, washing was carried out with the buffer only.

Following centrifugation of the TCA-precipitated protein, the supernatant was removed and 1 cm^3 of ice-cold distilled, deionized water was added to the tubes. The tubes were centrifuged and the supernatant removed as before. The protein pellets were resuspended in 80 mm^3 of solubilization buffer containing 100 mol m^{-3} sodium phosphate buffer (pH 5.5), 10% (v/v) glycerol, 1% (w/v) SDS, and 0.005% (w/v) bromophenol blue. In addition, the solubilization buffer contained 10 mol m^{-3} DTT, 0.5 mol m^{-3} PMSF, and 0.05 mol m^{-3} chymostatin, added fresh. After incubation at room temperature for 15 min, the samples were subjected to electrophoresis on acidic 5.6% polyacrylamide gels (2.7% cross-linker) containing 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate, and 0.1% (v/v) TEMED, buffered to pH 5.5 with 100 mol m^{-3} sodium phosphate. The electrophoresis running buffer contained 100 mol m^{-3} sodium phosphate (pH 5.5) and 0.1% SDS. Electrophoresis was carried out under constant current conditions at 170 mA per gel with a cooled aggregate for 2.5 to 3 h until the tracking dye reached the bottom of the gel. Immediately following electrophoresis, the gels were dried down onto filter paper using a Bio-Rad model 543 gel dryer. The dried gels were exposed to Kodak X-Omat AR 5 autoradiography film in the presence of Dupont Cronex Lightning Plus intensifying screens at −70°C for about 18 h unless otherwise indicated. The autoradiographs were then developed and the molecular masses of the phosphorylated bands were estimated by comparison with Sigma prestained molecular mass markers (27,000–180,000 D) run in adjacent lanes. The masses of the unbound-dye form of the molecular mass standards were used to obtain the molecular masses of the phosphorylated proteins, since, following regression analysis, the prestained markers were observed to separate in a manner similar to other Sigma unstained molecular mass markers on these acidic, low-percentage gels.

A modification of the method described by Briskin and Poole (1983) for the demonstration of the phosphorylated

intermediate of the plasma membrane proton pump was also used. The procedure was similar to that described above except for some minor differences. The reaction was carried out over a 20-s incubation period in the presence of 30 mol m^{-3} Tris-Mes (pH 6.5), 60 mmol m^{-3} $MgSO_4$, and 40 to 80 μg of membrane protein and was started by the addition of 20 mmol m^{-3} ATP-BTP (containing 185 kBq of [γ-^{32}P]ATP). The TCA-precipitated protein was solubilized in 1% (w/v) LDS, 50 mol m^{-3} citric acid-Tris (pH 2.4), 2% (v/v) β-mercaptoethanol, 20% (v/v) glycerol, 4×10^3 mol m^{-3} urea, and 0.001% (w/v) pyronin Y. The gels used contained 5.6% (w/v) acrylamide (2.7% [w/v] cross-linker), 0.2% (w/v) LDS, 50 mol m^{-3} citrate-Tris (pH 2.4), 0.016% ascorbate, and 0.0048% (w/v) $FeSO_4$. Polymerization was initiated by the addition of hydrogen peroxide to a final concentration of 0.006% (v/v). The electrophoresis running buffer contained 50 mol m^{-3} citrate-Tris (pH 2.4) and 0.2% (w/v) LDS. Electrophoresis was performed in a cold room under constant current conditions at 25 mA per slab gel for 2.5 to 3 h or until the pyronin Y tracking dye reached the bottom of the gel. Phosphorylation experiments were carried out at least three times on different membrane preparations and the results shown are those from a representative experiment.

Western Transfer and Immunoblotting

Immunoblotting was performed with the polyclonal antibody to the maize plasma membrane proton pump (kindly provided by Dr. R. Serrano of the European Molecular Biology Laboratory, Heidelberg, Germany). For details of the antibody, see Parets-Soler et al. (1990). Following electrophoresis, the gels were equilibrated in ice-cold transfer buffer containing 192 mol m^{-3} Gly, 25 mol m^{-3} Tris, and 20% (v/v) methanol (pH 8.3). The proteins were transferred to nitrocellulose using a Bio-Rad Trans-Blot electrophoretic transfer cell. Following transfer, the nitrocellulose was rinsed for 5 min in blotting buffer containing 150 mol m^{-3} NaCl and 10 mol m^{-3} NaH_2PO_4/NaOH (pH 7.2). The membrane was then blocked in the blotting buffer containing 5% (w/v) low-fat dried skimmed milk powder for 30 min. The membrane was incubated for 2 h with the polyclonal antibody at a 1:200 dilution in the blotting buffer with the addition of 8% (w/v) BSA. The membrane was then rinsed five times for 5 min each in blotting buffer, and the membrane was incubated for 2 h with protein A-alkaline phosphatase in blotting buffer containing 5% (w/v) dried skimmed milk powder. Following this, the membrane was rinsed five times for 5 min each with blotting buffer containing 1% (v/v) Triton X-100 and briefly with 100 mol m^{-3} diethanolamine-HCl (pH 9.8). The reaction of alkaline phosphatase was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. The immunoblotting experiments were repeated with at least two different membrane preparations and the results shown are from one representative experiment.

Protein Determination

Protein was measured with a modified Bradford (1976) procedure with BSA as standard.

RESULTS

Marker Enzyme Analysis of Membrane Fractions following Suc Gradient Centrifugation

This study aimed to produce enriched ER and plasma membrane fractions that showed little cross-contamination in order to characterize the respective calcium ATPases. Preparation of membrane fractions was initially carried out according to Giannini et al. (1987a), with 2 mol m^{-3} $MgSO_4$ in the homogenization medium but none in the Suc gradient (Fig. 1). This, however, resulted in an enrichment of the ER marker NADH-Cyt *c* reductase in the 10/22% interface (Fig. 1B) rather than in the 22/26% interface as previously described (Giannini et al., 1987a). The 10/22% fraction also showed the highest levels of the tonoplast marker nitrate-sensitive, azide-insensitive ATPase (results not shown). When magnesium was omitted from the homogenization medium and Suc gradient, the ER marker was seen to be dispersed throughout the gradient with a slight enrichment in the fraction from the 10/22% interface (Fig. 1A). However, with 5 mol m^{-3} magnesium in the homogenization medium and 3 mol m^{-3} in the gradient, the peak in NADH-Cyt *c* reductase activity shifted from the top of the gradient, giving a clear enrichment in the 22/26% interface (Fig. 1C). The reason for the difference in separation of the membranes from that previously reported (Giannini et al., 1987a) is uncertain but this difference may be due to possible differences in the growing conditions of the beets or in their developmental stage.

High levels of magnesium can lead to nonspecific membrane aggregation, but this does not appear to have occurred here, since the other cell membranes have peaked at their expected equilibrium densities (Table I). The distribution of

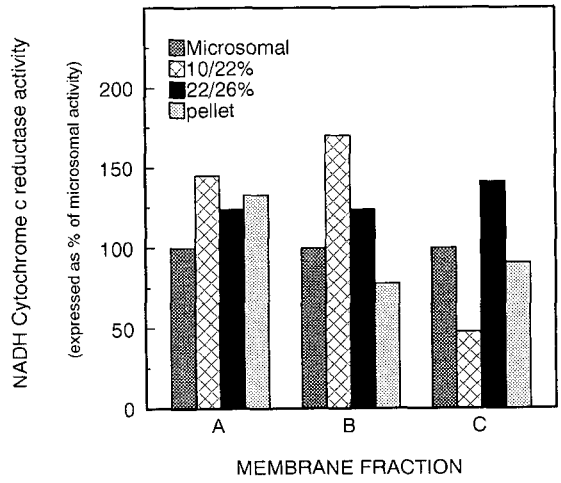

Figure 1. The effect of altering the concentration of magnesium in the homogenization medium and in the discontinuous Suc-density gradient on the distribution of the ER marker antimycin A-insensitive NADH-Cyt c reductase. A, No magnesium in the homogenization medium or gradient. B, Two mol m^{-3} magnesium in the homogenization medium, none in the gradient. C, Five mol m^{-3} magnesium in the homogenization medium, 3 mol m^{-3} magnesium in the gradient.

marker enzymes indicated that tonoplast was enriched at the 10/22% interface (tonoplast fraction), ER at the 22/26% interface (GER), plasma membrane at the 26/38% interface (GPM), and mitochondrial membranes in the pellet. However, the marker enzyme analysis shows that there was cross-contamination of the membrane fractions.

^{45}Ca Uptake into Vesicle Fractions Prepared by Suc Gradient Centrifugation

Calcium transport characteristics for the Suc gradient fractions gave further evidence that these were cross-contaminated (Table II). The strong inhibition by nitrate and protonophores and low inhibition by vanadate and EB in the tonoplast fraction is consistent with the presence of a Ca^{2+}/H^+ antiport at this membrane. For the GPM, the strong inhibition by EB and vanadate and low inhibition by nitrate and nigericin suggest that most of the transport in this fraction occurs by a direct calcium-pumping ATPase. Inhibition by nitrate and nigericin was lower in the GER than in the tonoplast fraction, whereas inhibition by vanadate was much stronger. This suggests that a proportion of the transport present is due to a calcium-transporting ATPase at this membrane. However, the presence of nitrate and nigericin inhibition is consistent with the presence in this fraction of antiport activity resulting from the presence of tonoplast. Although antiport activity could be eliminated in this ER fraction by the inclusion of nitrate and nigericin, the proportion of transport activity occurring due to any plasma membrane contamination cannot be readily assessed due to the expected similarity in transport properties of the two pumps. Therefore, further membrane purification was carried out.

Table II. *Calcium uptake into vesicles of red beet prepared by Suc gradient centrifugation, using 5 mol m^{-3} magnesium in the homogenization medium and 3 mol m^{-3} magnesium in the gradient*

Calcium uptake was measured at 22°C after 5 min in a 200 mm^3 reaction volume containing 250 mol m^{-3} Suc, 25 mol m^{-3} BTP-Mes (pH 7.25), 3.75 mol m^{-3} $MgSO_4$, 3.75 mol m^{-3} ATP/BTP (pH 7.25), 100 mol m^{-3} KCl, 0.4 mol m^{-3} NaN_3, 15 mol m^{-3} $CaCl_2$ with 3 μCi cm^{-3} $^{45}Ca^{2+}$. Uptake was initiated by the addition of 15 μg of membrane protein. Inhibitors were added as indicated, with KNO_3 replacing KCl when used.

Compound	Membrane Fraction			
	Microsomal	Tonoplast	ER	Plasma membrane
	% inhibition of ATP-stimulated calcium uptake			
Vanadate (100 mmol m^{-3})	61	24	57	44
EB (1 mmol m^{-3})	73	+13	40	94
Nitrate (100 mol m^{-3})	37	73	44	1
Nigericin (1 mmol m^{-3})	16	64	36	28

Phase Partitioning of a Microsomal Fraction and a GER

To reduce the level of cross-contamination in the fractions, aqueous two-phase partitioning was employed. Marker enzyme analysis (Table I) indicated that the upper phase (subsequently referred to as the PPM) obtained following phase partitioning of the microsomal fraction showed the highest vanadate-sensitive ATPase activity (plasma membrane marker) but negligible activity for other membrane markers, including the ER marker. To remove contaminating plasma membrane from the GER, phase partitioning of this fraction was performed. Marker enzyme analysis of the resulting upper and lower phases shows that plasma membrane (vanadate-sensitive ATPase) partitioned largely into the upper phase (Table I). The lower phase, subsequently referred to as the PER, showed an enrichment in ER (NADH-Cyt *c* reductase), although there was also an enrichment of other endomembranes in this fraction (Table I). As a consequence, although this fraction is enriched in the ER calcium-transporting system, it will also show an enrichment in the tono-

Table I. *Distribution of marker enzymes in membrane fractions of red beet*

The analyses of fractions shown are for the 10/22% interface (tonoplast), 22/26% interface (GER), 26/38% interface (GPM), and pellet, obtained using 5 mol m^{-3} magnesium in the homogenization medium and 3 mol m^{-3} magnesium in the Suc gradient. The GER was subjected to phase partitioning and the analyses of the resulting upper and lower phases are shown (PER [upper] and PER [lower]). Marker enzyme analysis of the upper phase, obtained following phase partitioning of the microsomal fraction, is also shown (PPM). For details of marker enzyme assays, see "Materials and Methods."

Membrane Fraction	Marker Enzyme			
	Vanadate-sensitive ATPase	K^+-stimulated PPase	NADH-Cyt c reductase	Cyt c oxidase
	μmol Pi mg^{-1} h^{-1}	*μmol PPi hydrolyzed mg^{-1} h^{-1}*	*μmol mg^{-1} min^{-1}*	*μmol mg^{-1} min^{-1}*
Microsomal	5.68	3.47	0.128	0.072
10/22% (tonoplast)	5.29	18.36	0.080	0.006
22/26% (GER)	4.67	7.79	0.219	0.024
26/38% (GPM)	19.34	2.91	0.120	0.00
Pellet	6.29	1.74	0.109	0.085
PER (upper)	13.41	0.64	0.01	0.00
PER (lower)	7.17	13.30	0.55	0.05
PPM	47.19	0.00	0.01	0.00

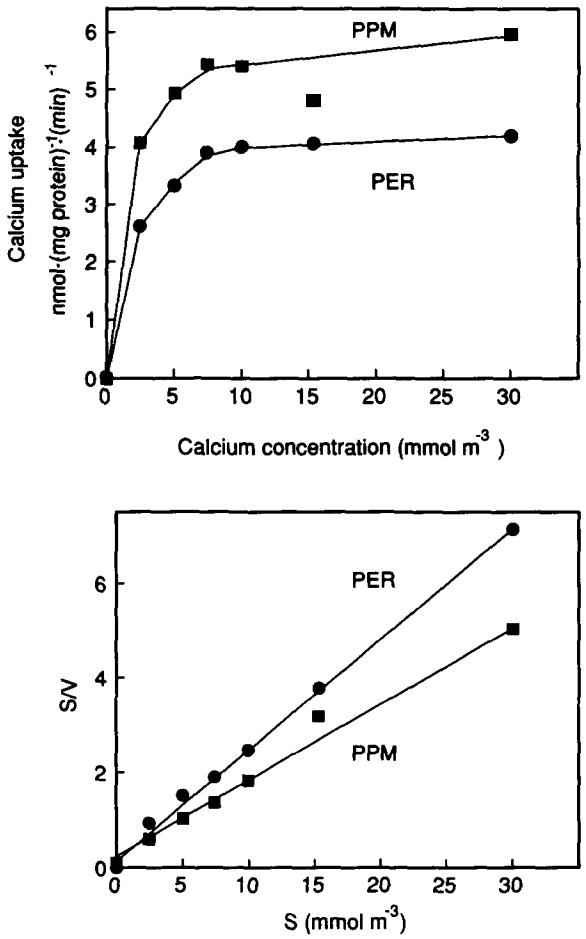

Figure 2. Calcium concentration dependence of ^{45}Ca uptake into the PPM and PER of red beet (upper). A Hanes-Woolf plot of the data is also shown (lower). K_m values of 0.92 mmol m^{-3} (PER) and 1.24 mmol m^{-3} (PPM) were obtained from the transformation.

plast-transporting system with respect to the GER. However, any calcium uptake occurring in the PER due to the operation of the tonoplast calcium antiporter was eliminated by inclusion of nitrate and nigericin at concentrations that totally inhibit the generation of a proton motive force required for the antiport to operate.

Following immunoblotting with an antibody raised to the maize plasma membrane proton pump (see "Materials and Methods" for details), a specific reaction was seen in the PPM but not in the PER (see below, Fig. 8ii), demonstrating that the PER shows little plasma membrane contamination.

Characteristics of ^{45}Ca Uptake into the PER and PPM

Calcium uptake into the PER and PPM was measured in the presence of 100 mol m^{-3} nitrate, 1 mmol m^{-3} nigericin, and 0.4 mol m^{-3} azide to avoid interference by tonoplast or mitochondrial systems. The ATP-driven uptake of calcium into the PER and PPM was A23187 reversible (results not shown). When calcium concentration dependence was determined, the uptake into the PER and PPM showed saturation kinetics with K_m and V_{max} values of 0.92 mmol m^{-3} and 4.3 nmol mg^{-1} min^{-1} for the PER, and 1.24 mmol m^{-3} and 6.0 nmol mg^{-1} min^{-1} for the PPM (Fig. 2).

Table III. *Effect of inhibitors on ATP-driven calcium uptake into the PER and the PPM*

Calcium uptake was measured at 22°C after 5 min in a 200-mm^3 reaction volume containing 250 mol m^{-3} Suc, 25 mol m^{-3} BTP-Mes (pH 7.25), 3.75 mol m^{-3} $MgSO_4$, 3.75 mol m^{-3} ATP/BTP (pH 7.25) (0.6 mol m^{-3} ATP/BTP for CPA), 100 mol m^{-3} KNO_3, 0.4 mol m^{-3} NaN_3, 1 mmol m^{-3} nigericin, 15 mol m^{-3} $CaCl_2$, with 3 μCi cm^{-3} $^{45}Ca^{2+}$. Uptake was initiated by the addition of 3 to 4 μg of membrane protein.

Inhibitor	Concentration	PER	PPM
		% inhibition	
Vanadate	100 mmol m^{-3}	39	67
EB	1 mmol m^{-3}	100	100
EB	0.05 mmol m^{-3}	65	83
CPA	100 nmol/mg protein	+5	9
Thapsigargin	0.2 mmol m^{-3}	0	0

Vanadate and EB both inhibited calcium transport into the PER and PPM; however, the plasma membrane system appeared to be more sensitive (Table III). Thapsigargin, a tumor-promoting sesquiterpene lactone that is a specific inhibitor of the ER/SR type calcium ATPase in animal cells (Thastrup et al., 1990; Lytton et al., 1991; Sagara and Inesi, 1991), had no effect on calcium uptake into either PER or PPM (Table III). CPA is an indole tetramic acid produced by certain fungi that, at low concentrations, specifically inhibits the SR calcium ATPase (Campbell et al., 1991) by stabilizing the calcium pump in an enzymically inactive conformation (Seldler et al., 1989). This inhibitor had no effect at concentrations used to inhibit the SR enzyme (Table III); it was effective only at high concentrations (5 μmol/mg protein), where it caused an inhibition in both the PER and PPM of about 30%.

The substrate specificity of calcium transport was com-

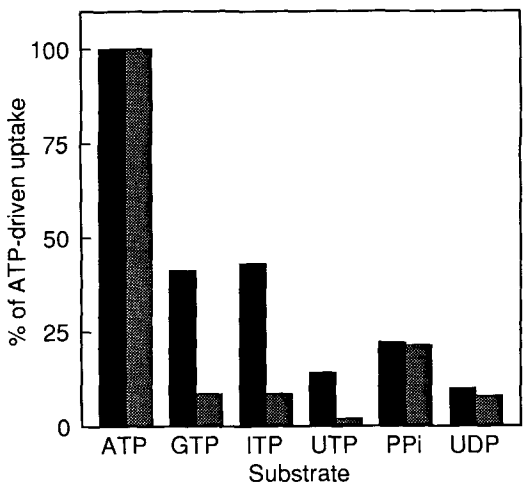

Figure 3. Substrate specificity of ^{45}Ca uptake into the PPM (black bars) and PER (shaded bars) of red beet. Substrate-driven uptake was determined after 10 min, and results are expressed as percentage of ATP-driven uptake (see "Materials and Methods" for details).

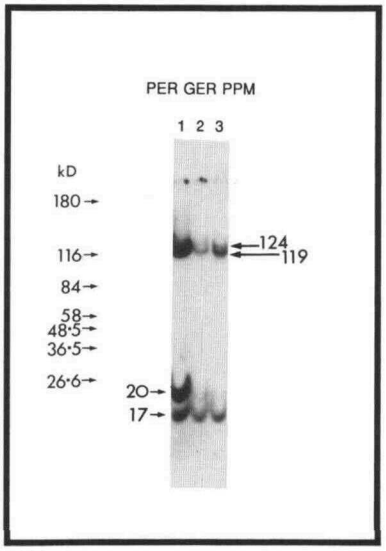

Figure 4. Comparison of phosphoproteins formed in the presence of calcium in the PER, GER, and PPM, fractions of red beet using equal protein loading. Phosphorylation, in the presence of calcium, and electrophoresis were carried out as described in "Materials and Methods." The molecular masses for the phosphoproteins, calculated after regression analysis, are given in the figure together with the position of the molecular mass standards.

pared in the PPM and PER (Fig. 3). GTP- and ITP-driven calcium transport in the PPM was approximately 40% of that obtained using ATP, whereas the other substrates tested were used at less than 25%. In contrast, in the PER, calcium transport was less than 25% of the ATP-driven uptake for all the substrates tested.

Phosphoenzyme Formation

The ^{45}Ca-uptake studies indicated that there were distinct calcium-transporting enzymes at the ER and plasma membrane of red beet. The inhibition of calcium transport in the PER and PPM by vanadate indicates that the enzymes responsible for transport form phosphorylated intermediates during their reaction cycles. To establish whether there are any major differences in the molecular properties of the plasma membrane and ER calcium-pumping enzymes, the formation of phosphorylated proteins in the presence of calcium, following incubation at 4°C for short time intervals with [γ-^{32}P]ATP, was studied in the PPM, GER, and PER. The relative molecular masses determined for the phosphorylated proteins in the three fractions varied slightly between experiments; therefore, those given here are the values obtained from one representative gel on which samples of the three membrane fractions were separated in adjacent lanes (Fig. 4). A 119-kD phosphoprotein was observed in all fractions. For longer exposure times, a second, 124-kD phosphoprotein was seen in the PPM and the GER. Two low molecular mass proteins of 20 and 17 kD were observed in the GER and PER and a single low molecular mass phosphoprotein of 17 kD was observed in the PPM. When equal amounts of membrane protein from the three membrane fractions were phosphorylated, the 119-kD phosphoprotein appeared to be most highly enriched in the PER, and yet the PPM showed a higher degree of phosphorylation of this polypeptide relative to the GER. This suggests that the ER and plasma membrane both possess a 119-kD phosphoprotein and that its presence in each fraction was not the result of cross-contamination. The 124-kD phosphoprotein was more strongly labeled in the PPM than in the GER and was not evident in the PER, suggesting that this protein was plasma membrane in origin and was present in the GER due to plasma membrane contamination (Fig. 4). The exposure time of the autoradiographs was very important. After short exposure times, only the 119-kD band was seen, but as the time was increased the 124-kD band became evident. For longer exposure times, it was difficult to distinguish the 119-kD band from the 124-kD band, and they appeared as one broad band.

The high molecular mass phosphoproteins have properties consistent with their representing phosphorylated intermediates of calcium ATPases. They were calcium dependent (Fig. 5i) and showed rapid turnover, as indicated by the reduction in labeling when excess unlabeled ATP and GTP was supplied as a chase (Fig. 5ii). Incubation with hydroxylamine following phosphorylation decreased the labeling relative to the buffer control (Fig. 5iii), indicating that the protein phosphate bond is an acyl phosphate linkage (Briskin and

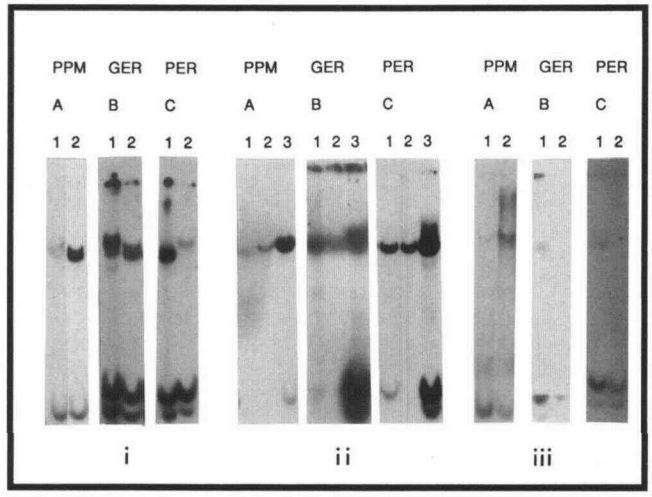

Figure 5. Characteristics of phosphoprotein formation in the PPM, the PER, and the GER of red beet. Phosphorylation, chases with ATP and GTP, hydroxylamine treatment, and electrophoresis were carried out as described in "Materials and Methods," i, Calcium dependence. A, PPM: lane 1, phosphorylation in the absence of calcium (0.5 mol m^{-3} EGTA present); lane 2, phosphorylation in the presence of calcium (no EGTA). B, GER: lane 1, −calcium; lane 2, +calcium; C, PER: lane 1, +calcium; lane 2, −calcium. ii, Chases with ATP and GTP following phosphorylation in the presence of calcium. A, PPM: lane 1, ATP chase; lane 2, GTP chase; lane 3, control. B, GER: lane 1, GTP chase; lane 2, ATP chase; lane 3, control. C, PER; lane 1, ATP chase; lane 2, GTP chase; lane 3, control. iii, Hydroxylamine treatment. A, PPM: lane 1, hydroxylamine treatment; lane 2, buffer control. B, GER: lane 1, buffer control; lane 2, hydroxylamine treatment. C, PER: lane 1, buffer control; lane 2, hydroxylamine treatment.

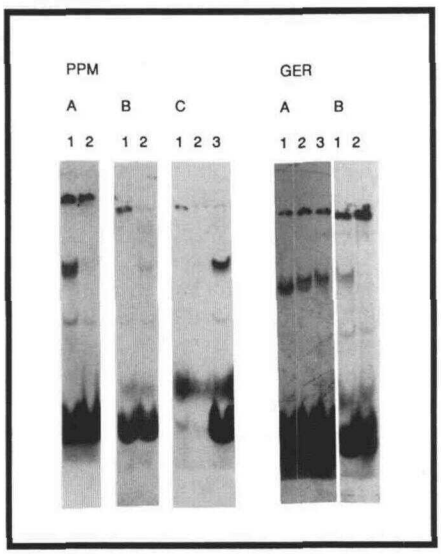

Figure 6. Phosphorylation of PPM and GER fraction using [γ-^{32}P]-GTP. Phosphorylation and electrophoresis were carried out as described in "Materials and Methods." Gels were exposed for 5 d. PPM: A, Calcium dependence of phosphorylated intermediates in PPM. Lane 1, +calcium; lane 2, −calcium. B, Hydroxylamine treatment of PPM following phosphorylation in the presence of calcium. Lane 1, hydroxylamine treatment; lane 2, buffer control. C, Chases with ATP and GTP following phosphorylation in the presence of calcium in PPM. Lane 1, GTP chase; lane 2, ATP chase; lane 3, control. GER: A, Calcium dependence of phosphorylated intermediates in GER. Lane 1, +calcium + KCl; lane 2, +calcium; lane 3, −calcium. B, Hydroxylamine treatment of GER following phosphorylation in the presence of calcium. Lane 1, buffer control; lane 2, hydroxylamine treatment.

Poole, 1983, and refs. therein). In contrast, the low molecular mass proteins generally showed no calcium dependence or hydroxylamine sensitivity, but they did chase out with ATP.

In the absence of calcium, two high molecular mass proteins were apparent in the PPM, but only after longer exposure times and the bands were still very faint (Fig. 5i, lane A1). These phosphoproteins appeared to have a very slightly higher molecular mass than those seen in the presence of calcium. This was also observed for the GER (Fig. 5i, lane B1); however, in this case, the proteins observed in the absence of calcium showed stronger labeling. In addition, a phosphoprotein of 98 kD was observed in the absence of calcium in the GER. For the PER (Fig. 5i, lane C2), in the absence of calcium, either no high molecular mass, band was visible or, in some cases, a weakly phosphorylated protein of 127 kD was observed.

The substrate specificity of the phosphoenzymes was investigated using GTP. When excess unlabeled GTP was used as a chase following phosphorylation of the fractions with ATP, there was a decrease in the intensity of the phosphorylated proteins in all the fractions (Fig. 5ii). When [γ-^{32}P]GTP was used as substrate in the phosphorylation reactions with the PPM and GER, 10 times higher levels of radioactivity and longer exposure times were necessary before the high molecular mass phosphoproteins could be seen, and the bands were still very faint (Fig. 6). Calcium-dependent phosphoproteins of 119 and 124 kD were seen in PPM and GER with properties similar to those observed when ATP was used as substrate (calcium dependence, rapid turnover, and hydroxylamine sensitivity) (Fig. 6). Unlike the high molecular mass phosphoproteins, the 17- and 20-kD proteins were strongly labeled with GTP. These phosphoenzymes showed properties similar to those observed when ATP was used as substrate. In some experiments using GTP as substrate, other phosphorylated proteins were seen, but these did not appear to have the properties of calcium-dependent, phosphorylated intermediates.

To ascertain whether the high molecular mass phosphoproteins had any distinguishing properties, the effect of a number of inhibitors and the response to various salts present in the assay medium were investigated (Fig. 7). Generally, 100 mmol m^{-3} vanadate and 50 mmol m^{-3} EB reduced the labeling of the calcium-dependent phosphoproteins in all the fractions (Fig. 7A). At 1 mmol m^{-3}, EB reduced the labeling of these proteins more than vanadate. Thapsigargin and CPA,

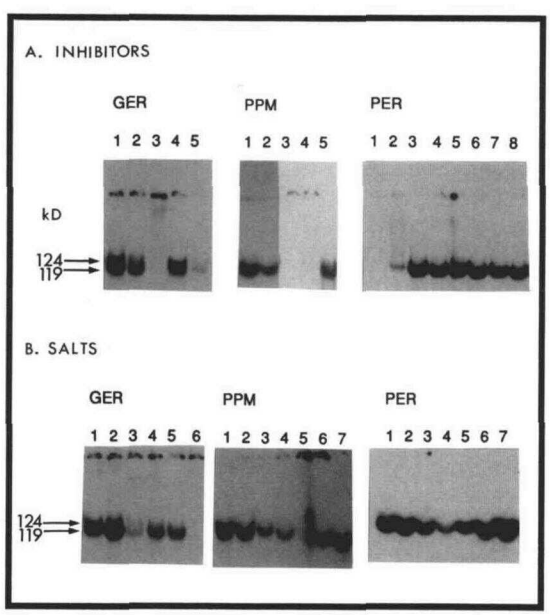

Figure 7. Effect of inhibitors and salts on high molecular mass phosphoproteins following phosphorylation in the presence of calcium. Phosphorylation and electrophoresis were carried out as described in "Materials and Methods." A, Inhibitors. GER: Lane 1, control (+calcium); lane 2, 1 mmol m^{-3} EB; lane 3, 50 mmol m^{-3} EB; lane 4, 1 mmol m^{-3} vanadate; lane 5, 100 mmol m^{-3} vanadate. PPM: Lane 1, control; lane 2, 100 mmol m^{-3} vanadate; lane 3, 50 mmol m^{-3} EB; lane 4, 1 mmol m^{-3} EB; lane 5, control (+calcium). PER: Lane 1, 50 mmol m^{-3} EB; lane 2, 1 mmol m^{-3} EB; lane 3, control (+calcium); lane 4, 0.2 mmol m^{-3} thapsigargin (in DMSO); lane 5, 0.1 mmol m^{-3} thapsigargin (in DMSO); lane 6, +calcium +DMSO (control); lane 7, 100 mmol m^{-3} CPA (in DMSO); lane 8, 10 mmol m^{-3} CPA (in DMSO). B, Salts (present at 50 mol m^{-3}). GER: Lane 1, control (+calcium); lane 2, +KCl; lane 3, +KNO_3; lane 4, +KBr; lane 5, +K_2SO_4 (25 mol m^{-3}); lane 6, membrane protein boiled for 5 min prior to phosphorylation. PPM: Lane 1, control (+calcium); lane 2; +KCl; lane 3; +K_2SO_4 (25 mol m^{-3}); lane 4, +KNO_3; lane 5, +KBr; lane 6, +NH_4Cl; lane 7, +NaCl. PER: Lane 1, +NaCl; lane 2, +NH_4Cl; lane 3, +KBr; lane 4, +KNO_3; lane 5, +K_2SO_4 (25 mol m^{-3}); lane 6, +KCl; lane 7, control (+calcium).

at concentrations used to inhibit the SR enzyme, had no marked effect on any phosphoproteins (results shown for PER only, Fig. 7A).

Potassium ions are reported to reduce the amounts of the phosphorylated intermediate of the red beet plasma membrane proton pump (Briskin, 1988), the Na^+/K^+-ATPase (Cantley 1981), and the Ca^{2+}-ATPase of animal cells (Shikegawa and Pearl, 1976). This is thought to occur by stimulation of the shift between the two conformations of the phosphoenzyme from E_1-P to E_2-P and hence the subsequent breakdown of the phosphorylated intermediate, a rate-limiting step in the reaction cycle of E_1E_2-type ATPases. The 119- and 124-kD phosphoproteins were affected differently by the various salts present in the assay (Fig. 7B). The 124-kD phosphoprotein, seen on longer exposure times in the PPM and GER, was generally reduced in the presence of all of the salts tested, whereas for the 119-kD phosphoprotein, only KNO_3, K_2SO_4, and KBr markedly reduced the labeling. KCl consistently enhanced phosphorylation of the 119-kD phosphoprotein in the GER but not the PPM or PER; the reason for this is not clear at present, but it is possible that the phase-partitioning procedure had some effect on the proteins.

Location of the Proton Pump

The phosphorylated intermediate of the proton pumping H^+-ATPase has previously been demonstrated to be a 100-kD protein in red beet plasma membrane fractions (Briskin and Poole, 1983), but in the present study the proton pump phosphorylated intermediate was not readily apparent. Previously, different assay and electrophoresis conditions were used: acidic LDS gels (pH 2.4) at low temperature and phosphorylation at pH 6.5 in the presence of higher MgATP concentrations but in the absence of added calcium ("proton pump conditions"). Using proton pump conditions and longer exposure time (3 d), a single high molecular mass phosphoprotein was observed in the PPM (Fig. 8i), but no reaction was seen in the PER (results not shown). The molecular mass markers ran diffusely on the LDS gels, making it difficult to determine the relative molecular mass accurately, but the phosphoprotein occurred in a position close to the calcium-dependent, 119-kD protein (results not shown). Because of the poor resolution using LDS gels, the molecular mass of the calcium-dependent phosphoproteins was compared with the plasma membrane proton pump by carrying out simultaneous phosphorylation and immunoblotting with the proton pump antibody, using acidic 5.6% SDS gels. A reaction to the proton pump antibody was seen only in the PPM and not in the PER (Fig. 8ii). By overlaying the resulting autoradiographs on the corresponding immunoblots, the position of the 119-kD calcium-dependent phosphorylated proteins formed in the PPM (Fig. 8iii) using calcium-pump conditions (50 mmol m^{-3} calcium, pH 7.4, low MgATP concentrations) could be compared with the cross-reacting protein. The antibody reaction on the nitrocellulose occurred at the lower edge of the 119-kD phosphorylated intermediate band on the autoradiograph.

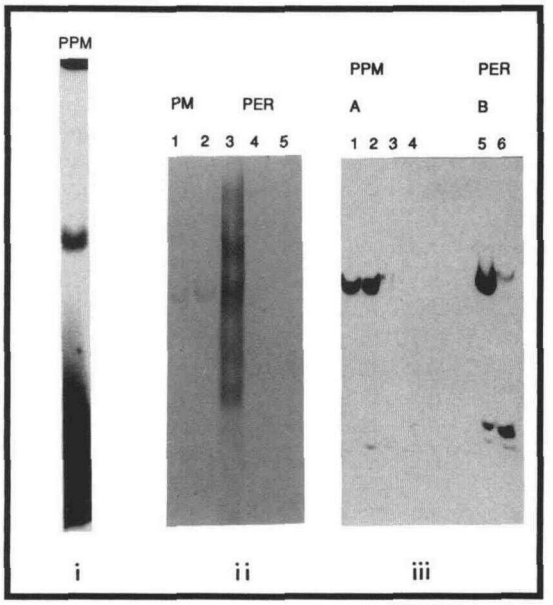

Figure 8. i, Formation of phosphoprotein in the PPM under proton-pump conditions. Phosphorylation and electrophoresis on acidic 5.6% LDS polyacrylamide gels were carried out as described in "Materials and Methods." Arrow indicates high molecular mass phosphoprotein. ii, Western blotting of PPM (lanes 1 and 2) and PER (lanes 4 and 5) with a maize H^+-ATPase antibody. Lane 3, Markers. Electrophoresis and immunoblotting were carried out as described in "Materials and Methods." Acidic 5.6% SDS gels were used. iii, Formation of phosphorylated intermediates in PPM and PER and transfer to nitrocellulose. Phosphorylation, electrophoresis (using acidic 5.6% SDS gels), and transfer of proteins to nitrocellulose were carried out as described in "Materials and Methods." Nitrocellulose was exposed for 7 d. A, PPM: lanes 1 and 2, +calcium; lanes 3 and 4, −calcium. B, PER: lane 5, +calcium; lane 6, −calcium.

DISCUSSION

The results of this study, using membrane fractions that show little cross-contamination, suggest that there are distinct calcium-transporting ATPases at the ER and plasma membrane of red beet. The apparent calcium affinities of the plasma membrane and ER calcium pumps, determined by ^{45}Ca uptake, were similar and suggest that these pumps would both function efficiently in vivo to maintain low cytosolic calcium concentrations. In this study, the K_m was determined by measuring uptake in the presence of calcium-EGTA buffers. The values obtained were lower (although in the same range) than those obtained previously (Giannini et al., 1987a, 1987b), in which buffers were not used.

There appear to be at least two different calcium-dependent phosphorylated intermediates in red beet with molecular masses of about 119 and 124 kD. The evidence suggests that the plasma membrane and ER both contain a 119-kD calcium-ATPase and that its presence in each fraction is not due to cross-contamination. Although the characteristics demonstrated for the phosphorylated intermediate of this protein in the PPM and PER are broadly the same, further work is necessary to determine whether these are identical proteins. The 124-kD phosphorylated intermediate appears to be present only at the plasma membrane. The possibility exists that the 119-kD phosphoprotein is a proteolytic product of the 124-kD enzyme. However, the different response by the two proteins to various salts in the phosphorylation assay and the

apparent lack of the 124-kD protein in the PER suggest that this is unlikely.

There have now been a number of reports using various plant tissues identifying calcium-dependent phosphorylated intermediates. These have tentatively been suggested as forms of either ER or plasma membrane calcium pumps, although this has not been shown unequivocally in any of the studies. The reported molecular masses for these intermediates vary quite widely. A 140-kD phosphoprotein, observed in maize, was initially postulated to be the plasma membrane calcium pump (Briars and Evans, 1989), whereas in carrot cells a 120-kD phosphoprotein was identified (Hsieh et al., 1991). In cauliflower, the 115-kD phosphoprotein observed in a reconstituted fraction was suggested to represent the calmodulin-stimulated calcium ATPase; this activity separated with the ER after Suc-gradient centrifugation of a microsomal fraction (Askerlund and Evans, 1992). In red beet, the phosphoproteins observed previously under calcium-pump assay conditions in ER- and plasma membrane-enriched Suc gradient fractions were determined to be around 100 kD (Giannini et al., 1987a; Williams et al., 1990). The reason for the differences in the molecular masses in the studies on red beet is not clear at present but this difference may be due to differences in the membrane preparations used or the conditions for gel electrophoresis. To ascertain whether there are such marked variations in the size of the calcium pumps between plant species, and whether the varied results are not instead due to different experimental procedures, it would be useful to compare directly the phosphorylated intermediates of the calcium-ATPases from these species under the same experimental conditions. It is interesting that preliminary results comparing red beet and maize indicate that the maize phosphorylated intermediate has a slightly higher molecular mass than the beet enzymes (L.J. Thomson and L.E. Williams, unpublished data).

In this study and in previous reports (Briars and Evans, 1989; Hsieh et al., 1991) investigating phosphorylated intermediates of calcium pumps, no phosphoenzyme representing the plasma membrane proton pump was identified under the assay conditions used (higher pH, the presence of calcium, low MgATP). However, red beet has been used previously in a number of studies to identify the phosphorylated intermediate of the proton pump (Briskin and Poole, 1983). In the present study, it was found that if different assay conditions were used (lower pH, no calcium, higher MgATP) a phosphorylated protein was seen in the PPM, but not in the PER, and this had a mobility on gels similar to the protein in the PPM that cross-reacted with the proton pump antibody. This, therefore, may be the proton pump, which may require different conditions than the calcium pump for optimum phosphorylation. The immunoreaction with the proton pump antibody occurred at the lower edge of the 119-kD band seen after phosphorylation in the presence of calcium, indicating that, using these gels, the proton pump has a slightly lower molecular mass than the calcium pump. However, further studies, possibly with gradient gels, are required to resolve these proteins clearly.

The very faint high molecular mass bands observed in the absence of calcium in the PER and PPM ran at a slightly higher molecular mass than those seen in the presence of calcium. It is possible that these were the calcium-dependent phosphorylated intermediates running differently in the presence of EGTA. A similar effect has been reported previously for phosphoproteins formed in maize coleoptile microsomes (Briars and Evans, 1989). The molecular mass of the phosphorylated intermediate of the erythrocyte calcium pump appeared to shift in the presence of EGTA (Szasz et al., 1978). It is also possible, however, that the phosphoproteins formed in the absence of calcium are not related to the calcium-dependent phosphoproteins and are, in fact, phosphoproteins that are inhibited by micromolar concentrations of calcium.

The identity of the two low molecular mass phosphoproteins (20 and 17 kD) observed in red beet in this study is not clear. In maize coleoptile microsomes, the rapidly turning over, hydroxylamine-sensitive 28-kD phosphoprotein was suggested to be present as a result of partial proteolysis of the 140-kD phosphorylated intermediate (Briars and Evans, 1989). However, the 20- and 17-kD phosphoproteins observed in the PER and PPM were generally insensitive to hydroxylamine treatment and are therefore unlikely to represent proteolytic products of the red beet calcium-ATPases. It is possible that they are the result of protein kinase activity in these membrane fractions. Rapidly turning-over kinases have been demonstrated in other plant tissues (Blowers and Trewavas, 1989; Garbarino et al., 1991).

None of the inhibitors used in this study clearly distinguished between the phosphorylated intermediates. Although EB was inhibitory at micromolar concentrations, any differences in the sensitivity to this inhibitor of the intermediates in the PER and PPM were not readily apparent. Measurements of ^{45}Ca uptake indicated that the plasma membrane transporter may be slightly more sensitive, but further studies, with a range of concentrations, are required to confirm this. EB inhibition of plasma membrane calcium uptake has been reported for a number of species, but the reported sensitivity to this compound varies (Graf and Weiler, 1989; Rasi-Caldogno et al., 1989; Olbe and Sommarin, 1991). Perhaps the suggestion of using the sensitivity of calcium transport to low concentrations of EB as a marker for the plasma membrane calcium-ATPase (Briskin, 1990) should be regarded with caution until more studies are carried out with purified ER fractions from more plant species. However, EB does appear to be a useful marker at low concentrations (1 mmol m^{-3}) for distinguishing primary calcium-transport systems (calcium-ATPases) from secondary calcium-transport mechanisms, because the tonoplast Ca^{2+}/H^+ antiporter is not significantly affected by EB at these concentrations (Table II, this study; Chanson, 1991). The strong inhibition by 1 mmol m^{-3} EB in the PER and PPM observed in this study confirms that there is no residual transport by the tonoplast antiporter under the conditions of these experiments. EB has also been shown to cause differential inhibition of plant calcium-ATPases and H^+-ATPases depending on the concentration used. The radish plasma membrane proton pump is half-maximally inhibited by 10 mmol m^{-3} EB (Coccuci, 1986), whereas the plasma membrane calcium pump from the same plant material has an I_{50} for EB of 0.2 mol m^{-3} (Rasi-Caldogno et al., 1987). The marked sensitivity of phosphoenzyme formation in the PER and PPM to 1 mol m^{-3} EB argues against

their representing forms of the plasma membrane proton pump. The mode of action of EB is unclear, but our studies suggest that it may be capable of directly blocking the formation of phosphorylated intermediates of calcium pumps. This might be achieved by hindering the access of ATP to the nucleotide-binding site on the enzymes or by preventing covalent linkage of the terminal phosphate from ATP to the acyl residue in the protein. Binding studies with the SR calcium-ATPase suggest that EB binds to a site near to, but not identical with, the nucleotide-binding site (Morris et al., 1982).

More is known about the mode of action of vanadate, which is thought to inhibit E_1E_2-type ATPases by blocking the shift in conformation states during the reaction cycle of these enzymes. It is thought to bind to the enzyme and trap it in its E_2 conformation. Vanadate inhibits both ATP-hydrolytic activity and ion-transporting action. It has previously been shown partially to inhibit phosphorylated intermediates of the plasma membrane proton pump from red beet (Briskin and Poole, 1983), the plasma membrane proton pump from corn roots (Briskin and Leonard, 1982), the calcium-dependent phosphorylated intermediate in fractions from cultured carrot cells (Hsieh et al., 1991), and the plasma membrane calcium pump from rat intestinal basolateral membranes (Wajsman et al., 1988). Although in red beet calcium transport into PPM was more sensitive to 100 mmol m^{-3} vanadate than transport into PER, no marked differences in sensitivity were observed for the phosphorylated intermediates in each fraction, although a concentration range for vanadate was not tested. Reports on vanadate inhibition of calcium uptake into clearly defined plasma membrane vesicles are few. In wheat root plasma membrane vesicles (Olbe and Sommarin, 1991) and radish microsomes (Rasi-Caldogno et al., 1987), the inhibition values were similar (I_{50} approximately 50 mmol m^{-3}), whereas the *Corydalis sempervirens* system was less sensitive (I_{50} of 95 mmol m^{-3}), especially after solubilization and reconstitution of the enzyme (I_{50} of 450 mmol m^{-3}) as reported by Liß et al. (1991). In *Commelina communis* plasma membrane vesicles, the vanadate sensitivity was extremely low, with an I_{50} of 500 mmol m^{-3} (Graf and Weiler, 1989).

There are several possible reasons for the marked variations in the values for vanadate sensitivity; it is possible that measurements were made after different incubation times or under different incubation conditions with vanadate or that in certain cases preincubation was carried out (Olbe and Sommarin, 1991). It is also possible that some preparations may have contained contaminating ER, which may show a different sensitivity to this inhibitor. For calcium uptake into the ER, again there are few studies using highly purified ER fractions. In corn roots and zucchini hypocotyls, ER calcium transport (as measured by chlorotetracycline fluorescence changes) was inhibited totally by 100 mmol m^{-3} vanadate (Lew et al., 1986; Brauer et al., 1990). Vanadate inhibited the ER calcium transport system in cultured carrot cells with an I_{50} of 12 mmol m^{-3} (Bush and Sze, 1986).

The lack of inhibition of transport and phosphoenzyme formation observed in red beet and in cauliflower (Askerlund and Evans, 1992), using low concentrations of thapsigargin and CPA (specific inhibitors of the ER/SR-type calcium-ATPase in animal cells), suggests that these compounds may not be specific inhibitors of the ER uptake system in plants. This lack of inhibition may reflect possible differences in the plant and animal SR/ER-type calcium-ATPases. It is interesting that we have found no specific cross-reaction to beet ER membrane fractions with monoclonal antibodies (generously provided by Dr. M. East, Southampton University, UK) raised to the SR calcium-ATPase (T. Xing, unpublished data). Only at higher concentrations did CPA inhibit transport, and the inhibition of uptake occurred in both fractions. At this higher concentration, inhibition may be due to a nonspecific action of the compound, possibly affecting the permeability of membrane vesicles, although this has not been investigated in red beet. CPA-sensitive calcium transport observed in membrane fractions of cultured carrot cells (Hsieh et al., 1991) deserves further attention to determine the membrane location of this activity.

A number of workers have shown that the plasma membrane calcium-pumping ATPase can use GTP as an alternative substrate (Rasi-Caldogno, 1989; Williams et al., 1990; Olbe and Sommarin, 1991); however, it is not clear, as yet, whether this is also the case for the ER pump. Although in certain species GTP supported calcium uptake in ER-enriched fractions (Buckhout, 1983), this may have been a result of plasma membrane contamination. In red beet, although GTP can drive calcium uptake into the PPM at approximately 40% of the level obtained with ATP, in the phosphorylation studies performed here with GTP, 10-fold higher levels of radiolabel and 5 times longer exposure times were required before the 119- and 124-kD phosphorylated intermediates were visible in this fraction. Therefore, it is possible that the enzyme kinetics are different in the presence of GTP. Faint bands were also seen in the GER, and GTP chased out the PER phosphoprotein in a manner similar to ATP. Thus, it appears that phosphorylation of the ER protein may also be achieved with GTP to some extent, even though transport rates with GTP were very low. Further studies are needed with ER fractions from other species, since substrate specificity may be one of the major criteria for differentiating between the PM and ER pumps. Also, the response to the presence of various salts may be useful for distinguishing between different phosphorylated intermediates, since the 119- and 124-kD phosphoproteins observed in this study showed differing responses to the presence of various salts in the phosphorylation assay. The reasons for this are not clear at present, but there may be a direct anion interaction with the proteins or some type of charge compensatory mechanism in operation.

In summary, this study provides further evidence for the presence of distinct calcium-transporting ATPases at the plant ER and plasma membrane, which form phosphorylated intermediates. The possibility of two different calcium-ATPases, or possible isoforms, at the plasma membrane is particularly interesting in light of the varying reports of the effects of calmodulin on calcium-ATPases in different plant species. The results obtained with inhibitors, particularly CPA and thapsigargin, suggest that it is too soon to conclude that the calcium-transporting ATPases in plant cells are closely similar to those occurring in animal cells.

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