Plant Physiol. (1987) 85, 1137–1142
0032-0889/87/85/1137/06/$01.00/0

Calcium Transport in Sealed Vesicles from Red Beet (*Beta vulgaris* L.) Storage Tissue[1]

II. CHARACTERIZATION OF $^{45}Ca^{2+}$ UPTAKE INTO PLASMA MEMBRANE VESICLES

Received for publication May 5, 1987 and in revised form August 31, 1987

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ABSTRACT

Calcium uptake was examined in sealed plasma membrane vesicles isolated from red beet (*Beta vulgaris* L.) storage tissue using $^{45}Ca^{2+}$. Uptake of $^{45}Ca^{2+}$ by the vesicles was ATP-dependent and radiotracer accumulated by the vesicles could be released by the addition of the calcium ionophore A23187. The uptake was stimulated by gramicidin D but slightly inhibited by carbonylcyanide *m*-chlorophenylhydrazone. Although the latter result might suggest some degree of indirect coupling of $^{45}Ca^{2+}$ uptake to ATP utilization via $\Delta\mu H^+$, no evidence for a secondary H^+/Ca^{2+} antiport in this vesicle system could be found. Following the imposition of an acid-interior pH gradient, proton efflux from the vesicle was not enhanced by the addition of Ca^{2+} and an imposed pH gradient could not drive $^{45}Ca^{2+}$ uptake. Optimal uptake of $^{45}Ca^{2+}$ occurred broadly between pH 7.0 and 7.5 and the transport was inhibited by orthovanadate, *N*,*N*′-dicyclohexylcarbodiimide, and diethylstilbestrol but insensitive to nitrate and azide. The dependence of $^{45}Ca^{2+}$ uptake on both calcium and Mg:ATP concentration demonstrated saturation kinetics with K_m values of 6 micromolar and 0.37 millimolar, respectively. While ATP was the preferred substrate for driving $^{45}Ca^{2+}$ uptake, GTP could drive transport at about 50% of the level observed for ATP. The results of this study demonstrate the presence of a unique primary calcium transport system associated with the plasma membrane which could drive calcium efflux from the plant cell.

In animal cells, the efflux of calcium across the plasma membrane is mediated by two possible transport mechanisms. One mechanism, ubiquitous in mammalian cell membranes, involves a Ca^{2+}-ATPase which functions as an ATP dependent, primary calcium transport pump (16, 21, 24). The other mechanism, present primarily in cells from excitable tissue, involves a Na^+/Ca^{2+} exchanger which utilizes the sodium gradient produced by the Na^+,K^+-ATPase to drive calcium efflux (16, 21). In terms of the physiological roles for these two systems, it appears that the Na^+/Ca^{2+} exchanger may represent a high capacity, low affinity efflux mechanism while the Ca^{2+} pumping ATPase may represent a high affinity efflux mechanism responsible for reducing intracellular calcium to its final resting level in the cell. Of the animal plasma membrane Ca^{2+}-pumping ATPases examined so far, the erythrocyte enzyme has been the most extensively studied. This enzyme has a subunit mol wt of 130 to 150 kD and forms a phosphorylated intermediate during the course of ATP hydrolysis (16, 24). It is also stimulated by calmodulin and inhibited by calmodulin antagonists (16).

[1] Supported by funds from the University of Illinois Agricultural Experiment Station (Hatch Project No. 15-0327).

As in animal cells, plant cells maintain low cytoplasmic calcium levels by various transport processes (13) and there is evidence of a role for this cation in signal transduction (15 and references therein). Studies with isolated membrane vesicles from plant cells have suggested the possibility of both a Ca^{2+}-pumping ATPase (9–11, 27) and a H^+/Ca^{2+} exchanger (27) being associated with the plasma membrane. However, these studies were carried out with microsomal membrane fractions where both the degree of sealing of the plasma membrane and relative contamination by sealed vesicles from other membrane components were uncertain. Indeed, recent studies have shown the presence of an ATP driven calcium transport system (Ca^{2+}-pumping ATPase) associated with the ER (6, 7, 13, 19) and a H^+/Ca^{2+} exchanger associated with the tonoplast (1, 25) which could have been present as contaminants in the previous work on putative plasma membrane calcium transport systems using microsomal membranes. Therefore, these proposals for calcium transport systems at the plasma membrane need to be confirmed using a vesicle system with defined sealing of the plasma membrane and where the contamination by vesicles from other cellular components is known.

In a previous report from this laboratory (12), methodology was developed for the isolation of sealed plasma membrane vesicles from red beet (*Beta vulgaris* L.) storage tissue. The final plasma membrane vesicle fraction, isolated on a sucrose density gradient, was relatively free of contamination by vesicles from the ER and tonoplast. In the previous paper in this series (13), calcium transport was examined in ER vesicles isolated using a similar procedure (0.25 M KI absent) but recovered at a different region of the sucrose gradient. The use of chlorotetracycline as a probe for calcium transport (19) appeared to be selective for ER calcium transport in this system. In the present study, calcium uptake into sealed plasma membrane vesicles from red beet was examined using $^{45}Ca^{2+}$. The results suggest that calcium efflux at the plasma membrane may be mediated by a primary, ATP-dependent Ca^{2+} pump and that H^+/Ca^{2+} exchange may be absent.

MATERIALS AND METHODS

Plant Material. Red beet (*Beta vulgaris* L., cv Detroit Dark Red) storage roots were purchased commercially. The tops of the plants were removed and the storage roots were stored in moist vermiculite at 2 to 4°C until use. All root tissue was stored at least 10 d prior to use to ensure uniformity in membrane isolation (22).

Membrane Isolation. Sealed plasma membrane vesicles were isolated from red beet storage tissue as previously described (12, 13). For the routine preparation of plasma membrane vesicles, the microsomal membrane pellet was suspended in 250 mM sucrose, 10% glycerol, 0.2% BSA, 2 mM BTP[2]/Mes (pH 7.0), 1

mM DTE (suspension buffer), and layered on a discontinuous sucrose density gradient consisting of 15 ml of 26% (w/w) sucrose layered over 18 ml of 38% (w/w) sucrose. Both sucrose solutions were buffered to pH 7.2 with 1 mM Tris/Mes and contained 1 mM DTE. The gradient was centrifuged for 2 h at 100,000*g* (25,000 rpm) in a Beckman SW28 rotor and the membranes at the 26/38% (w/w) sucrose interface were collected. The gradient purified membranes were diluted in suspension buffer and concentrated by centrifugation at 80,000*g* for 30 min. The membrane pellet was suspended in suspension buffer to a protein concentration of 4 mg/ml and either used immediately or frozen under liquid N_2 and stored at −80°C. Membrane vesicles stored in this manner retained transport activity for up to 3 months.

Uptake of Radiolabeled Calcium. Calcium uptake was measured at 22°C in a 1 ml reaction volume containing 250 mM sorbitol, 25 mM BTP/Mes (pH 7.0), 3.75 mM $MgSO_4$, 3.75 mM ATP, 100 mM KNO_3, 10 μM $CaCl_2$ (containing 2.5 μCi $^{45}Ca^{2+}$), and 0.4 mM NaN_3 (transport buffer). The uptake was initiated by adding plasma membrane vesicles to a final concentration of 33 to 35 μg protein/ml. At timed intervals following the initiation of uptake, 150 μl of the media was removed and the membrane vesicles were collected by vacuum filtration onto 0.45 μm metricel membrane filters (Gelman, Inc.). The filters were then rapidly washed four times each with 1 ml aliquots of transport buffer without ATP and $^{45}Ca^{2+}$ (washing buffer). Filtration was carried out using a manifold system consisting of six individual scintered glass filter support units (Microfiltration, Inc.), individually valved to a common vacuum line. The filters were incubated in washing buffer for at least 20 min under vacuum prior to use. Radioactivity associated with the filters was determined by liquid scintillation spectroscopy in 5 ml of scintillation cocktail (Aquasol, New England Nuclear). Any variations in these conditions are indicated in "Results."

Production of Acid Interior pH Gradients. Acid interior pH gradients were produced by rapidly adding aliquots of 1 M BTP to 1 ml of buffer containing 250 mM sorbitol, 2.5 mM BTP/Mes (pH 5.5) and plasma membrane vesicles (about 35 μg protein). When the artificial pH gradient was monitored by fluorescence spectroscopy, the medium contained 5 μM acridine orange as a pH sensitive fluorescent probe (17). Fluorescence measurements were carried out at 25°C using a Perkin-Elmer model 203 spectrofluorimeter with the excitation monochronometer set at 472 nm and the emission monochronometer set at 525 nm. Any variation in these reaction conditions is indicated in "Results."

Protein Assay. Protein was determined by the method of Bradford (2) using BSA as a protein standard. The Bradford assay reagent was filtered prior to use.

All data shown are for representative experiments which have been repeated at least three separate times. Within each experiment the individual data points represent the mean of two determinations.

RESULTS AND DISCUSSION

$^{45}Ca^{2+}$ Uptake in Sealed Red Beet Plasma Membrane Vesicles. In previous studies carried out in this laboratory (12), a method was developed for the isolation of sealed plasma membrane vesicles from red beet storage tissue. The assignment of a plasma membrane origin for the sealed vesicles was supported by the observation that proton transport displayed properties similar to those of the plasma membrane ATPase with respect to pH optimum, substrate specificity, and inhibitor sensitivity. In addition, the distribution of proton transporting vesicles on sucrose density gradients followed the distribution of plasma membrane as indicated by marker enzyme activities. When red beet plasma membrane vesicles isolated in this manner were incubated with $^{45}Ca^{2+}$, an ATP-dependent increase in radiolabel associated with the vesicles was observed (Fig. 1). Since this increase in vesicle associated $^{45}Ca^{2+}$ could be discharged by the addition of the calcium ionophore A23187, the timecourse presented in Figure 1 represents the uptake of calcium into the vesicles. If the addition of ATP only energizes those plasma membrane vesicles in the inside-out orientation (26 and references therein), the transport observed in these assays would correspond to calcium efflux from the intact plant cell. This orientation of the calcium transport system in the vesicle preparation would be consistent with its possible function in calcium removal from the cytoplasm as previously proposed (8–11, 15, 27).

While the gradient purified vesicles used in these studies may be fairly free of cross-contamination by calcium transporting vesicles from other membrane components (mitochondria, tonoplast, and ER vesicles), specific inhibitors were added to all assays to ensure that the observed calcium uptake reflected transport at the plasma membrane. Transport assays were generally carried out in the presence of 100 mM KNO_3 and 0.4 mM NaN_3 to eliminate the possibility of calcium transport occurring in tonoplast (1, 25) and mitochondrial (20) membrane vesicles, respectively. Although we have demonstrated ATP-dependent calcium transport in ER vesicles in the previous paper in this series (13), sufficient differences in the properties of calcium transport suggest that ER vesicles cannot account for the uptake observed in this study (see subsequent sections).

In order to determine whether $^{45}Ca^{2+}$ uptake in the vesicles was directly coupled to ATP utilization or involved the use of the proton electrochemical gradient established by the plasma membrane H^+-ATPase (*i.e.* H^+/Ca^{2+} antiport), transport was examined in the presence of either gramicidin D or CCCP (Fig. 1). Both of these compounds would eliminate the proton electrochemical gradient ($\Delta\mu H^+$) so that any residual calcium uptake would indicate direct coupling to ATP utilization. Calcium uptake was enhanced in the presence of 5 μM gramicidin D, but slightly decreased in the presence of 10 μM CCCP. These results were conflicting and difficult to interpret. While these concentrations of the ionophores were found to be optimal for fully collapsing $\Delta\mu H^+$ in our previous studies with red beet plasma membrane vesicles (12), it should be noted that at this CCCP

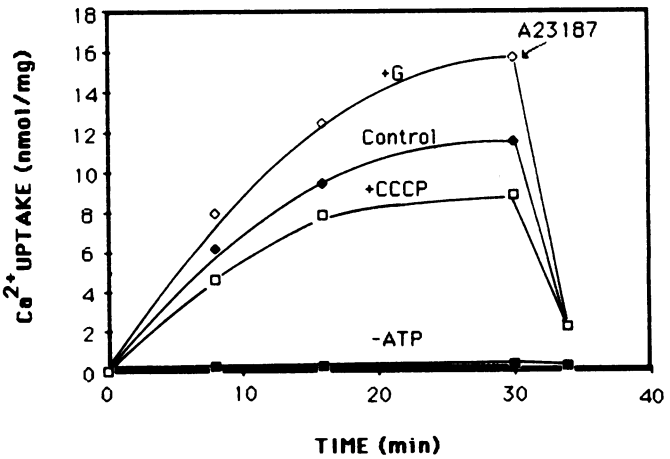

FIG. 1. Timecourse of $^{45}Ca^{2+}$ uptake by plasma membrane vesicles from red beet storage tissue. The uptake of $^{45}Ca^{2+}$ was carried out as described in "Materials and Methods." As indicated, 0.3 μg/ml A23187 was added to release calcium gradients produced by ATP-dependent uptake. Gramicidin D (G) and CCCP, when added, were present at 5 μM and 10 μM, respectively.

[2] Abbreviations: BTP, bis-tris propane; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; DES, diethylstilbestrol; G, gramicidin D; Mes, 4-morpholinoethanesulfonic acid.

concentration, direct inhibitory effects on transport systems separate from uncoupling have been observed (23 and references therein). However, even if this effect of CCCP were to reflect the inhibition of secondary Ca^{2+} transport, these results would imply that primary transport represents the major pathway for Ca^{2+} uptake in these vesicles, since inhibition by this protonophore was at most 25% when $\Delta\mu H^+$ would be fully collapsed. In order to investigate the possibility of secondary calcium transport with the vesicles in a more direct manner, the approach of using artificial pH gradients was employed.

Imposition of Artificial pH Gradients and Their Effect on $^{45}Ca^{2+}$ Uptake. Acid-interior pH gradients were produced in the plasma membrane vesicles by rapidly changing the exterior pH from 5.5 to 7.3 by the addition of BTP. If a secondary system for the transport of calcium (H^+/Ca^{2+} antiport) was associated with the plasma membrane, then this imposed pH gradient should have the capacity to drive calcium uptake and calcium should affect H^+ flux. The pH difference produced in this manner would also be within the expected range produced as a result of ATP-dependent H^+-pumping in inside-out plasma membrane vesicles which could occur under the conditions of ATP-driven $^{45}Ca^{2+}$ uptake (*e.g.* Fig. 1). A similar type of approach has been used to demonstrate the presence of a H^+/Ca^{2+} antiport associated with vesicles derived from the tonoplast (1, 25).

When the vesicle pH gradient was monitored using acridine orange, a pH sensing fluorescent probe (17), transient quenching of the probe fluorescence occurred which was followed by a biphasic recovery (Fig. 2). If gramicidin D was added to the vesicles during the slower phase of recovery, the fluorescence was rapidly restored. This ionophore effect is consistent with the observed quenching of probe fluorescence corresponding to the production of an acid interior pH gradient. This response of the fluorescent probe during transient pH gradient production is similar to that presented by Blumwald and Poole (1) for red beet tonoplast vesicles except that fluorescence recovery is more rapid. This would indicate that the plasma membrane vesicles used in this study may be more leaky to protons than the tonoplast vesicles used in previous studies by these workers. However, the imposed pH gradient is present over a sufficient duration that secondary calcium transport processes could be investigated. If calcium was transported into the vesicle through the action of a secondary H^+/Ca^{2+} antiport, then the addition of Ca^{2+} to vesicles during the slower phase of recovery should enhance proton flux to the cell exterior (1). However, in the red beet plasma membrane vesicles used in this study, the addition of calcium at concentrations up to 100 μM had no effect upon the the recovery of fluorescence, which would suggest that proton flux was unaffected by this cation.

To investigate further the possibility of a secondary H^+/Ca^{2+} system in the red beet plasma membrane vesicles, the effect of imposed acid interior pH gradients on $^{45}Ca^{2+}$ uptake was examined (Fig. 3). The exterior of the vesicle was made alkaline to pH 7.3 by the addition of BTP (as above) and $^{45}Ca^{2+}$ uptake was examined over the time that the transient pH gradient was present as observed using acridine orange (lower panel). In

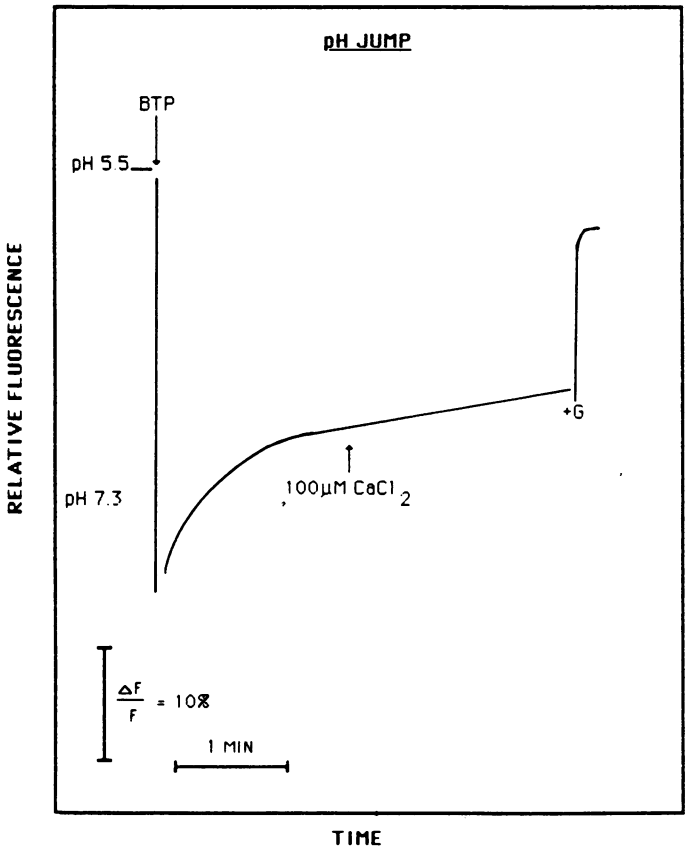

FIG. 2. Quenching of acridine orange fluorescence during the imposition of an artificial pH gradient and the lack of an effect of Ca^{2+} on H^+ flux. At the indicated time, 4 μl of 1 M BTP was added to a 1 ml volume containing 250 mM sorbitol, 2.5 mM BTP/Mes (pH 7.0), plasma membrane vesicles (35 μg protein), and 5 μM acridine orange. $CaCl_2$ and gramicidin D (5 μM) were added to the vesicles as indicated.

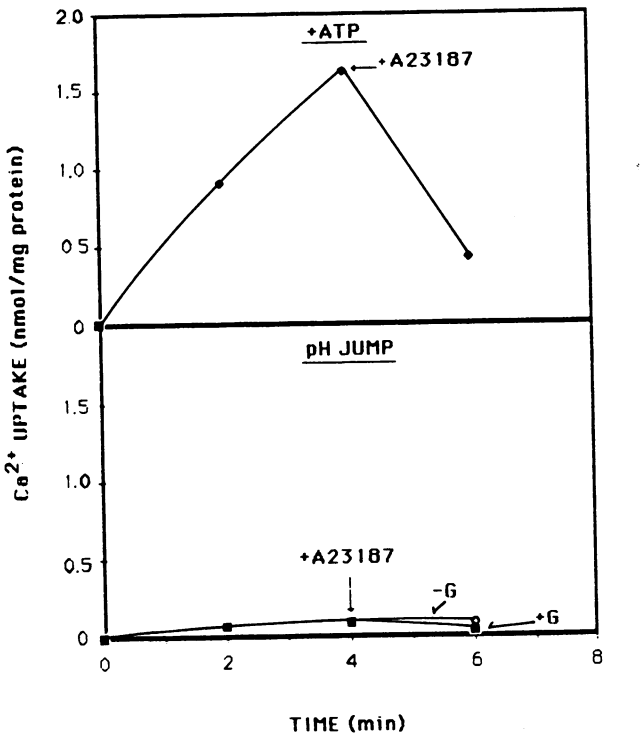

FIG. 3. Time course of $^{45}Ca^{2+}$ uptake in the presence of ATP or an imposed pH gradient. Upper panel: the uptake of $^{45}Ca^{2+}$ was carried out as described in "Materials and Methods" in the presence of 250 mM sorbitol, 25 mM BTP/Mes (pH 7.0), and plasma membrane vesicles (35 μg membrane protein). As indicated, 0.3 μg/ml A23187 was added to release the accumulated calcium. Lower panel: an artificial pH gradient was imposed under the conditions described for Figure 2 and 50 μM $CaCl_2$ containing 2.5 μCi of $^{45}Ca^{2+}$ was added during the slow phase of proton efflux. At the indicated times, vesicles were collected by filtration and radioactivity associated with the vesicles was determined as described in "Materials and Methods." When gramicidin D was present the final concentration was 5μM and A23187 (0.3 μg/ml) was added as indicated.

addition, $^{45}Ca^{2+}$ uptake in the presence of ATP was carried out with the vesicles over the same time period for comparison (upper panel). In vesicles where an artificial pH gradient was imposed, a low level of $^{45}Ca^{2+}$ accumulation occurred which was uneffected by the presence of gramicidin D. The addition of A23187, 4 min after the imposition of a pH gradient, caused little change in $^{45}Ca^{2+}$ content. In contrast, $^{45}Ca^{2+}$ was rapidly taken up by vesicles supplied with ATP and discharged by the addition of A23187.

Taken together, these results would suggest that our observed ATP-dependent $^{45}Ca^{2+}$ uptake in the red beet plasma membrane primarily reflects Ca^{2+} transport directly coupled to ATP utilization and that secondary $^{45}Ca^{2+}$ transport driven by the proton electrochemical gradient could not be observed in our assays. If such a system is associated with red beet plasma membrane *in vivo*, its contribution to the total $^{45}Ca^{2+}$ uptake in these assays must be extremely low. Alternatively, such a transport system could be damaged during the vesicle isolation procedure which requires treatment with 0.25 M KI to cause the formation of sealed plasma membrane vesicles (12). Therefore, these results should be viewed with caution in regard to proposals for possible *in vivo* modes of calcium transport across the plasma membrane of cells from this tissue. The properties of the primary calcium transport system, observable in the red beet plasma membrane vesicles, were then examined.

General Characteristics of $^{45}Ca^{2+}$ Uptake in Red Beet Plasma Membrane Vesicles. When the effect of assay pH on $^{45}Ca^{2+}$ uptake was examined for the red beet plasma membrane vesicles, the optimal pH range for activity was broadly distributed between pH 7.0 and 7.5 (Fig. 4). While red beet ER calcium transport was optimal in the pH 7 range, this activity differed in showing a sharp decrease at pH 7.5 (13).

The effect of phosphohydrolase inhibitors on $^{45}Ca^{2+}$ uptake is shown in Table I. Since all transport assays were carried out in the presence of both nitrate and azide, these inhibitors were not considered. It should be noted that there was no difference in uptake activity when the nitrate supplied as KNO_3 in the assay was replaced by an equal concentration of KCl (data not shown). This would indicate that even in the absence of nitrate, the calcium uptake cannot be attributed to tonoplast vesicles. Furthermore, since our studies with ER vesicles indicated nitrate sensitivity in the absence of KCl (13), this would also indicate that vesicles from the endoplasmic reticulum cannot account for the $^{45}Ca^{2+}$ transport observed in these assays. The uptake of $^{45}Ca^{2+}$ in the red beet vesicles was further inhibited by orthovanadate, DCCD, and DES. While DES and DCCD have been shown to be inhibitors of a number of phosphohydrolases (18, 26), the inhibition by orthovanadate suggests that the enzyme responsible for driving calcium transport forms a phosphorylated intermediate during the course of ATP hydrolysis (2, 3). When $^{45}Ca^{2+}$ uptake was assayed in the presence of increasing concentrations of orthovanadate, 50% inhibition of uptake occurred at a concentration of about 30 μM (Fig. 5). In comparison to the calcium transport system associated with red beet ER vesicles (13), this plasma membrane associated system is less sensitive to these inhibitory compounds. However, the $^{45}Ca^{2+}$ uptake was more sensitive to orthovanadate than H^+-transport and phosphohydrolase activity (50% inhibition at 50 μM orthovanadate) associated with the plasma membrane ATPase in these vesicles (4, 12).

The substrate specificity of $^{45}Ca^{2+}$ uptake for various nucleoside phosphates is shown in Table II. While ATP was the preferred substrate for driving $^{45}Ca^{2+}$ uptake, GTP could partially substitute for ATP. Transport in the presence of GTP was about 50% of that observed with ATP. This differed substantially from the red beet ER calcium transport system which was almost entirely substrate specific for ATP (13). In addition, the H^+-transporting ATPase associated with these vesicles is substrate specific for ATP (12)

The uptake of $^{45}Ca^{2+}$ was examined in the presence of either increasing concentrations of calcium (Fig. 6) or Mg:ATP (1:1 concentration ratio) (Fig. 7). Over the concentration range of each substrate tested, both kinetic plots followed simple Michaelis-Menten type profiles. The K_m for the calcium was about 6 μM, consistent with the role of this transport system in maintaining low cytoplasmic calcium levels through calcium extrusion (15 and references therein). The K_m for Mg:ATP was about 0.37 mM which is similar to what was observed for the ER calcium transport system from the same tissue (13). However, this value

Table I. *Effect of Various Inhibitors on $^{45}Ca^{2+}$ Uptake in Sealed Plasma Membrane Vesicles from Red Beet Storage Tissue*

The data presented represents the mean of three separate experiments.

Treatment	$^{45}Ca^{2+}$ Uptake
	nmol/mg protein
Control	3.24 (100)[a]
+100 μM Na_3VO_4	0.81 (25)
+50 μM DCCD	1.72 (53)
+30 μM DES	1.29 (40)

[a] Values in parentheses represent the percent of the control assay carried out in the presence of 100 mM KNO_3 for 8 min.

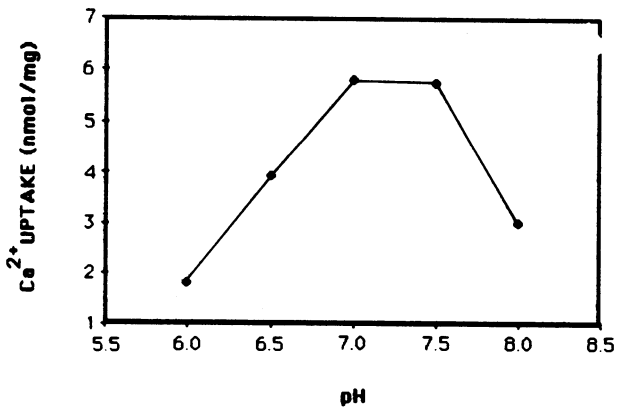

FIG. 4. Effect of assay pH on ATP-dependent $^{45}Ca^{2+}$ uptake in plasma membrane vesicles from red beet storage tissue. Uptake of $^{45}Ca^{2+}$ was carried out as described in "Materials and Methods" at the indicated assay pH.

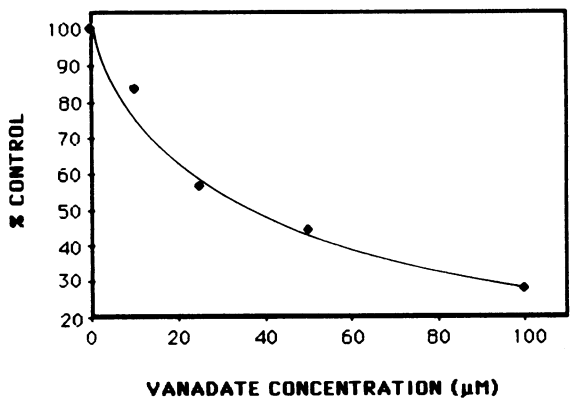

FIG. 5. Effect of orthovanadate on ATP-dependent $^{45}Ca^{2+}$ uptake in plasma membrane vesicles from red beet storage tissue. Uptake of $^{45}Ca^{2+}$ was carried out as described in "Materials and Methods" at the indicated concentration of Na_3VO_4.

Table II. *Substrate Specificity of $^{45}Ca^{2+}$ Uptake for Phosphorylated Compounds in Sealed Plasma Membrane Vesicles from Red Beet*

The data presented represents the mean of three separate experiments.

Substrate[a]	$^{45}Ca^{2+}$ Uptake
	nmol/mg protein
ATP	3.81
GTP	2.00
AMP	0.00
CDP	0.00
CMP	0.00

[a] Phosphate substrates were present in the assay for $^{45}Ca^{2+}$ at a concentration of 4 mM.

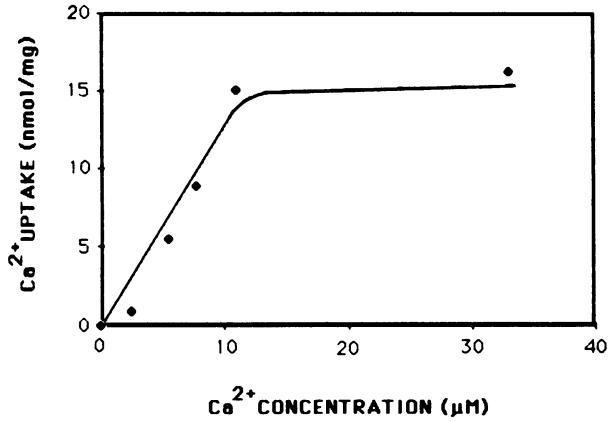

FIG. 6. Uptake of $^{45}Ca^{2+}$ as a function of calcium concentration in plasma membrane vesicles from red beet storage tissue. Uptake of $^{45}Ca^{2+}$ was carried out as described in "Materials and Methods" in the presence of the indicated concentration of $CaCl_2$ (each assay contained 2.5 μCi).

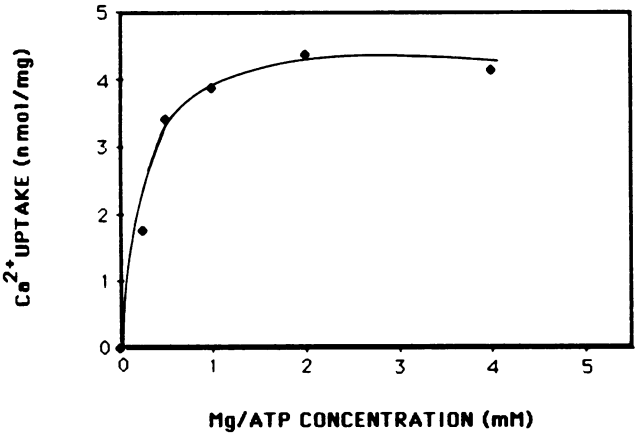

FIG. 7. Uptake of $^{45}Ca^{2+}$ as a function of Mg:ATP concentration in plasma membrane vesicles from red beet storage tissue. Uptake of $^{45}Ca^{2+}$ was carried out as described in "Materials and Methods" in the presence of the indicated concentration of Mg:ATP (1:1 concentration ratio).

is slightly lower than the K_m for Mg:ATP observed for the red beet plasma membrane H^+-ATPase (5).

GENERAL DISCUSSION

The results of this study suggest that an ATP-dependent, primary calcium transport system is associated with the plasma membrane of red beet storage tissue. Using selectively sealed plasma membrane vesicles that have been characterized previously (12), it is possible to demonstrate ATP-dependent $^{45}Ca^{2+}$ uptake which can be discharged by the addition of the calcium ionophore, A23187. This calcium uptake appeared to predominately reflect a direct coupling of $^{45}Ca^{2+}$ movement to ATP utilization and attempts to directly demonstrate the action of a secondary Ca^{2+} transport system (*e.g.* H^+/Ca^{2+} antiport) were unsuccessful with this vesicle system. However, it is uncertain as to whether this apparent absence (or low level) of secondary Ca^{2+} transport indicates that such a transport system is not associated with the red beet plasma membrane *in vivo* or whether this simply reflects a limitation of the vesicle system used to study this process. Since only those plasma membrane vesicles with the cytoplasmic facing membrane surface on the outside (inside-out) will be active in these transport assays (see Ref. 26 for discussion), this transport of calcium observed in these assays would correspond to ATP driven calcium efflux from the plant cell.

When the plasma membrane calcium transport system was characterized, properties were demonstrated which allowed this transport system to be distinguished from the calcium transport system associated with ER vesicles isolated from the same tissue (13). The plasma membrane calcium transport system was less sensitive to DCCD, DES, and orthovanadate than the ER system. In addition, calcium transport at the plasma membrane could use GTP as a substrate at a level 50% of that observed with ATP. In contrast, the ER system is entirely substrate specific for ATP (13). These results argue against the notion that the calcium transport observed in these assays could be related to the presence of ER vesicles as a minor contaminant in the plasma membrane fractions. Furthermore, since all assays were carried out in the presence of 100 mM nitrate and 0.4 mM azide, calcium transport systems associated with the tonoplast (1, 25) and mitochondria (20) cannot account for the calcium uptake observed in these studies.

These results confirm earlier proposals for the presence of an ATP-dependent primary calcium efflux system at the surface of plant cells, separate from transport driven by the plasma membrane H^+-ATPase (8 and references therein). These proposals, however, were based upon the observation of calcium transport in crude microsomal membrane fractions where both the relative quantity and degree of sealing of the plasma membrane were uncertain (9, 10, 14, 27). Subsequent studies by Dieter and Marmé (11) involved the purification of a Ca^{2+}-ATPase from a corn root microsomal membrane fraction by solubilization and calmodulin-affinity chromatography. However, due to the observation of primary calcium transport systems at the endoplasmic reticulum (6, 7, 13, 19) and plasma membrane (this paper) of plant cells, it is uncertain as to the origin of the calcium transport systems observed in these initial reports. The presence of these primary calcium transport systems at the ER and plasma membrane, together with transport systems at the tonoplast and mitochondria, would provide an integrated system for maintenance of low cytoplasmic calcium levels important for the function of this cation as a second messenger in signal transduction (15, 25).

Our future studies will focus on the identification and characterization of an ATPase activity responsible for ATP dependent primary calcium transport at the plasma membrane. This may be a challenging problem because of the requirement to distinguish this calcium ATPase from the plasma membrane H^+-ATPase. One property of this plasma membrane calcium transport system, which may prove useful in this respect, is the observation of partial activity using GTP as a substrate. Preliminary work has shown that under the optimal conditions for calcium transport, GTP hydrolysis is observed which is inhibited by orthovanadate (I Niesman, DP Briskin, unpublished results). Thus, it may be possible to use [γ-^{32}P]GTP to identify the

phosphorylated intermediate of this enzyme since phosphorylation of the plasma membrane H^+-ATPase shows absolute specificity for ATP (3).

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