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Ca^{2+} Pump and Ca^{2+}/H^+ Antiporter in Plasma Membrane Vesicles Isolated by Aqueous Two-Phase Partitioning from Corn Leaves

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Summary. Plasma membrane vesicles, which are mostly right side-out, were isolated from corn leaves by aqueous two-phase partitioning method. Characteristics of Ca^{2+} transport were investigated after preparing inside-out vesicles by Triton X-100 treatment. $^{45}Ca^{2+}$ transport was assayed by membrane filtration technique. Results showed that Ca^{2+} transport into the plasma membrane vesicles was Mg-ATP dependent. The active Ca^{2+} transport system had a high affinity for $Ca^{2+}(K_m(Ca^{2+}) = 0.4$ μM) and ATP(K_m(ATP) = 3.9 μM), and showed pH optimum at 7.5. ATP-dependent Ca^{2+} uptake in the plasma membrane vesicles was stimulated in the presence of Cl^- or NO_3^-. Quenching of quinacrine fluorescence showed that these anions also induced H^+ transport into the vesicles. The Ca^{2+} uptake stimulated by Cl^- was dependent on the activity of H^+ transport into the vesicles. However, carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and VO_4^{3-} which is known to inhibit the H^+ pump associated with the plasma membrane, canceled almost all of the Cl^--stimulated Ca^{2+} uptake. Furthermore, artificially imposed pH gradient (acid inside) caused Ca^{2+} uptake into the vesicles. These results suggest that the Cl^--stimulated Ca^{2+} uptake is caused by the efflux of H^+ from the vesicles by the operation of Ca^{2+}/H^+ antiport system in the plasma membrane. In Cl^--free medium, H^+ transport into the vesicles scarcely occurred and the addition of CCCP caused only a slight inhibition of the active Ca^{2+} uptake into the vesicles. These results suggest that two Ca^{2+} transport systems are operating in the plasma membrane from corn leaves, i.e., one is an ATP-dependent active Ca^{2+} transport system (Ca^{2+} pump) and the other is a Ca^{2+}/H^+ antiport system. Little difference in characteristics of Ca^{2+} transport was observed between the plasma membranes isolated from etiolated and green corn leaves.

Key Words Ca^{2+} transport · plasma membrane · Ca^{2+} pump · pH gradient · Ca^{2+}/H^+ antiporter · *Zea mays*

Introduction

In higher plants, Ca^{2+} plays an important role in regulating a large number of cellular processes (Hepler & Wayne, 1985; Kauss, 1987). It is believed that a transient rise in the cytoplasmic Ca^{2+} level which responds to external stimulus serves as a second messenger in signal transduction (Hepler & Wayne 1985; Poovaiah & Reddy, 1987). Cytoplasmic Ca^{2+} concentration is reported to be in the micromolar range or lower (Williamson & Ashley, 1982; Gilroy, Hughes & Trewavas, 1986; Bush & Jones, 1987). The maintenance of this low cytoplasmic Ca^{2+} level should be due to sequestration of Ca^{2+} into the intracellular organelles, such as ER[1], vacuoles and mitochondria and extrusion of Ca^{2+} through the plasma membrane. To date, two Ca^{2+} transporting systems have been found in the microsomal membrane. One is an ATP-dependent Ca^{2+} transport system in ER (Buckhout, 1984; Giannini et al., 1987*a*) and the plasma membrane (Giannini, Ruiz-Cristin & Briskin, 1987*b*; Rasi-Caldogno, Pugliarello & De Michaelis, 1987), and another is a Ca^{2+}/H^+ antiport system in the tonoplast (Bush & Sze, 1986; Schumaker & Sze, 1986).

Ca^{2+} transport characteristics in the plasma membrane have been studied using membranes isolated mostly with the sucrose density gradient method. In most reports, the degree of contamination of membranes from other organelles in the plasma membrane fraction was not tested and hence Ca^{2+} transport inhibitors for vesicles from different membrane origins were used. Indeed, Hodges and Mills (1986) and Bérczi and Møller (1986) showed that the phase partitioning method gave much purer preparations than the discontinuous sucrose density gradient centrifugation method. Bérczi and Møller (1986) suggested that the plasma

[1] *Abbreviations:* ER, endoplasmic reticulum; MES, 2-(N-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl) aminomethane; EGTA, ethyleneglycol *bis*(β-aminoethyl-ether)N,N,N',N'-tetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; SHAM, salicylhydroxamic acid; BSA, bovine serum albumin; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; BTP, 1,3-*bis*[tris(hydroxymethyl)methylamino]-propane; and CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

membrane vesicles obtained by the sucrose density method are likely to be leaky, and accordingly less suitable for transport studies. Therefore, the use of vesicles which are sealed and essentially free of other organelle membranes is needed for the studies of characteristics and the intracellular distribution of the active Ca^{2+} transport system. We have purified right side-out vesicles of the plasma membrane from corn leaves using the aqueous two-phase partitioning method, and obtained inside-out membrane vesicles by Triton X-100 treatment, which was suggested to increase membrane tightness to H^+ (Grouzis et al., 1987). Using the detergent-treated plasma membrane vesicles, we report here evidence suggesting that active Ca^{2+} transport system in the plasma membrane from corn leaves is composed of two systems, i.e., a Ca^{2+} pump and a Ca^{2+}/H^+ antiporter dependent on a proton motive force driven by H^+ pump.

Materials and Methods

Materials

SHAM, CCCP and nigericin were purchased from Sigma Chemical (St. Louis, MO). $^{45}Ca^{2+}$ (1 mCi/ml) was obtained from New England Nuclear (Boston, MA). All other reagents used were of the highest grade commercially available.

Plants

Corn (*Zea mays* L. var. Indentata) seeds purchased from Tokita Shubyo (Omiya, Japan) were sown in moist vermiculite fertilized with 1,000 times diluted Hyponex (Murakami Bussan, Tokyo, Japan) and grown in darkness or under natural daylight at 27°C in a glasshouse. Leaves from 14–15 day-old etiolated or green plants were used for isolation of the plasma membrane.

Isolation of Plasma Membrane

All steps for membrane preparation were carried out at 0–4°C. Leaves were cut into small pieces and homogenized for 30 sec at position 6 with a Polytron homogenizer (Kinematica GmbH, Switzerland) equipped with a PTA 36/2 generator shaft, in an isolation medium containing 0.3 M sucrose, 50 mM MES-Tris (pH 7.6), 5 mM EGTA, 5 mM EDTA, 10 mM NaF, 2.5 mM $Na_2S_2O_5$, 1 mM DTT, 2 mM PMSF, 4 mM SHAM, 0.5% (wt/vol) BSA, and 1.5% (wt/vol) Polyclar AT (insoluble polyvinylpyroridone, Gokyo Industries, Osaka, Japan) at a medium-to-tissue ratio of 5. The homogenate was filtered through nylon cloth (opening, 80 μm) and centrifuged for 10 min at 10,000 × g_{max}. The resulting pellet was discarded and the supernatant was centrifuged for 30 min at 50,000 × g_{max} to obtain microsomal fraction, which was suspended in a suspension medium containing 0.25 M sucrose and 10 mM K-phosphate (pH 7.8). The plasma membrane was purified from the microsomal fraction by the aqueous two-phase partitioning according to the method of Yoshida et al. (1986), except that final concentration of NaCl in the phase system was 50 mM, at which concentration the highest purity of the membrane was obtained. An upper phase after the second partition was diluted with 4 volumes of 5 mM MES-Tris buffer (pH 7.0) containing 0.25 M sucrose and 0.1 mM DTT, and centrifuged for 40 min at 80,000 × *g*. The resulting pellet was suspended in the same medium and used as the plasma membrane.

Preparation of Inside-Out Membrane Vesicles

As sidedness of the plasma membrane vesicles purified by the phase partitioning was right side out (*see below*), these were treated with Triton X-100 according to the method of Grouzis et al. (1987) to obtain inside-out vesicles for Ca^{2+} transport experiments.

To portions of the plasma membrane fraction was added an equal volume of 4 mM MES-BTP buffer (pH 7.0) containing 0.2% (wt/vol) Triton X-100, 0.25 M sucrose, 20% (vol/vol) glycerol, 2 mM DTT, and 0.4% (wt/vol) BSA (inversion medium) at a Triton X-100-to-protein ratio of approximately 1.5. After incubation for 10 min on ice, this mixture was diluted with 10 volumes of 5 mM MES-Tris buffer (pH 7.0) containing 0.25 M sucrose and 0.1 mM DTT, followed by centrifugation at 80,000 × *g* for 40 min at 4°C. The resulting pellet was washed again with the same buffer to eliminate Triton X-100 and resuspended in the same buffer solution. This was used as the inside-out plasma membrane vesicles.

Loading of Sucrose and KCl into the Plasma Membrane Vesicles

Sucrose- and KCl-loaded vesicles were prepared by the following procedures. The upper phase containing the plasma membrane vesicles after the phase partitioning was diluted with 4 volumes of a suspension medium containing 250 mM sucrose (or 125 mM KCl), 2.5 mM HEPES-BTP (pH 7.0), and 0.1 mM DTT, and centrifuged at 80,000 × *g* for 30 min. The pellet was suspended in a small volume of the suspension medium and an equal volume of the inversion medium containing 250 mM sucrose (or 125 mM KCl), in which HEPES-BTP (final concentration, 2.5 mM, pH 7.0) was substituted for MES-BTP, was added to each medium and incubated for 10 min at 0°C. The mixture was diluted with the suspension medium, washed once with the same medium as described above. The plasma membrane vesicles were finally resuspended in a small volume of the suspension medium.

Enzyme Assay

ATPase activity was assayed as described (Staal, Hommels & Kuiper, 1987) with some modifications. Unless otherwise indicated, the standard assay medium consisted of 5 mM $MgSO_4$, 0.1 mM Na_2MoO_4, 3 mM Na_2-ATP, 1 mM EGTA, 0.02% (wt/vol) Triton X-100, 30 mM MES-Tris (pH 6.75) and the plasma membrane (10–20 μg protein) in 0.5 ml. Reaction was started by the addition of ATP. IDPase activity was determined as described by Bowles and Kauss (1976), except that the assay medium contained 0.02% (wt/vol) Triton X-100. P_i released from the substrates was assayed with the method of Heinonen and Lahti (1981). NADPH-cytochrome *c* reductase activity was assayed as

Table 1. Distribution of marker enzymes in microsome and plasma membrane fractions from etiolated and green corn leaves

Marker enzyme	Specific activity (μmol/mg protein/hr)		Recovery in plasma membrane (%)
	Microsome	Plasma membrane	
(A)			
NADPH-cyt.*c* reductase	6.3	6.6	6
IDPase	2.7	0.9	2
(K^+-Mg^{2+})-ATPase	16.0	86.0	33
VO_4^{3-}-sensitive ATPase	13.0	68.4	32
(B)			
NADPH-cyt.*c* reductase	5.3	11.5	12
IDPase	2.5	2.0	4
(K^+-Mg^{2+})-ATPase	10.6	81.7	41
VO_4^{3-}-sensitive ATPase	6.1	70.2	61

(A): etiolated corn leaves; (B): green corn leaves. Total protein contents of microsome and plasma membrane fractions are 13.6 and 0.8 mg in (A), and 19.3 and 1.0 mg in (B), respectively. (K^+-Mg^{2+})-ATPase activity was assayed in the presence of 50 mM KCl and its VO_4^{3-}-sensitive activity was calculated as the difference between the activities measured in the presence and in the absence of 100 μM vanadate. Other assay conditions were similar to that described in Materials and Methods. The data are means of two replicates.

described by Bowles and Kauss (1976) in the presence of 0.1% (wt/vol) Triton X-100.

H^+ Transport Assay

H^+ transport into the plasma membrane vesicles was determined by quenching of quinacrine fluorescence. The excitation and emission wavelengths were 420 and 495 nm, respectively. The assay medium contained 250 mM sucrose, 10 mM HEPES-BTP (pH 7.0), 5 mM $MgSO_4$, 3 mM Na_2-ATP, 1 mM EGTA, 10 μM quinacrine and vesicles (10–20 μg protein), unless otherwise indicated. The reaction was started by the addition of ATP.

Ca^{2+} Transport Assay

Ca^{2+} uptake by the plasma membrane vesicles was determined by Millipore filtration technique essentially as previously described (Reddy & Poovaiah, 1987). Ca^{2+} transport was assayed in the presence of 0.25 M sucrose, 30 mM MES-Tris (pH 7.0), 5 mM $MgSO_4$, 1 mM Na_2-ATP and 70 μM $CaCl_2$ (2–4 μCi $^{45}Ca^{2+}$/ml) at 30°C unless otherwise indicated.

Ca^{2+} uptake was started by adding membrane vesicles (final protein concentration, approximately 0.1 mg/ml). At the desired time, portions (40 μl each) were quickly transferred onto Millipore filter (pore size, 0.22 μm) and the reaction medium was immediately filtered. After washing three times with 1 ml of 0.25 M sucrose containing 2 mM EGTA (pH 7.0), the filter was dried and the radioactivity determined by a Gas flow counter (LBC-453, Aloka, Tokyo, Japan).

Total Ca concentration in the assay medium was determined with an atomic absorption spectrophotometer (Model 370, Perkin-Elmer, CT). Free Ca^{2+} concentration in the assay medium was varied with Ca^{2+}-EGTA buffer system and calculated from the apparent association constant for Ca^{2+}-EGTA complex (Pershadsingh & McDonald, 1980).

Protein Determination

Protein was determined by the method of Bradford (1976) with BSA as standard.

Results

Purity of Plasma Membrane

Table 1 shows the distribution of marker enzymes in the microsome and the plasma membrane fraction from etiolated and green corn leaves. Specific activity of NADPH-cytochrome *c* reductase slightly increased and that of IDPase decreased in the plasma membranes from both leaves. Recoveries of these enzymes in the plasma membrane were very low. These results indicate that contaminations of ER and Golgi in the plasma membrane fraction are low. Specific activities of (K^+-Mg^{2+})-ATPase and VO_4^{3-}-sensitive (K^+-Mg^{2+})-ATPase markedly increased and the recoveries of these activities were higher in the plasma membranes from both leaves. To test the contamination of tonoplasts and mitochondria in the plasma membrane the effect of various ions on Mg^{2+}-ATPase activity was examined (Table 2). The addition of potassium salts (KCl, KNO_3; 50 mM) more or less stimulated Mg^{2+}-ATPase activity. Five repeated measurements in separate experiments showed that the stimulation is 10–20%. Similar low stimulation of the Mg^{2+}-ATPase by K^+-salts was reported in corn shoot plasma membrane isolated

Table 2. Effect of various salts on Mg^{2+}-ATPase in plasma membranes from etiolated and green corn leaves

Addition	Mg^{2+}-ATPase activity (%)	
	Etiolated leaves	Green leaves
None (control)	100	100
KCl (50 mM)	106	107
KNO_3 (50 mM)	118	109
NaN_3 (1 mM)	102	105
Na_3VO_4 (100 μM)	29	24
KCl (50 mM), Na_3VO_4 (100 μM)	22	15

Control values of Mg^{2+}-ATPase are 81.4 and 76.1 μmol/mg protein/hr in plasma membranes from etiolated and green corn leaves, respectively. The data are means of two replicates.

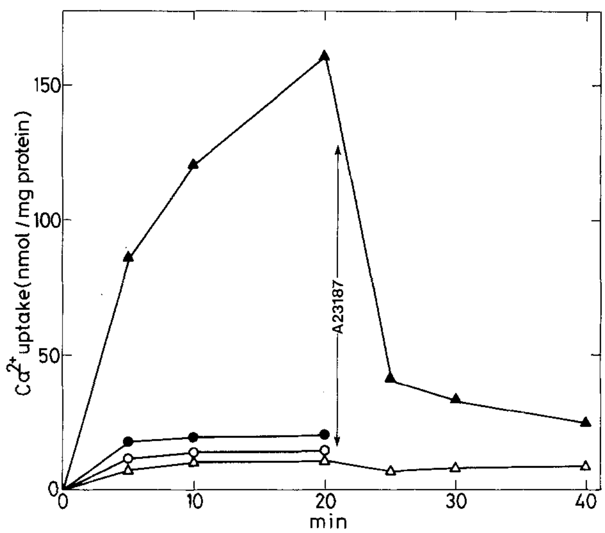

Fig. 1. Ca^{2+} uptake in the plasma membrane vesicles from corn leaves before and after Triton X-100 treatment. Triton X-100 treatment was carried out with BTP-MES buffer containing Triton X-100 as described in detail in Materials and Methods. Ca^{2+} uptake into plasma membrane vesicles was assayed in the presence of 0.25 M sucrose, 30 mM Tris-MES (pH 7.0), 5 mM $MgSO_4$, 70 μM $CaCl_2$ (open symbols) and 1 mM Na_2-ATP (filled symbols). Circles and triangles indicate Ca^{2+} uptake before and after Triton X-100 treatment, respectively. Other details are described in Materials and Methods. A23187 (5 μM) was added when indicated by arrows. Each point is the mean of two replicates in one representative experiment

by the aqueous two-phase partitioning (Clement et al., 1986), while Perlin and Spanswick (1981) reported stronger effect of potassium ion on Mg^{2+}-ATPase activity in the plasma membrane fraction prepared by the sucrose density gradient method. No inhibition of Mg^{2+}-ATPase activity by NO_3^- or azide was observed in both plasma membranes, while the activity was significantly inhibited by VO_4^{3-} (>70%). This inhibition was further pronounced in the presence of KCl (50 mM). These results indicate that the plasma membranes from both etiolated and green leaves are essentially free from contaminations of mitochondria and tonoplasts, and both plasma membranes can be isolated with the same procedure.

Sidedness of the Plasma Membrane Vesicles

Essentially no quenching of quinacrine fluorescence was observed with the plasma membranes from etiolated and green corn leaves, however, active MgATP-dependent quenching was observed after treating the plasma membranes with Triton X-100 (*data not shown*). This indicates that the plasma membranes purified by the phase partitioning are right side-out vesicles. The sidedness of the plasma membrane vesicles before and after detergent treatment was further tested based on latency of Mg^{2+}-ATPase as described by Grouzis et al. (1987). The proportion of vesicles with right-side-out orientation were 95 and 65% before and after detergent treatment, respectively.

Triton X-100 treatment for the plasma membrane also caused ATP-dependent Ca^{2+} uptake (Fig. 1). All further experiments were thus carried out using the Triton X-100 treated plasma membrane vesicles.

Essentially the same results were obtained with the plasma membranes isolated from etiolated and green leaves in the following experiments, and thus only the results with green leaves are presented.

Requirement of Mg^{2+} and ATP for Ca^{2+} Transport

Triton X-100 treated plasma membrane vesicles showed ATP-dependent Ca^{2+} uptake in the presence of Mg^{2+}; this was an active process, since addition of A23187, after Ca^{2+} uptake had almost attained to the summit, caused a rapid release of Ca^{2+} taken up (Fig. 1). A little ATP-dependent Ca^{2+} uptake occurred in the absence of Mg^{2+} (*data not shown*). In the following experiments, ATP-dependent Ca^{2+} uptake was therefore expressed as the difference between the values determined in the presence of Mg^{2+} plus ATP and in the presence of Mg^{2+} alone.

Ca^{2+} and ATP Concentration Dependency of Ca^{2+} Transport

Dependency of Ca^{2+} concentration on ATP-dependent Ca^{2+} uptake was examined with Ca-EGTA buffer in the presence of 5 mM Mg^{2+} and 1 mM ATP. Since 1 mM ATP binds a negligible amount of Ca^{2+} in the presence of 5 mM Mg^{2+}, free-Ca^{2+} concentration was calculated based on the association constant for Ca-EGTA complex as described in Materials and Methods. Ca^{2+} uptake showed Michaelis-Menten type saturation kinetics. Hanes-Wolf plot of the data gave a linear plot (*data not shown*), and the $K_m(Ca^{2+})$ and V_{max} values obtained from the linear regression analysis were 0.41 ± 0.04 μM and 28.48 ± 0.16 nmol/mg protein/min (mean $\pm$ SE, $n = 4$), respectively. The $K_m(Ca^{2+})$ value is in the vicinity of cytoplasmic free-Ca^{2+} concentration (0.1–1 μM) (Gilroy et al., 1986; Bush & Jones, 1987) and is similar to that reported for ER (Buckhout, 1984), but one order of magnitude lower than those of tonoplast vesicles (Bush & Sze, 1986; Schumaker & Sze, 1986).

In the presence of 5 mM Mg^{2+} and 70 μM Ca^{2+}, the effect of increasing concentration of ATP on Ca^{2+} uptake was examined. Over the concentration range of ATP, Ca^{2+} uptake exhibited typical Michaelis-Menten type kinetics. The K_m(ATP) and V_{max} values were 3.9 ± 0.6 μM and 26.9 ± 1.0 nmol/mg protein/min (mean $\pm$ SE, $n = 4$), respectively.

The optimal pH for ATP-dependent Ca^{2+} uptake was 7.5 (*data not shown*).

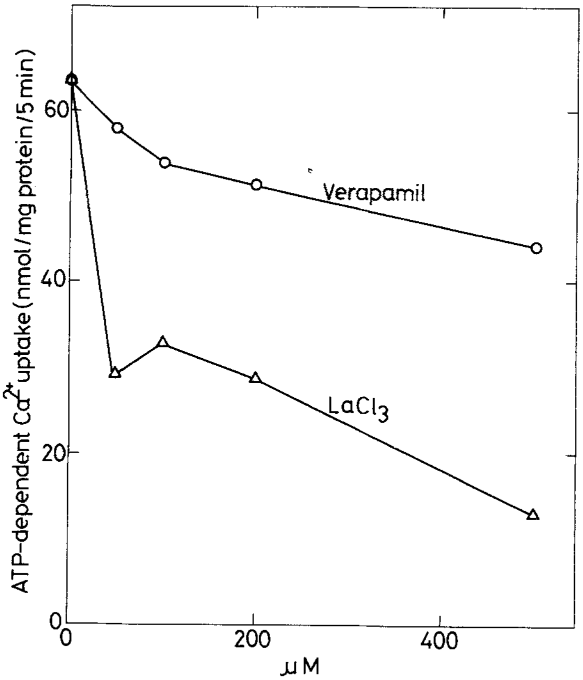

Fig. 2. Effect of verapamil and $LaCl_3$ on ATP-dependent Ca^{2+} uptake in the plasma membrane vesicles from corn leaves. ATP-dependent Ca^{2+} uptake was calculated as the difference between the values determined in the presence and in the absence of 1 mM ATP. Circles and triangles indicate ATP-dependent Ca^{2+} uptake in the presence of verapamil and $LaCl_3$, respectively. Other assay conditions were as described in Fig. 1. Each point is the mean of two replicates in one representative experiment

Effect of Verapamil and La^{3+} on ATP-Dependent Ca^{2+} Uptake

Ca^{2+} channel has been studied extensively in animal cells, while very little is known in higher plants. Recently, Graziana et al. (1988) examined the effect of various series of Ca^{2+} channel inhibitors on Ca^{2+} influx of carrot protoplasts and showed that phenylalkyl amine drugs such as verapamil were effective inhibitors.

To test involvement of the Ca^{2+} channel in Ca^{2+} transport of the plasma membrane, the effect of verapamil on ATP-dependent Ca^{2+} uptake was examined (Fig. 2). If Ca^{2+} channel is involved in Ca^{2+} transport of the plasma membrane vesicles, verapamil should inhibit Ca^{2+} release from the vesicles and might result in an increase of net Ca^{2+} uptake. Fivefold higher concentration of verapamil (500 μM) than the concentration which completely inhibits Ca^{2+} influx of carrot protoplasts (Graziana et al., 1988) inhibited ATP-dependent Ca^{2+} uptake by 31.5%. These results appear to eliminate the involvement of Ca^{2+} channel in ATP-dependent Ca^{2+} uptake that we determined. Verapamil, therefore, seems to affect ATP-dependent Ca^{2+} uptake itself.

Robinson, Larsson & Buckhout (1988) reported that La^{3+} inhibited calmodulin-stimulated (Ca^{2+}-Mg^{2+})-ATPase in maize leaf plasma membrane, which constituted only a minor portion of the total plasma membrane Mg^{2+}-ATPase. They assumed that this ATPase was an active Ca^{2+} pump. Over the similar range of La^{3+} concentration which they examined, La^{3+} inhibited ATP-dependent Ca^{2+} uptake much more effectively than verapamil (Fig. 2), suggesting that the inhibition site of this cation is ATP-dependent Ca^{2+} uptake but not the Ca^{2+} channel. The inhibition of Ca^{2+} uptake was biphasic, showing a small increase of Ca^{2+} uptake at 50–100 μM. Its reason is not clear; however, this small increase may be attributable to a stimulation effect of Cl^- as the counter ion of La^{3+} (*see below*).

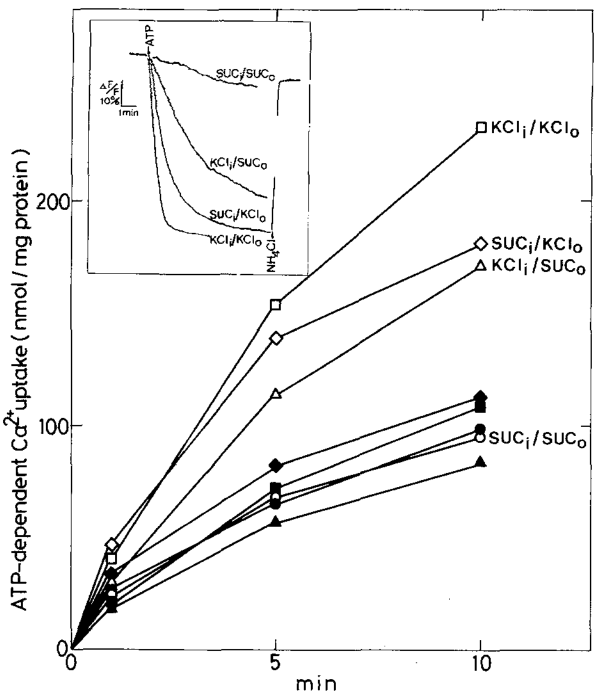

Fig. 3. Effect of CCCP on ATP-dependent Ca^{2+} uptake in the plasma membrane vesicles from corn leaves. KCl-loaded or sucrose-loaded vesicles were prepared as described in Materials and Methods, and each plasma membrane vesicle was then diluted 50 times into assay medium containing 125 mM KCl or 250 mM sucrose. ATP-dependent Ca^{2+} uptake was assayed in the presence (filled symbols) or absence of 10 μM CCCP (open symbols). Other assay conditions were same as in Table 3. Circles, triangles, rhombuses and squares indicate the Ca^{2+} uptake under SUC_i/SUC_o, KCl_i/SUC_o, SUC_i/KCl_o and KCl_i/KCl_o conditions, respectively. Each point is the mean of two replicates in one representative experiment. Inset demonstrates ATP-dependent H^+ uptake into the vesicles under the four conditions described above. Other assay conditions are described in Materials and Methods. NH_4Cl (10 mM) was added at the end of all traces (shown only for SUC_i/KCl_o condition)

Effect of Various Salts on ATP-Dependent Ca^{2+} Uptake

Table 3 shows the effect of various salts on ATP-dependent Ca^{2+} uptake in the plasma membrane vesicles. KCl, KNO_3, BTP-Cl and BTP-NO_3 stimulated ATP-dependent Ca^{2+} uptake 65–94% when added to the sucrose medium at 20 mM, while 20 mM K-HEPES partially inhibited the Ca^{2+} transport. These results indicate that Cl^- and NO_3^- are stimulatory on the Ca^{2+} transport. Essentially no H^+ transport was observed in the sucrose medium (*see* Fig. 3) or in the presence of K-HEPES in the same medium, while addition of Cl^- or NO_3^- markedly stimulated H^+ transport (*data not shown*). Clement et al. (1986) reported similar results with maize shoot plasma membrane. These results suggest that H^+ transport in the presence of these anions into the vesicles causes the stimulation of Ca^{2+} transport activity. No inhibition of H^+ transport activity by NO_3^- indicates that the tonoplast is not contaminated in our plasma membrane vesicles. To evaluate the contribution of ER (Martonosi & Feretos, 1964) or mitochondrial ATP-dependent Ca^{2+} transport (Dieter & Marmé, 1980), the effect of phosphate and azide on Ca^{2+} transport activity were also examined. Phosphate (1–10 mM) increased the activity by 14–17%, while azide (1–10 mM) had no effect. These results suggest that a little part of the Ca^{2+} transport is due to the transporter of ER but not to the mitochondrial transporter.

Table 3. Effect of various salts on ATP-dependent Ca^{2+} uptake in plasma membrane vesicles from corn leaves

Medium	ATP-dependent Ca^{2+} uptake (nmol/mg protein/5 min)	% of control
0.25 M Sucrose	61.7	100
+20 mM KCl	104.6	170
+20 mM KNO_3	115.9	188
+20 mM BTP-Cl	101.5	165
+20 mM BTP-NO_3	119.4	194
+20 mM K-HEPES	45.8	74

Sucrose-loaded plasma membrane vesicles were used. The procedure for sucrose-loading is described in Materials and Methods. Ca^{2+} transport assays were carried out under the same conditions as described in Materials and Methods, except that 10 mM HEPES-BTP (pH 7.0) was substituted for 30 mM MES-Tris (pH 7.0) in the assay medium and various salts were added to the assay medium when indicated. The data are means of two replicates.

The Effect of CCCP on ATP-Dependent Ca^{2+} Uptake

KCl- or sucrose-loaded vesicles were prepared as described in Materials and Methods, and H^+ or Ca^{2+} uptake was measured in the KCl or sucrose (SUC) medium (Fig. 3). The initial rate of H^+ transport was the following order: $KCl_i/KCl_o > SUC_i/KCl_o > KCl_i/SUC_o$, and Ca^{2+} uptake was stimulated by KCl in the same sequence. KCl-stimulated Ca^{2+} uptake in each condition was reduced by the addition of CCCP to almost the level in SUC_i/SUC_o condition in which essentially no H^+ transport occurred. These results suggest that stimulation of Ca^{2+} uptake in the presence of Cl^- may be attributable to the H^+ gradient across the membrane (acid inside).

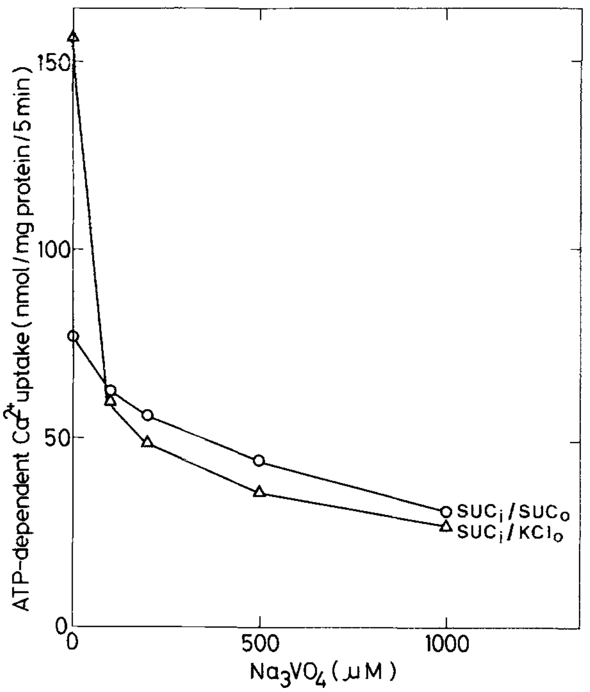

Fig. 4. Effect of VO_4^{3-} on ATP-dependent Ca^{2+} uptake in the plasma membrane vesicles from corn leaves. Assay conditions were essentially the same as in Fig. 3, except that VO_4^{3-} (0–1000 μM) was added to the assay medium. Circles and triangles indicated ATP-dependent Ca^{2+} uptake under SUC_i/SUC_o, and SUC_i/KCl_o conditions, respectively. Each point is the mean of two replicates in one representative experiment

Effect of VO_4^{3-} on ATP-Dependent Ca^{2+} Uptake

VO_4^{3-} at 100 μM greatly inhibited H^+ transport into the plasma membrane vesicles (*data not shown*). Figure 4 shows the effect of VO_4^{3-} concentration on ATP-dependent Ca^{2+} uptake into the plasma membrane vesicles. In the absence of KCl (SUC_i/SUC_o condition), Ca^{2+} uptake was not markedly inhibited by VO_4^{3-}. On the other hand, Ca^{2+} uptake in the presence of KCl (SUC_i/KCl_o condition) was double that in its absence and this KCl-activated Ca^{2+} uptake was totally inhibited by VO_4^{3-}. These results further suggest that H^+ transport into the vesicles contributes to the Cl^--stimulated Ca^{2+} transport.

Effect of Artificially Imposed pH Gradient on Ca^{2+} Uptake

When nigericin which electroneutrally exchanges K^+ for H^+ was added to K^+-loaded vesicles in sucrose medium, very rapid quenching of quinacrine fluorescence was observed and this was followed by a slow decrease of the quenching (Fig. 5). The magnitude of H^+ gradient imposed by nigericin was similar to that in the steady state during ATP-dependent H^+ uptake under KCl_i/KCl_o condition (*see* Fig. 3). Under the similar condition, Ca^{2+} was rapidly taken up reaching plateau within 10 min. Addition of A23187 after 30 min, caused rapid release of Ca^{2+}. These results indicate that artificially imposed outward H^+ gradient causes Ca^{2+} uptake into the plasma membrane vesicles.

Discussion

Marker enzyme analysis showed the lack of mitochondria and tonoplast membranes but low contaminations of Golgi and ER membranes in the plasma membrane fraction obtained by the aqueous two-phase partitioning method. However, the contamination of ER marker in our plasma membrane fraction was considerably higher than those reported in the plasma membrane isolated by similar methods (Buckhout & Hrubec, 1986; Robinson et al., 1988). We failed to reduce this ER contamination even when exactly the same phase partitioning method as reported by Robinson et al. (1988) was applied. We measured NADPH-cytochrome *c* reductase activity as a marker enzyme for ER, while antimycin A-insensitive NADH-cytochrome *c* reductase activity was measured in the reported assay (Buckhout & Hrubec, 1986; Robinson et al., 1988). This might be a reason why the apparent high contamination of ER was observed in our plasma membrane. With respect to the judgment of contamination, the following points should be noted: (*i*) NADPH-cytochrome *c* reductase is not an absolute marker enzyme for ER. The presence of this enzyme in the plasma membrane from higher plants was suggested by recent reports (Strobel & Dignam, 1983; Kjellbom & Larsson, 1984). (*ii*) Recovery of ER marker was less than one-fifth that of plasma membrane marker (VO_4^{3-}-sensitive (K^+-Mg^{2+})-ATPase) in both plasma membrane fractions from etiolated and green leaves. (*iii*) Maximal Ca^{2+} transport activity of the plasma membrane fraction was about one order of magnitude higher than that of ER (Buckhout, 1984; Bush & Sze, 1986; Giannini et al., 1987*a*). (*iv*) Phosphate, which markedly stimulates ATP-dependent Ca^{2+} transport of ER (Martonosi & Feretos, 1964), stimulated Ca^{2+} transport activity of the plasma membrane fraction only 14–17%.

Ca^{2+} transport of plasma membrane vesicles was MgATP-dependent. Affinity for Ca^{2+} of the active Ca^{2+} transporter was similar to that of ER (Buckhout, 1984; Giannini et al., 1987*a*) but higher than those of mitochondria (Dieter & Marmé, 1983; Martins, Carnieri & Vercesi, 1986), vacuoles (tono-

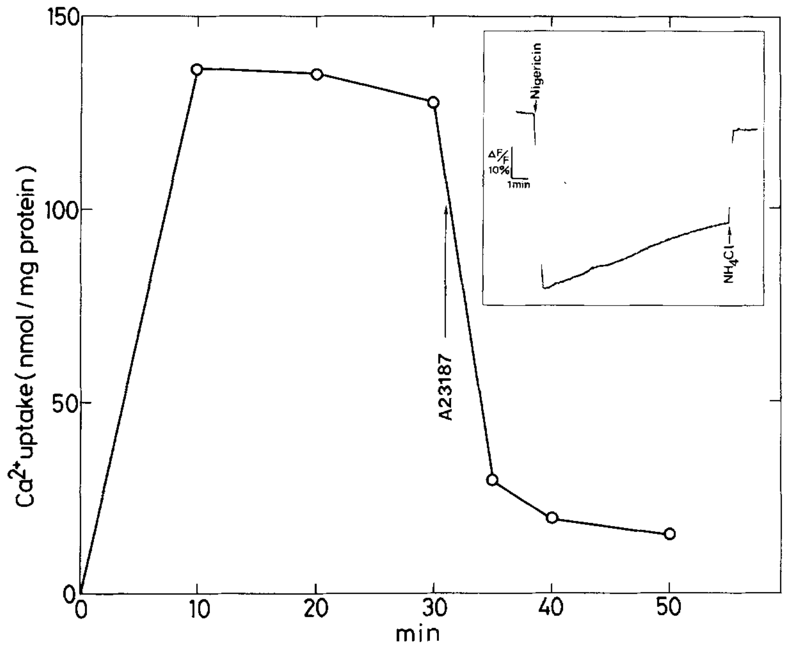

Fig. 5. Ca^{2+} uptake into the plasma membrane vesicles from corn leaves by artificially imposed pH gradient. Assay conditions were the same as KCl_i/SUC_o condition in Fig. 3, except that no ATP was contained in the assay medium. Reaction was initiated by the addition of vesicles to the assay medium containing nigericin (1 μM). A23187 (final 5 μM) was added where indicated. Each point is the mean of two replicates in one representative experiment. Inset demonstrates H^+ uptake into the vesicles by the addition of nigericin under the similar condition. Assay medium is described in the Materials and Methods. Nigericin (final 1 μM) and NH_4Cl (10 mM) was added where indicated with arrows

plasts) (Bush & Sze, 1986; Schumaker & Sze, 1986) and chloroplasts (Muto, Izawa & Miyachi, 1982; Kreimer et al., 1985*a*). On the other hand, the transport capacity was higher than those of ER (Buckhout, 1984; Bush & Sze, 1986; Giannini et al., 1987*a*) and tonoplasts (Bush & Sze, 1986; Schumaker & Sze, 1986) but lower than those of mitochondria (Martins et al., 1986) and chloroplasts (Kreimer et al., 1985*a*). The plasma membrane vesicles used in the present study had only 30% inside-out sidedness. If all vesicles were inside-out, the transport capacity would be comparable to those of mitochondria and chloroplasts, and also intact cell membranes. Thus the active Ca^{2+} transport system of the plasma membrane may play the most important role in maintaining low cytoplasmic Ca^{2+} levels which is essential for the function as a second messenger of this ion in signal transduction.

In the presence of Cl^- in the assay medium, ATP-dependent Ca^{2+} uptake into the vesicles was significantly stimulated. This Cl^--stimulated Ca^{2+} uptake could be caused by the operation of a Ca^{2+}/H^+ antiport system associated with the plasma membrane but not with tonoplast because of the following observations: (*i*) Neither Mg^{2+}-ATPase activity in the plasma membrane fraction nor ATP-dependent H^+ uptake into the vesicles were not inhibited by NO_3^-, which is an inhibitor for tonoplasts H^+-ATPase (Sze, 1985; Rea & Sanders, 1987). (*ii*) When ATP-dependent H^+ uptake into the vesicles induced in the presence of Cl^- was inhibited by VO_4^{3-} or by collapsing the pH gradient with CCCP, Cl^--stimulated Ca^{2+} uptake was decreased nearly to the level in the absence of Cl^-. (*iii*) In the absence of ATP, an outward H^+ gradient which was imposed artificially by nigericin resulted in Ca^{2+} uptake into the vesicles. Our results indicate the presence of Ca^{2+}/H^+ antiporter in the plasma membrane and its contribution in the total Ca^{2+} transport was about a half under the best condition for H^+ transport that we measured (*see* Fig. 3). On the contrary, Giannini et al. (1987*b*) and Rasi-Caldogno et al. (1987) indicated that the majority of Ca^{2+} transport system at the plasma membrane from higher plants is a Ca^{2+} pump and a Ca^{2+}/H^+ antiporter may be absent. This contradiction may be attributable to the difference of the plant materials used for the preparation of membrane vesicles. However, it should be noted that in the former studies, the plasma membrane vesicles, which were prepared by the sucrose density gradient method, were possibly more leaky to H^+, and in the latter, microsome fraction was used and the effect of artificially imposed H^+ gradient on Ca^{2+} transport was not investigated.

In the absence of Cl^- in the assay medium where essentially no active H^+ uptake was observed, CCCP only slightly inhibited Ca^{2+} transport of the plasma membrane vesicles. This result indicates that in Cl^--free medium, the contribution of Ca^{2+}/H^+ antiporter driven by H^+ gradient is very small if any, and the majority of Ca^{2+} transport is due to the active Ca^{2+} pump.

Vanadate was much less effective on Ca^{2+} transport in the absence of Cl^- where Ca^{2+} is mainly transported by the Ca^{2+} pump. The sensitivity of Ca^{2+} pump associated with our corn leaf plasma membrane to VO_4^{3-} is obviously different from that associated with ER (Bush & Sze, 1986; Giannini et al., 1987*a*), in which Ca^{2+} transport was greatly inhibited at 100 μM VO_4^{3-}. Vanadate is known to inhibit the H^+ pump in the plasma membrane of higher plants (Sze, 1985; Maria, Michaelis & Spanswick, 1986) and Ca^{2+} pump in animal cells (Carafoli & Zurini, 1982; Birch-Machin & Dawson, 1988) by inhibiting the formation of phosphorylated intermediate (Briskin & Leonard, 1982). Insensitivity of the Ca^{2+} pump to VO_4^{3-} in our plasma membrane suggests that this pump is not a typical pumping system which forms the phosphorylated intermediate.

In summary, our results strongly suggest the presence of two Ca^{2+} transport systems: one is an active Ca^{2+} pump, the other is a Ca^{2+}/H^+ antiporter driven by the pH gradient. Characteristics of the corn leaf plasma membrane Ca^{2+} transport system representing the high affinities for Ca^{2+} and ATP, the stimulation by Cl^- or NO_3^- and the sensitivity to VO_4^{3-} were different from those of Ca^{2+} transport systems associated with the tonoplasts (Bush & Sze, 1986; Schumaker & Sze, 1986), the mitochondria (Yamaya, Oaks & Matsmoto, 1984; Martins et al., 1986), ER (Buckhout, 1984; Bush & Sze, 1986; Giannini et al., 1987*a*), and the chloroplasts (Kreimer et al., 1985*a*; Kreimer, Melkonian & Latzko, 1985*b*) from higher plants. The presence of two active Ca^{2+} transport systems in the plasma membrane is very unique. In order to further elucidate the mechanism and the regulation of the Ca^{2+} transport systems in corn leaf plasma membrane which is composed of at least three transporters, i.e., Ca^{2+} pump, H^+ pump and Ca^{2+}/H^+ antiporter, specific inhibitors for each transporters are valuable tools. Biochemical separation and reconstitution into liposomes of these transporters may bring out much information.

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