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Solubilization and Reconstitution of Ca^{2+} Pump from Corn Leaf Plasma Membrane[1]

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ABSTRACT

The Ca^{2+} transport system of corn (*Zea mays*) leaf plasma membrane is composed of Ca^{2+} pump and Ca^{2+}/H^+ antiporter driven by H^+ gradient imposed by a H^+ pump (M Kasai, S Muto [1990] J Membr Biol 114: 133–142). It is necessary for characterization of these Ca^{2+} transporters to establish the procedure for their solubilization, isolation, and reconstitution into liposomes. We attempted to solubilize and reconstitute the Ca^{2+} pump in the present study. A nonionic detergent octaethyleneglycol monododecyl ether ($C_{12}E_8$) was the most effective detergent for a series of extraction and functional reconstitution of the Ca^{2+} pump among seven detergents examined. This was judged from activities of ATP-dependent $^{45}Ca^{2+}$ uptake into liposomes reconstituted with the respective detergent-extract of the plasma membrane by the detergent dilution method. $C_{12}E_8$-extract of the plasma membrane was subjected to high performance liquid chromatography using a DEAE anion exchange column. Ca^{2+}-ATPase was separated from VO_4^{3-}-sensitive Mg^{2+}-ATPase. These ATPases were separately reconstituted into liposomes, and their ATP-dependent Ca^{2+} uptake was measured. The liposomes reconstituted with the Ca^{2+}-ATPase, but not with the VO_4^{3-}-sensitive Mg^{2+}-ATPase, showed ATP-dependent Ca^{2+} uptake. Nigericin-induced pH gradient (acid inside) caused only a little Ca^{2+} uptake into liposomes reconstituted with the Ca^{2+}-ATPase, suggesting that the Ca^{2+}/H^+ antiporter was not present in the preparation. These results indicate that the Ca^{2+}-ATPase actually functions as Ca^{2+} pump in the corn leaf plasma membrane.

In higher plants, Ca^{2+} is believed to serve as a second messenger in signal transduction (20). Cytosolic Ca^{2+} concentration is usually maintained at less than 10^{-7} M by the Ca^{2+} extrusion pump of the plasma membrane and the Ca^{2+}-sequestering activities of the organelles (20). Calmodulin-stimulated, ATP-dependent Ca^{2+} uptake into microsomal membrane vesicles from some plants has been reported (6). Recently, a calmodulin-stimulated Ca^{2+}-ATPase was highly purified from corn (*Zea mays*) coleoptiles as a 140-kD polypeptide cross-reacting to anti-erythrocyte Ca^{2+}-pumping ATPase (2). This suggests that the calmodulin-stimulated Ca^{2+}-ATPase is the Ca^{2+} pump. No indication of calmodulin stimulation in Ca^{2+} transport has been reported with the plasma membrane (8, 22) and the endoplasmic reticulum (3) from higher plants. Recently, Rasi-Caldogno *et al.* (22) identified a calmodulin-independent plasma membrane Ca^{2+}-ATPase from radish seedlings as a Ca^{2+} pump based on the similarities between kinetics of the Ca^{2+}-ATPase and Ca^{2+} uptake with Ca^{2+}, Mg-ATP, and erythrocin B.

In erythrocyte and heart sarcolemma, the Ca^{2+}-ATPase has been verified to have Ca^{2+}-pumping function with the reconstitution system (4). However, there has not been such a report in higher plants. In a previous paper, we (12) reported that the Ca^{2+} transport system of corn leaf plasma membrane is composed of a Ca^{2+} pump and Ca^{2+}/H^+ antiporter driven by H^+-gradient, and we partially characterized it. For further characterization, it is necessary to establish the procedure for solubilization and isolation of the Ca^{2+} pump and Ca^{2+}/H^+ antiporter and their reconstitution into liposomes with high activities. In the present work, solubilization and isolation of Ca^{2+}-pumping ATPase from the corn leaf plasma membrane, and subsequently its reconstitution into liposomes, were tried. Though the quantity and recovery of the enzyme reconstituted was not sufficient, experimental evidence indicated that Ca^{2+}-ATPase of corn leaf plasma membrane actually functioned as a Ca^{2+} pump.

MATERIALS AND METHODS

Chemicals

$C_{12}E_8$[3] and Chaps were purchased from Wako Chemical Co., lysolecithin (L-α-monopalmitoyllecithin) from Funakoshi Chemical Co. Zwittergent 3-14 from Calbiochem-Boehring, *n*-octylglucoside from Nakarai Chemical Co., acridine orange from Daiichi Chemical Co., soybean phospholipids (type II-S) and nigericin were from Sigma, and Chelex-100 from Bio-Rad. $^{45}Ca^{2+}$ (28.7 mCi/mg) and [γ-^{32}P]ATP (3,000 Ci/mmol) were obtained from New England Nuclear. All other chemicals were of analytical grade.

Plant Materials and Preparation of Plasma Membrane

Corn (*Zea mays* L. var Indentata) seeds were sown in moist vermiculite and grown in a greenhouse as described previously (12). The plasma membrane was isolated from 14- to 15-d-old leaves by the aqueous two-phase partitioning method (12) and stored in a solution containing 0.25 M sucrose, 2.5 mM

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[3] Abbreviations: $C_{12}E_8$, octaethyleneglycol monododecylether; Chaps, 3-[(3-cholamidopropyl)dimethylammonio}-1-propanesulfonate; BTP, 1,3-bis[tris(hydroxymethyl)methylamino]-propane.

Hepes-BTP (pH 7.0), and 0.1 mM DTT (protein concentration, 2–4 mg/mL) at −80°C until use.

Extraction and Reconstitution of Active Ca^{2+} Transport Activity

To portions of plasma membrane suspension was added an equal volume of extraction buffer containing 0.25 M sucrose, 20% (v/v) glycerol, 2.5 mM Hepes-BTP (pH 7.0), 0.1 mM DTT, and applied detergent (final concentration, see "Results"). After incubating on ice for 15 min, the mixture was centrifuged at 450,000*g* (Beckman TL100.2 rotor) for 15 min at 3°C. The resulting detergent-extract was added to portions of liposomal suspensions including glycerol (phospholipid concentration, 120 mg/mL). The liposomal suspension was prepared by sonicating a solution containing 0.25 M sucrose, 2.5 mM Hepes-BTP (pH 7.0), 4 mM DTT, and acetone-washed soybean phospholipids (10) with a bath sonicator (type 2N-100, Toyoriko Co.), and then glycerol was added to 10% (v/v). After incubation for 20 to 30 min on ice, concentration of detergent in the mixture was diluted according to the detergent dilution method (21) below the critical micelle concentration with a dilution buffer containing 0.25 M sucrose, 2.5 mM Hepes-BTP (pH 7.0), and 0.1 mM DTT (when KCl-loaded reconstituted liposomes were required, sucrose was replaced by 0.125 M KCl). The diluted mixture was centrifuged at 200,000*g* for 1 h to precipitate-reconstituted liposomes. To eliminate $C_{12}E_8$ from the preparation, the liposomal pellet was suspended in a bulk of dilution buffer and centrifuged at 450,000*g* for 15 min and then resuspended in a small volume of dilution buffer and used for Ca^{2+} transport assay.

Fractionation and Reconstitution of Ca^{2+}-ATPase

The $C_{12}E_8$ extract of plasma membrane was applied to an HPLC system (Tosoh Co.), which was equipped with a DEAE-5PW anion exchange column (7.5 × 75 mm, Tosoh Co.) preequilibrated with 10 mM Hepes-BTP (pH 7.0) containing 0.25 M sucrose, 10% (v/v) glycerol, 0.1% (w/v) $C_{12}E_8$, and 0.1 mM DTT. Elution was carried out with a linear gradient of NaCl concentration (0–0.5 M) within 20 min (flow rate, 1 mL/min) at room temperature. Eluate was collected in 1-mL fractions. Reconstitution of proteins in the eluate into liposomes was carried out with the procedures described for detergent-extract of the plasma membrane.

Ca^{2+} Transport Assay

The Ca^{2+} transport assay was carried out at 30°C. The medium included 0.25 M sucrose, 10 mM Hepes-BTP (pH 7.0), 5 mM $MgSO_4$, 0.5 mM Na_2-ATP adjusted to pH 7.0 with BTP, and 70 μM $CaCl_2$ (2–4 μCi $^{45}Ca^{2+}$/mL). Ca^{2+} uptake was started by adding reconstituted liposomes. At the desired time, portions (50 μL each) were quickly transferred into 200 μL of assay medium in which Ca^{2+} was withdrawn and Chelex-100 was included (0.5 g/mL). The mixture was immediately vortexed three times and then centrifuged at 1,000*g* for 2 to 3 s at room temperature to precipitate Ca^{2+} remaining outside the liposomes with resin (Chelex-100 treatment always eliminated >98% of Ca^{2+} in control experiments without liposomes). Portions of the supernatant containing liposomes were placed on Whatman 3MM chromatography paper and dried. Radioactivity associated with the filter paper was determined by a gas flow counter (12). Chelex 100 treatment was evaluated to be satisfactory for the Ca^{2+} transport assay since values of Ca^{2+} uptake into inside-out plasma membrane vesicles were comparable with those determined with Millipore filtration technique (12) and thus was used in this study.

Artificial pH Gradient

KCl-loaded reconstituted liposomes were prepared and the suspension was diluted 20 times into the Ca^{2+} transport assay medium (sucrose medium) without ATP, in which acridine orange (final concentration, 10 μM) was included when H^+ uptake was determined. Gradient of pH (acid inside) on reconstituted liposomes was imposed by adding nigericin (final concentration, 1 μM). H^+ uptake was determined by quenching of acridine orange fluorescence with the excitation and emission wavelength of 493 and 540 nm, respectively.

ATPase Assay

ATPase activity was assayed essentially as described by Iggo and Lane (9). Standard assay medium included 35 mM Mes-Tris (pH 7.0), 5 mM $MgSO_4$, 1 mM Na_2-[γ-^{32}P]ATP (2–4 μCi/mL), 0.005% (w/v) $C_{12}E_8$ at which concentration the maximal activity was obtained, and the plasma membrane or eluate of HPLC in total volume of 0.1 mL. Free Ca^{2+} concentration of the assay medium was adjusted with Ca-EGTA buffer system (12). Reaction was started by addition of ATP and stopped with 200 μL of 7.5% (w/v) TCA containing 1.5 mM KH_2PO_4. After incubating for 10 min, about 25 mg of charcoal (chromatography grade, Wako Chemical Co.) was added to adsorb unreacted ATP. The mixture was placed on ice for about 30 min, shaken vigorously several times at intervals, and then centrifuged at 16,000*g* for 15 min at 4°C to remove charcoal. The radioactivity of liberated Pi in the supernatant was determined as described in Ca^{2+} transport assay.

Protein Determination

Protein was determined by the method of Bradford with BSA as standard (1). For the samples containing detergents, the Amido black 10B procedure (11) was used, since detergents such as $C_{12}E_8$ interfered with the Bradford method.

RESULTS

Selection of Detergent

To find a suitable detergent for extraction of active Ca^{2+} transport activity from the plasma membrane and the subsequent reconstitution into liposomes, plasma membrane proteins were extracted with a fixed concentration of various detergents, and then subjected to reconstitution into liposomes, in the presence of the same detergent and concentration. As far as examined, liposomes prepared with $C_{12}E_8$ showed the highest ATP-dependent Ca^{2+} transport activity (Fig. 1). Addition of A23187 released most of the Ca^{2+} taken up, indicating that Ca^{2+} was indeed taken up into the lipo-

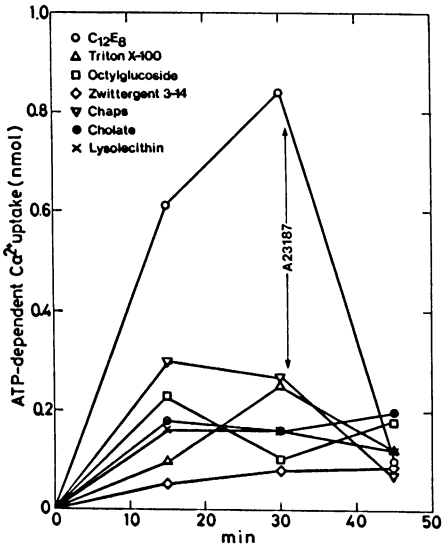

Figure 1. ATP-dependent Ca^{2+} uptake into reconstituted liposomes from various detergent extracts. Proteins of plasma membrane (240 μg) were individually extracted with seven detergents (each 0.4% [w/v]) as indicated. Respective extracts were added to liposomal suspension of soybean phospholipids, and the detergent concentration was adjusted to 0.4% (phospholipids-to-detergent ratio, 8.4) by adding concentrated detergent solution. This mixture was subjected to reconstitution into liposomes by dilution as described in "Materials and Methods." ATP-dependent Ca^{2+} uptake was expressed assuming that total preparation was used at each point of assay. A23187 (final concentration, 10 μM) was added as indicated.

somes. In this way, $C_{12}E_8$ was chosen as a suitable detergent for extraction and reconstitution of Ca^{2+} transport activity of the plasma membrane.

Optimal Concentration of $C_{12}E_8$

To prepare liposomes with more functionally active Ca^{2+} transport activity, optimal concentrations of $C_{12}E_8$ during extraction of the plasma membrane proteins and incubation of the detergent-extract with liposomal suspension were examined in separate experiments. Figure 2A shows the effect of $C_{12}E_8$ concentration during the extraction of plasma membrane proteins on ATP-dependent Ca^{2+} uptake into liposomes. The highest Ca^{2+} transport activity was obtained at 0.4%. In the following experiments, therefore, the extraction of the Ca^{2+} transport activity was carried out with 0.4% $C_{12}E_8$. This concentration gives $C_{12}E_8$-to-plasma membrane protein ratio of approximately 1.

To evaluate recovery of Ca^{2+} transport activity of the plasma membrane into reconstituted liposomes, the activity in the plasma membrane residue remaining after extraction with 0.4% $C_{12}E_8$ was measured (Fig. 2B). The Ca^{2+} transport activity was 10 to 15 times higher than that of liposomes reconstituted with $C_{12}E_8$ extract. This suggests that the recovery of Ca^{2+} transport proteins from the plasma membrane into reconstituted liposomes was within the range of 5 to 10%.

Concentrations of $C_{12}E_8$ for reconstitution were next examined. Within the concentrations examined, the highest Ca^{2+} transport activities were shown at 0.5% and 1% (Fig. 3). As the detergent dilution method was used for reconstitution, 0.5% was preferred to reduce the volume of preparation after dilution. This concentration gives a phospholipids-to-$C_{12}E_8$ ratio of approximately 8. Extraction and reconstitution were carried out with the following detergents at the respective optimal concentrations reported for Ca^{2+} pump or H^+ pump: Triton X-100 (18), Zwittergent 3–14 (24), and *n*-octylglucoside (13, 21), but none of them gave a higher Ca^{2+} transport activity than $C_{12}E_8$.

Fractionation and Reconstitution of Ca^{2+}-ATPase

To determine if Ca^{2+}-ATPase of the plasma membrane is the Ca^{2+} pump, isolation of Ca^{2+}-ATPase from the $C_{12}E_8$-extracted plasma membrane was attempted with HPLC. Figure 4 shows the elution pattern of $C_{12}E_8$-extracted proteins from DEAE-5PW column. Mg^{2+}-ATPase activity was separated into three peaks. Activities of the first two peaks were inhibited by VO_4^{3-}, indicating that these are the plasma membrane H^+-pumping ATPase. Ca^{2+}-ATPase activity was eluted following the second peak of VO_4^{3-}-sensitive Mg^{2+}-ATPase. This fraction (fraction 14) contained a low activity of VO_4^{3-}-insensitive Mg^{2+}-ATPase. Recoveries of total Mg^{2+}-ATPase and Ca^{2+}-ATPase activities from the plasma membrane were 1.9% and 18.2%, respectively. Specific activity of Ca^{2+}-ATPase in fraction 14 was increased by only twice of that in the plasma membrane, though the ATPase was separated by HPLC from the major portion of the proteins extracted with $C_{12}E_8$. The sensitivity of Mg^{2+}-ATPase to VO_4^{3-} was decreased after solubilization, *i.e.* the enzyme in the plasma membrane was inhibited by 80% with 100 μM VO_4^{3-}, while inhibition in the $C_{12}E_8$ extract and HPLC eluate was only 50%. The reason for this is unknown.

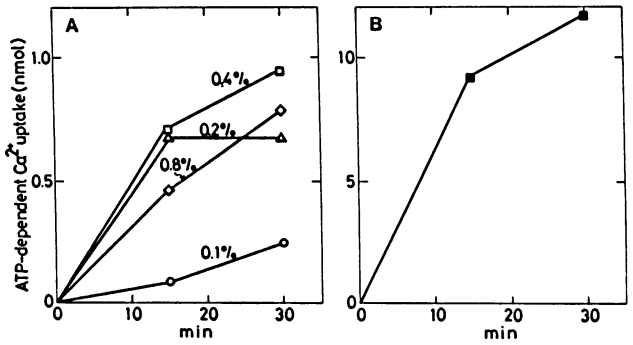

Figure 2. Effect of $C_{12}E_8$ concentration during extraction of plasma membrane proteins on ATP-dependent Ca^{2+} uptake into reconstituted liposomes. A, Reconstituted liposomes. A plasma membrane preparation was divided into four parts (each 280 μg protein) and individually incubated at the indicated concentration of $C_{12}E_8$. The concentration was adjusted to 0.4% after each extract was mixed with the liposomal suspension. B, Plasma membrane residue after extraction with 0.4% $C_{12}E_8$. The residues were washed once with an excess of dilution buffer to eliminate detergent associated with the preparation and then assayed for Ca^{2+} transport. Ca^{2+} uptake was expressed as in Figure 1.

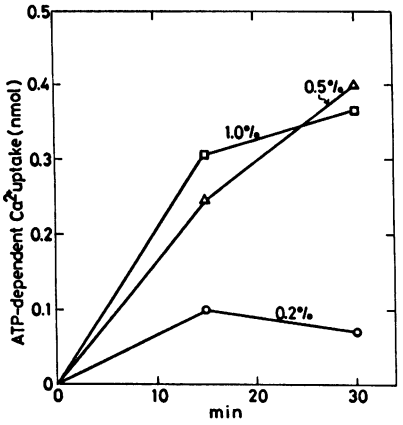

Figure 3. Effect of $C_{12}E_8$ concentration on reconstitution of ATP-dependent Ca^{2+} uptake. Proteins of plasma membrane (1035 μg) were extracted with 0.4% $C_{12}E_8$. The extract was divided into three parts and mixed with liposomal suspension of soybean phospholipids. $C_{12}E_8$ concentration in each mixture was adjusted as indicated by adding concentrated $C_{12}E_8$ solution when needed. Ca^{2+} uptake of reconstituted liposomes was expressed as in Figure 1.

Peak fractions of Ca^{2+}-ATPase (fraction 14) and VO_4^{3-}-sensitive Mg^{2+}-ATPase (fractions 6 and 10) were subjected to reconstitution into liposomes at the optimal $C_{12}E_8$ concentration. Significant Ca^{2+} uptake activity was observed with the former liposomes but little in the latter (Fig. 5). Addition of A23187 almost completely released Ca^{2+} taken up in liposomes, indicating that Ca^{2+} was indeed accumulated.

Effect of Artificially Imposed pH Gradient

There is a possibility that the Ca^{2+}/H^+ antiporter is coordinately extracted and fractionated together with the Ca^{2+}-ATPase and reconstituted into liposomes. As the Ca^{2+}-ATPase fraction was still contaminated with Mg^{2+}-ATPase, ATP-dependent Ca^{2+} uptake into the reconstituted liposomes could have been caused by the operation of Ca^{2+}/H^+ antiporter driven by a pH gradient formed by the Mg^{2+}-ATPase. Upon addition of ATP to the liposomes, no quenching of acridine orange fluorescence was observed. This indicates that the Mg^{2+}-ATPase which could transport H^+ was not active or present in the liposomes, and thus the Ca^{2+}/H^+ antiporter could not operate even if it was reconstituted into the liposomes.

To examine whether the Ca^{2+}/H^+ antiporter was reconstituted into liposomes from the Ca^{2+}-ATPase fraction, a pH gradient (acid inside) was artificially imposed using nigericin. KCl-loaded, reconstituted liposomes were prepared with the Ca^{2+}-ATPase fraction and suspended in K^+-free assay medium. Addition of nigericin, which exchanges K^+ for H^+ electroneutrally, caused very rapid quenching of acridine orange fluorescence (Fig. 6A, inset), which was followed by a decrease in quenching at a rate greater than that observed in plasma membrane vesicles (see Fig. 5 in ref. 12). This indicates that the reconstituted liposome preparation was more leaky to H^+ than were the plasma membrane vesicles. Addition of A23187, which exchanges Ca^{2+} or Mg^{2+} for H^+, restored the fluorescence to the level before addition of nigericin. These results indicate that, with K^+/nigericin treatment, a pH gradient (acid inside) was indeed imposed on the reconstituted liposomes, and thus the preparation was vesicular. As the reconstituted liposomes were leaky to H^+ and the pH gradient disappeared within 15 min after the addition of nigericin, Ca^{2+} uptake was assayed immediately. The nigericin-induced pH gradient caused a Ca^{2+} uptake into the reconstituted liposomes for 2 min, but no accumulation of Ca^{2+} was observed at 4 min (Fig. 6A). In a separate similar experiment, the nigericin-induced Ca^{2+} uptake was not observed 2 and 4 min after the addition of nigericin (data not shown). Thus, the observed nigericin-induced Ca^{2+} uptake may be attributable to an experimental error. In contrast, ATP greatly increased Ca^{2+} uptake into the liposomes in the absence of a pH gradient (Fig. 6B). These results indicate that ATP-de-

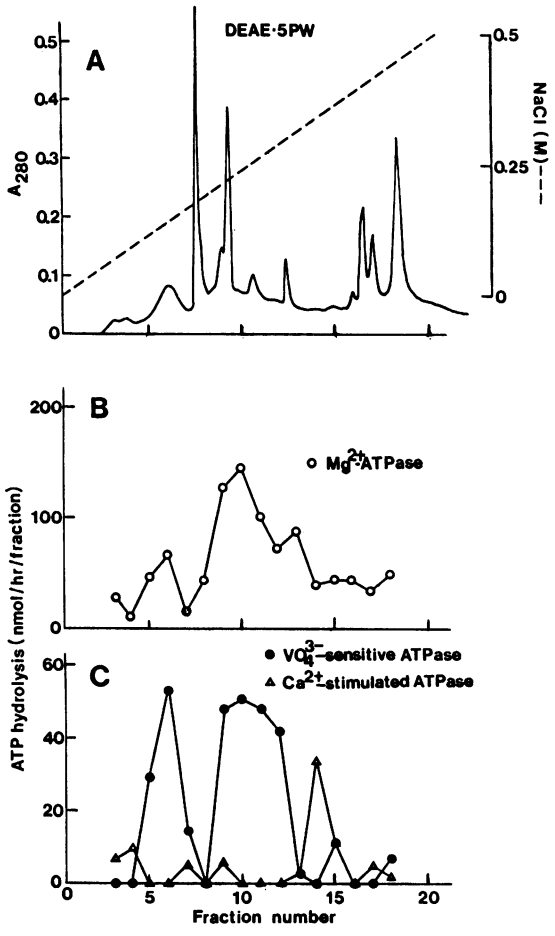

Figure 4. HPLC of a $C_{12}E_8$ extract on a DEAE-5PW anion exchange column. Plasma membrane proteins (390 μg) were extracted with 0.4% $C_{12}E_8$ and fractionated with HPLC as described in "Materials and Methods." A, Absorbance at 280 nm; B, Mg^{2+}-ATPase activity. Assay was carried out in the presence of 0.3 mM EGTA without adding Ca^{2+}. C, VO_4^{3-}-sensitive ATPase and Ca^{2+}-stimulated ATPase activities. Mg^{2+}-ATPase activity was determined in the presence of 100 μM VO_4^{3-} or in the presence of 10 μM Ca^{2+} The activities inhibited by VO_4^{3-} (closed circles) and stimulated by Ca^{2+} (open triangles) were plotted.

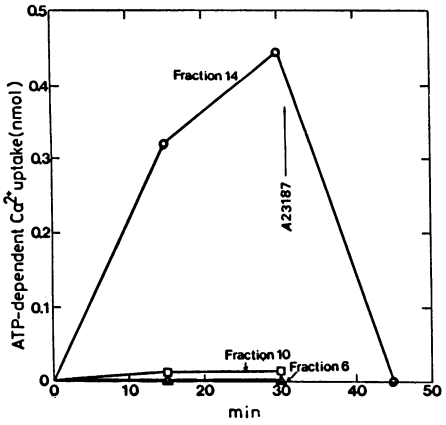

Figure 5. ATP-dependent Ca^{2+} uptake into reconstituted liposomes with VO_4^{3-}-sensitive and Ca^{2+}-stimulated ATPase fractions. Peak fractions (each 1 mL) of VO_4^{3-}-sensitive ATPase (fractions 6 and 10) and Ca^{2+}-stimulated ATPase (fraction 14) (see Fig. 4) were individually subjected to reconstitution into liposomes. A23187 (final concentration, 10 μM) was added as indicated. Expression of the activity was as in Figure 1.

pendent Ca^{2+} uptake into the reconstituted liposomes from the Ca^{2+}-ATPase fraction is due to the Ca^{2+}-ATPase and not a Ca^{2+}/H^+ antiporter.

DISCUSSION

$C_{12}E_8$ was selected from the various kinds of detergents as a suitable detergent for extraction and reconstitution of active Ca^{2+} transport activity in the plasma membrane from corn leaves. The detergent concentrations for the procedures were established. This detergent had already been used for solubilization of Ca^{2+}-pumping ATPase in animal cells (16) and indicated as an optimal detergent for function and stabilization of the enzyme among various detergents examined (15). Recovery of active Ca^{2+} transport proteins from the plasma membrane into the reconstituted liposomes was estimated to be only about 5 to 10%, judging from the activity of ATP-dependent Ca^{2+} uptake. However, it should be noted that the reconstituted liposomes were more leaky to H^+ than plasma membrane vesicles, and thus may be also to Ca^{2+}. Attempts to increase recovery by increasing $C_{12}E_8$ concentration during extraction were unsuccessful (see Fig. 2). This may be due to inactivation of the active Ca^{2+} transporting enzyme in the presence of high levels of detergent which causes dissociation of oligomeric proteins (14, 17, 25) and the removal of phospholipids required for the activity (15, 19).

The nonionic detergent $C_{12}E_8$ was also useful for the preparation of Ca^{2+}-ATPase, which was considered as the active Ca^{2+} transport protein (Ca^{2+} pump), using an anion exchange DEAE-5PW column. Active Ca^{2+} transport activity was successfully shown with the reconstituted liposomes with the Ca^{2+}-ATPase fraction, indicating that the Ca^{2+}-ATPase functioned as a Ca^{2+} pump.

Calmodulin-stimulated Ca^{2+}-ATPase has been purified by calmodulin affinity chromatography from corn coleoptiles (2, 7). Preliminary experiments showed that Ca^{2+}-ATPase of the plasma membrane from etiolated and green corn leaves was not stimulated by calmodulin, even after separating the enzyme from the endogenous calmodulin by a DEAE-Cellulofine (Seikagaku-Kogyo Co.) anion exchange column, and that Ca^{2+} transport activity of the plasma membrane was only slightly stimulated (less than 10%) by exogenous calmodulin (data not shown). Similar results were obtained in plant membranes (8, 22): no stimulatory effect of exogenous calmodulin on Ca^{2+} uptake; no activation or erratic activation by calmodulin of Ca^{2+}-ATPase which had been freed from endogenous calmodulin; and no reversal of the inhibition of Ca^{2+} uptake by calmodulin antagonists by exogenous calmodulin. This experimental evidence may indicate that calmodulin-dependent and -independent Ca^{2+}-ATPases are present in plant plasma membrane and that distribution of these enzymes is different among tissues and/or plant species. Stosic *et al.* (26) reported that ATP-dependent Ca^{2+} transport capacity and the effect of calmodulin on it were different in different tissues of spinach.

Calmodulin has recently been reported to be present in highly purified plasma membrane from pea leaves (5). Thus, Ca^{2+} uptake might be inhibited by calmodulin inhibitors if calmodulin stimulates plasma membrane Ca^{2+}-ATPase. However, cautious evaluation should be done on the specificity of calmodulin inhibitors. Gräf and Weiler (8) reported that trifluoperazin and W-7 strongly stimulated Ca^{2+} release from preloaded plasma membrane vesicles.

In contrast to H^+-pumping ATPase, which is a relatively abundant protein in plant plasma membrane (23), Ca^{2+}-

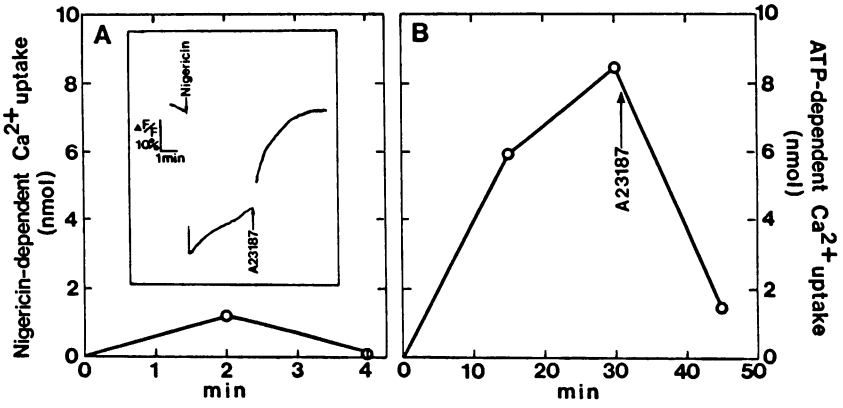

Figure 6. Effect of artificially imposed pH gradient on Ca^{2+} uptake into reconstituted liposomes with Ca^{2+}-ATPase fraction. Fraction 14 of Figure 4 was subjected to reconstitution into KCl-loaded liposomes as described in "Materials and Methods." A pH gradient was imposed with nigericin. A, Ca^{2+} uptake is expressed as the difference between the values determined in the presence and in the absence of nigericin; inset, H^+ uptake into the reconstituted liposomes induced with nigericin. B, ATP-dependent Ca^{2+} uptake without treating with nigericin. Expression of the activity was as in Figure 1.

pumping ATPase is obviously a minor protein (see Fig. 4). This may be the main reason why progress in studies of Ca^{2+} transport has been slow. In the present study, we succeeded in isolating Ca^{2+}-pumping ATPase and functionally reconstitute it into liposomes; however, more improved method for solubilization and purification of the enzyme will be required for further characterization of Ca^{2+}-pumping ATPase.

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