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Functional Reconstitution of an ATP-Driven Ca^{2+}-Transport System from the Plasma Membrane of *Commelina communis* L.[1]

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ABSTRACT

The protein(s) that constitute(s) the ATP-driven Ca^{2+}-translocator of plasma membrane enriched vesicles obtained by aqueous two-phase partitioning from leaves of *Commelina communis* L. has/have been solubilized and reincorporated into tightly sealed liposomes. The reconstituted Ca^{2+}-transport system was studied using ATP-driven $^{45}Ca^{2+}$ import into the proteoliposomes as a measure of activity. The detergent, 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate proved to be the most suitable and was used at 10 millimolar concentration, *i.e.* just above its critical micellar concentration. The presence of additional phospholipid (2 milligrams phosphatidylcholine per milliliter) and ATP (5 millimolar) improved the solubilization and/or reconstitution. The characteristics of the reconstituted system were similar to those of the plasma membrane-bound activity, including the apparent K_m for Ca^{2+} (5.2 micromolar), inhibition by relatively high levels of vanadate (IC_{50} = 500 micromolar) and lacking response to added calmodulin. The reconstituted transport system was very strongly inhibited by erythrosine B (IC_{50} = 0.01 micromolar) and had a low apparent K_m for ATP (11.4 micromolar). As in the plasma membrane vesicles, the protonophore carbonylcyanide m-chlorophenyl hydrazone did not affect Ca^{2+}-transport detectably in the reconstituted system. However, low levels of the Ca^{2+}-ionophore A 23187 instantaneously discharged 90% of the Ca^{2+} associated with the vesicles, proving that it had been accumulated in the intravesicular volume in soluble, freely exchangeable form. Ca^{2+}-transport in the reconstituted system was thus primary active, through a Ca^{2+}-translocating ATPase. The system reported here may serve as a valuable tool for purifying the Ca^{2+}-ATPase and for studying structural and functional aspects of the purified enzyme.

The role of Ca^{2+} as an intra- and perhaps an intercellular regulator of cell function is not clearly understood in higher plants. Available evidence (8–10) suggests that plant cells usually maintain submicromolar levels of free cytoplasmic Ca^{2+} against a large opposing gradient of extracellular Ca^{2+}. This requires efficient systems for Ca^{2+} sequestration within the cell and also for export of the ion from the cell.

While a number of studies have provided evidence for the presence of ATP-dependent Ca^{2+} transport systems in microsomal fractions or partially purified plasma membrane fractions from several higher plants (1, 2, 6, 7, 13, 18, 22, 27), only few studies have used highly pure plasma membranes. Robinson *et al.* (24) have shown a CM-[2] and Ca^{2+} stimulated ATPase in maize plasma membranes obtained by aqueous two-phase partitioning. Recently, Gräf and Weiler (11) characterized in detail a Ca^{2+}-translocating ATPase in the plasma membrane of *Commelina communis* L.. The molecular study of this enzyme and its regulation should contribute to our understanding of the role of plasma membrane Ca^{2+} transport systems in the regulation of cytoplasmic Ca^{2+} levels and provide further insights into the structure-function relationships and evolutionary aspects of the E_1/E_2-ATPases in eucaryotic cells.

Any approach toward a molecular study of the enzyme relying only on sequence similarities between the plant enzyme and Ca^{2+} ATPases of known sequence via either protein chemistry or DNA analysis, ultimately will require proof of function. Purification of the Ca^{2+} ATPase with retention of function seems the most direct and the only conclusive approach toward the identification of the polypeptide component(s) of the enzyme. This means, that the ATP-driven translocation of Ca^{2+}, rather than just a Ca^{2+}-stimulated ATPase activity, must be shown for the enzyme. Toward this end, the Ca^{2+} ATPase needs to be solubilized, reconstituted into sealed proteoliposome vesicles, and shown to transport Ca^{2+} in the presence of ATP after reconstitution. We report here on an efficient technique for the reconstitution of the *C. communis* Ca^{2+} ATPase, as well as on the characteristics of the reconstituted enzyme as compared to the plasma membrane system. Our data show that the enzyme can be reconstituted in highly functional form. The technique should allow the purification of the enzymatically active Ca^{2+} ATPase from higher plant plasma membranes.

MATERIALS AND METHODS

Plant Material

Plants of *Commelina communis* L. were grown as described previously (11) in a growth chamber for 3 weeks at 25°C (day)

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[2] Abbreviations: CM, calmodulin; CHAPS, 3-((3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate; CCCP, carbonylcyanide *m*-chlorophenyl hydrazone; CMC, critical micellar concentration; IC_{50}, effective concentration giving 50% inhibition; MEGA-9, nonanoyl-*N*-methylglucamide; PC, phosphatidylcholine.

and 20°C (night) with 16 h photoperiod from fluorescent tubes, Osram cool-white NT6 (PAR: 200 $\mu E\ m^{-2}\ s^{-1}$) and a RH of 80%.

Reagents

Dextran T-500 was obtained from Pharmacia (Freiburg, FRG), PEG 3350 from Union Carbide (Düsseldorf, FRG). Trypsin, calmodulin (spinach), and soybean phosphatidylcholine (type II-S) were purchased from Sigma (Munich, FRG). Asolectin was prepared as described (5) from soybean phosphatidylcholine type IV-S (Sigma). CHAPS was obtained from Biomol (Hamburg, FRG), sodium cholate from Serva (Heidelberg, FRG), MEGA-9 from Oxylchemie (Bobingen, FRG), and $^{45}CaCl_2$ (15–60 TBq mol^{-1}) from Amersham Buchler (Braunschweig, FRG).

Preparation of Membranes

Plasma membrane vesicles were prepared from leaves of 3 week old *C. communis* plants using the aqueous two-phase partitioning method initially introduced by Larsson (17). The characteristics of the *Commelina* plasma membrane fraction and of the plasma membrane Ca^{2+} ATPase have been detailed previously (11).

In brief, microsomal pellets from leaves of *C. communis* were produced by differential centrifugation at 10,000*g* and subsequently 50,000*g*, after homogenization of the tissue in 30 mM Hepes-KOH (pH 7.2), containing 3 mM DTT, 3 mM EDTA, 500 mM sucrose, and 1% (w/v) insoluble PVP. Two-phase partitioning was carried out using 6.4% (w/w) dextran T-500 and polyethylene glycol 3350. The final upper phases ($U_3 + U_3'$) were combined, diluted at least three fold using 250 mM sucrose, 2 mM $MgSO_4$, 25 mM Hepes-KOH (pH 7.2) and collected by centrifugation for 45 min at 100,000*g*. The resulting pellets were resuspended in 250 mM sucrose, 2 mM $MgSO_4$, 25 mM Hepes-KOH (pH 7.2), pelleted again, and finally resuspended in the same buffer at 1 mg mL^{-1} of protein. Membranes were either kept on ice and used immediately or were stored frozen at −20°C. Storage up to 2 weeks did not affect the Ca^{2+} ATPase activity.

Solubilization and Reconstitution

Solubilization and reconstitution of the plasma membrane Ca^{2+} ATPase were carried out using either freshly prepared or frozen (−20°C) plasma membrane enriched vesicles without any evident difference.

Except when otherwise stated, solubilization was carried out at 4°C using 10 mM CHAPS, 2 mg phosphatidylcholine mL^{-1}, 5 mM ATP, 2 mM $MgSO_4$, 250 mM sucrose, and 25 mM Hepes-KOH (pH 7.2) at a protein concentration of 0.5 mg mL^{-1}. After 30 min of incubation with occasional mixing, the samples were centrifuged for 1 h at 100,000*g*. The supernatants and, for some experiments, the pellets obtained from the detergent-extracted plasma membranes, were reconstituted into asolectin liposomes by the detergent dialysis technique (21). For this purpose, asolectin and CHAPS were added to either supernatants or the resuspended pellets to a final concentration of 1 mg asolectin mL^{-1} and 10 mM CHAPS. Dialysis was carried out against two 500-fold volumes of 25 mM Hepes-KOH (pH 7.2), containing 2 mM $MgSO_4$ and 250 mM sucrose for 8 h each time and at 4°C. Aliquots of the dialyzed preparations containing the proteoliposomes were either assayed directly for Ca^{2+} ATPase activity or the dialysates were diluted threefold with 25 mM Hepes-KOH, 2 mM $MgSO_4$, and 250 mM sucrose (pH 7.2). The proteoliposomes were then collected by centrifugation (1 h, 130,000*g*) and assayed for enzyme activity. Variations in the procedures are indicated in the legend of each experiment.

ATP-Driven $^{45}Ca^{2+}$ Transport

Assays were carried out exactly as described previously (11) following the ATP-dependent $^{45}Ca^{2+}$ uptake into plasma membrane vesicles or reconstituted proteoliposomes. The standard incubation mixture consisted of 25 mM Hepes-KOH, 2 mM $MgSO_4$, 250 mM sucrose, 1 mM ATP, and 10 μM $^{45}CaCl_2$ (67–130 MBq, 15–60 TBq mol^{-1}) (pH 7.2), in a reaction volume of 0.1 mL. Variations in protein, $^{45}CaCl_2$, and ATP concentrations as well as the conditions of incubation with trypsin, CM, and inhibitors are detailed in "Results." Except when otherwise indicated, minus-ATP controls were included in each experiment to determine the amount of $^{45}Ca^{2+}$ bound or unspecifically associated with the membrane vesicles or the proteoliposomes. All data were corrected for this contribution. After 10 min at 20°C, the reaction was terminated by diluting with 0.6 mL of 25 mM Hepes-KOH, 2 mM $MgSO_4$, 250 mM sucrose, and 1 mM EGTA (pH 7.2) (stop buffer). The samples were filtered under vacuum using Schleicher & Schüll BA85 nitrocellulose membrane filters. The membranes were then washed three times with 1 mL stop buffer each time. Dry filters were immersed in 5 mL scintillation cocktail (Hydroluma, Baker) and the radioactivity was measured in a Philips PW 4700 liquid scintillation counter (window settings 0.4 to 1990 keV).

RESULTS

In a first series of experiments, a range of detergents was tested for their ability to solubilize the ATP-driven Ca^{2+} transport system and allow its reconstitution with retention of catalytic activity (Table I). All of the detergents, when added to the plasma membrane preparations at the indicated concentrations, reduced ATP-dependent $^{45}Ca^{2+}$ transport, indicating permeabilization of the vesicles, solubilization of the Ca^{2+} transporter, and/or denaturation of the enzyme. After centrifugation of these preparations and reconstitution of the supernatants, ATP-driven Ca^{2+} transport activity was detected in the proteoliposomes. The yield varied with the detergent used. In terms of activity recovered after reconstitution, the zwitterionic detergent CHAPS proved to be most suitable.

Based on these initial findings, the process of solubilization and reconstitution of the Ca^{2+} transporter was further optimized using CHAPS as the detergent. When CHAPS was added to plasma membrane vesicles below its CMC (approximately 8 mM, 0.49% w/v [15]), no effect on the ATP-driven uptake of $^{45}Ca^{2+}$ into the vesicles was observed (Fig. 1). Concentrations slightly above the CMC (≥10 mM, however,

effectively abolished the ATP-driven $^{45}Ca^{2+}$ transport. In these experiments, it was not possible to distinguish between a permeabilization of the vesicles, the solubilization of the transport system and/or denaturation of the enzyme. Evidence for solubilization of protein by CHAPS can be seen in Figure 2. When used above its CMC, the residual pelletable material of the CHAPS-treated membranes contained little protein while the detergent extract contained most (up to 85%) of the initial protein (Fig. 2A). ATP-driven $^{45}Ca^{2+}$ transport activity appeared in the proteoliposomes after reconstitution with the CHAPS extract of plasma membranes while it disappeared from the residue from CHAPS extraction (Fig. 2B). The recovery of ATP-driven Ca^{2+} transport constituted proof of reconstitution of the transport system because Ca^{2+} transport and accumulation, in order to take place, require the correct transmembrane orientation of the transporter in vesicles tightly sealed for Ca^{2+} as well as functionality of the structure(s) linking ATP hydrolysis to ion translocation. Comparing Figure 2A with Figure 2B, it will be noted, that the optimum concentration of CHAPS with respect to ATP-driven $^{45}Ca^{2+}$ transport reconstituted into the proteoliposomes is 10 mM. Solubilization of the total membrane protein appears to continue up to 15 to 20 mM detergent. This suggests that at concentrations higher than 10 mM CHAPS, the solubilized enzyme is either destabilized or denatured, thus preventing its functional reconstitution.

The phospholipid environment as well as other factors that might stabilize the catalytic function of the ATPase were tested for their effectiveness in recovering activity after reconstitution. The addition of PC affected the yield of functionally reconstituted Ca^{2+} transport activity (Fig. 3). The optimum was achieved when 2 mg PC per mL were added during solubilization while at higher concentrations, ATP-driven uptake of Ca^{2+} into the proteoliposomes decreased again. Similar phospholipid effects were observed during the reconstitution of other plant and nonplant proteins (5), but are not completely understood (23). The effect has been attributed partially to decreasing protein solubilization when the detergent to lipid ratio is decreased (5). We have further noted that the addition of ATP (optimum 5 mM) during the solubilization

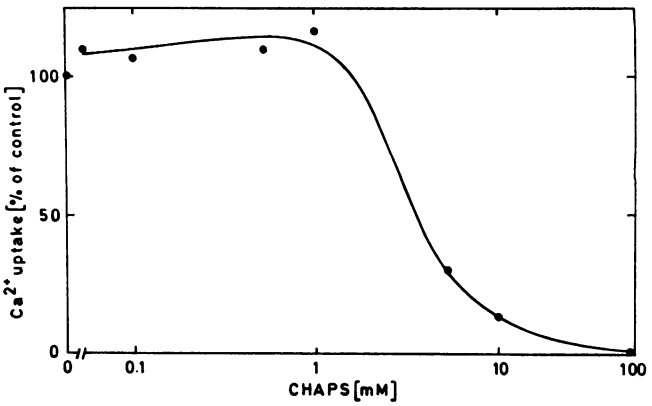


Figure 1. Effect of increasing CHAPS concentrations on the Ca^{2+} uptake by plasma membrane vesicles from the leaves of *C. communis* L. ATP-driven uptake of $^{45}Ca^{2+}$ was determined in the presence of the detergent (final concentrations as indicated) using 10 μg of protein per assay; 100% = 0.69 nmol Ca^{2+} uptake min^{-1} (mg protein)$^{-1}$.

improved the yield of reconstituted, active enzyme, presumably by stabilizing the catalytic site.

From the results presented so far, the optimum conditions for solubilization of the Ca^{2+} transport system in functional form are a protein concentration of 0.5 mg mL^{-1}, a detergent concentration of 10 mM and a detergent: exogeneous PC ratio of 3.07:1 (w/w) in the buffer specified. Under those conditions, the stability of the reconstituted Ca^{2+}-transport activity was quite high. At 4°C, no loss of Ca^{2+} transport activity was observed within 6 h. When kept frozen at −20°C, no loss of activity was observed for up to 4 d of storage. The proteoliposomes were tightly sealed with respect to Ca^{2+}. An average of 10% of the loaded $^{45}Ca^{2+}$ was lost during 0.5 h of subsequent incubation at 4°C, and 84.2% of the $^{45}Ca^{2+}$ was retained inside the vesicles even after one freeze-thaw cycle. Thus, the possibility that the strong retention of Ca^{2+} by the vesicles was due to binding of the ion to vesicle components had to be considered. However, 90.4% of the accumulated $^{45}Ca^{2+}$ could be discharged instantaneously by incubation of the

Table I. *Effect of Different Detergents on ATP-Driven $^{45}Ca^{2+}$ Transport in Plasma Membrane Vesicles and in the Proteoliposomes after Reconstitution*

ATP-dependent $^{45}Ca^{2+}$ transport into the plasma membrane vesicles (250 μg of protein in a final volume of 0.5 mL) was measured after the addition of the respective detergent in the final concentration as given. In the absence of any detergent (100%), 0.69 nmol Ca^{2+} min^{-1} (mg protein)$^{-1}$ were translocated. After centrifugation of the detergent-treated samples and reconstitution of the supernatants, ATP-driven $^{45}Ca^{2+}$ uptake recovered in the proteoliposomes was determined. The total activity recovered in each reconstituted sample is given.

Detergent	Concentration used	ATP-Dependent Transport of $^{45}Ca^{2+}$ in the Plasma Membrane Vesicles	ATP-Dependent Transport of $^{45}Ca^{2+}$ in the Proteoliposomes
	mM	%	*pmol Ca^{2+} min^{-1}*
None		100	0
CHAPS	10	14	14.8
Cholate	20	14	7.1
MEGA-9	20	10	9.7
Triton X-100	0.2	0	0

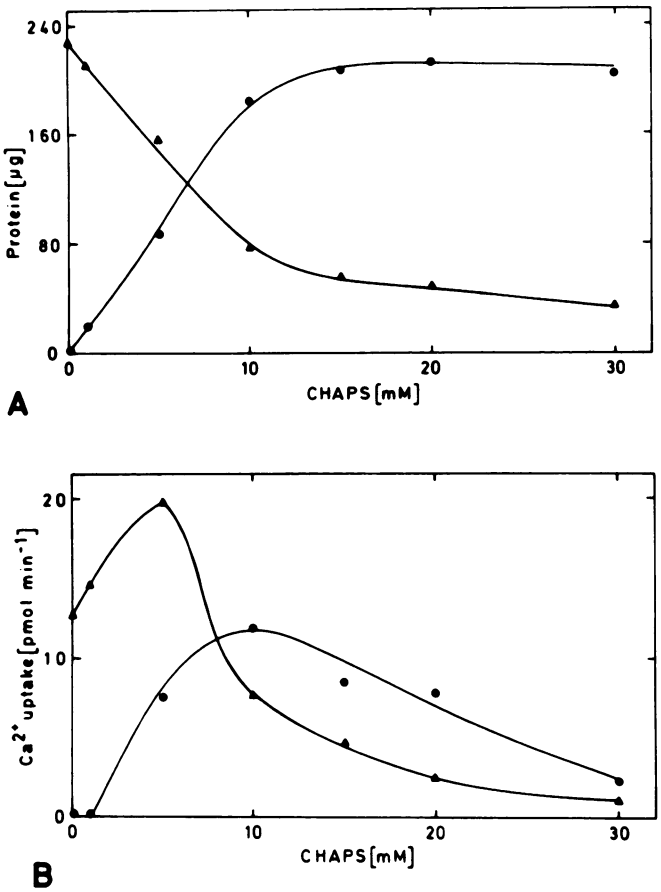


Figure 2. Reconstitution of supernatants and pellets derived from the solubilization of 250 µg plasma membrane vesicles (0.5 mg of protein mL^{-1}) with the indicated final concentrations of CHAPS in the presence of 2 mg PC mL^{-1} and 5 mM ATP. After centrifugation, the supernatants (0.5 mL) were collected and the pellets were resuspended in 0.5 mL detergent-free solubilization buffer (25 mM Hepes-KOH [pH 7.2], 2 mM $MgSO_4$, 250 mM sucrose containing 2 mg mL^{-1} PC and 5 mM ATP). Reconstitution of both fractions was then carried out by the detergent dialysis technique as described in "Materials and Methods." A, Total protein recovered in the supernatants (●) and pellets (▲); B, total ATP-driven $^{45}Ca^{2+}$ uptake recovered in the proteoliposomes reconstituted from supernatants (●) and pellets (▲).

proteoliposomes in the presence of 5 µM of the Ca^{2+} ionophore, A23187 (see Fig. 4A). Thus, only 9.6% of the total $^{45}Ca^{2+}$ associated with the vesicles was bound to the vesicle surfaces with the remainder being accumulated in the intravesicular volume in soluble, freely exchangeable form.

The yield of the reconstituted ATP-driven Ca^{2+} transport system can only be estimated roughly, because only the fraction of enzyme, oriented with its catalytic domain outward can be assayed. While approximately 30 to 35% of plasma membrane vesicles have such an inside-out orientation, the percentage of inside-out or right-side-out reconstituted Ca^{2+} transporter is not known. Therefore, only rough estimates can be derived. Usually, 10% of the total initial Ca^{2+} transport activity of inside-out plasma membrane vesicles could be recovered inside-out oriented in the proteoliposomes. However the efficiency of the reconstitution step is likely to be much higher. When the reconstituted Ca^{2+} transport activity was compared to controls processed identically with the sole exception that the detergent was omitted, the reconstituted activity amounted to 90% of the residual activity of these controls. This proved that the observed losses in activity resulted from procedural steps other than the reconstitution.

Having optimized the conditions for solubilization and reconstitution of the ATP-driven Ca^{2+} transporter, it was important to characterize the properties of the reconstituted enzyme and compare these with the known (11) properties of the plasma membrane embedded Ca^{2+} ATPase. The data are shown in Figure 4, A to D, and in Table II. The loading of $^{45}Ca^{2+}$ into the reconstituted vesicles was clearly ATP (Fig. 4A) and protein dependent (Fig. 4B) and proceeded at a constant rate for 10 to 12 min (see Fig. 4A). Therefore, 10 µg of protein and a standard assay time of 10 min was chosen for all subsequent experiments. The specific activity of ATP-driven $^{45}Ca^{2+}$ transport in the reconstituted system was identical to that of plasma membrane vesicles (Table II) and the apparent K_m for Ca^{2+} remained practically unchanged. We had earlier seen no indication for a secondary active Ca^{2+}/H^+ exchange in the *Commelina* plasma membrane (11). In agreement with this observation, there was no effect of the protonophore, CCCP, on ATP-driven $^{45}Ca^{2+}$ uptake into the vesicles (see Table II). This finding ruled out the possibility that the Ca^{2+} loading we have observed in the presence of ATP was driven by a proton gradient generated by the action of a H^+-ATPase and finally proved that the Ca^{2+} transport measured in the reconstituted vesicles was due to the action of a primary active Ca^{2+} ATPase. The apparent K_m for Mg-ATP was drastically lower for the reconstituted ATPase compared to the plasma membrane system (Table II). The second significant difference was the higher sensitivity of the reconstituted ATPase to the inhibitor, erythrosine B, while the enzyme's pH-optimum, vanadate-sensitivity, its susceptibility toward short tryptic treatments and the absence of any stimulatory effect

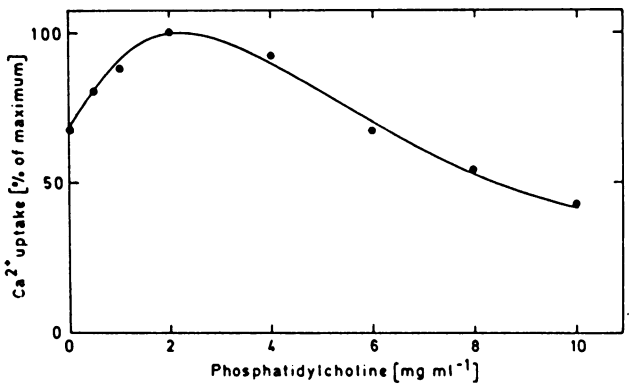
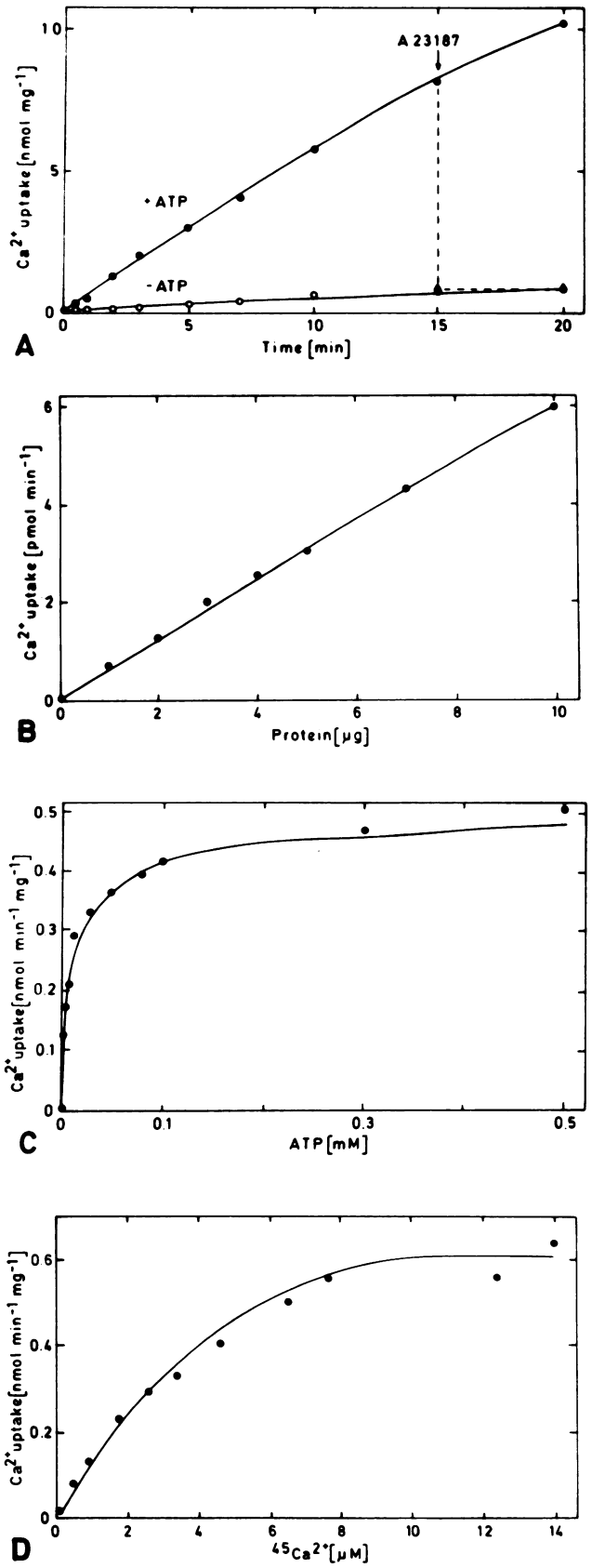


Figure 3. Effect of phosphatidylcholine on the reconstitution of Ca^{2+} transport activity. Plasma membranes (0.5 mg of protein in 1 mL of solubilization buffer) were solubilized with 10 mM CHAPS in the presence of the indicated final concentrations of PC, centrifuged and the supernatants were reconstituted by the detergent dialysis technique. These fractions were then assayed for ATP-driven $^{45}Ca^{2+}$ transport (100% = 20.1 pmol Ca^{2+} uptake min^{-1}).



of exogenously added CM were indistinguishable from the corresponding properties of the enzyme in the plasma membrane (Table II).

DISCUSSION

The Ca^{2+} transporting ATPase at the plasma membrane of plant cells is thought to be important because of its involvement in the maintenance of low cytoplasmic levels of Ca^{2+}. The availability of detailed enzymatic data about the *Commelina communis* plasma membrane Ca^{2+} translocating ATPase and the high purity of plasma membranes obtained from this species (11) provided the basis for our attempts to identify and purify this enzyme. Although it is tempting to isolate the ATPase based on labeling or inhibitor binding studies, or relying on similarities in protein sequence/structure or nucleotide sequences using heterologous probes, the only definitely conclusive approach will be to purify the enzyme in functional form. Only in this way is it ascertained that all elements required for function will be obtained in the process of purification. The most discriminative assay of the Ca^{2+} ATPase (*i.e.* to distinguish it from other ATPases and phosphatases) is ATP-driven Ca^{2+} translocation, and this parameter, using $^{45}Ca^{2+}$ as tracer, was therefore used for probing enzyme function. It seemed inappropriate to rely on the determination of Ca^{2+}-stimulated hydrolysis of ATP as a measure of activity of this enzyme, because we (11) and others (24) had already shown that this activity was small and not readily detected against the high background of other ATPases present in the plasma membrane.

Invariably the most critical step in the purification procedure of an integral membrane enzyme is the proper solubilization and reinsertion in an artificial membrane with retention of function. With two notable differences, the plasma membrane-bound Ca^{2+} ATPase and the reconstituted enzyme exhibit practically identical properties (Table II). However, the reconstituted Ca^{2+} ATPase showed a much lower apparent K_m for ATP and also for the inhibitor erythrosine B (Table II) while the K_m for Ca^{2+}, the vanadate sensitivity and the pH optimum remained unaffected. Thus, a conformational change of the ATP-binding domain seems unlikely. Rather, we conclude that the differences observed in the K_m for ATP and IC_{50} for erythrosine B are mainly due to the elimination of other ATP-consuming enzymes (*e.g.* kinases, phosphatases) and of erythrosine-binding components, during solubilization

Figure 4. Characteristics of the ATP-driven Ca^{2+} transport activity of proteoliposomes reconstituted from the CHAPS-solubilized plasma membrane protein fraction. The proteoliposomes were, at the end of the dialysis procedure, collected by centrifugation as described in "Materials and Methods" and the Ca^{2+} transport activity was measured, if not stated otherwise, using 10 µg protein per assay under standard conditions, A, Kinetics of $^{45}Ca^{2+}$ uptake in the presence (●) or in the absence (○) of ATP (1 mM). After 15 min, A 23187 (5 µM final concentration) was added and the kinetics of $^{45}Ca^{2+}$ release (▲) were determined. B, ATP-dependent $^{45}Ca^{2+}$ uptake as a function of the amount of reconstituted protein used per assay. C, $^{45}Ca^{2+}$ uptake as a function of Mg^{2+} ATP (2:1 molar ratio) concentration. D, $^{45}Ca^{2+}$ uptake as a function of $^{45}CaCl_2$ concentration.

Table II. *Characteristics of the ATP-Driven Ca^{2+} Transport System Reconstituted into Proteoliposomes as Compared with the Characteristics of the Enzyme in Plasma Membrane Vesicles*

Assays were performed under standard conditions using 10 μg protein per sample. The specific activity of the transport system was derived from the measurement of ATP-driven $^{45}Ca^{2+}$ uptake as in Gräf and Weiler (11) and is given as nmol Ca^{2+} uptake min^{-1} (mg protein)$^{-1}$. The effect of tryptic digestion was measured after 5 min preincubation of the vesicles with trypsin (trypsin:protein = 1:1, w/w). The influence of calmodulin was tested after 15 min preincubation of the vesicles with 0.5 μM spinach calmodulin followed by the addition of ATP. The protonophore, CCCP, was tested at 1 to 100 μM concentrations and in this concentration range it did not affect Ca^{2+} transport. Only the data for 10 μM CCCP are shown.

Parameter	Plasma Membrane Vesicles	Reconstituted Liposomes
Specific activity (nmol min^{-1} mg^{-1})	0.69	0.66
K_m ATP app. (μM)	300	11.4
K_m Ca^{2+} app. (μM)	4.4	5.2
Optimum pH	7.2	7.2
IC_{50} vanadate (μM)	500	500
IC_{50} erythrosine B (μM)	0.1	0.01
Activity in the presence of 10 μM CCCP (% of control)	99.8	100.1
Residual activity after treatment with trypsin (% of control)	7	15
Activity in the presence of calmodulin (% of control)	104	88

and reconstitution. If this is so, then the data in Table II show a remarkable retention of enzyme characteristics, indicative of a native conformation of the reconstituted Ca^{2+} ATPase. The effectivity of the process of solubilization and reconstitution is furthermore documented by the fact that 90% of the ATP-driven $^{45}Ca^{2+}$ transport activity of treated controls (only detergent omitted) was recovered in the proteoliposomes, representing an overall 10% yield of functionally reconstituted enzyme based on the initial transport activity. This yield compared well with the recovery obtained for the reconstituted H^+ ATPase from oat roots (25).

Reconstitution of an integral membrane protein in functional form is obtained only, if its polypeptide chain(s) reorganize properly. To achieve this, several critical factors need to be optimized including (a) the choice of an appropriate detergent, (b) the inclusion of stabilizing factors which help to prevent denaturation of the solubilized enzyme, (c) the choice of an appropriate lipid or lipid mixture for the reconstitution, and (d) a proper reconstitution protocol (15). The detergent dialysis technique in conjunction with steroidal detergents is known to produce vesicles tightly sealed for calcium in contrast to, *e.g.* column reconstitution in the presence of Triton X-100 (20) and was thus selected for our study. It is, furthermore, well known that acidic phospholipids, such as *e.g.* phosphatidylinositolphosphates or cardiolipin strongly activate E_1/E_2-type ATPases (16, 20). Asolectin was chosen as source of lipid for the reconstitution because it contains numerous acidic phospholipids including cardiolipin and is known to form tightly sealed vesicles (20). We also expected, that treatments preventing excessive delipidation in the presence of the detergent as well as a stabilization of the enzyme's catalytic domain by adding ATP to the solubilization buffer would enhance the yield of reconstituted, catalytically active enzyme, and this was indeed observed. The detergent to lipid ratio found optimum in our case (3:1 by weight) is close to the optimum values observed in other systems, too (5, 23). As Figure 2 shows, this is a compromise between total solubilized enzyme activity and structural integrity in the solubilized state, which is impaired by higher detergent concentrations.

A final consideration to be made was the nature of the detergent to be used. Among the detergents reported to yield active E_1/E_2-ATPases are Triton X-100 (3), Zwittergent 3-14 (26), cholate or desoxycholate (19) and lysolecithin (25). Since detergents with low CMC and high micellar weights cannot readily be dialyzed (20), Zwittergent 3-14 and Triton X-100 seemed less suitable for our purposes. Triton X-100, furthermore, quickly inactivated the *Commelina* Ca^{2+} ATPase (*cf.* Table I). CHAPS proved to be the most suitable detergent in our hands (*cf.* Table I). The compound combines structural elements of two classes of detergents, which are by themselves highly effective in solubilizing E_1/E_2-type ATPases, namely the steroidal nucleus and the substituents of cholate and the zwitterionic (dimethylammonio)-1-propane sulfonate head group of Zwittergent 3-14 (14). With its relatively high CMC (8 mM) and low micellar weight (6150 D) (15), it can effectively be removed from the preparations. This facilitates the purification process and minimizes detergent interference when studying the characteristics of the reconstituted enzyme.

As with the Ca^{2+} ATPase embedded in its native environment (11), the reconstituted enzyme was unaffected by added calmodulin. It is known that the CM stimulation of the plasma membrane Ca^{2+} ATPases from animal cells is lost after reconstitution into asolectin liposomes due to the maximum activation of the enzyme by the cardiolipin present (20). Interestingly, we observed that the specific catalytic activity of the reconstituted enzyme and the enzyme in the plasma membrane were identical (*cf.* Table II). This suggests that the Ca^{2+} ATPase obtained after preparation of the plasma membrane vesicles might already be maximally activated. This could be through Ca^{2+} CM tightly bound to the membrane as in peas (4) or as a result of sufficient acidic phospholipids in the lipid environment in the vesicles. The question of CM regulation of the *Commelina* plasma membrane Ca^{2+} pump thus awaits purification of the enzyme and a study of its molecular architecture. With the aid of the reconstitution assay described in this paper, the purification of the *Commelina* Ca^{2+} ATPase in now in progress.

ACKNOWLEDGMENTS

Some experiments described here have been reported in preliminary form elsewhere (12).

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