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Calmodulin-stimulated Ca^{2+}-ATPases in the Vacuolar and Plasma Membranes in Cauliflower[1]

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The subcellular locations of Ca^{2+}-ATPases in the membranes of cauliflower (*Brassica oleracea* L.) inflorescences were investigated. After continuous sucrose gradient centrifugation a 111-kD calmodulin (CaM)-stimulated and CaM-binding Ca^{2+}-ATPase (BCA1; P. Askerlund [1996] Plant Physiol 110: 913–922; S. Malmström, P. Askerlund, M.G. Palmgren [1997] FEBS Lett 400: 324–328) co-migrated with vacuolar membrane markers, whereas a 116-kD CaM-binding Ca^{2+}-ATPase co-migrated with a marker for the plasma membrane. The 116-kD Ca^{2+}-ATPase was enriched in plasma membranes obtained by aqueous two-phase partitioning, which is in agreement with a plasma membrane location of this Ca^{2+}-ATPase. Countercurrent distribution of a low-density intracellular membrane fraction in an aqueous two-phase system resulted in the separation of the endoplasmic reticulum and vacuolar membranes. The 111-kD Ca^{2+}-ATPase co-migrated with a vacuolar membrane marker after countercurrent distribution but not with markers for the endoplasmic reticulum. A vacuolar membrane location of the 111-kD Ca^{2+}-ATPase was further supported by experiments with isolated vacuoles from cauliflower: (a) Immunoblotting with an antibody against the 111-kD Ca^{2+}-ATPase showed that it was associated with the vacuoles, and (b) ATP-dependent Ca^{2+} uptake by the intact vacuoles was found to be CaM stimulated and partly protonophore insensitive.

Ca^{2+} is an essential intracellular messenger in plant cells and is involved in metabolic and developmental regulation. Maintenance of a low free cytosolic concentration (about 0.1 μM) of Ca^{2+} is necessary for its function as a messenger in signal transduction (Gilroy et al., 1993; Bush, 1995). A variety of stimuli can trigger the opening of Ca^{2+} channels in the plasma membrane and in internal membranes, causing Ca^{2+} influx and accumulation in the cytosol. The cytosolic Ca^{2+} concentration is restored and maintained at the low level by extrusion of Ca^{2+} from the cell or by sequestration into intracellular organelles, mainly the vacuole (Canut et al., 1993).

Two classes of active Ca^{2+} transporters have been shown to be present in plant membranes: Ca^{2+}-ATPases and nH^+/Ca^{2+} antiporters (Evans et al., 1991; Hirschi et al., 1996). The Ca^{2+}-ATPases, some of which are stimulated by CaM, have been shown to be responsible for Ca^{2+} extrusion across the plasma membrane as well as transport of Ca^{2+} into the ER lumen (Buckhout, 1983; Robinson et al., 1988; Rasi-Caldogno et al., 1992; Gilroy and Jones, 1993). Uptake of Ca^{2+} into the vacuole has long been thought to be catalyzed by an nH^+/Ca^{2+} antiporter only. More recently it was suggested that Ca^{2+}-ATPases also take part in the transport of Ca^{2+} into the vacuole (DuPont et al., 1990; Gavin et al., 1993; Pfeiffer and Hager, 1993; Bush and Wang, 1995; Ferrol and Bennett, 1996; for a review, see Askerlund and Sommarin [1996]). The subcellular location of CaM-stimulated Ca^{2+}-ATPases in plants has long been a matter of controversy. In contrast to the situation in animal cells (Carafoli, 1994), CaM-stimulated Ca^{2+}-ATPases in plants are present in intracellular membranes as well as in the plasma membrane (Askerlund and Sommarin, 1996). CaM-stimulated, ATP-dependent Ca^{2+} uptake in maize root membranes was found to be associated with markers for vacuolar membranes (Gavin et al., 1993). In studies with maize shoots the same activity was proposed to be located in the ER (Logan and Venis, 1995). In barley aleurone the CaM-stimulated, ATP-dependent Ca^{2+} uptake was localized to the ER (Gilroy and Jones, 1993), whereas in wheat aleurone a much greater fraction of the CaM-stimulated activity was associated with the vacuolar membrane (Bush and Wang, 1995). Previously, a 111-kD CaM-stimulated Ca^{2+}-ATPase was shown to be enriched in a low-density intracellular membrane fraction from cauliflower (*Brassica oleracea* L.) inflorescences (Askerlund and Evans, 1992; Askerlund, 1996). The cDNA corresponding to this pump (*BCA1*) was recently cloned (based on the sequence of tryptic peptides derived from the purified protein), showing that it represents a novel class of CaM-stimulated Ca^{2+}-ATPases (Malmström et al., 1997). The main objective of this study was to accurately determine the intracellular location of this 111-kD CaM-stimulated Ca^{2+}-ATPase.

MATERIALS AND METHODS

Suc Gradient Centrifugation

All operations were carried out at 0 to 4°C, and the outermost part of the cauliflower (*Brassica oleracea* L.) inflorescence was used. This tissue contains a large proportion of immature cells in the process of vacuolation but also differentiated cells of different types. The plant material was homogenized as described previously (Askerlund, 1996), except that BSA was omitted from the homogeniza-

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Abbreviations: BiP, ER luminal binding protein; BTP, 1,3-bis-(tris[hydroxymethyl]methylamino)propane; CaM, calmodulin; CCD, countercurrent distribution; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

tion medium. The homogenate was filtered through a nylon cloth (140 μm, Lockertex, Warrington, UK) and centrifuged at 10,000*g* for 10 min. About 80 mL of the supernatant was carefully pipetted onto a cushion of 4 g of 2 M Suc in a gradient buffer (25 mM Mops-BTP, pH 7.2, 50 mM KCl, and 2 mM Na_2-EDTA) in two centrifuge tubes and was centrifuged at 100,000*g* for 45 min in an SW-28 rotor (Beckman). The microsomal membranes at the interface (total volume 5 mL) were collected and mixed with 6 mL of 2 M Suc in the gradient buffer. One-milliliter fractions (about 4.5 mg of protein) of the membrane suspension were pipetted into 13-mL centrifuge tubes and were overlaid with a continuous Suc gradient (1.7–0.6 M Suc in 11 mL of the gradient buffer). The tubes were centrifuged in a swing-out rotor (RPS40T, Hitachi, Mountain View, CA) for 16 h at 150,000g. Fractions (0.8 mL) were collected from the bottom of the gradient with a peristaltic pump and were aliquoted and stored at −20°C until analysis. The Suc concentration was measured with a refractometer (Atago 1T, Atago, Tokyo, Japan).

CCD of Low-Density Intracellular Membranes in an Aqueous Polymer Two-Phase System and Preparation of Plasma Membranes

Low-density intracellular membranes were collected at the 10/32% (w/w) interface after discontinuous Suc gradient centrifugation of a microsomal membrane fraction, as described previously (Askerlund, 1996). After the sample was pelleted, the low-density membranes were resuspended in 5 mM potassium phosphate, pH 7.8, 330 mM Suc, 1 mM DTT, and 0.5 mM PMSF and were again pelleted at 100,000g for 45 min. The final washed membrane pellet was suspended in the same buffer and stored at −80°C. The low-density membranes (about 8 mg of protein) were further fractionated by CCD in an aqueous polymer two-phase system (8 g, final weight, of 6.2% [w/w] dextran T-500, 6.2% [w/w] PEG 3350, 330 mM Suc, 1 mM KCl, and 5 mM potassium phosphate, pH 7.8), essentially as described for the crude microsomal membranes by Bérczi et al. (1989; for a detailed description of CCD, see Larsson [1983]). A total of five transfers of the upper phase were made, resulting in a total of six final tubes. After CCD the total content in each tube was diluted to 70 mL with 25 mM Mops-BTP, pH 7.2, 0.33 M Suc, 1 mM DTT, and 0.5 mM PMSF and pelleted at 100,000*g* for 45 min. Because of the high content of polymers the following procedure was necessary to pellet the CCD fractions: After the sample was centrifuged about 90% of the supernatant was carefully removed with a pipette. A new buffer was then added, the content was mixed, and the tubes were centrifuged at 100,000*g* for 30 min. The final membrane pellets were suspended in 25 mM Mops-BTP, pH 7.2, 0.33 M Suc, and 0.5 mM PMSF, aliquoted, and stored at −80°C. All operations were carried out at 0 to 4°C. Preparation of plasma membranes from cauliflower by aqueous two-phase partitioning was carried out as described by Askerlund and Evans (1992).

Preparation of Intact Vacuoles

Intact vacuoles from cauliflower inflorescences were isolated by slicing and flotation, essentially as described by Bennett et al. (1983). All of the operations were performed at 4°C or on ice. The plant material (650 g) was sliced using a blender (HR 2375/D, Philips Eindhoven, The Netherlands) in 0.5 L of 50 mM Tris-Mes, pH 8.0, 1 M sorbitol, 5 mM Na_2-EDTA, 0.5% (w/v) polyvinylpolypyrrolidone, 4 mM DTT, and 0.5 mM PMSF. The extract was filtered through a nylon cloth and centrifuged at 1380*g* for 20 min in two buckets. Each pellet was carefully suspended in 10 mL of 15% (w/v) sodium diatrizoate in 25 mM Tris-Mes, pH 7.0, and 1.2 M sorbitol. Each resuspension was placed in a centrifuge tube and was overlaid with 10 mL of 10% (w/v) sodium diatrizoate in the same buffer followed by 5 mL of the buffer without sodium diatrizoate and was centrifuged at 400*g* in a swing-out centrifuge for 15 min. The intact vacuoles (weak white band; about 0.13 mg of protein in 1.3 mL) at the 0/10% sodium diatrizoate interface were collected and placed on ice. The purity and intactness of the vacuoles were determined with a phase-contrast microscope (Nikon).

Enzyme Activities

PPi-dependent H^+ pumping and ATP-dependent Ca^{2+} pumping (radioactive filter assay) with membrane fractions were measured as described earlier (Askerlund, 1996). Antimycin A-insensitive NADH-Cyt *c* reductase was measured in the absence of detergent as described by Askerlund et al. (1991). Measurement of UDP-Gal:diacylglycerol galactosyltransferase (galactolipid synthase activity) was as described by Douce and Joyard (1980). Radioactive Ca^{2+} uptake with intact vacuoles was measured in a mixture of 50 μL of 25 mM Tris-Mes, pH 7.0, 200 mM KCl, 10 mM $MgCl_2$, 1.2 M sorbitol, 2 mM DTT, 0.2 mM sodium molybdate, 2 mM NaN_3, 10 μM $CaCl_2$ (36 Bq $^{45}CaCl_2$ $pmol^{-1}$), and 50 μL of vacuoles (about 5 μg of protein, see above) for 30 min at 30°C in a final volume of 0.1 mL. The mixture was preincubated for 10 min prior to starting the Ca^{2+} uptake with 2.5 mM ATP-BTP, pH 7.0. The reactions were stopped by the addition of 0.6 mL of Tris-Mes, pH 7.0, 1 mM EGTA, and 1.2 M sorbitol, and the mixtures were immediately filtered through 0.45-μm pore-size cellulose nitrate membrane filters (Gelman Sciences, Ann Arbor, MI) using a vacuum-sampling manifold (Millipore). After they were washed four times with 1 mL of a stop solution, the filters were dried and the amount of $^{45}Ca^{2+}$ was measured by scintillation counting.

Protein Analysis and Determination

Protein was measured with a modified Bradford procedure (Stoscheck, 1990), except for preparations of intact vacuoles, for which protein was measured with the bicinchoninic acid protein assay (Pierce, Rockford, IL), since sodium diatrizoate interferes with the Bradford procedure. BSA was used as a standard. SDS-PAGE was carried out on linear minigels as described earlier (Askerlund, 1996). To avoid proteolysis samples were TCA-precipitated prior to

solubilization with SDS. Vacuoles were precipitated with methanol-chloroform-H_2O (Pohl, 1990). A sample volume corresponding to an 11-μL Suc gradient fraction or 6 μg of protein was applied to each lane unless otherwise indicated.

Western Analysis

Western blotting was carried out as described earlier (Askerlund, 1996) using ^{125}I-labeled or alkaline-phosphatase-conjugated secondary antibodies. Radioactive blots were first analyzed with a phosphor imager (Molecular Dynamics, Sunnyvale, CA) to quantify the amount of radioactivity in each band. To obtain sharper images the blots were later exposed for about 2 d at 20°C with Hyperfilm-βmax (Amersham) in the presence of an intensifier screen (Hyperscreen, Amersham). An antiserum against the 111-kD, CaM-stimulated cauliflower Ca^{2+}-ATPase (Askerlund, 1996) and an antiserum against the putative chloroplast envelope Ca^{2+}-ATPase in *Arabidopsis thaliana* (Huang et al., 1993; a gift from Dr. N.E. Hoffman, Department of Plant Biology, Carnegie Institute of Washington, Stanford) were both used at a dilution of 1:2000, whereas an antiserum against the *A. thaliana* plasma membrane H^+-ATPase (no. 761; a gift from Prof. R. Serrano, Department of Biotechnology, University of Valencia, Spain) was diluted 1:3300. Antisera against the 57-kD subunit of the vacuolar H^+-ATPase from *Beta vulgaris* L. (Manolson et al., 1989; a gift from Prof. R.J. Poole, Department of Biology, McGill University, Montreal, Quebec, Canada) and against the tobacco BiP (Denecke et al., 1991; a gift from Dr. J. Denecke, Department of Biology, University of York, UK) were both diluted 1:2000.

^{125}I-CaM Overlays and Phosphorylated Intermediate Formation and Analysis

^{125}I-CaM overlays were prepared and analyzed as described earlier (Askerlund, 1996). For studies of the phosphorylated intermediate of Ca^{2+}-ATPases, membrane fractions were phosphorylated with 1 nM [γ-^{32}P]ATP for 15 s on ice at pH 7.4, in the absence or presence of 50 μM $LaCl_3$, and analyzed as described earlier (Askerlund and Evans, 1993).

RESULTS

Distribution of ATP-Dependent Ca^{2+} Uptake and Enzymic Membrane Markers after Continuous Suc Gradient Centrifugation

Crude microsomes were fractionated by continuous Suc gradient centrifugation and the distribution of ATP-dependent Ca^{2+} uptake was compared with various enzymic membrane markers (Fig. 1). Membrane protein was mainly found in a broad peak in the middle of the gradient. Since membranes were loaded from the bottom of the tube, soluble protein was found in fraction 1 (Fig. 1A). Antimycin A-insensitive NADH-Cyt *c* reductase activity (ER marker), galactolipid synthase (plastid inner envelope; Douce and Joyard, 1980), PPi-dependent H^+ pumping (vacuolar

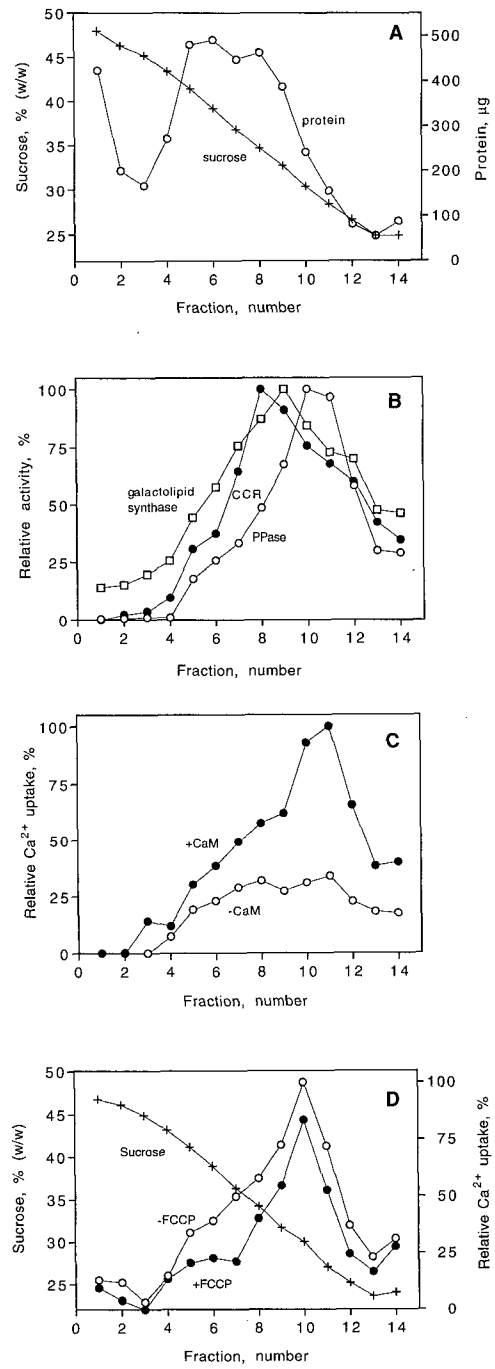

Figure 1. Distribution of ATP-dependent, CaM-stimulated Ca^{2+} uptake and different marker enzyme activities after continuous Suc gradient centrifugation of a microsomal membrane fraction from cauliflower inflorescences. Membranes were loaded from the bottom (fraction 1). A, +, In percentage (w/w) of Suc; ○, in micrograms of protein. B, ●, Antimycin A-insensitive NADH-Cyt *c* reductase activity (CCR; 100% = 68 nmol min^{-1}); ○, PPi-dependent H^+ pumping (PPase; 100% = a change in A_{495} min^{-1} of 0.86); □, galactolipid synthase (100% = 12 nmol h^{-1}). C, ATP-dependent Ca^{2+} uptake (100% = 1.0 nmol min^{-1}) in the presence (●) or absence (○) of 1 μM CaM. D, ATP-dependent Ca^{2+} uptake (100% = 1.2 nmol min^{-1}) measured in the presence of 1 μM CaM in the presence (●) or absence (○) of 5 μM FCCP. The data shown in A to C were obtained from the same Suc gradient separation, and the data shown in D are from a separate experiment.

membrane marker; Chanson, 1990), and ATP-dependent Ca^{2+} uptake were all located mainly in the lighter part of the gradient (Fig. 1, B and C). The protonophore FCCP had only a small effect on Ca^{2+} uptake, indicating that the major part of the Ca^{2+} uptake was catalyzed by a Ca^{2+}-ATPase rather than by a Ca^{2+}/nH^+ antiporter (Fig. 1D; see also "Discussion"). The distribution of Ca^{2+} uptake (clear optimum visible only when measured in the presence of CaM) correlated very closely with PPi-dependent H^+ pumping but was different from that of NADH-Cyt *c* reductase (Fig. 1, B and C). This is in contrast with the earlier report in which the major part of the CaM-stimulated Ca^{2+} uptake was suggested to be located in the ER (Askerlund and Evans, 1992). In the earlier experiments, PPi hydrolysis rather than PPi-dependent H^+ pumping was measured. The current view is that PPi hydrolysis is a much less specific marker for the vacuolar membrane than PPi-dependent H^+ pumping, possibly due to binding of soluble PPiases to membranes. In contrast, PPi-dependent H^+ pumping may be considered a relatively specific marker for the vacuolar membrane, although this activity has been reported to be present in the plasma membrane (Chanson, 1990; Robinson, 1996).

Use of Antibodies and ^{125}I-CaM to Study the Distribution of Ca^{2+}-ATPases and Membrane Markers after Continuous Suc Gradient Centrifugation

Antibodies were also used as probes for Ca^{2+}-ATPases and different membranes on the Suc gradient (Fig. 2). Antibodies against the BiP detected a polypeptide of 78 kD, in agreement with the molecular mass of BiP (Denecke et al., 1991; Fig. 2, A and B). The BiP that was found at the bottom of the gradient (fraction 1) may have been released from the ER lumen during homogenization (the membranes applied to the Suc gradient were not extensively washed, since pelleting increases the risk of membrane aggregation). Like the amount of antimycin A-insensitive NADH-Cyt *c* reductase, the amount of BiP was maximal in fraction 8 (34.76% [w/w] Suc); however, BiP showed a wider distribution on the gradient (compare Figs. 1B and 2B). Antibodies against the 57-kD subunit of the vacuolar H^+-ATPase detected a 57-kD polypeptide, which peaked in fractions 9 and 10 (Fig. 2, A and B). The amount of plasma membrane H^+-ATPase (100 kD) was maximal in fraction 5 (Fig. 2, A and C). Finally, antibodies against the 111-kD CaM-stimulated Ca^{2+}-ATPase from cauliflower (Askerlund, 1996) detected a polypeptide of 111 kD, which peaked in fraction 10 (Fig. 2, A and C).

In agreement with an earlier report (Askerlund, 1996), the same antiserum also recognized a minor polypeptide band at 102 kD (Fig. 2A). The 102-kD band was suggested to be a truncated form of the intact 111-kD Ca^{2+}-ATPase, since it accumulated during the trypsin treatment of low-density membranes (Askerlund, 1996). This hypothesis is supported by the fact that the 111- and 102-kD polypeptides showed a perfect correlation on the Suc gradient (Fig. 2A), indicating that they are present on the same membrane. In addition to the 111- and 102-kD bands, the antiserum against the cauliflower Ca^{2+}-ATPase recognized a

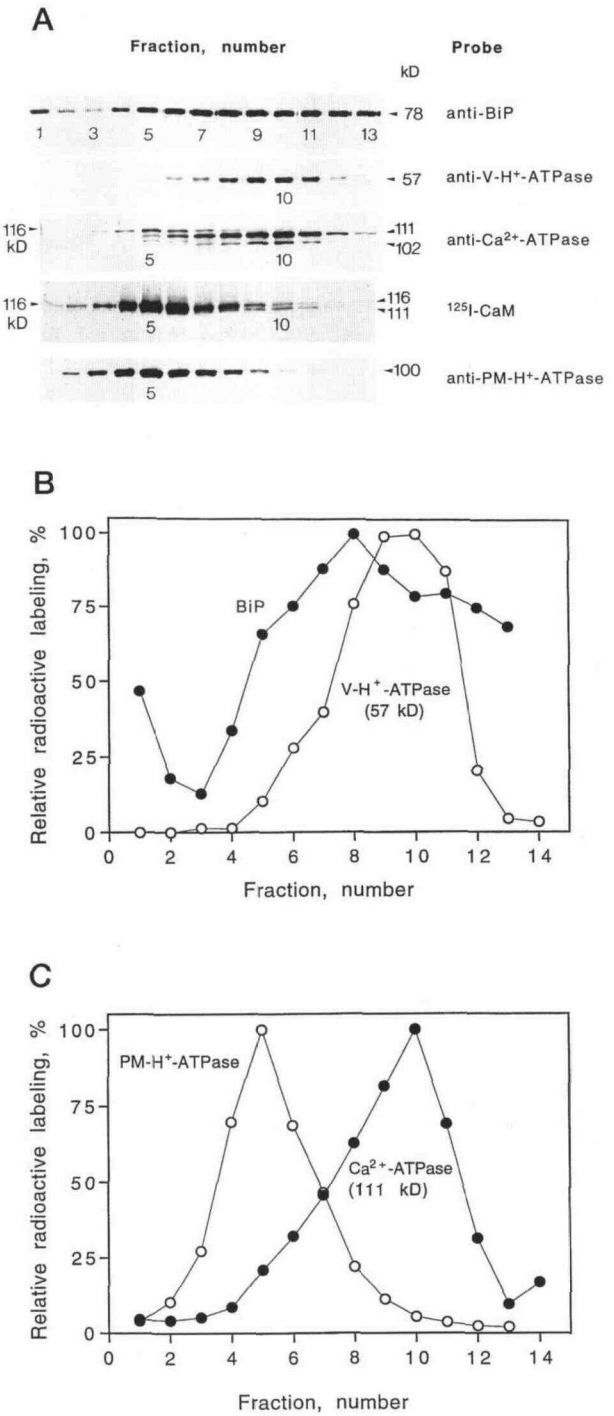

Figure 2. Western analysis of fractions after Suc gradient centrifugation of a microsomal fraction from cauliflower inflorescences. A, Autoradiographs of a ^{125}I-CaM blot and immunoblots with antisera against BiP (anti-BiP), the 57-kD subunit of the vacuolar H^+-ATPase (anti-V-H^+-ATPase), the 111-kD CaM-stimulated Ca^{2+}-ATPase in low-density membranes from cauliflower (anti-Ca^{2+}-ATPase), and the plasma membrane H^+-ATPase (anti-PM-H^+-ATPase). ^{125}I-labeled secondary antibodies were used. A sample volume corresponding to an 11-μL Suc gradient fraction was loaded in each lane. B and C, Phosphor imager quantification of immunoblots in A. Data were obtained from the same Suc gradient separation that is shown in Figure 1, A to C.

116-kD polypeptide, which peaked in fraction 5 (Fig. 2A). The co-localization of this polypeptide with the plasma membrane H^+-ATPase (Fig. 2A) agrees with the presence of a 116-kD Ca^{2+}-ATPase in plasma membranes from cauliflower (Askerlund and Evans, 1993). The same antiserum also detected a minor polypeptide band at about 107 kD (clearly visible in fractions 5 and 6 only). This band peaked in fraction 5 and may therefore be a truncated form of the 116-kD Ca^{2+}-ATPase (see also the data for phase-partitioned plasma membranes below).

CaM-binding polypeptides of 116 (very strong) and 111 kD were identified in ^{125}I-CaM overlays (Fig. 2A). The 116-kD CaM-binding band peaked in fraction 5, whereas the much weaker 111-kD CaM-binding band peaked in fraction 10. These results agree with the suggestion (Askerlund, 1996) that the 116-kD CaM-binding polypeptide represents a plasma membrane Ca^{2+}-ATPase with very high affinity for CaM and that the 111-kD CaM-binding polypeptide represents the Ca^{2+}-ATPase in low-density membranes.

CCD of Low-Density Intracellular Membranes

Low-density membranes from cauliflower, prepared by discontinuous Suc gradient centrifugation (Askerlund, 1996), were further fractionated by CCD in an aqueous two-phase system (Fig. 3). The ER membranes (as detected by anti-BiP and antimycin A-insensitive NADH-Cyt *c* reductase) had a high affinity for the bottom phase and ended up mainly in CCD fractions 1 to 4 (Fig. 3, A and B). In contrast, the vacuolar membranes (as detected by antibodies against the 57-kD subunit of the vacuolar H^+-ATPase) had a higher affinity for the upper phase and ended up mainly in CCD fractions 3 to 6 (Fig. 3, A and B). Thus, CCD resulted in a relatively good separation of the ER and vacuolar membranes. The 111-kD Ca^{2+}-ATPase showed an almost perfect correlation with the 57-kD subunit of the vacuolar H^+-ATPase (Fig. 3, A and B), in agreement with a vacuolar membrane location of this Ca^{2+}-ATPase. The co-migration of the 111- and 102-kD polypeptides after CCD further support the suggestion (Askerlund, 1996) that the 102-kD polypeptide is a truncated form of the intact 111-kD Ca^{2+}-ATPase.

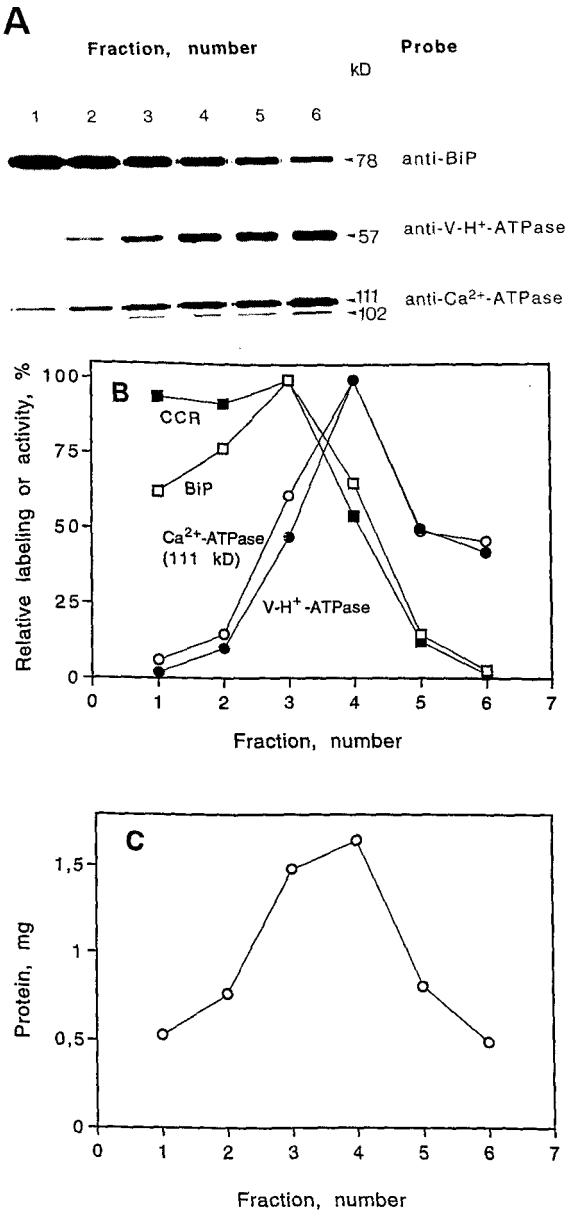

Figure 3. Analysis of fractions after CCD of low-density membranes from cauliflower inflorescences. A, Autoradiographs of immunoblots with antisera against BiP (anti-BiP), the 57-kD subunit of the vacuolar H^+-ATPase (anti-V-H^+-ATPase), and the 111-kD CaM-stimulated Ca^{2+}-ATPase in low-density membranes from cauliflower (anti-Ca^{2+}-ATPase). ^{125}I-labeled secondary antibodies were used. All lanes received 6 μg of protein. B, Phosphor imager quantification of immunoblots in A and antimycin A-insensitive NADH-Cyt *c* reductase activity (CCR; 100% = 0.65 μmol min^{-1}) expressed as relative amount of labeling or activity in each CCD fraction: □, BiP; ●, V-H^+-ATPase; ○, Ca^{2+}-ATPase; and ■, CCR. C, Distribution of protein between CCD fractions.

Comparison of the Ca^{2+}-ATPases in Low-Density Intracellular Membranes and Phase-Partitioned Plasma Membranes

The Ca^{2+}-ATPases in cauliflower plasma membranes (prepared by two-phase partitioning) and low-density membranes (prepared as described by Askerlund, 1996) are compared in Figure 4. As shown earlier (Askerlund, 1996), the antiserum against the 111-kD CaM-stimulated Ca^{2+}-ATPase detected the intact Ca^{2+}-ATPase and the 102-kD degradation product when reacted with low-density membranes (Fig. 4, lane A). With plasma membranes, the same antiserum detected a band at 116 kD and weaker bands at 111 and about 107 kD (Fig. 4, lane B). A comparison with Figure 2A suggests that the 116-kD band observed with plasma membranes represents the plasma membrane Ca^{2+}-ATPase and that the 111-kD band is due to contamination by the vacuolar membranes. The presence of a 107-kD band (probably a degradation product of the plasma membrane Ca^{2+}-ATPase) is also in agreement with

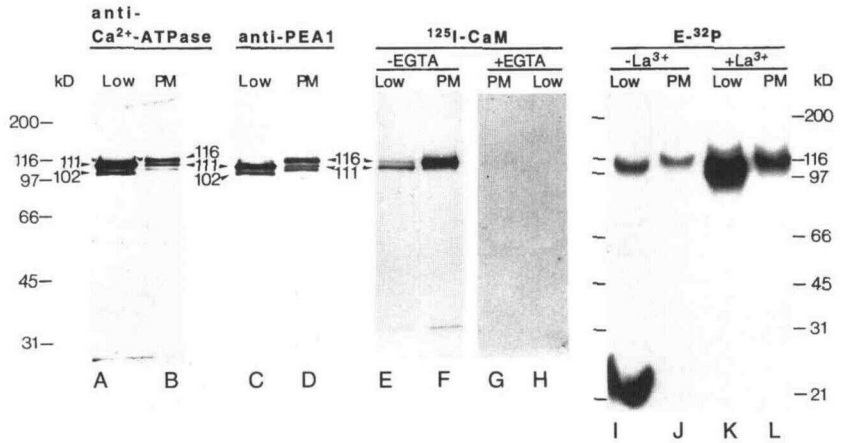

Figure 4. Characterization of Ca^{2+}-ATPases in low-density intracellular membranes (Low) and phase-partitioned plasma membranes (PM). Lanes A and B, Immunoblot with antibodies against the 111-kD Ca^{2+}-ATPase in low-density membranes (anti-Ca^{2+}-ATPase; Askerlund, 1996); lanes C and D, immunoblot with antibodies against the putative Ca^{2+}-ATPase from *A. thaliana* chloroplast envelope (anti-PEA1; Huang et al., 1993); lanes E and F, autoradiograph of ^{125}I-CaM overlay; lanes G and H, same as E and F, but blot was incubated with ^{125}I-CaM in the presence of 1 mM EGTA; lanes I and J, autoradiograph showing phosphorylated intermediates of Ca^{2+}-ATPases (E-^{32}P); and lanes K and L, same as I and J, but phosphorylation was carried out in the presence of $LaCl_3$. Lanes A to H received 6 μg of protein, and lanes I to L received 80 μg of protein.

the data from the Suc gradient centrifugation (compare Figs. 2A and 4, lane B).

Antibodies against a region of PEA1 (ACA1), the putative Ca^{2+}-ATPase in the *A. thaliana* chloroplast envelope (Huang et al., 1993), detected the 111- and 102-kD bands when reacted with low-density membranes (Fig. 4, lane C). This is in agreement with the relatively high homology between the 111-kD intracellular Ca^{2+}-ATPase in cauliflower (BCA 1; Malmström et al., 1997) and the *A. thaliana* Ca^{2+}-ATPase. With plasma membranes these antibodies gave essentially the same result as the antibodies against the 111-kD Ca^{2+}-ATPase in cauliflower, except that the 116- and 107-kD bands were more strongly stained and the 111-kD band was more weakly stained than when reacted with the antiserum against the cauliflower Ca^{2+}-ATPase (Fig. 4, lane D). ^{125}I-CaM overlays of low-density intracellular membranes and phase-partitioned plasma membranes confirmed the presence of CaM-binding Ca^{2+}-ATPases with apparent masses of 111 and 116 kD in low-density membranes and plasma membranes, respectively (Fig. 4). As was indicated by the data in Figure 2A, the plasma membrane Ca^{2+}-ATPase was more strongly labeled by ^{125}I-CaM. As was suggested earlier (Askerlund, 1996), the 116-kD CaM-binding band visible in the low-density membrane fraction therefore probably represents contamination by a very small amount of plasma membranes. The stronger labeling of the plasma membrane Ca^{2+}-ATPase may reflect a higher affinity for CaM. Analysis of phosphorylated intermediates also indicated that the plasma membrane Ca^{2+}-ATPase is slightly larger than the intracellular Ca^{2+}-ATPase (Fig. 4, lanes I–L). [^{32}P]ATP labeling of phosphoproteins was intensified by La^{3+} in both fractions, in agreement with earlier reports (Askerlund and Evans, 1993; Askerlund, 1996). Furthermore, labeling of both bands were Ca^{2+} dependent and sensitive to hydroxylamine (data not shown).

Experiments with Isolated Vacuoles

To further establish that the 111-kD CaM-stimulated Ca^{2+}-ATPase is situated in the vacuolar membrane of cauliflower inflorescences, intact vacuoles were isolated from cauliflower by slicing and flotation. This resulted in a preparation that looked very pure by light microscopy (not shown). Antimycin A-insensitive NADH-Cyt *c* reductase activity in vacuole preparations was only 20 nmol min^{-1} mg^{-1} protein or less (data not shown), indicating that the levels of contaminating ER were very low. The vacuoles were small, only 4 to 15 μm in diameter, compared with vacuoles isolated from red beet roots using the same technique (15–30 μm). The cauliflower vacuoles were able to accumulate $^{45}Ca^{2+}$ in the presence of ATP (Table I). Possibly because of their small size, at least part of the vacuoles remained intact during the filtration step used in the $^{45}Ca^{2+}$ uptake procedure. Ca^{2+} uptake by the vacuoles was CaM stimulated and only partly sensitive to the protonophore FCCP (Table I), indicating that a CaM-stimulated

Table I. *The effect of CaM (1 μM) and FCCP (5 μM) on $^{45}Ca^{2+}$ uptake in intact cauliflower vacuoles in the presence (+) and absence (−) of ATP*

The assay included 50 μL of vacuoles corresponding to about 5 μg of protein. Data are means ± SD of three Ca^{2+} uptake measurements with a single vacuole preparation. A typical experiment is shown.

Addition			Ca^{2+} Uptake
ATP	CaM	FCCP	
			pmol min^{-1}
+	−	−	0.306 ± 0.036
+	+	−	0.407 ± 0.009
+	−	+	0.186 ± 0.001
+	+	+	0.235 ± 0.021
−	+	−	0.026 ± 0.002

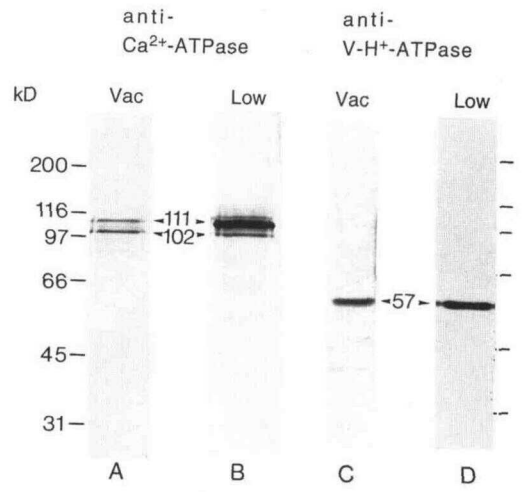

Figure 5. Immunodetection of Ca^{2+}-ATPase and vacuolar H^+-ATPase in isolated vacuoles (Vac) and low-density membranes (Low) from cauliflower. Lanes A and B, Blots incubated with an antiserum against the 111-kD Ca^{2+}-ATPase from cauliflower; lanes C and D, blots incubated with an antiserum against the 57-kD subunit of the vacuolar H^+-ATPase. Alkaline-phosphatase-conjugated secondary antibodies were used. Lanes A and C received 200 μL of cauliflower vacuoles (about 20 μg), and lanes B and D received 6 μg of protein.

Ca^{2+}-ATPase was responsible for at least part of the Ca^{2+} accumulation (see also "Discussion"). The highest degree of CaM stimulation seen in any preparation (n = 5) was 112%, but usually it was much lower (Table I).

The vacuole preparation was probed with the antiserum against the 111-kD Ca^{2+}-ATPase and with the antiserum against the vacuolar H^+-ATPase. Because of the very small yield of vacuoles, the whole-vacuole preparation, rather than the vacuolar membrane, was analyzed. In all of the preparations that were tested, the 111-kD Ca^{2+}-ATPase and the 102-kD degradation product were detected (Fig. 5 lane A). In the vacuoles the relative amount of the 111-kD band in comparison with the 102-kD band was usually smaller than in the low-density membranes (Fig. 5, lanes A and B). This is in agreement with the lower degree of CaM stimulation of Ca^{2+} uptake that was observed with the intact vacuoles (Table I) than with the low-density membranes (Askerlund, 1996) and the fact that the CaM-binding region is lost when the size of the Ca^{2+}-ATPase is reduced from 111 to 102 kD (Askerlund, 1996). The Ca^{2+}-ATPase in isolated vacuoles is probably very susceptible to attack by proteases released from broken vacuoles. Indeed, the largest relative amount of the 111-kD band, in comparison with the 102-kD band, was observed with the vacuole preparation that showed the highest degree of CaM stimulation. The antiserum against the 57-kD subunit of the vacuolar H^+-ATPase detected a 57-kD band in the isolated vacuoles, as well as in the low-density membranes (Fig. 5, lanes C and D). In the vacuole preparation the labeling of Ca^{2+}-ATPase (the sum of the 111- and 102-kD bands) was somewhat weaker than the labeling of the 57-kD vacuolar H^+-ATPase subunit, whereas in the low-density membranes this relation was the opposite (Fig. 5). The reason for this discrepancy is probably that the Ca^{2+}-ATPase in the vacuole preparation is proteolyzed to a greater extent than the vacuolar H^+-ATPase. The fragments resulting from proteolysis may rapidly be degraded to even smaller fragments and thus be undetected in the immunoblot.

DISCUSSION

In this paper several lines of evidence for a vacuolar membrane location of the 111-kD CaM-stimulated Ca^{2+}-ATPase in cauliflower are presented. First, CaM-stimulated Ca^{2+} uptake and the 111-kD Ca^{2+}-ATPase showed the best correlation with the vacuolar membrane markers PPi-dependent H^+ pumping and vacuolar H^+-ATPase after continuous Suc gradient centrifugation of microsomal membranes (Figs. 1 and 2). The vacuolar H^+-ATPase and PPiase have both been reported to be present in membranes other than the vacuolar membrane (Herman et al., 1994; Robinson, 1996), but the main location of both of these proteins is the vacuolar membrane. The fact that both of these markers were used must have reduced the risk of incorrectly identifying the vacuolar membrane to a minimum. Second, the 111-kD Ca^{2+}-ATPase followed the vacuolar H^+-ATPase after CCD of low-density membranes in an aqueous two-phase system but showed a completely different distribution than the ER markers BiP and NADH-Cyt *c* reductase (Fig. 3). This is a new application of aqueous two-phase partitioning and shows that the composition of the two-phase system can be adjusted to give separation of vacuolar and ER membranes. Third, ATP-dependent Ca^{2+} uptake by intact vacuoles was found to be CaM-stimulated and partly protonophore-insensitive (Table I). Fourth, immunoblotting showed that the 111-kD Ca^{2+}-ATPase (and the 102-kD degradation product) was present in the isolated vacuoles (Fig. 5).

PEA1 (ACA1), the putative Ca^{2+}-ATPase in *A. thaliana*, was suggested to be located in the chloroplast inner envelope (Huang et al., 1993). Antibodies against PEA1 reacted with the intracellular (and plasma membrane) Ca^{2+}-ATPase in cauliflower (Fig. 4, C and D), in agreement with the relatively high homology between PEA1 and BCA1 (62% identity at the amino acid level; Malmström et al., 1997). Thus, the possibility existed that the 111-kD Ca^{2+}-ATPase in cauliflower was located in the plastid envelope. However, galactolipid synthase, a marker for the plastid inner envelope, did not correlate with the 111-kD Ca^{2+}-ATPase after Suc gradient centrifugation (Fig. 1). Furthermore, attempts to identify the Ca^{2+}-ATPase in a crude preparation of plastids from cauliflower (prepared as described by Journet, 1987) by immunoblotting were negative (data not shown).

After Suc gradient fractionation a 116-kD band was identified that bound ^{125}I-CaM and showed a cross-reaction with the antiserum against the 111-kD CaM-stimulated Ca^{2+}-ATPase (Fig. 2). The 116-kD band correlated with the plasma membrane H^+-ATPase, indicating that it represented a CaM-binding Ca^{2+}-ATPase in plasma membranes (Fig. 2). This was confirmed by experiments with high-purity plasma membranes obtained by two-phase partitioning (Fig. 4) and is in agreement with earlier observations (Askerlund and Evans 1993; Askerlund, 1996). The

much stronger labeling of the 116-kD Ca^{2+}-ATPase than of the 111-kD Ca^{2+}-ATPase by ^{125}I-CaM indicates that the plasma membrane Ca^{2+}-ATPase has a higher affinity for CaM (at least CaM from bovine brain) than the vacuolar Ca^{2+}-ATPase (Figs. 2A and 4, lanes E and F). This is consistent with the difficulties in observing the CaM stimulation of Ca^{2+} uptake in plasma membrane vesicles (for reviews, see Briskin [1990]; Evans et al. [1991]; Askerlund and Sommarin [1996]); the plasma membrane Ca^{2+}-ATPase in many preparations is probably saturated with endogenous CaM. In some cases, CaM may even be permanently bound to the Ca^{2+} pump, as was suggested for the hepatocyte plasma membrane Ca^{2+}-ATPase, which shows a much higher CaM affinity than most other animal plasma membrane Ca^{2+}-ATPase isoforms (Carafoli, 1994).

It has been reported that a CaM-stimulated Ca^{2+}-ATPase is present in the ER from barley aleurone (Gilroy and Jones, 1993) and carrot (Hsieh et al., 1991). In contrast, the major part of the CaM-stimulated Ca^{2+}-ATPase in wheat aleurone was localized to the vacuolar membrane (Bush and Wang, 1995). A recent study (Hwang et al., 1997) suggested that both the ER and vacuolar membrane in carrot may harbor CaM-stimulated Ca^{2+}-ATPases. The data shown in Figure 3 indicates that the 111-kD CaM-stimulated Ca^{2+}-ATPase in cauliflower is enriched in the vacuolar membrane, although the presence of a small amount of the 111-kD ATPase in the ER cannot be excluded. However, if a more abundant Ca^{2+}-ATPase is present in the ER, it must show little homology with the 111-kD Ca^{2+}-ATPase, since it was not detected by the antiserum (Fig. 3). Ca^{2+}-ATPases insensitive to CaM have been identified in the ER (Buckhout, 1983; Thomson et al., 1993; Hwang et al., 1997). The Ca^{2+}-ATPase activity in the absence of CaM was low and much more difficult to localize than the activity in the presence of CaM (Fig. 1C). Therefore, Ca^{2+}-ATPases that are not stimulated by CaM may be present in several different membranes in cauliflower, including the ER. Also, if such pumps were immunologically distinct from the 111-kD Ca^{2+}-ATPase, they would not have been detected in the western blots (Figs. 2A and 3A).

The presence of a CaM-stimulated Ca^{2+}-ATPase in the vacuolar membrane agrees with the demonstration by Fukumoto and Venis (1986) that accumulation of Ca^{2+} into tonoplast vesicles from apple fruit was CaM-stimulated and directly coupled to ATP hydrolysis. Additional reports supporting the presence of a Ca^{2+}-ATPase in the vacuolar membrane have appeared (Malatialy et al., 1988; DuPont et al., 1990; Gavin et al., 1993; Pfeiffer and Hager, 1993). Recently, it was reported that antibodies against a fusion protein encoding a portion of LCA1, the Ca^{2+}-ATPase previously cloned in tomato (Wimmers et al., 1992), reacted specifically with two polypeptides of 116 and 120 kD that were localized in the vacuolar and plasma membrane, respectively (Ferrol and Bennett, 1996). This is very similar to the situation in cauliflower (Fig. 2). This, however, is surprising, since LCA1 is related to the sarco/ER-type Ca^{2+}-ATPases (Wimmers et al., 1992), whereas the 111-kD CaM-stimulated Ca^{2+}-ATPase (BCA1) in cauliflower is more closely related to the plasma membrane-type Ca^{2+}-ATPases (Malmström et al., 1997). It was suggested by Ferrol and Bennett (1996) that both Ca^{2+}-ATPase polypeptides detected in tomato are encoded by the same gene. At present it is not possible to say if the 111- and 116-kD CaM-binding Ca^{2+}-ATPases in cauliflower are encoded by the same or different genes.

The partial inhibition of ATP-dependent Ca^{2+} uptake by FCCP (Fig. 1D and Table I) indicates that secondary as well as primary Ca^{2+} pumps are present in cauliflower vacuolar membranes. However, since it has been suggested that Ca^{2+}-ATPases show an obligatory transport of protons in the opposite direction (Rasi-Caldogno et al., 1987; Da Costa and Madeira, 1994), the vacuolar Ca^{2+}-ATPase may itself be inhibited by protonophores (Rooney and Gross, 1992; Rooney et al., 1994; Bush and Wang, 1995); the observed inhibition by FCCP may therefore not be conclusive evidence for the presence of a secondary Ca^{2+}/nH^{+} antiporter. This may explain why the CaM-stimulated part of the Ca^{2+} uptake by membrane vesicles (Fig. 1D) and intact vacuoles (Table I) was partly inhibited by FCCP. Most likely, however, both a Ca^{2+}/nH^{+} antiporter (Hirschi et al., 1996) and a Ca^{2+}-ATPase are present in cauliflower vacuolar membranes, in analogy to the situation in *Saccharomyces cerevisiae* (Cunningham and Fink, 1994). These transporters may be active under different conditions, and the much higher Ca^{2+} affinity of the Ca^{2+}-ATPase than of the secondary Ca^{2+}/nH^{+} antiporter may be necessary to deplete the cytosol of Ca^{2+}.

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