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Modulation of an Intracellular Calmodulin-Stimulated Ca^{2+}-Pumping ATPase in Cauliflower by Trypsin[1]

The Use of Calcium Green-5N to Measure Ca^{2+} Transport in Membrane Vesicles

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The effect of controlled trypsin digestion of a calmodulin-stimulated Ca^{2+}-ATPase in low-density intracellular membranes from cauliflower (*Brassica oleracea* L.) inflorescences was investigated. Ca^{2+} uptake into vesicles was measured either continuously with the fluorescent Ca^{2+} indicator Calcium Green-5N or with a radioactive filter technique. Trypsin treatment of vesicles resulted in a 3-fold activation of Ca^{2+} uptake and loss of calmodulin sensitivity. Immunoblotting experiments with an antiserum raised against the Ca^{2+}-ATPase showed that the trypsin activation was accompanied by a decrease in the amount of intact Ca^{2+}-ATPase (111 kD) and by successive appearances of polypeptides of 102 and 99 to 84 kD. ^{125}I-Calmodulin overlays showed that only the intact Ca^{2+}-ATPase bound calmodulin. Removal of the calmodulin-binding domain (about 9 kD) was not enough to obtain full activation. Trypsin proteolysis resulted in a Ca^{2+} concentration necessary for half-maximal activity of 0.5 μM, whereas a value of about 2 μM was obtained with untreated membranes in the presence of calmodulin. Without trypsin treatment or calmodulin the activity was not saturated even at 57 μM free Ca^{2+}. The data suggest that trypsin digestion and calmodulin activate the cauliflower Ca^{2+}-ATPase by at least partly different mechanisms.

Ca^{2+} plays an important role as an intracellular mediator in plant cells. Physiological processes that appear to be associated with a change in the cytosolic Ca^{2+} concentration include gravitropism, stomatal responses, mitosis, polarized growth, responses to hormones, and phytochrome-related events (Gilroy et al., 1993; Bush, 1995). For Ca^{2+} to function as an intracellular mediator, the resting cytosolic Ca^{2+} concentration must be kept low (about 0.1 μM free Ca^{2+}). This can be achieved by active extrusion of Ca^{2+} from the cell or by sequestration of Ca^{2+} into intracellular organelles. Active Ca^{2+} transporters in plants fall into two categories: primary Ca^{2+}-ATPases and Ca^{2+}/nH^{+} antiporters (where n is an integer). A Ca^{2+}/nH^{+} antiporter is responsible for Ca^{2+} transport into the vacuole, whereas Ca^{2+}-ATPases are located in the plasma membrane and the ER (Evans et al., 1991; Chanson, 1993; Askerlund and Sommarin, 1996). Ca^{2+}-ATPases may also be present in the vacuolar membrane and the inner plastid envelope (Gavin et al., 1993; Huang et al., 1993; Pfeiffer and Hager, 1993). In animals, only the plasma-membrane Ca^{2+}-ATPase is directly stimulated by CaM (Carafoli, 1994). In contrast, CaM-stimulated Ca^{2+}-ATPases in plants have been reported to be present in the ER (Hsieh et al., 1991; Askerlund and Evans, 1992; Gilroy and Jones, 1993; Liss and Weiler, 1994; Logan and Venis, 1995), the vacuolar membrane (Gavin et al., 1993), and the chloroplast envelope (Nguyen and Siegenthaler, 1985), as well as the plasma membrane (Robinson et al., 1988; Rasi-Caldogno et al., 1993, 1995; M. Olbe and M. Sommarin, unpublished data; for a review, see Askerlund and Sommarin, 1996). CaM-stimulated Ca^{2+}-ATPase in cauliflower (*Brassica oleracea* L.) was earlier found to be located mainly in a low-density intracellular membrane fraction that was suggested to be the ER, but also in the plasma membrane (Askerlund and Evans, 1992). More recent investigations have shown that the distribution of the intracellular CaM-stimulated Ca^{2+}-ATPase in cauliflower correlates much better with vacuolar membrane markers than with ER markers, suggesting that it may be located in the vacuolar membrane (P. Askerlund, unpublished data).

Controlled proteolysis has been used extensively as a tool to identify and characterize different functional domains of the CaM-dependent Ca^{2+}-ATPase in the plasma membrane of animals (Enyedi et al., 1987; Papp et al., 1989; Carafoli, 1994). In this paper I describe the effects of controlled trypsin proteolysis on the CaM-stimulated Ca^{2+}-ATPase in low-density intracellular membranes from cauliflower. For this purpose, an antiserum was raised against the CaM-stimulated Ca^{2+}-ATPase from the low-density membranes, and a fluorometric method for continuous measurement of Ca^{2+} transport in membrane vesicles was developed. The results are discussed in relation to what is known about the Ca^{2+}-ATPase in animal plasma membranes and to recent reports of the effect of trypsin treatment of the CaM-stimulated Ca^{2+}-ATPase in plasma membranes from radish seedlings (Rasi-Caldogno et al., 1993, 1995).

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Abbreviations: BTP, 1,3-bis(tris[hydroxymethyl]methylamino)-propane; CaM, calmodulin; $K_{½}$, concentration necessary for half-maximal activity; TBS, Tris-buffered saline.

MATERIALS AND METHODS

Preparation of Low-Density Intracellular Membranes

Cauliflower (*Brassica oleracea* L.) inflorescences were purchased locally. Preparatory work was carried out at 0 to 4°C. The outermost part of the inflorescence (180 g) was homogenized using a blender fitted with razor blades (Kannangara et al., 1977) in 200 mL of 50 mM Mops-BTP, pH 7.5, 0.33 M Suc, 5 mM Na_2-EDTA, 5 mM DTT, 0.2% (w/v) casein (boiled enzymic hydrolysate), 0.2% (w/v) BSA (Sigma catalog No. A3294, protease free), 0.6% (w/v) PVP, 1 mM benzamidine-HCl, and 0.5 mM PMSF. The homogenate was filtered through a nylon cloth (140 μm, Lockertex, Warrington, UK) and centrifuged at 10,000*g* for 15 min. The supernatant was carefully pipetted onto a cushion of 4 g of 2 M Suc in gradient buffer (25 mM Mops-BTP, pH 7.2, 50 mM KCl, and 2 mM Na_2-EDTA) in six centrifuge tubes and was centrifuged at 100,000*g* for 45 min in a Beckman SW-28 rotor. The microsomal membranes at the interface (total volume 15–20 mL) were collected, diluted to 50 mL with 2 M Suc, 1 mM PMSF in gradient buffer, and divided between six new centrifuge tubes. The membrane suspensions (8 mL/tube) were overlaid sequentially with 20 mL of 32% (w/w) Suc and 10 mL of 10% (w/w) Suc, each containing 0.5 mM PMSF in gradient buffer. The tubes were centrifuged for 5 h or overnight at 100,000*g* in a Beckman SW-28 rotor. The membranes at the 10/32% interface from all tubes were pooled, diluted to 140 mL with 25 mM Mops-BTP, pH 7.5, 0.33 M Suc, 0.5 M NaCl, 1 mM DTT, 0.5 mM PMSF, and 1 mM Na_2-EDTA, and pelleted at 100,000*g* in a fixed-angle rotor for 1 h.

In experiments that were carried out for marker enzyme analysis, the high-density membranes that partitioned at the lower interface and in the bottom of the tube, as well as a small amount of the microsomal membranes, were diluted and pelleted in the same way as the low-density membranes at the 10/32% interface. The membranes were resuspended in 25 mM Mops-BTP, pH 7.5, 0.33 M Suc, and 1 mM DTT to a protein concentration of 3 to 7 mg mL^{-1} and stored in aliquots at −80°C until analysis.

Affinity Purification of Ca^{2+}-ATPase and Production of Antibodies

The CaM-stimulated Ca^{2+}-ATPase was purified with CaM-affinity chromatography as described by Askerlund and Evans (1992), but from the low-density intracellular membranes (10/32% Suc interface) instead of a crude microsomal fraction. For antibody production the column eluate was precipitated with methanol-chloroform-H_2O (Pohl, 1990), dissolved in solubilization buffer (Laemmli, 1970), and fractionated by SDS-PAGE on a 7.5 to 15% gradient gel. The gel was carefully washed in deionized H_2O and stained briefly with 0.05% Coomassie R-250 dissolved in H_2O as described by Harlow and Lane (1988). The 111-kD Ca^{2+}-ATPase band was cut out from the gel, washed repeatedly with deionized H_2O, and emulsified in a 1.5-mL microcentrifuge tube using a micropestle. A female rabbit was given a subcutaneous injection of 90 μg of purified Ca^{2+}-ATPase (in emulsified polyacrylamide) mixed 1:1 (v/v) with Freund's complete adjuvant. Following the first injection, two booster injections, each with 60 μg of antigen with incomplete Freund's adjuvant, were given at 3-week intervals, after which the serum was collected. A preimmune serum was collected from the same rabbit prior to injection of antigen.

Phosphorylated Intermediate Formation and Analysis

The fractions eluted from the CaM-affinity column that showed the highest ATPase activity were pooled. Phosphorylation was carried out for 15 s by the addition of 1 nM [γ-^{32}P]ATP (74 kBq in 10 μL) to a 0.6-mL reaction mixture at 0°C. The reaction mixture consisted of 0.5 mL of column eluate (with 12.5 mM added $CaCl_2$; see Askerlund and Evans, 1992) plus 100 μL of either (a) H_2O; (b) 3 mM $LaCl_3$; (c) 100 mM EGTA-BTP, pH 7.5; or (d) 100 mM EGTA-BTP, pH 7.5, 3 mM $LaCl_3$. The reaction was stopped by the addition of 0.6 mL of 20% (w/v) ice-cold TCA. Subsequent steps were as described previously (Askerlund and Evans, 1993).

Measurement of Ca^{2+}-Pumping Activity

Ca^{2+} pumping was measured either with a newly developed continuous fluorometric assay or with a discontinuous radioactive filter assay. In the fluorometric assay the complete medium contained 25 mM Mops-BTP, pH 7.2, 100 mM KCl, 0.1% (w/v) BSA (Sigma catalog No. A7030), 2.5 mM $MgCl_2$, 1 mM DTT, 1 mM NaN_3, 50 μM $CaCl_2$, 1 μM Calcium Green-5N (hexapotassium salt; Molecular Probes, Eugene, OR), 1 mM ATP-BTP, pH 7.2, and 0.18 mg of membrane protein in 0.4 mL. Fluorescence emission quenching was recorded continuously at 20°C in an RF-1501 spectrofluorophotometer (Shimadzu, Kyoto, Japan) with excitation and emission wavelengths set at 506 and 550 nm, respectively.

Radioactive Ca^{2+} uptake was measured essentially as described earlier (Askerlund and Evans, 1992). The medium contained 25 mM Mops-BTP, pH 7.2, 100 mM KCl, 0.1% BSA, 5 mM $MgCl_2$, 0.33 M Suc, 1 mM DTT, 0.1 mM Na_2-molybdate, 1 mM NaN_3, 50 μM $CaCl_2$ (0.7–3 Bq $^{45}CaCl_2$ $pmol^{-1}$), 2.5 mM ATP-BTP, pH 7.2, and 5 μg of membrane protein in 0.1 mL. The sample was preincubated with assay medium for 10 min prior to starting Ca^{2+} uptake with ATP. During investigations of the effect of free [Ca^{2+}] on Ca^{2+} uptake activity, measurements were carried out in a slightly different way (see "Results"). Controls without ATP were run in parallel. The reactions were stopped by addition of 0.6 mL of Mops-BTP, pH 7.2, 1 mM EGTA, 0.33 M Suc, and aliquots were immediately filtered through 0.45-μm pore-size cellulose nitrate membrane filters (Gelman Sciences, Ann Arbor, MI) using a Millipore vacuum sampling manifold. After they were washed four times with 1 mL of stop solution, the filters were dried and the amount of $^{45}Ca^{2+}$ was measured by scintillation counting.

Other Enzyme Activities

PPi-dependent H^+ pumping was measured as the change in A_{495} using a DW 2 spectrophotometer (Aminco,

Silver Spring, MD). The assay was run at 20°C in 1 mL of 25 mM Mops-BTP, pH 7.5, 0.33 M Suc, 0.1 M KCl, 0.1% (w/v) BSA, 20 μM acridine orange, 7.5 mM $MgCl_2$, 0.2 mM $Na_2P_2O_7$, and 40 to 80 μg of membrane protein. The reaction was started by the addition of $Na_2P_2O_7$. Measurement of UDP-Gal:diacylglycerol galactosyltransferase (galactolipid synthase activity) was as described by Douce and Joyard (1980). Antimycin A-insensitive NADH-Cyt *c* reductase, glucan synthase II, and Cyt *c* oxidase activities were measured as described by Widell and Larsson (1990).

Controlled Trypsin Proteolysis

Trypsin digestion was carried out in two different ways. To investigate the effect of trypsin on Ca^{2+} pumping (V_{max}) and the corresponding degradation pattern of the Ca^{2+}-ATPase, the fluorometric Ca^{2+}-pumping assay was used. Trypsin (1–20 μg in 5 μL of H_2O; from bovine pancreas; Boehringer Mannheim) was added directly to the fluorometer cuvette (under the conditions for Ca^{2+} pumping as described above) and the stimulatory effect of trypsin digestion on V_{max} was recorded without prior addition of trypsin inhibitor. In some experiments a sample (22 μg of membrane protein in 50 μL) was collected from the fluorometer cuvette exactly 2 min after the addition of trypsin (when the rate of fluorescence quenching had become linear) and was mixed with 100 μL of 10% ice-cold TCA in a microcentrifuge tube. After centrifugation for 8 min at 16,000*g* and 4°C, the supernatant was carefully removed and the pellet resuspended with 0.1 mL of Laemmli (1970) solubilization buffer supplemented with 5% (v/v) 0.5 M NaOH, 1 mM PMSF, and bromphenol blue. Polypeptides were fractionated by SDS-PAGE on linear minigels for subsequent analysis with immunoblotting or ^{125}I-CaM overlay (see below).

Investigations of the effect of trypsin and CaM on the Ca^{2+} affinity of the Ca^{2+}-ATPase involved the radioactive filter technique for Ca^{2+} uptake. Trypsin digestion was carried out in 25 mM Mops-BTP, pH 7.2, 100 mM KCl, 0.1% (w/v) BSA, 2.5 mM $MgCl_2$, 1 mM DTT, 1 mM NaN_3, 0.1 mM EGTA-BTP, pH 7.2, 1 mM ATP-BTP, pH 7.2, and 0.27 mg of membrane protein in 0.6 mL at 20°C. Proteolysis was started by the addition of 30 μg of trypsin in 7.5 μL of H_2O. The reaction was stopped after 2 min by the addition of 0.6 mg of soybean trypsin inhibitor (Biomol, Hamburg, Germany) in 15 μL of H_2O, and the sample was placed on ice until it was analyzed for Ca^{2+} uptake activity. Controls were treated in exactly the same way except that trypsin was substituted with H_2O.

Protein Analysis and Determination

Protein was measured with a modified Bradford procedure with BSA as standard (Stoscheck, 1990). SDS-PAGE was carried out in the buffer system of Laemmli (1970) on gradient gels (concentration of monomers, 7.5–15%; cross-linking, 2.7%; 5% stacking gel; dimensions 160 × 160 × 1.5 mm) with a Bio-Rad Protean II apparatus or on linear minigels (concentration of monomers, 8%; cross-linking, 2.7%; 5% stacking gel; dimensions 80 × 73 × 0.75 mm; Bio-Rad Mini-Protean II apparatus). To avoid proteolysis of the Ca^{2+}-ATPase during solubilization, it was necessary to precipitate protein with TCA prior to SDS treatment.

Western Immunoblotting Analysis

Gradient gels were incubated for about 0.5 h in 25 mM Tris, 0.15 M Gly, 0.02% (w/v) SDS, pH 8.3. Polypeptides were then transferred to an Immobilon polyvinylidene difluoride transfer membrane (Millipore) at a current of 150 mA per gel for about 1.5 h under semidry conditions (Kyhse-Andersen, 1984; apparatus from Bio-Rad). Polypeptides separated on linear minigels were transferred to Immobilon polyvinylidene difluoride for 15 h at 12 V with a Mini Trans-Blot transfer cell (Bio-Rad) using a buffer containing 10 mM $NaHCO_3$, 3 mM Na_2CO_3, 0.01% (w/v) SDS, pH 9.6. After transfer, the membranes were blocked for 2 h or overnight in 2% (w/v) BSA in TBS. The BSA-coated membranes were incubated with primary antibodies in TBS plus 1% (w/v) BSA on a shaker for 2 h at 20°C or overnight at 4°C. The membranes were then carefully washed four times in TBS (the second wash also included 0.05% [v/v] Nonidet P-40) and incubated with secondary antibodies (alkaline-phosphatase-conjugated goat anti-rabbit IgG [Promega] or ^{125}I-labeled donkey anti-rabbit immunoglobulin [Amersham]) in TBS plus 1% (w/v) BSA for 1 h at 20°C. Finally, the membranes were washed four times (see above). Blots that had been incubated with alkaline-phosphatase-conjugated secondary antibodies were developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Protoblot kit, Promega).

Radioactive blots were first analyzed with a Phosphorimager (Molecular Dynamics) 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 a Hyperscreen intensifier screen (Amersham). Antiserum against the CaM-stimulated cauliflower Ca^{2+}-ATPase and preimmune serum were diluted 2000 times, and antiserum against *Arabidopsis thaliana* plasma-membrane H^+-ATPase (No. 761; a gift from Prof. R. Serrano, Department of Biotechnology, University of Valencia, Spain) was diluted 3300 times. Alkaline-phosphatase-conjugated secondary antibodies were diluted 7500 times, and ^{125}I-labeled secondary antibodies were diluted 250 times (148 kBq mL^{-1}).

Iodination of CaM and ^{125}I-CaM Overlays

CaM (Sigma catalog No. P2277) was iodinated as described by Chafouleas et al. (1979) using Bolter and Hunter reagent (Amersham). Peak fractions containing ^{125}I-CaM (20 pmol; 1.5 MBq in 2 mL) were mixed with an equal volume of TBS plus 0.2 mM $CaCl_2$, 1% (w/v) BSA and incubated with BSA-coated blots (see above) on a shaker table for 1 h at 20°C. In experiments in which the Ca^{2+} dependency of CaM binding was investigated, 1 mM EGTA-BTP, pH 7.2, was added to the incubation buffer to complex all free Ca^{2+}. After they were washed for several hours with several changes of TBS plus 0.2 mM $CaCl_2$ and 1% (w/v) BSA, blots were analyzed with a Phosphorimager followed by autoradiography (see above).

RESULTS

Characterization of the Low-Density Membrane Fraction

After gradient centrifugation, 16% of the protein that was applied to the gradient was found in the low-density membrane fraction (10/32% interface; Table I). The specific ATP-dependent Ca^{2+} uptake activity in the low-density membranes was 260 and 360% of that in the microsomal fraction measured in the absence and presence of CaM, respectively. The low-density membrane fraction was also enriched in the vacuolar membrane marker PPi-dependent H^+-pumping (specific activity 286% that of the microsomal fraction), antimycin A-insensitive NADH-Cyt *c* reductase (ER marker; 154%) and in galactolipid synthase activity (plastid inner envelope; 188%). The low-density membranes were depleted in the plasma-membrane marker glucan synthase II (37%) and in Cyt *c* oxidase, a marker for mitochondrial inner membranes (27%).

Purification of Ca^{2+}-ATPase from Low-Density Membranes

The proteins eluted from the CaM-affinity column were fractionated by SDS-PAGE in a 7.5 to 15% gradient gel. Coomassie staining revealed polypeptides at 111, 46, 42, 28, and 22 to 15 kD (Fig. 1A). This polypeptide pattern was very similar to that observed when a crude microsomal fraction was used as starting material for purification (Askerlund and Evans, 1992), but the apparent molecular masses differed slightly in some cases. For example, a polypeptide from the crude fraction that was identified as a CaM-stimulated Ca^{2+}-ATPase migrated with an apparent molecular mass of 115 kD (Askerlund and Evans, 1992), whereas in the present investigation the same polypeptide had a molecular mass of 111 kD (Fig. 1A). The difference in apparent molecular mass is probably due to the use of different molecular weight markers in the two investigations. The 111-kD polypeptide in the present investigation was identified as a Ca^{2+}-ATPase by its ability to form a Ca^{2+}-dependent, hydroxylamine-sensitive phosphorylated intermediate in the presence of [γ-^{32}P]ATP (Fig. 2; Briskin, 1990; Garrahan and Rega, 1990). The amount of phosphorylated intermediate of the partly purified Ca^{2+}-ATPase increased in the presence of La^{3+} (Fig. 2C). This was also found for the Ca^{2+}-ATPase in cauliflower plasma membranes (Askerlund and Evans, 1993). The effect of La^{3+} results from an increase in steady-state level of phosphoenzyme and was reported to be characteristic of plasma-membrane Ca^{2+}-ATPases in animals (Carafoli, 1994). In plants, La^{3+} is also effective with intracellular Ca^{2+}-ATPases (Fig. 2; Chen et al., 1993) but possibly only of the CaM-stimulated type.

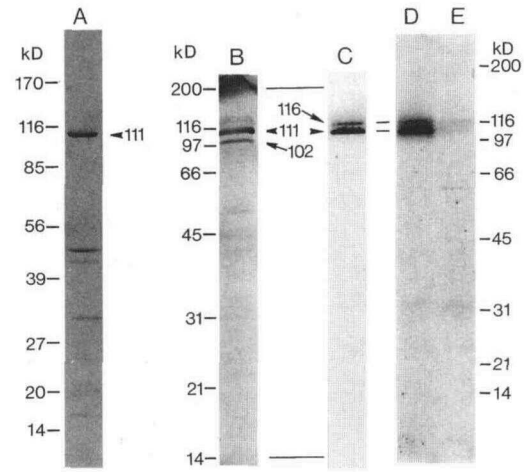

Figure 1. Polypeptide pattern of partly purified Ca^{2+}-ATPase and immunoblot and ^{125}I-CaM-overlay analysis of low-density membranes from cauliflower inflorescences. A, SDS-PAGE of the fraction eluted from CaM-affinity column visualized by Coomassie staining; B, immunoblot of low-density membranes with antiserum against cauliflower Ca^{2+}-ATPase; C and D, Phosphorimager scans of ^{125}I-CaM blots of low-density membranes; E, as D but blot was incubated with ^{125}I-CaM in the presence of EGTA. Lane A received 6 μg of protein; lanes B to E received 80 μg of protein. Separation of polypeptides was on 7.5 to 15% gradient gels. Alkaline-phosphatase-conjugated secondary antibodies were used for the immunoblot.

Table I. *Distribution of ATP-dependent Ca^{2+} uptake, marker enzyme activities, and total protein between microsomal membrane, low-density membrane, and high-density membrane fractions*

The low- and high-density membrane fractions were obtained by discontinuous Suc gradient centrifugation of the microsomal fraction as described in "Materials and Methods." Calcium uptake was measured with the radioactive filter method for 5 min at 30°C. All activities are specific and expressed in nmol min^{-1} mg^{-1} protein, except for PPi-dependent H^+ pumping and galactolipid synthase activity, which are expressed as the change in A_{495} h^{-1} mg^{-1} protein and nmol h^{-1} mg^{-1} protein, respectively. Data are means ± SD from two different membrane preparations.

Parameter	Microsomal Membranes	Low-Density Membranes	High-Density Membranes
Total protein (mg)	104 ± 39.7	16.4 ± 7.9	90.0 ± 20.9
Ca^{2+} uptake			
−CaM	2.5 ± 0.4	6.3 ± 0.9	1.8 ± 0.2
+0.5 μM CaM	5.2 ± 1.9	17.9 ± 0.9	3.0 ± 0.6
PPi-dependent H^+ pumping	4.3 ± 2.1	12.3 ± 0.3	0.9 ± 0.2
Glucan synthase II	95.1 ± 14.8	35.4 ± 11.9	90.2 ± 10.4
Antimycin A-insensitive NADH-Cyt *c* reductase	144 ± 0.7	222 ± 13.4	87.8 ± 25.0
Galactolipid synthase	48.2 ± 0.9	90.6 ± 11.3	40.1 ± 15.0
Cyt *c* oxidase	148 ± 3.8	40.4 ± 15.3	131 ± 9.1

Western Immunoblotting Analysis and ^{125}I-CaM Overlay

The antiserum produced against the CaM-stimulated Ca^{2+}-ATPase proved to be very specific for the Ca^{2+}-ATPase in immunoblots. The antiserum recognized the 111-kD ATPase band and a weaker band at 102 kD in blots of polypeptides from low-density membranes (Fig. 1B). The minor band was always present and was probably a degradation product of the Ca^{2+}-ATPase, since it accumulated during trypsin treatment (see below).

An ^{125}I-CaM overlay of the same kind of blot revealed a strong CaM-binding band at 111 kD and a weak band at

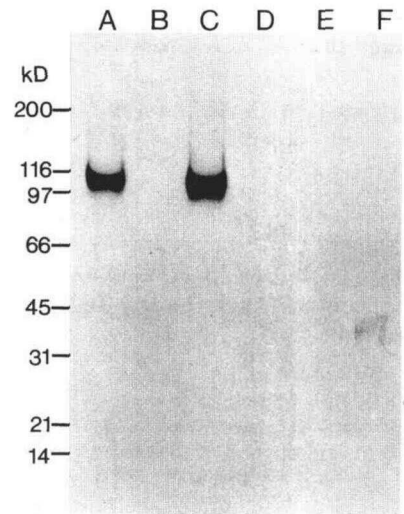

Figure 2. Analysis of the phosphorylated intermediate of the partly purified Ca^{2+}-ATPase from low-density membranes after SDS-PAGE in an acidic gel. Phosphorylation was carried out with [γ-^{32}P]ATP in the presence (A, C, E, and F) or absence (B and D; Ca^{2+} complexed with EGTA) of free Ca^{2+}. C and F, $LaCl_3$ included in the phosphorylation buffer; E and F, hydroxylamine included in the SDS-PAGE solubilization buffer. See "Materials and Methods" for details.

116 kD (Fig. 1, C and D). These bands were detected only when EGTA was absent from the incubation buffer, indicating that the binding of ^{125}I-CaM was Ca^{2+} dependent (Fig. 1E). The 111-kD CaM-binding band confirms the presence of a main CaM-binding Ca^{2+}-ATPase in the low-density membrane fraction. The 116-kD polypeptide probably represents the plasma-membrane Ca^{2+}-ATPase, since a CaM-binding polypeptide of this size was found to be strongly enriched in high-purity cauliflower plasma membranes prepared by two-phase partition (data not shown). In support of this, the Ca^{2+}-ATPase in cauliflower plasma membranes was earlier identified as a 116-kD polypeptide by its phosphorylated intermediate (Askerlund and Evans, 1993). Preliminary data suggest that, although the plasma-membrane Ca^{2+}-ATPase in cauliflower is stimulated to a lesser degree by CaM (Askerlund and Evans, 1992), it has a stronger affinity for CaM from bovine brain than the Ca^{2+}-ATPase in the low-density membranes. Large variations in CaM affinity have been found between different plasma-membrane Ca^{2+}-ATPase isoforms in animals (Carafoli, 1994). A higher CaM affinity of the cauliflower plasma-membrane Ca^{2+}-ATPase may explain why it was also detectable in the low-density membrane fraction, which contained relatively little plasma membrane (Table I; also see below). It is surprising that no other ^{125}I-CaM-binding bands were seen in the blot, even though several low-M_r polypeptides were detected in the fraction eluted from the CaM-affinity column (Fig. 1A). One possible reason why these low-M_r polypeptides do not show up on the ^{125}I-CaM blot may be that they bind CaM with lower affinities than the Ca^{2+}-ATPases.

For practical reasons all subsequent SDS-PAGE runs were carried out on 8% linear minigels instead of large gradient gels. Also, under these conditions the main Ca^{2+}-

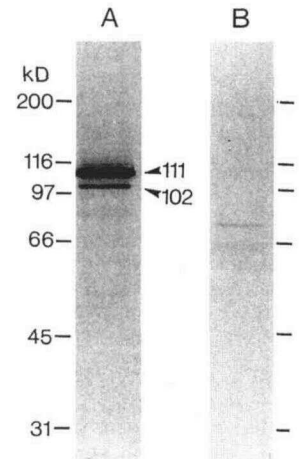

Figure 3. Western immunoblotting analysis of low-density membranes separated on an 8% linear minigel. A, Immunoblot with antiserum against cauliflower Ca^{2+}-ATPase; B, immunoblot with preimmune serum. Both A and B received 6 μg of protein. Alkaline-phosphatase-conjugated secondary antibodies were used.

ATPase (as detected by immunoblotting; Fig. 3A) migrated at an apparent molecular mass of 111 kD and the weaker band migrated at 102 kD. The preimmune serum did not recognize any bands at approximately 100 kD but did recognize a few very weak bands with apparent molecular masses of 50 to 70 kD (Fig. 3B).

The Ca^{2+}-ATPase recognized by the antiserum was much more abundant in the low-density membranes than in the microsomal and high-density membrane fractions (Fig. 4). The weak band at 116 kD that is visible in the microsomal and high-density membrane fractions may correspond to the plasma-membrane Ca^{2+}-ATPase for reasons discussed above. In contrast, immunoblotting with an an-

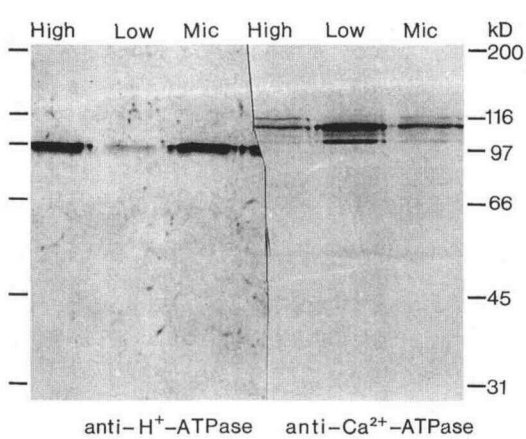

Figure 4. Western immunoblotting analysis of high-density membranes (High), low-density membranes (Low), and microsomal membranes (Mic) separated on an 8% minigel using antiserum against plasma-membrane H^+-ATPase from *A. thaliana* (left) and antiserum against cauliflower Ca^{2+}-ATPase (right). The high- and low-density membrane fractions were obtained by discontinuous Suc gradient centrifugation of the microsomal fraction as described in "Materials and Methods." All lanes received 6 μg of protein. Alkaline-phosphatase-conjugated secondary antibodies were used.

tiserum against the plasma-membrane H^+-ATPase gave a much stronger signal with the microsomal and high-density membranes than with the low-density membrane fraction (Fig. 4). This indicates that the low-density membranes were depleted in plasma membranes, in agreement with the distribution of glucan synthase II activity between the different fractions (Table I).

Continuous Measurement of ATP-Dependent Ca^{2+} Uptake using Calcium Green-5N

The fluorescent Ca^{2+} indicator Calcium Green-5N was used to continuously report the disappearance of Ca^{2+} from the medium when Ca^{2+} was pumped into the membrane vesicles. This method was less sensitive than the radioactive filter technique but greatly simplified the investigations, since the time between trypsin digestion and measurement was eliminated. Calcium Green-5N is a low-affinity Ca^{2+} indicator based on 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid ($K_d \cong 12$ μM at 22°C, pH 7.2; Molecular Probes Europe BV, 1995). To the author's knowledge, Calcium Green-5N has not previously been used to measure Ca^{2+} fluxes in cell-free systems, but its high sensitivity and specificity for Ca^{2+}, together with its relatively low affinity for Ca^{2+}, make it very useful for this purpose.

As demonstrated in Figure 5A, Calcium Green-5N was found to be useful for measurements of Ca^{2+} concentrations up to at least 50 μM. The fluorescence emission signal detected in the absence of added Ca^{2+} and membrane vesicles was probably due to contaminating Ca^{2+} in the assay buffer (at most a few micromolar) and to background fluorescence emission. The signal increased upon addition of membrane vesicles. Additions of small amounts of $CaCl_2$ gradually increased the signal further up to at least 50 μM added Ca^{2+} (Fig. 5A). Subsequent addition of ATP resulted in a continuous decrease in fluorescence, resulting from ATP-dependent pumping of Ca^{2+} into the membrane vesicles (Fig. 5, A and B). CaM increased the rate of fluorescence quenching more than 3-fold, and within 5 min following the addition of CaM the Ca^{2+} in the assay buffer was almost depleted (Fig. 5, A and B). All of the Ca^{2+} could be rapidly released by the addition of the Ca^{2+} ionophore A23187, showing that the decrease in fluorescence was due to accumulation of Ca^{2+} inside vesicles.

The Effect of Trypsin Digestion on ATP-Dependent Ca^{2+} Pumping, Apparent Molecular Mass, and CaM-Binding Properties of the Ca^{2+}-ATPase

In the absence of CaM, addition of trypsin resulted in a strong activation of Ca^{2+} pumping (Fig. 5C). For maximal activation, it was necessary to add trypsin after ATP. If the order of additions was reversed the degree of activation was lower, and the Ca^{2+} pumping leveled off much more rapidly (Fig. 5, C and D). Thus, ATP seemed to have a protective effect on the catalytic site of the Ca^{2+}-ATPase against proteolysis, in a manner similar to that reported for the plant plasma-membrane H^+-ATPase (Palmgren et al., 1990). At saturating concentrations of trypsin (20 μg/assay; Fig. 6), activation was equal to or stronger than that obtained with CaM (Fig. 5, B and C). However, the degree of both CaM and trypsin activation varied considerably between different membrane preparations.

The degradation pattern of the Ca^{2+}-ATPase was studied by immunoblotting (Fig. 7). In the experiment shown, the front was run out from the gel for optimal separation of high-M_r polypeptides. Samples were collected from the fluorometer cuvette exactly 2 min after addition of trypsin. At this time the rate of fluorescence quenching had reached a steady state (Fig. 5C). In the control (no trypsin added), the antiserum against the CaM-stimulated Ca^{2+}-ATPase recognized the 111-kD Ca^{2+}-ATPase band and the weak band at 102 kD, the latter constituting about 20% of the sum of the two bands (Fig. 7, 0 μg). Increasing amounts of trypsin resulted in a gradual decrease in the intact Ca^{2+}-ATPase (111 kD), and at 20 μg of trypsin/assay the amount was about half of that in the control. In contrast, the amount of the 102-kD band gradually increased up to 5 μg of trypsin, where it was about 2 times higher than in the control, and decreased again at higher trypsin concentrations (Fig. 7). This suggested that the 102-kD band represented a degradation product of the Ca^{2+}-ATPase, which was also present in a small amount in untreated membranes. In addition, there was a successive appearance of antibody-binding polypeptides of 99, 97, and 84 kD (in this

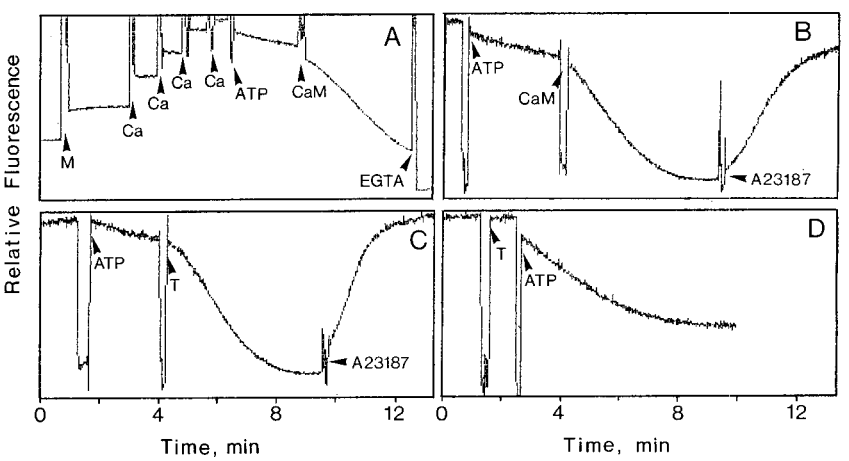

Figure 5. Continuous measurement of ATP-dependent Ca^{2+} uptake into low-density membrane vesicles using the fluorescent Ca^{2+} indicator Calcium Green-5N. Experimental conditions were as described in "Materials and Methods" with the following exceptions: A, Low-density membranes, $CaCl_2$ (each addition 10 μM), and ATP omitted from the start; B-D, Ca^{2+} at 50 μM, ATP omitted from the start. Additions (arrows) were as follows: low-density membranes (M), 0.18 mg in 34 μL; $CaCl_2$ (Ca), 5 nmol in 5 μL; ATP, 0.4 μmol in 4 μL; CaM, 1 nmol in 10 μL; A23187, 16 nmol in 4 μL; trypsin (T), 20 μg in 5 μL. Only 2 nmol of A23187 were required to release all of the Ca^{2+}, but a larger amount (16 nmol) enabled a quicker release as shown.

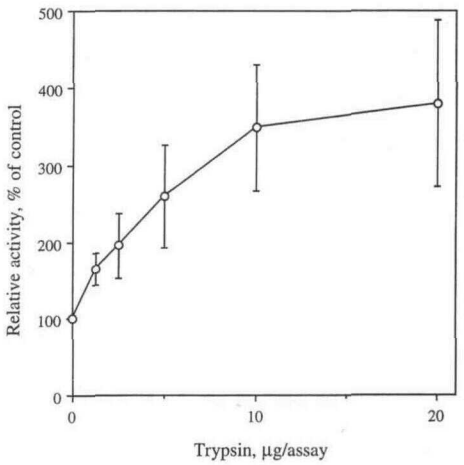

Figure 6. Effect of different concentrations of trypsin on ATP-dependent Ca^{2+} uptake into low-density membrane vesicles measured as quenching of Calcium Green-5N fluorescence emission. Data are means ± SD of three independent preparations.

order) during trypsin treatment (Fig. 7A). These polypeptides probably resulted from further degradation of the 102-kD polypeptide. There was also indication that trypsin treatment resulted in very weak polypeptides of 86, 78, and 72 kD (hardly visible in Fig. 7A). Smaller antibody-binding polypeptides than this did not appear. This was confirmed on gels in which the front was not run out (data not shown). There was, however, a generally higher background staining in all of the trypsin-treated samples than in the control (Fig. 7A).

The effect of trypsin treatment on CaM binding was studied in ^{125}I-CaM overlays. Blots of untreated membranes showed only two ^{125}I-CaM-binding polypeptides: one strong band at 111 kD and one weak band at 116 kD (Fig. 8). The 111-kD CaM-binding polypeptide represents the intact, main Ca^{2+}-ATPase in the low-density membrane fraction. The 116-kD polypeptide probably represents the plasma-membrane Ca^{2+}-ATPase for reasons already discussed above. The amount of ^{125}I-CaM binding at 111 kD gradually decreased during trypsin treatment. This was in agreement with the decreasing amount of intact Ca^{2+}-ATPase in the immunoblots (Fig. 7). However, for unknown reasons trypsin digestion seemed to have a slightly stronger effect on ^{125}I-CaM binding than on antibody binding (Fig. 9). The weaker ^{125}I-CaM-binding polypeptide at 116 kD also decreased during trypsin treatment (Fig. 8). No other CaM-binding polypeptides could be detected before or after trypsin treatment, even when polypeptides were separated on 7.5 to 15% gradient gels. This suggested that the 102- and 99- to 84-kD polypeptides, which accumulated during trypsin treatment (Fig. 7A), represented different truncated forms of the Ca^{2+}-ATPase with the CaM-binding region missing.

Effect of Trypsin Digestion and CaM on the Ca^{2+} Affinity of the Ca^{2+}-ATPase

For detailed studies of the Ca^{2+} dependence of trypsin- and CaM-activated Ca^{2+} uptake, the radioactive filter technique for Ca^{2+} uptake was used. The protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (5 µM) inhibited the activity by 10 to 20%, indicating that some of the ATP-dependent Ca^{2+} uptake was catalyzed by a Ca^{2+}/nH^+ antiport mechanism rather than by the Ca^{2+}-ATPase (data not shown). All of the accumulated Ca^{2+} could be released by the addition of 5 µM A23187 (data not shown).

Trypsin treatment was carried out under the same conditions that had been found to be optimal in the continuous Ca^{2+}-uptake measurements (Figs. 5 and 6), with the exceptions that free Ca^{2+} in the digestion buffer was complexed with EGTA to avoid buildup of high intravesicular levels of Ca^{2+} before starting the Ca^{2+}-uptake measurements and that proteolysis was stopped with trypsin inhibitor. With control membranes, the ATP-dependent Ca^{2+} uptake showed a significantly higher Ca^{2+} affinity [$K_{½}(Ca^{2+})$ 2

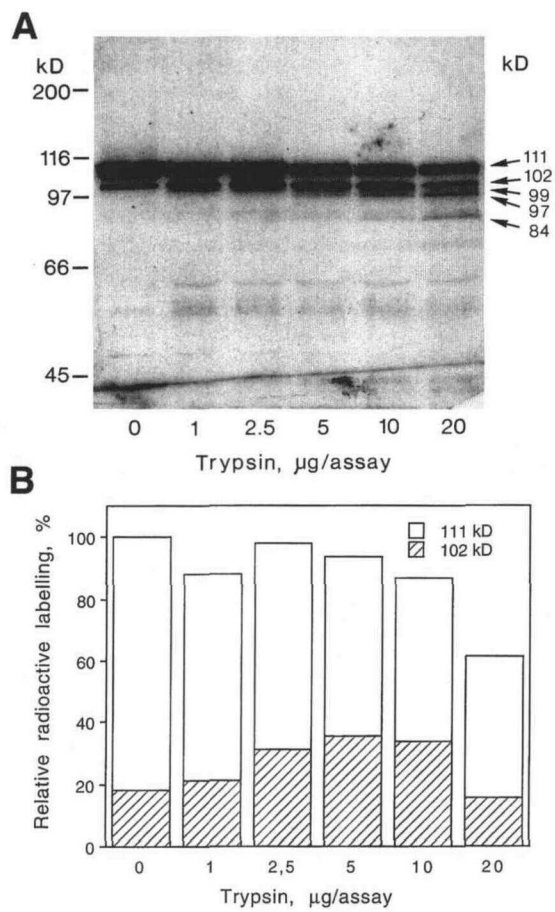

Figure 7. Effect of trypsin proteolysis on antibody-binding pattern of Ca^{2+}-ATPase in low-density membrane vesicles. Samples were collected from the complete assay mixture exactly 2 min after addition of trypsin (1–20 µg in 5 µL) and analyzed by immunoblotting with antiserum against cauliflower Ca^{2+}-ATPase and ^{125}I-labeled secondary antibodies as described in "Materials and Methods." A, Autoradiograph of a typical immunoblot; B, Phosphorimager quantification of the same blot showing relative amounts of radioactivity incorporated in the 111- and 102-kD polypeptides. The control received 5 µL H_2O instead of trypsin. In the experiment shown, the front was run out from the minigel for optimal separation of high-M_r polypeptides.

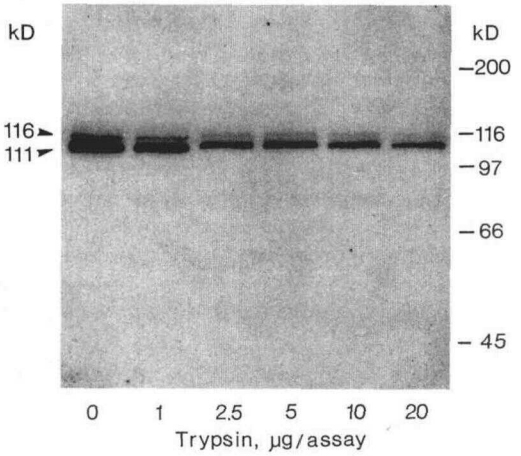

Figure 8. *Effect of trypsin proteolysis on* ^{125}I-CaM-binding pattern of Ca^{2+}-ATPase in low-density membrane vesicles. Samples were collected from the complete assay mixture exactly 2 min after addition of trypsin (1–20 μg in 5 μL) and analyzed by the ^{125}I-CaM-overlay technique as described in "Materials and Methods." An autoradiograph of an ^{125}I-CaM blot is shown. The control received 5 μL of H_2O instead of trypsin. In the experiment shown, the front was run out from the minigel for optimal separation of high-M_r polypeptides.

μM] in the presence of CaM than in its absence (activity not saturated at 57 μM free Ca^{2+}; Fig. 10). Tryptic proteolysis had a dramatic effect on the Ca^{2+} affinity and reduced the $K_{½}(Ca^{2+})$ to 0.5 μM. Furthermore, trypsin treatment changed the shape of the Ca^{2+}-activation curve from sigmoid in the presence of CaM to nonsigmoid after proteolysis. With trypsin-digested membranes, CaM had no further effect on the Ca^{2+} affinity and the activity was unaffected (or slightly inhibited) by CaM over the whole range of Ca^{2+} concentrations (Fig. 10). Maximal activity with control membranes in the presence of CaM was

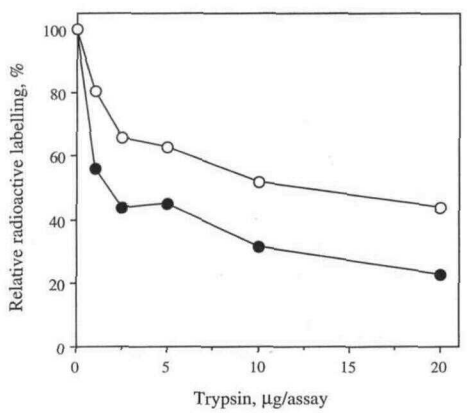

Figure 9. Phosphorimager quantification of 111-kD polypeptide in blots of low-density membranes after treatment with different amounts of trypsin. The amount of polypeptide was determined by immunoblotting using antiserum against the cauliflower Ca^{2+}-ATPase followed by treatment with ^{125}I-labeled secondary antibodies (○) and by ^{125}I-CaM binding (●). Data are means of trypsin treatment of two different membrane preparations, both of which showed approximately 20% lower relative ^{125}I-CaM binding than antibody binding at all trypsin concentrations. See Figures 7 and 8 for further details.

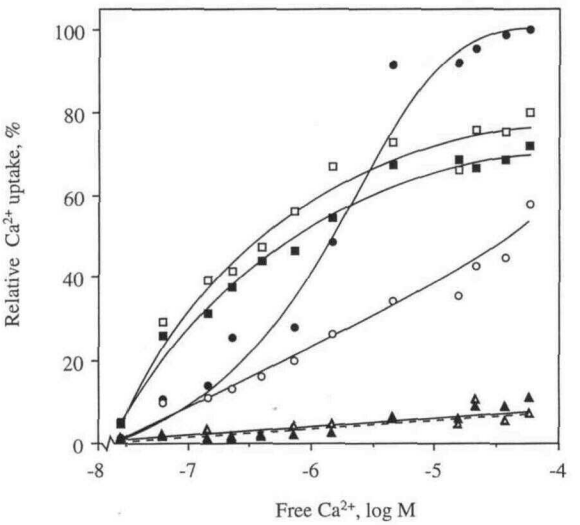

Figure 10. Kinetics of Ca^{2+} uptake in low-density membranes after or without trypsin digestion measured with the $^{45}Ca^{2+}$-filter technique. The assay medium contained 25 mM Mops-BTP, pH 7.2, 0.1 M KCl, 0.1% (w/v) BSA, 0.33 M Suc, 2.5 mM $MgCl_2$, 1 mM DTT, 2.5 mM ATP-BTP, pH 7.2, 5 μg of membrane protein, 0.25 mM EGTA, and varying amounts of $^{45}CaCl_2$ (0.74 Bq pmol^{-1}) in 0.1 mL. Reactions were started by addition of membrane vesicles and run for 6 min at 30°C. Free [Ca^{2+}] in assay buffers was measured with a Ca^{2+}-specific electrode (Radiometer, Copenhagen, Denmark) calibrated with the Ca^{2+} buffers of Tsien and Rink (1981). Filled symbols, Activity measured in the presence of 1 μM CaM; open symbols, no CaM; □, ■, membrane vesicles treated with 20 μg of trypsin for 2 min; ○, ●, no trypsin treatment; △, ▲, reaction run without ATP and no trypsin treatment. An activity of 100% corresponds to 23.6 nmol min^{-1} mg^{-1} protein.

reached at about 36 μM free Ca^{2+}, and the activity with trypsin-treated membranes saturated at about the same Ca^{2+} concentration. At the highest Ca^{2+} concentration tested (57 μM free Ca^{2+}), CaM approximately doubled the activity with control membranes. Maximal activity with trypsin-digested membranes in the absence of CaM was 75 and 130% of that with control membranes in the presence and absence of CaM, respectively (Fig. 10).

DISCUSSION

The objective of this study was not to determine the exact intracellular location of CaM-stimulated Ca^{2+}-ATPase in cauliflower. However, ATP-dependent Ca^{2+} uptake in the absence and presence of CaM was strongly enriched in the low-density membrane fraction (Table I), in agreement with previous results (Askerlund and Evans, 1992). Of the different marker enzymes tested, only PPi-dependent H^+ pumping showed as strong an enrichment as ATP-dependent Ca^{2+}-uptake, suggesting that the 111-kD CaM-stimulated Ca^{2+}-ATPase may be located in the vacuolar membrane. Results from preliminary investigations in which the low-density membranes have been further fractionated by countercurrent distribution in an aqueous two-phase system are in line with this conclusion (P. Askerlund, unpublished data). Although the 111-kD CaM-stimulated Ca^{2+}-ATPase is clearly not located in the plasma membrane (Fig.

4), strong indications exist that plasma membranes from cauliflower contain a 116-kD CaM-stimulated Ca^{2+}-ATPase (Figs. 1, C–E, and 4; Askerlund and Evans, 1993; P. Askerlund, unpublished data).

The Ca^{2+}-ATPase in red blood cells is by far the best studied of the CaM-stimulated (plasma membrane) Ca^{2+}-ATPases (Garrahan and Rega, 1990; Carafoli, 1994). The intact erythrocyte Ca^{2+}-ATPase has a molecular mass of 130 to 140 kD. Controlled trypsin proteolysis of inside-out erythrocyte ghosts, as well as of the purified Ca^{2+}-ATPase, results in fragments of 90, 85, 81, and 76 kD as indicated by SDS-PAGE. These fragments all retain ATPase as well as Ca^{2+}-pumping activity but differ with respect to CaM and phospholipid sensitivity (Carafoli, 1994). Similar to the case of animal plasma membranes, mild trypsin proteolysis of low-density intracellular membrane vesicles from cauliflower caused a strong activation of Ca^{2+} pumping (Figs. 5 and 6). Since the intact erythrocyte Ca^{2+}-ATPase is significantly larger than the cauliflower enzyme, a direct comparison of the size of tryptic fragments is meaningless. However, immunoblotting experiments showed that the trypsin activation with cauliflower membranes was accompanied by a decrease in the amount of intact Ca^{2+}-ATPase (111-kD band), by a transient increase of a 102-kD polypeptide, and by successive appearances of antibody-binding polypeptides of 99, 97, and 84 kD (Figs. 6 and 7). The increase in the 102-kD polypeptide was not alone in being responsible for the large activation by trypsin, since the amount of the 102-kD band was maximal at 5 μg of trypsin, where activation was only half-maximal (cf. Figs. 6 and 7). In contrast, the polypeptides with apparent molecular masses of 99 to 84 kD all showed maximal intensity at 10 to 20 μg of trypsin/assay, suggesting that any of these polypeptides could be responsible for the large activation of Ca^{2+} pumping observed at these trypsin concentrations. The 102-kD polypeptide could still be responsible for activation at low trypsin concentrations (1–5 μg/assay). The size reduction after trypsin treatment of the cauliflower Ca^{2+}-ATPase is similar to the 15-kD reduction in molecular mass observed with the plasma-membrane Ca^{2+}-ATPase from radish parallel to trypsin activation (Rasi-Caldogno et al., 1995).

In erythrocytes, the 90-kD Ca^{2+}-ATPase fragment, which results from trypsin cleavage at both the COOH- and NH_2-terminal ends of the molecule, behaves essentially like the intact Ca^{2+} pump with respect to CaM stimulation and lipid sensitivity (Carafoli, 1994). The 85- and 81-kD fragments result from further cuts at the COOH terminus. The 85-kD fragment has a somewhat impaired response to CaM, whereas the 81-kD fragment has a higher basal ATPase activity and is insensitive to CaM. The loss of CaM sensitivity is due to removal of the CaM-binding domain, which is located in the proximal portion of the COOH-terminal region (Carafoli, 1994). In cauliflower, only the intact Ca^{2+}-ATPase bound CaM (Figs. 1, C–E, and 8). The fragment that corresponds to the difference between the 111- and 102-kD polypeptides must therefore constitute at least part of the CaM-binding region. The fact that only the intact Ca^{2+}-ATPase was visible after purification on the CaM-affinity column (Fig. 1A) is in further support of this conclusion. Thus, tryptic removal of a much smaller fragment (about 9 kD) is needed for complete loss of CaM binding in cauliflower than in erythrocytes. Similar results were reported for the CaM-stimulated Ca^{2+}-ATPase in radish plasma membranes (Rasi-Caldogno et al., 1995). The CaM-binding fragment could not be detected in immunoblots or ^{125}I-CaM overlays, even after separation on gradient gels, probably because it was rapidly degraded into even smaller fragments. From the present investigation it is not possible to say whether the 9-kD CaM-binding fragment originated from the COOH- or the NH_2-terminal region of the ATPase, but the situation in animals suggests that it would be located at the COOH terminus. A more specific removal of the CaM-binding domain of the erythrocyte Ca^{2+}-ATPase can be achieved by treatment with calpain or chymotrypsin, which reduce the size of the Ca^{2+}-ATPase by 9 to 10 kD (from about 134 to about 125 kD) by a cut at the COOH terminus (Carafoli, 1994). The effect of these proteases on the erythrocyte Ca^{2+}-ATPase thus shows a great deal of similarity to the effect of trypsin on the cauliflower Ca^{2+}-ATPase.

Similar to the case when the animal Ca^{2+}-ATPase is digested to the 76-kD fragment (Enyedi et al., 1987; Papp et al., 1989; Carafoli, 1994), trypsin treatment had a very strong effect on the Ca^{2+} affinity of ATP-dependent Ca^{2+} uptake into cauliflower membrane vesicles and changed the shape of the Ca^{2+}-activation curve from sigmoid in the presence of CaM to nonsigmoid after trypsin proteolysis (Fig. 10). Tryptic digestion of the cauliflower Ca^{2+}-ATPase resulted in a $K_{1/2}(Ca^{2+})$ of 0.5 μM, whereas a value of about 2 μM was obtained with untreated membranes in the presence of CaM. In agreement with these results, it was reported that activation of the Ca^{2+}-ATPase in radish plasma membranes by trypsin was maximal at low Ca^{2+} concentrations (Rasi-Caldogno et al., 1993). CaM had no effect on the V_{max} or the Ca^{2+} affinity with trypsin-treated cauliflower membranes, as expected from the ^{125}I-CaM-binding studies, which showed that the CaM-binding region was rapidly lost during trypsin treatment (Fig. 8). The fact that CaM and trypsin digestion result in different Ca^{2+} activation curves suggests that these treatments activate the cauliflower Ca^{2+}-ATPase by at least partly different mechanisms. In support of this, removal of the CaM-binding domain was not enough to obtain full activation (cf. Figs. 6 and 7).

In summary, activation by controlled trypsin treatment of a CaM-stimulated Ca^{2+}-ATPase located in low-density intracellular membrane vesicles, probably of vacuolar origin, has been described. To my knowledge, this phenomenon has not previously been described in any organism. The data suggest that trypsin digestion and CaM activate the cauliflower Ca^{2+}-ATPase by at least partly different mechanisms.

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