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Controlled Proteolysis Activates the Plasma Membrane Ca^{2+} Pump of Higher Plants[1]

A Comparison with the Effect of Calmodulin in Plasma Membrane from Radish Seedlings

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The effects of calmodulin and of controlled trypsin treatments on the activity of the Ca^{2+} pump were investigated in plasma membrane purified from radish (*Raphanus sativus* L.) seedlings. Treatment of the plasma membrane with ethylenediaminetetraacetate (EDTA), which removed about two-thirds of the plasma membrane-associated calmodulin, markedly increased the stimulation of the Ca^{2+} pump by calmodulin. In EDTA-treated plasma membrane, stimulation by calmodulin of the Ca^{2+} pump activity was maximal at low free Ca^{2+} (2–5 μM) and decreased with the increase of free Ca^{2+} concentration. The Ca^{2+} pump activity was stimulated also by a controlled treatment of the plasma membrane with trypsin: the effect of trypsin treatment depended on the concentration of both trypsin and plasma membrane proteins and on the duration of incubation. Stimulation of the Ca^{2+} pump activity by trypsin treatment of the plasma membrane was similar to that induced by calmodulin both in extent and in dependence on the free Ca^{2+} concentration in the assay medium. Moreover, the Ca^{2+} pump of trypsin-treated plasma membrane was insensitive to further stimulation by calmodulin, suggesting that limited proteolysis preferentially cleaves a regulatory domain of the enzyme that is involved in its activation by calmodulin.

In plant cells, the extrusion of Ca^{2+} from the cytoplasm to the apoplast is catalyzed by a Mg-ATP-dependent Ca^{2+} pump. During the last few years, the transport and hydrolytic activities of the plant PM Ca^{2+} pump have been characterized in some detail both in native PM vesicles and in proteoliposomes reconstituted with the solubilized and partially purified enzyme (reviewed in Briskin, 1990; Evans et al., 1991; De Michelis et al., 1992a). The most striking characteristics of the plant PM Ca^{2+} pump are its ability to use ITP or GTP besides ATP as a substrate (Williams et al., 1990; Carnelli et al., 1992) and its high sensitivity to inhibition by fluorescein derivatives such as erythrosin B (Rasi-Caldogno et al., 1987, 1989; Graf and Weiler, 1989; Williams et al., 1990; Carnelli et al., 1992; De Michelis et al., 1993).

The plant PM Ca^{2+} pump shares a number of similarities with the Ca^{2+} pump of the PM of animal cells, among which the erythrocyte enzyme is the best known. Both the plant and the erythrocyte enzymes have high apparent affinity for Mg-ATP, a broad, slightly alkaline pH optimum, and a pH-dependent apparent K_m for free Ca^{2+} in the micromolar range; both are vanadate-sensitive ATPases that form a phosphorylated intermediate during the catalytic cycle; both enzymes catalyze a nH^+/Ca^{2+} exchange (reviewed in Briskin, 1990; Carafoli, 1991, 1992; Evans et al., 1991; De Michelis et al., 1992a). The functional molecular mass, as determined by the radiation inactivation technique, is around 270 kD for both the erythrocyte and the plant enzyme (Rasi-Caldogno et al., 1990; Carafoli, 1991, 1992). The erythrocyte enzyme is thought to work as a dimer of a 138-kD polypeptide (Carafoli, 1991, 1992; Coelho-Sampaio et al., 1991); molecular masses reported for the phosphorylated intermediate of the plant enzyme in SDS-PAGE range from 100 to 140 kD (Briars and Evans, 1989; Williams et al., 1990; Hsieh et al., 1991). It is interesting that a 140-kD polypeptide cross-reacting with antibodies against the erythrocyte Ca^{2+} pump was extracted from microsomes from maize coleoptiles (Briars et al., 1988).

Among the most striking characteristics of the erythrocyte Ca^{2+} pump is its sensitivity to regulation by the Ca^{2+}-binding protein CaM, which, upon binding to the enzyme, strongly lowers its K_m for free Ca^{2+} and less markedly increases its V_{max}. The effect of CaM on the erythrocyte Ca^{2+} pump can be mimicked by controlled proteolytic treatments: analysis of the products of proteolytic cleavage has shown that the enzyme contains an autoinhibitory C-terminal domain whose inhibitory action is hampered by binding of CaM to its binding site (Carafoli, 1991, 1992).

Stimulation by CaM of the plant PM Ca^{2+} pump has been a long-standing matter of controversy: stimulation was evident in some but not in other PM preparations (for a review,

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Abbreviations: Brij 58, polyoxyethylene-20-cetyl ether; BTP, bis-tris propane (1,3-bis[tris(hydroxymethyl)methylamino]-propane); CaM, calmodulin; FCCP, carbonylcyanide *p*-trifluoro-methoxyphenylhydrazone; PM, plasma membrane.

see Briskin, 1990; Evans et al., 1991; De Michelis et al., 1992a); in PM from radish (*Raphanus sativus* L.) or from *Arabidopsis thaliana* seedlings, we found that addition of CaM decreased the apparent K_m of the enzyme for Ca^{2+}, but its effect on V_{max} was small and erratic (Rasi-Caldogno et al., 1992; Rasi-Caldogno and De Michelis, 1992; De Michelis et al., 1993). The relatively low and quite variable stimulation of the plant PM Ca^{2+} pump by exogenous CaM might depend on the presence of CaM in the PM preparations. In fact, the presence of tightly bound CaM in PM preparations from plants is documented (Collinge and Trewavas, 1989; Evans et al., 1992); moreover, the activation of the PM Ca^{2+} pump by exogenous CaM could be increased by treatments of the PM fraction with the Ca^{2+} chelating agent EGTA (Williams et al., 1990).

In this paper, we show that about two-thirds of CaM tightly bound to the PM of radish seedlings can be removed by a drastic treatment of the PM with EDTA. In EDTA-treated PM, exogenous CaM markedly stimulates the activity of the PM Ca^{2+} pump not only at low, but also at saturating, free Ca^{2+} concentrations. Moreover, we show for the first time that the plant PM Ca^{2+} pump can be activated by a controlled treatment with trypsin, which makes the enzyme insensitive to further activation by CaM.

MATERIALS AND METHODS

Preparation of PM Vesicles

Methods for radish (*Raphanus sativus* L. cv Tondo Rosso Quarantino, Ingegnoli, Milano, Italy) seed germination, PM purification by the aqueous two-phase partitioning procedure, and protein determination have been described (Carnelli et al., 1992). The purified PM fractions were stored in aliquots at −80°C.

EDTA Treatment of the PM

Aliquots of freshly thawed PM were incubated for 5 min on ice in the presence of 20 mM BTP-Hepes (pH 7.5), 0.1 mg mL^{-1} Brij 58, 3 mM ITP, and 5 mM EDTA at a concentration of 0.3 to 1 mg protein mL^{-1}. The samples were diluted with 10 volumes of ice-cold medium containing 0.25 M Suc, 0.1 mM EGTA, 3 mM DTT, 1 mM PMSF, 0.1 mg mL^{-1} Brij 58, 1 mM Mes-Na (pH 6.0), and the PM was collected by centrifugation at 50,000*g* for 30 min at 3°C. The pellets were resuspended in 0.25 M Suc, 0.5 mM DTT, and 1 mM BTP-Mes (pH 6.0) at 0.5 to 1.5 mg protein mL^{-1} and immediately utilized.

Assay of CaM

CaM was assayed with a commercial radioimmunoassay, which we calibrated against CaM purified from radish seedlings (Cocucci, 1984). PM samples, treated with or without EDTA as described above, and reference CaM were boiled for 20 min in the presence of 0.25 M Suc, 0.5 mM DTT, 0.1 mg mL^{-1} Brij 58, 40 mM BTP-Hepes (pH 7.5), and then assayed according to the manufacturer's instructions. Figure 1 shows that radish CaM treated as above competes with $[^{125}I]$CaM from bovine brain for binding to antibodies against bovine brain CaM. The radioimmunoassay reliably measures radish CaM between 2 and 400 ng/sample.

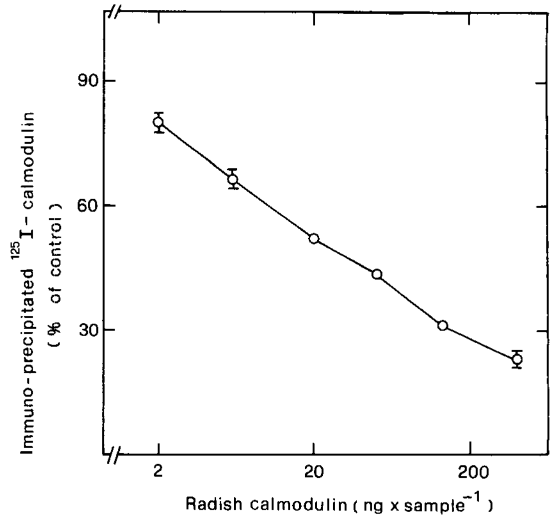

Figure 1. Calibration of the CaM radioimmunoassay with radish CaM. Immunoprecipitated $[^{125}I]$CaM in the presence of increasing amounts of radish CaM is expressed as a percent of the control run in the absence of added CaM. Data are from two experiments, each with three replicates, plus or minus SE.

Treatment of the PM with Trypsin

EDTA-treated PMs were incubated for 0.5 to 10 min on ice in 0.1 mM EDTA, 0.5 mM ITP, 20 mM BTP-Hepes (pH 7.0) in the presence of 5 to 50 $\mu g\ mL^{-1}$ trypsin. The reaction was blocked by addition of at least a 20-fold excess of soybean trypsin inhibitor. Controls run in the absence of trypsin or by adding the trypsin inhibitor prior to trypsin gave identical results.

Measurements of the Activity of the PM Ca^{2+} Pump

The hydrolytic activity of the PM Ca^{2+} pump was measured as Ca^{2+}-dependent ITPase activity (Carnelli et al., 1992). The standard assay medium contained 40 mM BTP-Hepes (pH 7.0), 50 mM KCl, 3 mM $MgSO_4$, 0.1 mM ammonium molybdate, 1 $\mu g\ mL^{-1}$ oligomycin, 5 mM $(NH_4)_2SO_4$, 5 μM FCCP, 2 μM A23187, 1 mM ITP, 1 mM EGTA plus or minus $CaCl_2$ to give the free Ca^{2+} concentrations specified in the legends; CaM was supplied at 20 $\mu g\ mL^{-1}$. Incubation was performed at 25°C for 30 to 90 min, with 20 to 80 μg PM protein mL^{-1}; under these conditions, the assay was linear with both time and protein concentration (data not shown). The released Pi was determined colorimetrically (De Michelis and Spanswick, 1986). The Ca^{2+}-dependent ITPase activity was evaluated as the difference between the activity measured in the presence of Ca^{2+} and that measured in the presence of EGTA alone, which did not exceed 8 nmol mg^{-1} protein min^{-1}.

When ITP-dependent Ca^{2+} uptake was measured, A23187 was omitted, calcium was labeled with 0.15 kBq/nmol of $^{45}Ca^{2+}$, and the PM vesicles were preincubated for 30 min at 25°C plus or minus CaM before initiating the reaction by

Table I. *Effect of EDTA treatment of PM on the activity of the Ca^{2+} pump and on the level of membrane-associated CaM*

Results are from two independent experiments performed on different PM preparations, each treated plus or minus EDTA as described in "Materials and Methods." All assays were performed with three replicates; results are given plus or minus SE. Ca^{2+}-dependent ITPase activity was measured in the presence of 10 μM free Ca^{2+}, plus or minus 20 μg mL^{-1} CaM.

Assays	Plasma Membrane Treatment	
	Minus EDTA	Plus EDTA
Ca^{2+}-dependent ITPase (nmol mg^{-1} protein min^{-1})		
Control	25.9 ± 1.1	14.0 ± 0.6
+CaM	33.6 ± 1.7	31.9 ± 1.6
PM-associated CaM (μg mg^{-1} protein)	2.0 ± 0.3	0.7 ± 0.1

addition of $MgSO_4$ and ITP. ITP-dependent Ca^{2+} uptake was evaluated as the difference between Ca^{2+} taken up after 2 min of incubation in the presence of Mg-ITP and that taken up in its absence. Other experimental details are given by Carnelli et al. (1992).

Free Ca^{2+} concentrations were computed using a value of the apparent association constant for the Ca-EGTA complex at pH 7.0 of 1.32×10^6 M^{-1}, experimentally determined in the assay buffer as described (Carnelli et al., 1992); calcium carry-over from the CaM stock solution and EDTA carry-over from the proteolytic treatment had negligible effects on the free Ca^{2+} concentration in the assay medium.

Unless otherwise specified, the reported results are from one experiment with three replicates, representative of at least three independent experiments conducted on different PM preparations.

Chemicals

Bovine brain CaM was purchased from Sigma (catalog No. P2277), dissolved at 0.4 mg mL^{-1} in 1 mM BTP-Hepes (pH 7.0) and 0.1 mM $CaCl_2$, and stored in aliquots at −20°C. CaM purified from radish seedlings (Cocucci, 1984) was a kind gift of Prof. M. Cocucci (Dipartimento di Fisiologia delle Piante Coltivate e Chimica Agraria, Universitá di Milano, Milano, Italy). Trypsin was purchased from Boehringer (catalog No. 109819), soybean trypsin inhibitor from Sigma (catalog No. T9003), and ^{45}Ca^{2+} (1.2 GBq/mg) and the CaM radioimmunoassay kit from New England Nuclear. All other chemicals were analytical grade or higher.

RESULTS

Removal of Endogenous CaM

Preliminary attempts to increase the CaM sensitivity of the Ca^{2+} pump in PM from radish seedlings by washing the PM in the presence of EGTA were unsuccessful (Rasi-Caldogno et al., 1992; Rasi-Caldogno and De Michelis, 1992). Thus, we decided to use EDTA, which, although less specific, is a stronger calcium chelator, especially at pH values around neutrality (Wolf, 1973; Pershadsingh and McDonald, 1980). The results in Table I show that treatment of the PM with 5 mM EDTA at pH 7.5 in the presence of 0.1 mg mL^{-1} of the detergent Brij 58 and of 3 mM ITP (for experimental details, see "Materials and Methods") strongly lowers the Ca^{2+}-dependent ITPase activity measured at 10 μM free Ca^{2+} in the absence of exogenous CaM. The activity of the EDTA-treated PM is strongly stimulated by CaM, whereas that of control PM, treated as above but in the absence of EDTA, is only very slightly stimulated under these experimental conditions. In the presence of CaM, the activities of PM treated with or without EDTA are very similar, indicating that the recovery of the PM Ca^{2+} pump in the EDTA-treated PM is nearly complete. Omission of ITP during the EDTA treatment of PM leads to a higher basal activity that is less stimulated by CaM (data not shown), possibly due to partial proteolysis of the enzyme (see below).

To check whether the increased sensitivity to exogenous CaM of the Ca^{2+} pump in EDTA-treated PM depended on removal of membrane-bound CaM, we measured the CaM content of purified PM treated with or without EDTA as above. Table I shows that PM treated without EDTA contains about 2 μg of bound CaM per mg of protein, a value that compares well with that determined in PM from pea (Collinge and Trewavas, 1989). After treatment with EDTA, PM-associated CaM decreases to about 0.7 μg per mg protein, the rest being recovered in the supernatant (data not shown).

Figure 2 shows the effect of CaM on the activity of the Ca^{2+} pump in EDTA-treated PM, as a function of free Ca^{2+} concentration; stimulation by CaM is evident at all the free Ca^{2+} concentrations tested. When computed on a percent basis, it is highest at the lowest free Ca^{2+} concentration tested (about 200% at 2 μM) and decreases with the increase of free Ca^{2+} concentration. In the presence of CaM, the activity is inhibited by increasing the concentration of free Ca^{2+} from 100 to 300 μM (Fig. 2), whereas in its absence inhibition of

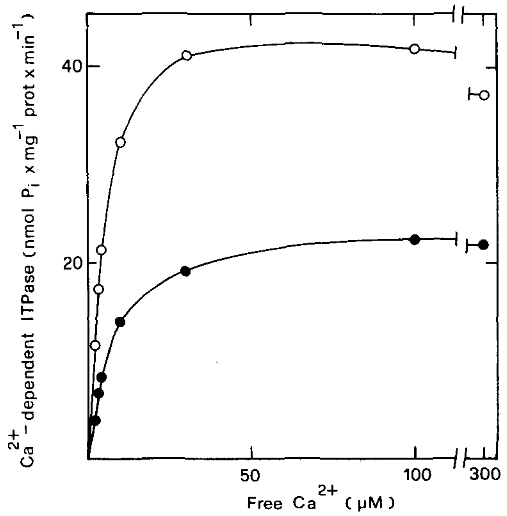

Figure 2. Effect of CaM on the Ca^{2+} pump activity of EDTA-treated PM as a function of free Ca^{2+} concentration. Ca^{2+}-dependent ITPase activity was assayed in the absence (●) or in the presence (○) of 20 μg mL^{-1} CaM.

the PM Ca^{2+} pump activity by free Ca^{2+} becomes evident only at higher concentrations (data not shown).

Effect of Controlled Trypsin Treatments on the Ca^{2+} Pump Activity of EDTA-Treated PM

Mild treatments of EDTA-treated PM with trypsin result in an increase of the activity of the Ca^{2+} pump; the effect depends on the concentration of trypsin, on the length of incubation, and on the concentration of PM proteins (Fig. 3). Maximal activation (about 100% stimulation of the activity measured in the presence of 10 μM free Ca^{2+}) is consistently obtained by incubating PM (0.3–1 mg protein mL^{-1}) for 5 min on ice in the presence of 25 to 50 μg trypsin mL^{-1}.

Analysis of the dependence of the Ca^{2+} pump activity of control and trypsin-treated PM on free Ca^{2+} concentration (Fig. 4) shows that the effect of trypsin is maximal on the activity measured in the presence of the lowest free Ca^{2+} concentration tested (2 μM) and decreases with the increase of free Ca^{2+} concentration; in trypsin-treated PM, but not in the controls, the PM Ca^{2+} pump activity decreases upon increasing the free Ca^{2+} concentration from 100 to 300 μM.

Interactions between the Effects of Controlled Proteolysis and of CaM on the Ca^{2+} Pump Activity of EDTA-Treated PM

In the experiment reported in Figure 5, we compared the effects of a controlled treatment with trypsin and of exogenous CaM on the activity of the PM Ca^{2+} pump. The stimulating effect of trypsin treatment of the PM on the activity of the Ca^{2+} pump is quantitatively similar to that of CaM. In both cases, in agreement with the results described in the previous sections, stimulation of the Ca^{2+} pump activity is higher at low (4 μM) than at saturating (100 μM) free Ca^{2+}. Most relevant, the data in Figure 5 also show that treatment of the PM with trypsin renders the Ca^{2+} pump insensitive to further activation by CaM.

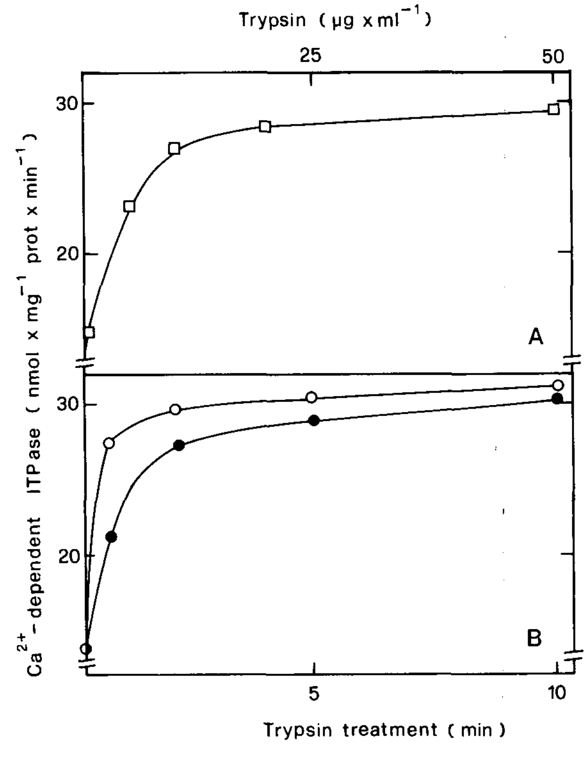

Figure 3. Activation of the Ca^{2+} pump of EDTA-treated PM by trypsin treatments as a function of trypsin concentration and of incubation length. A, PMs (0.5 mg protein mL^{-1}) were treated with the specified trypsin concentrations for 5 min. B, PMs were treated with 12.5 μg trypsin mL^{-1} for the specified times at a concentration of 0.3 mg PM protein mL^{-1} (○) or of 1 mg PM protein mL^{-1} (●). Free Ca^{2+} concentration in the assay medium was 10 μM.

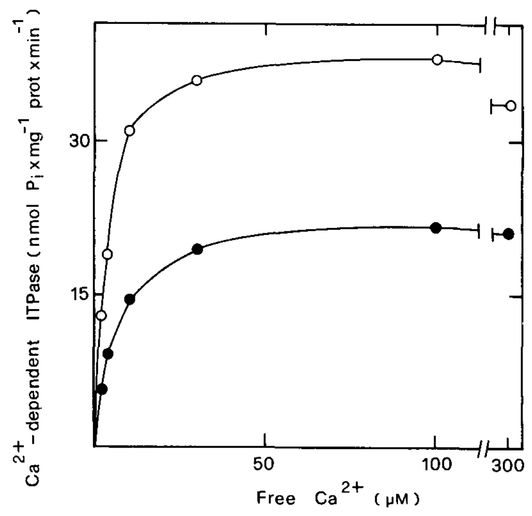

Figure 4. Effect of trypsin treatment of the PM on the activity of the Ca^{2+} pump as a function of free Ca^{2+} concentration. EDTA-treated PM (0.5 mg protein mL^{-1}) were treated with (○) or without (●) 25 μg trypsin mL^{-1} for 5 min.

The same pattern was observed when the effects of trypsin treatment of the PM and of CaM were measured on the transport activity of the PM Ca^{2+} pump (Table II); in this case

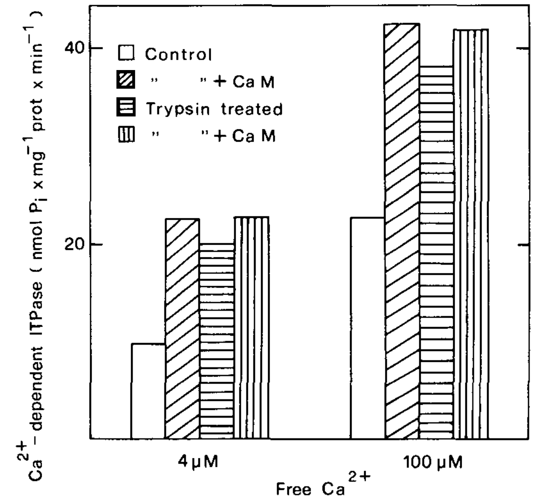

Figure 5. Effect of trypsin treatment of the PM on the sensitivity of the Ca^{2+} pump activity to CaM. EDTA-treated PM (1 mg protein mL^{-1}) were treated with or without 50 μg trypsin mL^{-1} for 5 min. Ca^{2+}-dependent ITPase activity was assayed in the presence or in the absence of 20 μg mL^{-1} CaM.

Table II. *Effects of trypsin treatment of the PM and of CaM on the transport activity of the Ca^{2+} pump*

PM was treated with EDTA as described in "Materials and Methods," but the dilution medium, adjusted to pH 7.0, was supplemented with 5 mM EDTA; after this treatment, stimulation by CaM of the Ca^{2+}-dependent ITPase activity was 345% at 3 μM free Ca^{2+} and 270% at 30 μM free Ca^{2+}. Treatment of the PM (1 mg protein mL^{-1}) with trypsin was for 5 min with 50 μg trypsin mL^{-1}. ITP-dependent Ca^{2+} uptake was assayed after 30 min of preincubation in the presence or in the absence of 20 μg mL^{-1} CaM, as described in "Materials and Methods."

Assay Conditions	ITP-Dependent Ca^{2+} Uptake	
	Control PM	Trypsin-treated PM
	nmol mg^{-1} protein min^{-1}	
3 μM free Ca^{2+}	1.25	4.81
3 μM free Ca^{2+} + CaM	5.38	6.43
30 μM free Ca^{2+}	2.46	7.66
30 μM free Ca^{2+} + CaM	8.98	9.15

as well, the effects of trypsin treatment and of CaM are similar in extent and are nonadditive. Stimulation of the PM Ca^{2+} pump by both trypsin treatment and CaM in the experiment of Table II is higher than in previous experiments because the PM fraction utilized was more extensively washed with EDTA (see the legend to Table II).

DISCUSSION

The presence of bound CaM in membranes isolated from plant cells has been suggested previously on the basis of the sensitivity to CaM antagonists of different CaM-stimulated activities measured in the absence of added CaM (Hsieh et al., 1991; Weiser et al., 1991; Rasi-Caldogno et al., 1992). In particular, the presence of CaM in purified PM fractions has been documented in a few instances (Collinge and Trewavas, 1989; Evans et al., 1992). The results reported in this paper show that the PM isolated from radish seedlings contains substantial amounts of tightly bound CaM that can be at least partially removed by drastic treatment with the calcium chelator EDTA. Previous failures to deplete the PM of endogenous CaM (Collinge and Trewavas, 1989; Rasi-Caldogno et al., 1992) were probably due to the use of EGTA, which is a more specific but weaker calcium chelator, especially at pH values around neutrality (Wolf, 1973; Pershadsingh and McDonald, 1980).

The presence of PM-associated CaM explains our previous observations that addition of CaM increased the activity of the Ca^{2+} pump of untreated PM only when assayed in the presence of low free Ca^{2+} concentrations (De Michelis et al., 1992b, 1993; Rasi-Caldogno et al., 1992; Rasi-Caldogno and De Michelis, 1992). In fact, given the relatively low dissociation constant of the calcium-CaM complex (Cohen and Klee, 1988), the quota of endogenous CaM present as the active calcium-CaM complex increases with the increase of free Ca^{2+} concentration in the micromolar range. So in the presence of low free Ca^{2+} concentrations, endogenous CaM of untreated PM would be only partially in the active calcium-bound form and thus may not be sufficient for maximal activation of the Ca^{2+} pump. On the other hand, in the presence of free Ca^{2+} concentrations required to saturate the Ca^{2+} pump activity, endogenous CaM of untreated PM may be calcium saturated, and thus may sustain maximal activation of the PM Ca^{2+} pump.

In CaM-depleted PM, both the hydrolytic and the transport activity of the Ca^{2+} pump are clearly stimulated by exogenous CaM, both at low and at saturating free Ca^{2+} concentrations. The basal activity of the PM Ca^{2+} pump can be further decreased and made more sensitive to stimulation by exogenous CaM by increasing the concentration of EDTA and/or the length of the EDTA treatment (see Table II). The presence of different amounts of PM-associated CaM is likely an important source of the variability of the reported effects of exogenous CaM on the activity of the Ca^{2+} pump in PM isolated from different plant materials through different procedures (reviewed in Briskin, 1990; Evans et al., 1991; De Michelis et al., 1992a). To conclude this point, we want to stress that stimulation by CaM is a characteristic of the PM Ca^{2+} pump, but cannot be taken as the sole criterion of identification of this enzyme, since the ER is also endowed with a CaM-stimulated Ca^{2+} pump (Brauer et al., 1990; Askerlund and Evans, 1992).

In CaM-depleted PM, the hydrolytic and the transport activity of the Ca^{2+} pump are stimulated also by a controlled treatment with trypsin. The effect of controlled proteolysis is similar to that of CaM. Moreover, the proteolyzed enzyme is insensitive to further activation by CaM. Understanding the molecular basis of the activation of the plant PM Ca^{2+} pump by controlled proteolysis requires the analysis of the changes in molecular mass of the enzyme induced by tryptic treatments. To this end, work is in progress in the authors' laboratories to develop highly specific methods of identification of the plant PM Ca^{2+} pump in SDS-PAGE, exploiting its ability to form a phosphorylated intermediate during the catalytic cycle (Briars and Evans, 1989; Williams et al., 1990; Hsieh et al., 1991) and/or its high sensitivity to inhibition by fluorescein derivatives such as eosin Y or erythrosin B (Rasi-Caldogno et al., 1987; De Michelis et al., 1993). However, in light of the close similarity between the plant PM Ca^{2+} pump and its animal counterpart, it is tempting to speculate that the plant PM Ca^{2+} pump also has an autoinhibitory C-terminal domain that can be cleaved by controlled proteolysis, leading to a CaM-insensitive activated form of the enzyme, with characteristics similar to those of the native enzyme activated by CaM (Carafoli, 1991, 1992).

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