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Plasma Membrane Ca-ATPase of Radish Seedlings[1]

II. Regulation by Calmodulin

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ABSTRACT

The effect of calmodulin on the activity of the plasma membrane Ca-ATPase was investigated on plasma membranes purified from radish (*Raphanus sativus* L.) seedlings. Calmodulin stimulated the hydrolytic activity and the transport activity of the plasma membrane Ca-ATPase to comparable extents in a manner dependent on the free Ca^{2+} concentration. Stimulation was marked at low, nonsaturating Ca^{2+} concentrations and decreased increasing Ca^{2+}, so that the effect of calmodulin resulted in an increase of the apparent affinity of the enzyme for free Ca^{2+}. The pattern of calmodulin stimulation of the plasma membrane Ca-ATPase activity was substantially the same at pH 6.9 and 7.5, in the presence of ATP or ITP, and when calmodulin from radish seeds was used rather than that from bovine brain. At pH 6.9 in the presence of 5 micromolar free Ca^{2+}, stimulation of the plasma membrane Ca-ATPase was saturated by 30 to 50 micrograms per milliliter bovine brain calmodulin. The calmodulin antagonist calmidazolium inhibited both basal and calmodulin-stimulated plasma membrane Ca-ATPase activity to comparable extents.

CaM[2] is a ubiquitous regulatory protein involved in regulatory events in animal and plant cells (22). The hypothesis that CaM regulated the activity of the PM Ca-ATPase of plant cells, similarly to that observed for the erythrocyte Ca-ATPase (8), has been proposed since 1980 (13). However, the available data on the effect of CaM on the activity of the PM Ca-ATPase of plants have been until now fragmentary and sometimes contradictory (reviewed in refs. 5, 12, 15).

The data on crude microsomes or on nonwell identified membrane fractions should be taken with caution, because CaM has been reported to stimulate also the active Ca^{2+} transport systems of the tonoplast (1, 21, 29) and of the endoplasmic reticulum (2).

A CaM-stimulated Ca-ATPase purified from maize microsomes by CaM-affinity chromatography (3, 4, 14, 16) has been proposed to represent the PM Ca-ATPase on the basis of its structural and immunological similarities with the erythrocyte Ca-ATPase (3, 4, 16). However, to our knowledge, its localization at the PM has not been directly demonstrated, nor has its involvement in the active transport of Ca^{2+} been shown by reconstitution in proteoliposomes. Moreover, CaM-stimulation of this purified enzyme could be observed only when ATP was supplied as a substrate (15), whereas the plant PM Ca-ATPase is able to utilize also ITP or GTP as substrates (5, 12, 15) and stimulation of its transport activity by CaM in PM from red beet has been observed also in the presence of GTP (28).

The response to CaM of the PM Ca-ATPase in PM isolated from different materials is quite variable, and in some instances no significant effect of CaM could be observed (7, 18–21, 24–26, 28, 29). A detailed analysis of the effect of CaM on the plant PM Ca-ATPase is lacking, because the only attempt to characterize the CaM-stimulated activity (26) was severely limited by the difficulties of analyzing PM Ca^{2+}-dependent ATPase activity in the presence of the much higher activity of the Ca^{2+}-inhibited PM H^+-ATPase (23, 24).

In this work, we have exploited the capability of the PM Ca-ATPase to utilize ITP as an alternative substrate (9) to analyze the effect of CaM on both the transport and the hydrolytic activity of the PM Ca-ATPase of radish seedlings. The reported results show that CaM activates the PM Ca-ATPase by shifting it to a higher affinity state.

MATERIALS AND METHODS

Preparation of PM Vesicles

Methods for seed germination, PM purification, and protein determination are given in the accompanying paper (9).

Measurements of Ca^{2+} Uptake

ATP- and ITP-dependent Ca^{2+} uptake were assayed as described in the accompanying paper (9). Free Ca^{2+} concentrations in media containing different $CaCl_2$ concentrations and 1 mM EGTA were computed using the apparent association constant of the Ca-EGTA complex at pH 6.9 (5×10^5 M^{-1}) determined in the accompanying paper (9). When present, CaM was supplied during the preincubation.

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[2] Abbreviations: CaM, calmodulin; PM, plasma membrane; BTP, bis-tris propane, (1,3-bis(tris(hydroxymethyl)methylamino)-propane; EB, erythrosin B; W_7, (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; W_5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide; Brij 58, polyoxyethylene 20 cetyl ether.

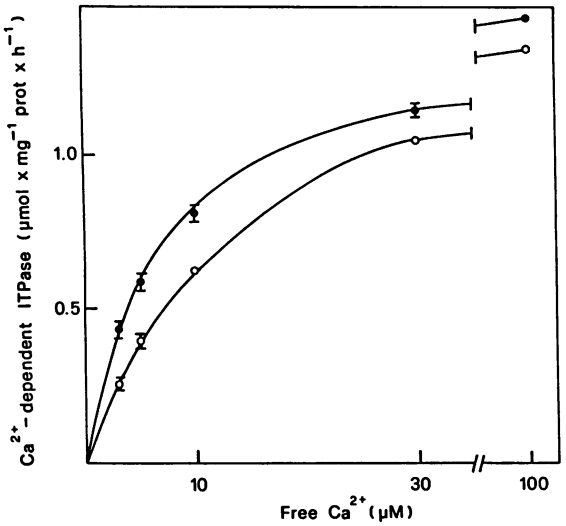

Figure 1. Dependence of Ca^{2+}-dependent ITPase activity on free Ca^{2+} concentration in the presence (●) and absence (○) of CaM. Assays were performed at pH 6.9; free Ca^{2+} was buffered at the specified concentrations with 1 mM EGTA. Bovine brain CaM was supplied at 20 μg mL^{-1}. Data are from three experiments, each with three replicates; bars are the standard error of the mean.

Measurements of Ca^{2+}-Dependent ITPase Activity

Assays were performed in the presence of 75 μg mL^{-1} Brij 58 essentially as described in the accompanying paper (9) in assay media buffered at pH 6.9 or 7.5 with 40 mM BTP-Hepes. Free Ca^{2+} concentrations in media containing different $CaCl_2$ concentrations and 1 mM (at pH 6.9) or 2 mM (at pH 7.5) EGTA were computed using the values of apparent association constant of the Ca-EGTA complex (5×10^5 M^{-1} at pH 6.9 and 7.6×10^6 M^{-1} at pH 7.5) determined in the accompanying paper (9).

Special Reagents

Bovine brain calmodulin was purchased from Sigma (cat. P2277), dissolved at 0.4 mg mL^{-1} in 1 mM BTP-Hepes, pH 6.9, and 10^{-4} M $CaCl_2$ and stored at −20°C. Calmodulin purified from radish seedlings (10) was a kind gift of Prof. M. Cocucci, Istituto di Chimica Agraria, Università di Milano, Milano, Italy. Calmidazolium (Sigma cat. C1278) was dissolved in ethanol at 10 mM concentration and added to the ITPase assay medium under continuous stirring.

RESULTS

Calmodulin-induced activation of the erythrocyte Ca-ATPase involves a dramatic increase of the apparent affinity of the enzyme for free Ca^{2+}, accompanied by a less important increase in V_{max} (8). We have analyzed the effect of exogenous CaM on the dependence of the PM Ca-ATPase from radish on free Ca^{2+} concentration. Figure 1 shows the dependence on free Ca^{2+} concentration of Ca^{2+}-dependent ITPase activity assayed in EGTA-buffered media at pH 6.9, in the presence and absence of bovine brain CaM. The results show that, in agreement with previously reported data on ATP-dependent Ca^{2+} uptake (25), stimulation by CaM of Ca^{2+}-dependent ITPase activity at saturating (100 μM) free Ca^{2+} is very small, albeit reproducible. Calmodulin-induced stimulation increases with the decrease of free Ca^{2+} concentration, so that CaM stimulates the activity measured in the presence of 3 μM free Ca^{2+} by up to 70%. As a consequence of this behavior, CaM lowers the free Ca^{2+} concentration required to half-saturate the Ca^{2+}-dependent ITPase activity from about 12 μM in its absence (9) to about 7 μM in its presence.

The pattern is similar when the assays are performed at the more physiologically relevant pH value of 7.5. At this pH, the apparent affinity of basal activity for Ca^{2+} is higher than at pH 6.9, the activity being half-saturated by about 3 μM free Ca^{2+} (9). Calmodulin markedly stimulates the Ca^{2+}-dependent ITPase activity measured in the presence of 1.2 or 3 μM free Ca^{2+}, and only slightly that measured in the presence of saturating Ca^{2+} concentration (Table I). Although the effect of CaM is somewhat higher at pH 7.5 than at pH 6.9, all the following experiments were performed at pH 6.9, where free Ca^{2+} concentration in the micromolar range can be more efficiently controlled by means of the Ca^{2+}-EGTA buffer system (9). On the other hand, it is essential to use EGTA-buffered media to avoid any change in free Ca^{2+} concentration caused by CaM itself.

Figure 2 shows that CaM stimulates also the initial rate of ATP- or ITP-dependent Ca^{2+} uptake into PM vesicles; the effect is similar to that on the Ca^{2+}-dependent ITPase activity both in extent and in being strong only in the presence of nonsaturating free Ca^{2+} concentration. The effects of CaM on Ca^{2+}-dependent ITPase activity and on ATP- or ITP-dependent Ca^{2+} uptake are abolished by 1 μM EB (Fig. 2A), indicating that they reflect the activation of the PM Ca-ATPase.

Calmodulin-induced stimulation of the PM Ca-ATPase activity depends on CaM concentration: in the presence of 5 μM free Ca^{2+} at pH 6.9, activation of the Ca^{2+}-dependent ITPase activity is maximal in the presence of 30 to 50 μg mL^{-1} of bovine brain CaM (Fig. 3).

Table II shows that the effect of CaM purified from radish seedlings is similar to that of bovine brain CaM, albeit somewhat stronger; also, homologous CaM strongly stimulates the Ca^{2+}-dependent ITPase activity in the presence of nonsaturating free Ca^{2+} concentration, but has little effect on the activity measured in the presence of saturating free Ca^{2+}.

The effects of the CaM antagonist calmidazolium on basal

Table I. *Effect of CaM on Ca^{2+}-Dependent ITPase Activity Measured at pH 7.5*

Free Ca^{2+} was buffered at the specified concentrations with 2 mM EGTA. Bovine brain CaM was supplied at 20 μg mL^{-1}. Results from four experiments, each with three replicates, are given plus or minus the standard error of the mean.

Free Ca^{2+}	Ca^{2+}-Dependent ITPase		
	Control	+ CaM	
μM	μmol mg^{-1} prot h^{-1}		% stimulation
1.2	0.29 ± 0.04	0.60 ± 0.06	107
3.0	0.79 ± 0.08	1.20 ± 0.14	52
50.0	1.60 ± 0.04	1.73 ± 0.05	8

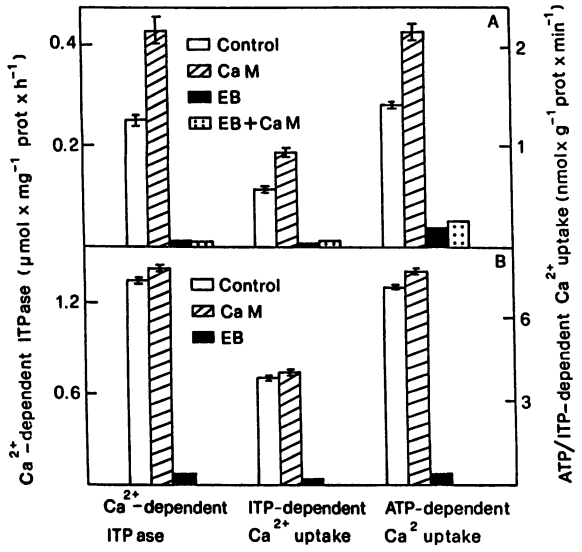

Figure 2. Comparison of the effects of CaM on the hydrolytic and the transport activity of the PM Ca-ATPase. Assays were performed at pH 6.9; free Ca^{2+} was buffered at 3 μM (A) or 100 μM (B) with 1 mM EGTA. Bovine brain CaM was supplied at 20 μg mL^{-1} and EB at 1 μM. Data are from three experiments, each with three replicates; bars are the standard error of the mean.

and CaM-stimulated Ca^{2+}-dependent ITPase activity are shown in Figure 4: calmidazolium inhibits basal and CaM-stimulated activities to similar extents. Similar results were obtained with a different CaM antagonist as W_7, whereas W_5 was nearly uneffective (data not shown). The effectiveness of calmidazolium drastically decreases with the increase of free Ca^{2+} concentration: in the presence of 3 μM free Ca^{2+} (Fig. 4A), the Ca^{2+}-dependent ITPase activity is 40% inhibited by 20 μM and more than 80% inhibited by 50 μM calmidazolium, whereas in the presence of 100 μM free Ca^{2+} (Fig. 4B), inhibition is only 12% with 20 μM and 45% with 50 μM calmidazolium.

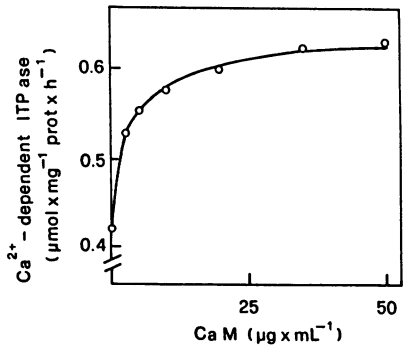

Figure 3. Dependence of the activation of Ca^{2+}-dependent ITPase on the concentration of CaM. Assays were performed at pH 6.9 in the presence of the specified concentrations of bovine brain CaM; free Ca^{2+} was buffered at 5 μM with 1 mM EGTA. Data are from two experiments, each with two replicates.

Table II. *Comparison of the Effect of CaM from Bovine Brain and from Radish Seeds on Ca^{2+}-Dependent ITPase Activity*

Assays were performed at pH 6.9; free Ca^{2+} was buffered at the specified concentrations with 1 mM EGTA. Data are the mean of two experiments, each with two replicates.

Treatment	Ca^{2+}-Dependent ITPase	
	5 μM free Ca^{2+}	100 μM free Ca^{2+}
	$\mu mol\ mg^{-1}\ prot\ h^{-1}$	
Control	0.42	1.35
+ 5 μg mL^{-1} bovine brain CaM	0.56	n.d.[a]
+ 20 μg mL^{-1} bovine brain CaM	0.59	1.43
+ 5 μg mL^{-1} radish seeds CaM	0.62	n.d.
+ 20 μg mL^{-1} radish seeds CaM	0.73	1.55

[a] n.d. = not determined.

DISCUSSION AND CONCLUSIONS

This is the first report in which activation by CaM of the plant PM Ca-ATPase has been demonstrated in parallel for the hydrolytic and transport activities of the enzyme. The results show that the effect of exogenous CaM on the PM Ca-ATPase from radish seedlings is the same when the hydrolytic or the transport activity of the enzyme is measured. Moreover, the effect of CaM is unchanged by substituting ITP for ATP as a substrate for the Ca-ATPase. This result is in agreement with that obtained on PM from red beet, using ATP or GTP to energize Ca^{2+} uptake (28). The different behavior of the affinity-purified Ca-ATPase from maize microsomes, which is CaM-stimulated only in the presence of ATP (15), awaits explanation.

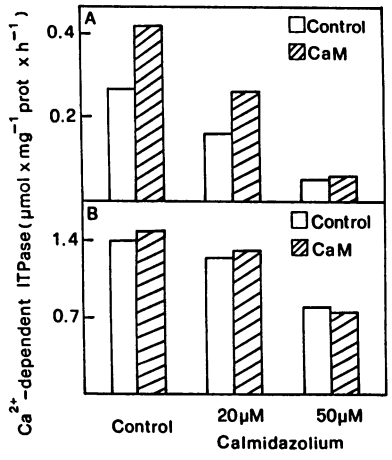

Figure 4. Effect of calmidazolium on Ca^{2+}-dependent ITPase activity in the absence and in the presence of CaM. Assays were performed at pH 6.9; free Ca^{2+} was buffered at 3 μM (A) or 100 μM (B) with 1 mM EGTA. Bovine brain CaM was supplied at 20 μg mL^{-1}. All the samples contained 0.25% (v/v) ethanol. Data are from two experiments, each with three replicates.

The fact that Ca^{2+}-dependent ITPase, ITP-, and ATP-dependent Ca^{2+} uptake show similar sensitivity to stimulation by CaM strengthen our previous conclusion that the hydrolytic activity of the PM Ca-ATPase can be fruitfully investigated in isolated PM by using ITP as a substrate (9), thus avoiding the errors due to the simultaneous operation of the Ca^{2+}-inhibited PM H^+-ATPase (see for example the different effects of CaM on Ca^{2+}-dependent ATPase activity and on ATP-dependent Ca^{2+} uptake in low salt media in ref. 25).

Calmodulin-induced activation of the plant PM Ca-ATPase is strongly dependent on the free Ca^{2+} concentration and results in an increase of the apparent affinity of the enzyme for free Ca^{2+}. The effect of CaM is similar at pH 6.9 and at pH 7.5, and the apparent affinity of the PM Ca-ATPase for free Ca^{2+} at pH 7.5 remains much higher than at pH 6.9 (9) also in the presence of CaM. The response of the plant PM Ca-ATPase to CaM qualitatively resembles that of the related enzyme of the erythrocytes, which shifts to a high affinity state upon activation by CaM (8). This observation points out once more the close similarity between the animal and plant PM Ca-ATPases (8, 12, 15).

The activation by exogenous CaM of the PM Ca-ATPase from radish seedlings described in this work is small compared with that of the erythrocyte Ca-ATPase, which under optimal conditions undergoes a CaM-induced decrease of the apparent K_m for free Ca^{2+} of up to 30-fold (8). The low degree of activation by exogenous CaM of the radish PM Ca-ATPase could depend on the presence of endogenous CaM in the PM fraction. In PM from red beet, CaM-stimulation of the PM Ca-ATPase could be observed only after extensive washing of the membranes with EGTA (28). Our PM fraction from radish was prepared and washed in the presence of EGTA, and no improvement of response to exogenous CaM could be obtained by further extensive washing of the PM with EGTA, even in the presence of the detergent Brij 58 (data not shown). However, it is possible that the PM contains some tightly bound CaM (11), capable of activating the PM Ca-ATPase. On the other hand, it is known that the erythrocyte Ca-ATPase can be shifted to the activated state also by other treatments such as limited proteolysis or changes of the phospholipid environment (8). Activation of the radish PM Ca-ATPase by partial proteolysis, albeit possible, seems unlikely because membrane extraction and purification were performed in the presence of BSA, casein, and the protease inhibitor PMSF. The lipid environment is the most likely candidate to control the enzyme activity in the native membrane, especially in the light of the high sensitivity of the PM Ca-ATPase activity to detergents (9).

The possibility that the radish PM Ca-ATPase is already in a partially activated state is suggested by the observation that the apparent K_m of the enzyme for free Ca^{2+} is relatively low also in the absence of exogenous CaM: at pH 7.5, half-saturation is reached in the presence of 3 μM free Ca^{2+} (9), but as much as 30 μM free Ca^{2+} is needed to half-saturate the erythrocyte Ca-ATPase in its inactive state (8). A partial activation of the radish PM Ca-ATPase is further suggested by the observation that CaM antagonists such as calmidazolium and W_7 inhibit basal and CaM-stimulated activity to comparable extents.

A different degree of activation of the PM Ca-ATPase in native membranes from different plant sources, as well as the different free Ca^{2+} concentrations utilized in the assays, probably explain at least in part the variability of response to exogenous CaM of the PM Ca-ATPase observed on PM isolated from various plant sources (7, 18–21, 24–26, 28, 29).

To conclude, we want to stress that CaM-induced activation enhances the capability of the plant PM Ca-ATPase to respond to an increase of cytoplasmic Ca^{2+} concentration and thus to substantially contribute to the reestablishment of cytoplasmic Ca^{2+} homeostasis. In fact, under physiological conditions, cytoplasmic free Ca^{2+} concentration varies in a range well below saturation of the activity of the plant PM Ca-ATPase: cytoplasmic free Ca^{2+} is about 0.1 μM in resting conditions, and can increase up to 1 or more μM in response to endogenous or exogenous stimuli (17, 22). An increase of cytoplasmic Ca^{2+} concentration can be expected to increase the level of the active Ca-CaM complex, which in turn would activate the PM Ca-ATPase, shifting it to the high affinity state, and thus allowing it to efficiently extrude Ca^{2+} from the cytoplasm to the apoplast. It is worth noting that the pH dependence of Ca^{2+} binding to CaM (27), together with the pH dependence of the apparent affinity of the PM Ca-ATPase for free Ca^{2+} (9), could constitute an important link between pH and Ca^{2+} homeostasis in the cytoplasm (6, 17).

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