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Identifying calcium channels and porters in plant membranes

Progress Report

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Summary of progress The overall objectives of the proposal submitted in 6/90 was to understand how Ca was transported across plant membranes, and how these transport pathways were regulated. Ca participates in many cellular processes, including the transduction of hormonal and environmental signals, secretion, and protein folding. These processes depend on the coordination of passive Ca fluxes via channels and active Ca pumps; however these transport pathways are poorly understood in plants. We had, therefore, proposed to identify and characterize Ca transport proteins, such as the inositol-1,4,5-trisphosphate (IP₃)-sensitive Ca channels and Ca pumps. We have had difficulties characterizing and cloning the IP₃-sensitive Ca channel, but have made considerable progress on the biochemical characterization, and partial purification of a 120 kD Ca-pumping ATPase. We have begun to determine the structure of Ca pumps by molecular cloning and have already obtained a partial cDNA with features characteristic of Ca pumps.

Progress

1. Ca-pumping ATPases in plants

(a) Calmodulin-stimulated Ca-ATPase is associated with the ER

Although plant cells lower cytoplasmic Ca levels by accumulating the ion in endomembrane compartments or by extruding Ca out of the cell; it is not known whether the plant Ca pumps fall into two distinct types as seen in animals. According to the model taken from animal cells, the SER-type Ca-pumping ATPases remove Ca from the cytoplasm by pumping Ca into the lumen of the sarcoplasmic/endoplasmic reticulum and the PM-type Ca-ATPase pump Ca out of the cell across the plasma membrane. The two types of Ca pumps are biochemically and structurally distinct. For example, the plasma membrane (PM-type) Ca-ATPase (mol mass ~134 kD) was stimulated by calmodulin directly; whereas the sarcoplasmic/endoplasmic reticulum type (SER-type) (mol mass ~110 kD) was insensitive to calmodulin.

My laboratory was one of the first to show that unlike animal cells, an ER-associated Ca pump in plant cells was stimulated by calmodulin (CaM). We showed active Ca transport was associated with the ER from carrot suspension-cultured cells since (i) Ca pumping activity comigrated on sucrose density gradients with the ER marker, antimycin A-insensitive NADH-dependent cytochrome c reductase; and (ii) Ca transport was shifted by Mg in parallel with the ER marker (Bush & Sze, 1986). This Ca transport activity was stimulated 2-4 fold by 1 μ M calmodulin (Pierce & Sze, 1987. Plant Physiol. abstract). However, these results appeared to contradict the commonly accepted view that CaM-stimulated ATPase were found on the plasma membrane. This idea taken from the animal model was perpetuated by plant biologists, although purified membranes were not used in many studies with plants (e.g. Evans et al., 1991)

We found that 90% of calmodulin-stimulated Ca transport was associated with endomembranes, like the ER because the CaM-stimulated activity comigrated on a linear sucrose gradient with an ER marker (Hsieh, Pierce, & Sze, 1991). A small peak (5%) of CaM-stimulated Ca transport activity was detected in vesicles at a density of 1.17 gm/ml. The results suggested that most of the CaM-stimulated activity was associated with endomembranes, such as ER vesicles and that a small proportion of the activity was associated with the plasma membrane. This conclusion has now been reported by several other laboratories working with plants (Evans, 1994).

The CaM-stimulated Ca pump could be inhibited by calmodulin antagonists, like W-7, and ML-7. L-Thyroxine, thought to bind at or near the calmodulin binding domain, effectively inhibited only the CaM-stimulated activity (Hsieh et al. 1991). One interpretation is that CaM is directly binding and stimulating a Ca-ATPase; however the possibility of indirect CaM

stimulation via a CaM-dependent protein kinase is also considered. Interestingly, like the PM-type Ca-ATPase from humans, the carrot Ca pump was stimulated by acidic phospholipids, like phosphatidylserine.

(b) Mol mass of an ER-associated Ca pump is 120 kD

To determine the molecular mass of Ca pumps, we took advantage of the phosphorylated enzyme intermediate (P-type) formed during the reaction mechanism of Ca-ATPases. This phosphorylation of usually an aspartate (D) residue forms an acyl phosphate bond that differs from the phosphoester bond formed with Ser or Thr by protein kinases. Interestingly, incubation of ER-enriched membranes with g[³²P]ATP for 15 sec resulted in the formation of a **single** Ca-dependent phosphoprotein with an apparent mol mass of 120 kD (Hsieh et al 1991).

All the properties of the 120 kD phosphoenzyme are consistent with it being a Ca-pumping ATPase. For example, formation of the 120 kD phosphoenzyme (PE) is absolutely dependent on Ca, but not on Mg. PE formation is enhanced by La and sensitive to hydroxylamine indicating this phosphoenzyme was formed from an acyl phosphate linkage characteristic of P-type transport ATPases.

It is not clear why we detect only one phosphoenzyme, as plant membranes clearly possess several P-type ion-pumping ATPases. It is possible that (i) phosphoenzyme formation of the PM H⁺-ATPase requires Mg, thus in the absence of Mg, activity of this H⁺ pump is not detected; (ii) in the absence of Mg, there is no Ser or Thr phosphorylation by protein kinases; and (iii) the conditions we use favor the detection of one type of Ca pump. Although the data show clearly one band of 120 kD, we cannot yet eliminate the possibility that the 120 kD phosphoprotein could reflect activity from a mixture of Ca pumps with the same mol mass.

(c) Properties of a 120 kD phosphoenzyme indicate it is a PM-type Ca-ATPase

The kinetic properties of a 120 kD Ca-dependent phosphoenzyme were determined as a step towards identifying the type of Ca pump. To my knowledge, this is the first time that the kinetic properties of a plant membrane Ca-ATPase has been characterized by determining phosphoenzyme activities quantitatively. This assay was possible because one major phosphoenzyme of 120 kD was formed in all membrane fractions under the conditions we used. Furthermore, the 120 kD phosphoenzyme was most abundant (65%) in ER-enriched membranes (Chen et al. 1993), consistent with the Ca pumping activity.

We showed that the amount of TCA-precipitable [³²P] in membrane proteins (Table I) that had been incubated with [³²P]ATP correlated with the amount of the 120 kD [³²P]phosphoprotein visualized by autoradiography (Fig. 1 from Chen et al 1993). Ca stimulated the levels of phosphoprotein by 5- to 8-fold over the EGTA control. Ca-dependent formation of the [³²P]phosphoprotein was stimulated 2-fold by La, and activity in the presence of La was 78% inhibited by vanadate. Importantly, the phosphoprotein levels were decreased 63% in vesicles treated with hydroxylamine. Therefore, the formation of the phosphorylated intermediate of a Ca-ATPase can be directly quantitated by the measurement of Ca-dependent phosphoprotein in carrot ER-enriched membranes.

Using this approach, we found that the properties of the Ca-dependent EP are similar in general, but not identical, to the PM-type Ca-ATPase of animal cells (Table I).

Table I. Properties of a 120 kDa Ca-dependent phosphoenzyme from endomembranes of carrot cells

Mol. Mass	120 kD
Mechanism	formation of a phosphorylated intermediate
Inhibitors	Vanadate La stimulate steady state EP by inhibiting dephosphorylation Erythrosin B ($I_{50} < 100$ nM) No effect by SERCa pump inhibitors: cyclopiazonic acid, thapsigargin
ATP affinity	K_m of EP formation = 67 nM
Substrate specificity	ATP > ADP > ITP > GTP
K_m	0.075 1 100 250 μ M
Ca affinity	K_m of EP formation = 2 μ M

One major difference between a carrot Ca-ATPase of 120 kD and the PM-type Ca pump from animals is the affinity for CaM. The CaM concentration required for 50% stimulation of Ca pumping is about 200 nM (Hsieh et al 1991) which is 100X higher than the 1-2 nM needed to stimulate the red cell pump. Furthermore, although added Mg was not required for the formation of Ca dependent EP, its presence resulted in the reduction of steady state level of EP in carrot membranes (Chen et al 1993), but an increase in human red cells. Thus the structure and regulation of a carrot 120 kD Ca-ATPase may differ in some features from animal Ca pumps.

(d) Relative low abundance of a 120 kD Ca pump in endomembranes

To our knowledge, the relative abundance of Ca pumps in plant membranes has not been estimated before. Based on the specific activities of Ca-dependent ATP hydrolysis (1-2 μ mol/mg-h) (Hsieh et al 1991), the relative amount of Ca pumps was thought to be ~10X lower than PM H^+ -ATPases (sp. act = 10-20 μ mol/mg-h). We have estimated the relative abundance of a Ca pump by determining the steady state level of PE formed under saturating concentrations of ATP (200 nM) when dephosphorylation was blocked with 1 mM La. Under these conditions, the steady state level of Ca-dependent PE was about 9 pmol/mg protein. Assuming that there is only one acyl-linked phosphate per monomer of enzyme, there would be about 9 pmol of Ca-ATPase per mg protein. With a molecular mass of 120,000, the Ca-dependent PE would represent at least 0.1% of the total membrane protein (Chen et al 1993). This is the first estimate of Ca-pump abundance in a plant membranes. The amount of Ca pumps in membranes is low relative to the PM H^+ -ATPase and the vacuolar H^+ -ATPase which make up 3% and 5-8% of the plasma membrane and endomembranes, respectively. The low abundance of Ca pump in plants is, however, comparable with that of erythrocyte PM in which the Ca pump makes up 0.01-0.1% of the total membrane protein.

(e) Ca pump has two classes of ATP binding sites

Ca pumping into carrot vesicles showed biphasic dependence on ATP (Chen et al, 1993) characteristic of Ca pumps from the animal SER or PM. These results are consistent with the idea that the Ca pump has two classes of site for ATP binding. The K_m for ATP of the high-affinity and low-affinity site was about 10 μ M and 1.5 mM, respectively. The maximum activity elicited by ATP binding to the site with lower K_m was about 20% of the activity reached at non-

limiting ATP. The high affinity site is thought to be the catalytic site of the Ca ATPase and the binding of ATP at the low affinity site is thought to regulate the turnover of the pump.

(f) Partial purification of a 120 kDa Ca-ATPase

The biochemical characterization of a Ca-ATPase phosphoenzyme have provided us with tools to identify the pump during purification as a first step towards determining the structure and function of the Ca pump at the molecular level. A purified enzyme preparation could be used to generate antibodies and obtain partial amino acid sequences. We wanted to purify this pump using calmodulin-affinity chromatography but decided to take a novel approach to monitor its activity during purification. Because only one major 120 kD protein was phosphorylated in a Ca-dependent manner, we followed activity using this sensitive [32 P]PE assay during purification instead of measuring CaM-stimulated, Ca-dependent ATP hydrolysis. Ca pumps are not abundant, therefore determining CaM- or Ca-dependent ATP hydrolysis is less sensitive and would require much more starting material.

Using the Ca-dependent PE formation assay, we have partially purified a 120 kD Ca pump after solubilization with Triton and chromatography with a calmodulin-affinity column (Hwang et al, 1997). Microsomal membranes proteins were initially washed with EGTA to remove Ca and any endogenous membrane-bound calmodulin, and then solubilized with 1% Triton. The solubilized proteins were loaded on to a CaM-Sepharose column in the presence of 5 mM Ca. These conditions promote the binding of CaM-binding domains to the immobilized CaM. The column was washed to remove unbound proteins and to reduce Ca conc. to 0.1 mM. Bound proteins were then eluted from the CaM-Sepharose with EGTA. The major proteins eluted by EGTA were of 120 kD, 68, 54, 50 and 44 kD. Only the 120 kD polypeptide was phosphorylated with [γ - 32 P]ATP in the presence of Ca. The Ca-dependent phosphoenzyme of 120 kD is stimulated by La, sensitive to erythrosin B and inhibited by vanadate, confirming the identity of the 120 kD polypeptide as a Ca-ATPase. We conclude that we have partially purified a PM-type Ca pump of 120 kD.

Interestingly, we found that a majority (80%) of the carrot Ca-ATPase EP activity did not bind to the CaM-affinity column, in spite of efforts to increase binding by adjusting binding conditions. We think that the presence of a large amount of activity remaining unbound may be due to (i) a low affinity of the Ca-ATPase for CaM resulting in weak binding to the column; (ii) endogenous CaM remaining bound to the CaATPase even after EGTA washing; or (iii) the presence of another Ca-ATPases of 120 kD that does not bind to CaM and that accounts for most of the EP activity. The latter idea can be tested by further purification of Ca-ATPases as monitored by phosphoenzyme formation and by measuring CaM-binding activities (see Aims) during purification.

(g) Multiple CaM-binding proteins of ~110-125 kD are associated with plant membranes

To confirm the presence of distinct CaM-binding proteins in carrot membranes, proteins blotted to nitrocellulose was assayed for direct binding to [125 I]CaM. Major CaM-binding proteins of 110-125, 68, 50 and 44 kD were present in ER-enriched and in PM-enriched membranes. [125 I]CaM binding was Ca-dependent. The CaM-binding proteins purified by CaM-affinity chromatography were strikingly similar in mol mass to those that bound [125 I]CaM directly, suggesting that both methods are effective in identifying specific CaM-binding proteins.

As Ca-dependent EP activity is associated with a 120 kD protein that bound to a CaM-affinity column, our results would suggest that CaM-binding protein of ~116 kD in carrot membranes is a Ca pump. Instead of one major polypeptide, multiple [125 I]CaM-binding

proteins of 110-125 kD were observed, raising the interesting possibility of several CaM-binding Ca-ATPases which differ slightly in mol mass. If so, a molecular approach would be needed to distinguish among them.

We have recently confirmed the results in my laboratory using biotinylated CaM to study CaM-binding to membrane proteins. We will use this assay in our renewal proposal. The method is sensitive, specific and does not require isotopes. [The CaM-binding studies with [^{125}I]CaM were performed by V. Ling in S. Assmann's laboratory.]

(h) Cloning Ca pump genes.

We have made good progress in our efforts to determine the primary structures of Ca pumps by molecular cloning. A partial cDNA we have cloned from carrot has all the features of a primary Ca-pumping ATPase. This clone will serve as a basis for the structural and functional analyses of a carrot Ca pump in the renewal proposal.

2. Biochemical and molecular characterization of the inositol-1,4,5-trisphosphate receptor from plants

In spite of considerable efforts, these studies have been disappointing and so these experiments have been put on hold for the moment. Schumaker and Sze (1987) had demonstrated an IP_3 -sensitive Ca flux in endomembranes from oat roots, suggesting the presence of an IP_3 -receptor. We wanted to characterize the properties of this IP_3 -sensitive Ca channel by first characterizing [^3H] IP_3 binding activity to endomembranes from oat roots. For reasons we do not understand, the [^3H] IP_3 -binding activity we had detected consistently for about 6 months decreased to nearly undetectable levels. The properties of the binding activity resembled that of IP_3 receptors from animal cells. For example, [^3H] IP_3 binding was specific as it was displaced by excess non-radiolabeled IP_3 , but not by IP_2 or by inositol. IP_3 binding was detected mainly in the low-density membranes which showed a $K_D = 25 \text{ nM}$, and a $B_{\text{max}} = 17 \text{ pmol/mg protein}$. After repeated trials to measure IP_3 binding to either membranes or a crude solubilized fraction, we were still unable to reproduce our previous results. This is apparently not an isolated incident, as D. Sanders has also communicated with us about difficulties in reproducing IP_3 binding to solubilized membrane proteins from red beets (Brosnan & Sanders, 1993).

Efforts towards cloning the gene encoding the IP_3 receptor from plants have also been unsuccessful so far. We intend to come back to this project in the near future if personnel or funding permits.

Summary, Significance and Future Direction

The detailed biochemical studies of Ca-pumping ATPases in carrot endomembranes indicate that plant Ca pumps may differ in their structure and regulation from animal Ca pumps. A calmodulin-stimulated Ca pump is associated with the endomembranes of actively dividing carrot cells. These endomembranes are enriched in a Ca pump of 120 kD. Using Ca-dependent [^{32}P]phosphoenzyme formation as an assay, a 120 kD Ca pump has been characterized as a PM-like Ca pump. We have partially purified a 120 kD Ca ATPase by calmodulin-affinity chromatography; however most of the [^{32}P]phosphoenzyme activity of 120 kD did not bind to the column. Thus, our studies suggest that several Ca pumps with a similar mol mass of ~120 kD exist in plants. As Ca pumps cannot be easily separated using conventional biochemical methods, future studies to understand the structure, function and regulation of each Ca pump will depend on a combination of molecular, biochemical and cell biological approaches. One working model is that distinct Ca pumps could have evolved to serve the particular functions and

needs of plant cells, and to respond to environmental or developmental signals encountered by plants.

Publications (supported by DOE)

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