Plant Physiol. (1994) 104: 1295–1300

A Study of the Effect of Inhibitors of the Animal Sarcoplasmic/Endoplasmic Reticulum-Type Calcium Pumps on the Primary Ca^{2+}-ATPases of Red Beet

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The inhibitor sensitivity of the endoplasmic reticulum (ER) and plasma membrane (PM) calcium pumps of red beet (*Beta vulgaris* L.) were studied by measuring the ATP-driven accumulation of $^{45}Ca^{2+}$ into isolated membrane vesicles. Both transporters were strongly inhibited by 50 μmol m^{-3} erythrosin B, but only by 50% in the presence of 100 mmol m^{-3} vanadate. A number of inhibitors considered to be specific for the sarcoplasmic reticulum (SR)/ER-type calcium pump in animal cells were used to further characterize the PM and ER Ca^{2+}-ATPases in red beet and were compared with their effect on the transport and hydrolytic activities of the PM and tonoplast H^+-ATPases. The hydroquinones 2,5-di(*tert*-butyl)-1,4-benzohydroquinone and 2,5-di(*tert*-amyl)-1,4-benzohydroquinone produced around 20 and 40% inhibition of activity, respectively, of the PM and ER calcium pumps and the PM H^+-ATPase when present at concentrations of 30 mmol m^{-3}. In contrast, the vacuolar proton pump displayed a much higher sensitivity to these two compounds. Nonylphenol appeared to have a general inhibitory effect on all four membrane transport proteins and gave almost complete inhibition when present at a concentration of 100 mmol m^{-3}. Thapsigargin and the structurally related compound trilobolide produced 50% inhibition of both the ER and PM calcium pumps at concentrations of 12.5 and 24 mmol m^{-3}, respectively. The PM and tonoplast proton pumps were also sensitive to these compounds. The ER and PM calcium pumps were almost completely insensitive to cyclopiazonic acid (CPA) up to a concentration of 20 mmol m^{-3}. When present at 100 mmol m^{-3} CPA caused 30% inhibition of the transport properties of all four ATPases. The high concentrations of all of the inhibitors of the SR/ER Ca-ATPase required to inhibit the red beet ER calcium pump, together with the similar effects on the PM calcium pump and the PM and tonoplast proton pumps, suggests that these hydrophobic compounds have a general nonselective action in red beet, possibly through disruption of membrane lipid-protein interactions.

The ER and PM E_1E_2-type calcium pumps of higher plants are responsible, in part, for the regulation of cytosolic calcium concentrations. Since they share a number of biochemical and functional characteristics, it is difficult to discriminate between them (Evans et al., 1991; Thomson et al., 1993). Recently, it has been proposed that CPA, an indole tetramic acid mycotoxin, acts as a specific inhibitor of the carrot ER calcium pump (Hsieh et al., 1991). CPA is a specific inhibitor of the SR/ER Ca^{2+}-ATPase but at concentrations as high as 10 μmol mg^{-1}, it has little effect on the hydrolysis of ATP by the Na^+,K^+-ATPase, the H^+,K^+-ATPase, the mitochondrial F_1-ATPase, and the erythrocyte Ca^{2+}-ATPase (Seidler et al., 1989). CPA is believed to inhibit the conformational transition of the enzyme between the E_1 and E_2 states (Seidler et al., 1989). It prevents the binding of Ca^{2+} to the high-affinity site of the SR calcium pump and also inhibits Ca^{2+}-dependent formation of phosphoenzyme (Goeger and Riley, 1989). The hydrophobic reagents nonylphenol, BHQ, AHQ, thapsigargin, and trilobolide are also potent inhibitors of the SR calcium pump and are thought to interact with the E_2 conformation of the ATPase (Wictome, 1992). However, although the effects of CPA and these other inhibitors have been well characterized in animal systems and are used routinely as specific inhibitors of the SR/ER calcium pump, their effects on plant calcium transporters have not been investigated thoroughly.

The aim of this study was to evaluate the possible specificity of action of these compounds in a higher plant tissue, red beet (*Beta vulgaris* L.) storage roots. If there is sufficient conservation of structure and function between the animal and plant Ca^{2+}-ATPases, the animal SR/ER and plant ER calcium pumps may show selective sensitivity to the same compounds. Most importantly, the inhibitors have not yet been used in the study of the higher plant ER and PM calcium pumps using ER- and PM-enriched fractions, which show almost negligible cross-contamination. The emergence of a marker specific for the plant ER calcium pump, such as inhibitor sensitivity, would facilitate the biochemical characterization, the solubilization and reconstitution, and ultimate purification of the enzyme. Likewise, the use of such inhibitors in vivo may provide greater understanding of the signal transduction pathways involved in physiological responses (Sievers and Busch, 1992), since they could be used to selectively interfere with the ER store of Ca^{2+}.

MATERIALS AND METHODS

Plant Material

Red beet (*Beta vulgaris* L.) roots were obtained either commercially or harvested from the field. The tops of the

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Abbreviations: AHQ,2,5-di(*tert*-amyl)-1,4-benzohydroquinone; BHQ,2,5-di(*tert*-butyl)-1,4-benzohydroquinone; BTP, 1,3-bis[tris-(hydroxymethyl)-methylamino]propane; CPA, cyclopiazonic acid; EB, erythrosin B; PM, plasma membrane; SR, sarcoplasmic reticulum.

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plants were removed and the roots were stored in moist vermiculite at 4°C until use. All root tissue was stored for at least 10 d to ensure uniformity in membrane isolation.

Membrane Isolation

Microsomal fractions, Suc gradient ER- and tonoplast-enriched fractions, and phase-partitioned PM fractions were prepared as previously described, whereas the final ER fraction, used in calcium transport assays, was prepared by subjecting the Suc gradient ER-enriched fraction to two-phase aqueous partitioning and collecting the lower phase (Thomson et al., 1993).

Microsomal Fractions for Proton Transport Measurements

Proton transport activity due to the tonoplast H^+-ATPase was measured using a microsomal fraction prepared as described above. Proton pumping within this fraction showed no vanadate sensitivity (PM H^+-ATPase marker), but was highly sensitive to nitrate (tonoplast H^+-ATPase marker) (see Table I). To measure the proton transport activity due to the PM H^+-ATPase, the microsomal fraction was prepared according to the method of Giannini et al. (1987) with 250 mol m^{-3} KI added fresh to the homogenization medium. Proton pumping in this microsomal fraction was not inhibited by nitrate but was vanadate sensitive (see Table I).

Enzyme and Transport Assays

ATPase Activity

The ATP-hydrolytic activities of the PM and Suc gradient-prepared tonoplast-enriched fractions were assayed as previously described (Williams et al., 1990). The PM H^+-ATPase hydrolytic activity was determined at pH 6.5, whereas the tonoplast H^+-ATPase activity was measured at pH 8.0 in the presence of 1 mol m^{-3} NaN_3 and 100 mol m^{-3} vanadate. Under these conditions ATPase activity within the PM fraction was almost completely inhibited by 100 mol m^{-3} vanadate (Thomson et al., 1993), and for the tonoplast fraction more than 90% of ATPase activity was inhibited by 100 mol m^{-3} KNO_3 when included in the assay medium in the place of KCl (see Table I). All assays were performed in triplicate and the SE values within each experiment were less than 10% of the means. The results shown are the means of at least two experiments. The curve in Figure 2 has been fitted by eye.

Table I. *The effects of various SR/ER-type Ca^{2+}-ATPase inhibitors on the transport properties of the ER and PM calcium pumps, and on the transport and hydrolytic activities of the PM and tonoplast H^+-ATPases in red beet*

Assays were performed as described in "Materials and Methods." ATP-driven uptake into ER and PM fractions was measured over a 10-min incubation period at 22°C, whereas ATPase activity within the PM and tonoplast-enriched fractions was measured over a 1-h incubation period at 37°C. The inhibitors were included at the indicated concentrations. Results are expressed as the percentage of inhibition of control activity measured in the absence of any inhibitor, but in the presence of 1% (v/v) DMSO or methanol, as appropriate.

Inhibitor	PM Ca^{2+} Transport	ER Ca^{2+} Transport	PM ATPase	Tonoplast ATPase	PM H^+ Transport	Tonoplast H^+ Transport
			% Inhibition			
EB						
50 μmol m^{-3}	73	70	6	+11	3	2
Nonylphenol						
25 mmol m^{-3}	54	37	69	79	50	73
100 mmol m^{-3}	95	90	95	n.d.[a]	98	83
BHQ						
30 mmol m^{-3}	9	23	15	60	17	83
AHQ						
30 mmol m^{-3}	42	44	45	92	31	97
Thapsigargin						
0.2 mmol m^{-3}	9	+7	6	22	n.d.	8
30 mmol m^{-3}	48	81	75	88	70	61
Trilobolide						
30 mmol m^{-3}	57	70	20	39	43	50
100 mmol m^{-3}	100	97	67	91	93	97
CPA						
20 mmol m^{-3}	7	3	n.d.	n.d.	n.d.	n.d.
100 mmol m^{-3}	27	36	2	1	32	22
Vanadate						
200 mmol m^{-3}	61	69	95	1	55	0
Nitrate						
100 mol m^{-3}	n.d.	n.d.	0	94	+40	99

[a] n.d., Not determined.

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Proton Transport

Proton transport was determined by measuring quinacrine fluorescence quenching as previously described (Williams and Hall, 1989). For H^+ transport due to the tonoplast ATPase, the assay contained 250 mol m^{-3} sorbitol, 20 mmol m^{-3} quinacrine, 15 mol m^{-3} Hepes-BTP (pH 8.0), 200 mmol m^{-3} sodium orthovanadate, 5 mol m^{-3} $MgSO_4$, 100 mol m^{-3} KCl, 5 mol m^{-3} ATP, and 72 μg of protein. For H^+-transport due to the PM ATPase, the assay was carried out in the absence of vanadate at pH 6.5 and contained KNO_3 instead of KCl. The results shown in Table I are the means taken from at least two experiments and were calculated from the ionophore reversible quench values.

Calcium Transport

Uptake of radiolabeled calcium into the PM and ER fractions was determined as previously described (Thomson et al., 1993) with KNO_3 and nigericin included in the assay. All assays were performed in triplicate and the SE values within each experiment were less than 10% of the means. The results are the means of at least two experiments. The curves in Figure 1 have been fitted by eye.

Protein

Protein was measured with a modified Bradford (1976) procedure using BSA as standard.

Chemicals

Nonylphenol, trilobolide, BHQ, and AHQ were kind gifts from Prof. A.G. Lee (Biochemistry Department, Southampton University, UK). Thapsigargin was obtained from Calbiochem Novabiochem Ltd., (Nottingham, UK). CPA was obtained from Sigma (Dorset, UK). Nonylphenol, AHQ, and BHQ were made up as concentrated stocks in methanol and stored at −20°C. Thapsigargin, trilobolide, and CPA were made up as concentrated stocks in DMSO and stored at −20°C. All reagents were of analytical grade quality.

RESULTS

The procedure for the preparation of the ER fraction has been shown to remove virtually all contaminating PM vesicles, although there is some enrichment of tonoplast vesicles, as shown by marker enzyme analysis (Thomson et al., 1993). By measuring calcium uptake in the presence of 1 mmol m^{-3} nigericin and 100 mol m^{-3} KNO_3 to inhibit operation of the Ca^{2+}/H^+ antiport associated with the vacuolar membrane, it was possible to assay the ER calcium transport mechanism in relative isolation. Inhibitors specific for SR/ER calcium pumps in animals were tested on ATP-driven uptake of ^{45}Ca into the red beet PM and ER fractions (Fig. 1) and on the hydrolytic and proton-transporting activities of the PM H^+-ATPase and the tonoplast H^+-ATPase (Table I).

Nonylphenol appeared to have a general inhibitory effect on the four membrane enzymes assayed, inhibiting transport and hydrolytic activity of the calcium pumps and proton pumps by between 40 and 80% when present at a concentration of 25 mmol m^{-3} (Table I; Fig. 1). Inhibition of all four membrane transport proteins was complete in the presence of 100 mmol m^{-3} nonylphenol (Table I). At 30 mmol m^{-3}, BHQ produced around 20% inhibition of the P-type enzymes studied, i.e. the ER and PM calcium pumps and the PM proton pump (Table I). In contrast it had a marked inhibitory effect on the tonoplast proton pump, inhibiting its hydrolytic activity by 60% and its transport activity by 83% (Table I). AHQ is suggested to be a more potent inhibitor of the SR calcium pump relative to BHQ (Wictome, 1992), and in red beet AHQ produced greater inhibition than BHQ of all four of the plant membrane enzymes assayed (Table I). At 30 mmol m^{-3}, AHQ inhibited activity of the P-type transport

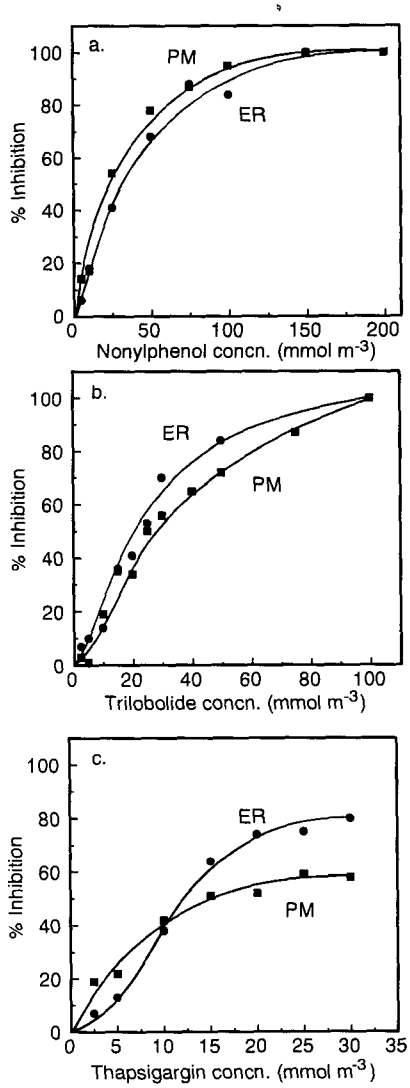

Figure 1. The relative sensitivities of the transport properties of the red beet ER (●) and PM (■) calcium pumps to (a) nonylphenol, (b) trilobolide, and (c) thapsigargin. ATP-driven $^{45}Ca^{2+}$ uptake was measured over a 10-min incubation period at 22°C, as described in "Materials and Methods." The results are expressed as the percentage inhibition of the control ATP-driven uptake measured in the absence of any inhibitor but in the presence of 1% (v/v) DMSO or methanol as appropriate.

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enzymes by around 40%, whereas for BHQ the tonoplast proton pump displayed relatively high sensitivity to AHQ, with more than 90% inhibition of hydrolytic and transport activity (Table I). Over the concentration range used, AHQ is a stronger inhibitor of the vacuolar H^+-ATPase than the PM H^+-ATPase, reflected in the concentrations producing 50% inhibition of ATPase activity, i.e. 3 mmol m^{-3} AHQ for the tonoplast H^+-ATPase compared with 30 mmol m^{-3} AHQ for the PM H^+-ATPase (Fig. 2).

At low concentrations (0.2 mmol m^{-3}), thapsigargin had no marked inhibitory effect on the PM and ER calcium pumps or the PM and tonoplast proton pumps (Table I). However, at the higher concentration of 30 mmol m^{-3}, it was a more potent inhibitor of the transport activity of the ER calcium pump than the PM calcium pump (Table I). Furthermore, it was consistently observed that at concentrations above 15 mmol m^{-3}, the ER calcium pump was the more sensitive, even though both pumps were inhibited 50% by 12.5 mmol m^{-3} thapsigargin (Fig. 1). However, at the high concentration of 30 mmol m^{-3}, thapsigargin produced levels of inhibition of the PM and tonoplast proton pumps of a similar order to that seen with the ER calcium pump (Table I). The structurally related compound trilobolide appeared to have a slightly greater inhibitory effect on the ER and PM calcium pumps than on the PM and tonoplast proton pumps (Table I). The former appeared to show similar sensitivities to this compound, with 24 mmol m^{-3} trilobolide producing 50% inhibition of both systems (Fig. 1).

The calcium pumps on the ER and PM of red beet were almost completely insensitive to CPA up to a concentration of 20 mmol m^{-3} (Table I). However, at 100 mmol m^{-3}, CPA produced around 30% inhibition of the transport activity of all the pumps assayed and the hydrolytic activity of the P-type and V-type H^+-ATPases were unaffected (Table I). This would suggest that at high concentrations, CPA may have a profound effect on membrane integrity.

The vanadate sensitivity of the ER and PM Ca^{2+}-ATPases was very similar over the 0 to 250 mmol m^{-3} range (results not shown), with vanadate producing 50% inhibition of ATP-driven uptake at 100 and 112 mmol m^{-3}, respectively. The ATP hydrolytic activity within the PM fraction was extremely sensitive to vanadate, providing further evidence that this fraction is highly enriched with PM vesicles (Table I). Proton pumping by the microsomal fraction, prepared in the presence of 250 mol m^{-3} KI, displayed 55% inhibition by 200 mmol m^{-3} vanadate and was stimulated by 100 mol m^{-3} nitrate, indicating that the activity assayed in this fraction was largely due to PM H^+-ATPase (Table I). In contrast, proton pumping in the microsomal fraction prepared in the absence of KI was almost completely blocked by 100 mol m^{-3} nitrate and was insensitive to vanadate, indicating that the observed activity was due to the tonoplast H^+-ATPase (Table I).

EB at 50 μmol m^{-3} inhibited ^{45}Ca uptake into the PM and ER by around 70% but had little effect on the hydrolytic and transport activities of the proton pumps of the PM and tonoplast (Table I). The ER and PM calcium pumps appeared to show similar inhibition profiles by EB over the concentration range used (results not shown), both being inhibited 50% by 22 μmol m^{-3} EB.

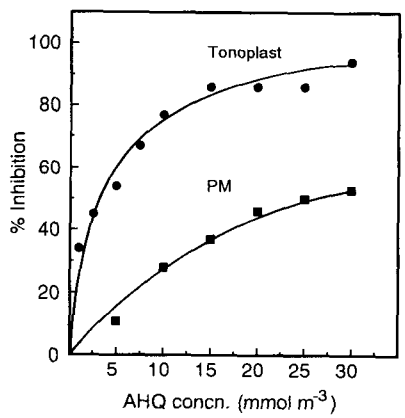

Figure 2. The relative sensitivities of the hydrolytic activities of the tonoplast (●) and PM (■) H^+-ATPases to AHQ. ATPase activity was measured as described in "Materials and Methods" over a 1-h incubation period at 37°C. The results are expressed as the percentage inhibition of the control ATPase activity measured in the absence of AHQ but in the presence of 1% (v/v) methanol.

DISCUSSION

The inhibitors specific for the ER/SR calcium pump in animal cells produce varying effects on the red beet ER Ca^{2+}-ATPase. The concentrations of nonylphenol, thapsigargin, and trilobolide producing 50% inhibition of ATP-driven $^{45}Ca^{2+}$ uptake were 35, 12.5, and 24 mmol m^{-3}, respectively, whereas the highest concentrations of CPA (100 mmol m^{-3}), BHQ (30 mmol m^{-3}), and AHQ (30 mmol m^{-3}) tested produced only about 20 to 30% inhibition. Although this may at the outset imply some structural and functional homology between the animal and plant calcium transporters, the relative concentrations of each compound used to produce inhibition should be noted. For the SR pump the concentrations producing 50% inhibition of activity are: 6 mmol m^{-3} nonylphenol (Michelangeli et al., 1990), 0.25 mmol m^{-3} CPA (Seidler et al., 1989; Mason et al., 1993), 0.035 mmol m^{-3} thapsigargin (Wictome, 1992), 0.04 mmol m^{-3} trilobolide (Wictome, 1992), 0.5 mmol m^{-3} BHQ (Mason et al., 1993), and 0.18 mmol m^{-3} AHQ (Wictome, 1992). Therefore, it is possible that the inhibition of the red beet ER calcium pump seen with high concentrations of these compounds is either nonselective or may reflect a difference in the structure and mechanism of action of this calcium pump. In support of a nonselective mode of action for some of these inhibitors at high concentrations, it was seen that the tonoplast and PM proton pumps were inhibited to approximately the same extent as the ER calcium pump.

The specificity of thapsigargin action in animal cells is believed to be conferred by a recognition site for the inhibitor on all the SR/ER ATPases that is absent from the PM Ca^{2+}-ATPase and other P-type ATPases (Wictome, 1992). It is unlikely that thapsigargin and, by analogy, trilobolide would be able to interfere with the binding and subsequent transport of protons as well as calcium ions. Thapsigargin and CPA have also been shown to prevent the phosphorylation of the

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SR/ER Ca^{2+}-ATPase as part of their mechanism of action (Goeger and Riley, 1989; Wictome et al., 1992). If 100 mmol m^{-3} CPA and 30 mmol m^{-3} thapsigargin, and by analogy trilobolide, are having similar effects on higher plant ion-transporting ATPases, the high inhibition of the tonoplast proton pump, which does not form a phosphorylated intermediate, would not be expected. The general nonselective action at high concentrations of thapsigargin and trilobolide are likely to be due to the highly hydrophobic nature of these compounds. Such compounds may partition selectively into the phospholipid component of membranes and/or directly interact with the hydrophobic domains of membrane proteins, thereby affecting lipid-protein interactions and general ATPase activity. The difference in apparent sensitivities of the ER and PM calcium pumps to the higher concentrations of thapsigargin may be related to differences in the hydrophobicity of the two types of membranes.

In the present study BHQ produced a low, nonselective inhibition of the P-type ATPases but had a much greater effect on the V-type ATPase. AHQ, being more hydrophobic in character, produced greater inhibition than BHQ of all the transport enzymes studied, and as for BHQ, AHQ appeared to be a fairly specific inhibitor of the vacuolar H^+-ATPase, with 3 mmol m^{-3} AHQ producing 50% inhibition. This V-type proton pump is a large multimeric structure composed of a peripheral hydrophilic catalytic complex and an integral proton channel (Hall and Williams, 1991). The hydrophobic nature of AHQ and BHQ may allow their nonselective interaction with hydrophobic portions of the enzyme or with membrane phospholipids, thereby disrupting the close association between the subunits required for ATP hydrolysis and proton transport, resulting in marked inhibition of enzyme activity.

In a study of primary Ca^{2+}-ATPases in cultured carrot cells, Hsieh et al. (1991) suggested that the calcium pump present on the ER is sensitive to CPA, with 100 nmol mg^{-1} CPA producing 30 to 57% inhibition of transport activity. This inhibition was described as specific since the transport of calcium by a calmodulin-stimulated calcium pump in the same fraction, suggested to be associated with the PM, was insensitive to such levels of CPA (Hsieh et al., 1991). Work on red beet membrane fractions has shown that CPA, at the equivalent concentration of 2 mmol m^{-3}, had no effect on the transport properties (present study) or the formation of phosphorylated intermediates (Thomson et al., 1993) of either the ER or PM calcium pump. This may reflect species differences in the CPA sensitivity of the ER calcium transporter, but further work in other species using vesicles with reduced cross-contamination is necessary to confirm this. In addition, as suggested by the present study, CPA seems to have nonselective effects on membrane permeability. This is seen in the apparent lack of effect of the mycotoxin, at 100 mmol m^{-3}, on the hydrolytic activities of the PM and vacuolar H^+-ATPases, which were assayed in the presence of permeabilizing concentrations of the detergent Triton X-100. If this is equally applicable to carrot cells, this might explain the apparent inhibition of Ca^{2+} transport by CPA and the absence of an effect on phosphoenzyme formation (Chen et al., 1993). In light of the results described in this present paper, the inhibition of the gravitropic response of cress roots by the high concentration of 20 mmol m^{-3} CPA (Sievers and Busch, 1992) may not necessarily provide further evidence for the involvement of the ER calcium pump in the transduction of the gravity stimulus. High concentrations of CPA may have profound effects on the permeability of membrane vesicles to ions, which may be equally applicable to membranes in intact plant organs, such as cress roots. Since the CPA sensitivity of the PM and ER calcium pumps in higher plants has not been previously compared, it is perhaps premature to assume significant conservation between plant and animal calcium transporters until further studies are carried out.

EB appears to be a useful tool for discriminating between calcium and proton pumps. The activity of both the PM and ER calcium pump was strongly inhibited by 50 μmol m^{-3} EB, whereas there was no marked effect on the transport and hydrolytic activity of the tonoplast and PM proton pumps. EB may also be used for distinguishing primary calcium pumps (i.e. Ca^{2+}-ATPases) from secondary calcium transport mechanisms (e.g. Ca^{2+}/H^+ antiports) when used at nanomolar concentrations (Thomson et al., 1993). The concentration dependence of inhibition of the ER calcium pump by EB has not been investigated in detail for other plants; however, our results indicate that the red beet ER calcium pump is as sensitive as the PM calcium pump to this inhibitor.

In conclusion, the selection of animal ER/SR-specific calcium pump inhibitors used in the present study of red beet were unable to distinguish clearly between the ER and PM Ca^{2+}-ATPases and were suggested to have only a general inhibitory effect when present at relatively high concentrations. The stoichiometric interaction of these inhibitors with the ER calcium pump protein may perhaps become apparent only upon solubilization and reconstitution of the transport enzyme. However, it should be noted that the ER-associated calmodulin-stimulated calcium pump from *Brassica oleracea* L. showed very low sensitivity to CPA and thapsigargin, even after solubilization and reconstitution (Askerlund and Evans, 1992). Recent evidence suggests that the higher plant ER calcium pump has the potential to be regulated by calmodulin (Hsieh et al., 1991; Askerlund and Evans, 1992; Chen et al., 1993). The presence of a calmodulin-binding domain on the ER calcium pump might imply higher structural similarity to the erythrocyte Ca^{2+}-ATPase than to the SR Ca^{2+}-ATPase, and may explain, in part, the lack of selective inhibition of transport activity of the red beet ER calcium pump by specific inhibitors of animal SR/ER-type calcium pumps.

ACKNOWLEDGMENTS

We wish to thank Prof. A.G. Lee, University of Southampton, for the kind gifts of nonylphenol, trilobolide, BHQ, and AHQ. We would also like to thank Dr. M. Wictome, University of Southampton, for advice concerning the handling and use of the inhibitors. We thank Prof. A.G. Lee, and Dr. D.E. Evans, Oxford Brooks University, for critical reading of the manuscript.

Received August 16, 1993; accepted December 21, 1993.
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