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The effect of calmodulin and far-red light on the kinetic properties of the mitochondrial and microsomal calcium-ion transport system from corn

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Abstract. The kinetic properties of active Ca^{2+} transport into mitochondria and microsomal membrane vesicles prepared from coleoptiles of dark- and light-grown corn seedlings have been studied. The apparent values for K_m and V_{max} for Ca^{2+} of the mitochondrial transport system from dark-grown plants are about one order of magnitude higher than those from the microsomal transport system. Calmodulin has no effect on the Ca^{2+} accumulation into mitochondria whereas the apparent maximum transport velocity and affinity for Ca^{2+} of the microsomal Ca^{2+}-transport system are both increased by calmodulin. When intact corn seedlings are irradiated with far-red light, the calmodulin-induced increase of the apparent maximum transport velocity and affinity for Ca^{2+} can no longer be observed. From these data it can be concluded that the low cytoplasmic Ca^{2+} concentration in the cytoplasm of coleoptile cells from dark-grown corn is maintained by a calmodulin-regulated Ca^{2+} pump. Irradiation with photomorphogenically active far-red light lowers the Ca^{2+}-transport activity and thus causes an increase of the cytoplasmic, free-Ca^{2+} concentration. The physiological implications will be discussed.

Key words: Calcium uptake – Calmodulin – Light, far-red – Microsome – Mitochondrion – *Zea* (calcium transport).

Introduction

Many enzymatic and physiological processes in plant cells are under the regulatory control of Ca^{2+} (Marmé 1982; Marmé and Dieter 1983). As in animal cells, in many cases calmodulin mediates the Ca^{2+}-dependent regulation supporting the hypothesis for Ca^{2+} being a second messenger in higher plants (Marmé and Dieter 1983). It has been reported previously that the control of cytoplasmic free Ca^{2+} in plant cells is achieved by accumulation of Ca^{2+} into intracellular organelles like mitochondria (Hodges and Hanson 1965; Dieter and Marmé 1980a), vacuoles and endoplasmic reticulum (Gross 1982) and by extrusion of Ca^{2+} through the plasma membrane (Gross and Marmé 1978; Dieter and Marmé 1980a). This extrusion out of the cell is driven by a plasma-membrane-located Ca^{2+}-transport ATPase which can be stimulated by calmodulin (Dieter and Marmé 1980b; Dieter and Marmé 1981a). This Ca^{2+}-transport ATPase is comparable to the Ca^{2+} pump in the plasma membrane of the red blood cell (Vincenzi and Hinds 1980).

The cytoplasmic concentration of the second messenger, Ca^{2+}, is affected by appropriate extracellular signals. In animal cells, such signals (e.g. hormones and neurotransmitters) exert their actions mainly by increasing the permeability of the plasma membrane to Ca^{2+} or by releasing Ca^{2+} from the membrane-bound state (Rasmussen 1981). Normally the Ca^{2+} pump is not affected. It is reported only for diseases like cystic fibrosis, muscular dystrophy and sickle-cell anemia that the properties of the active transport systems for Ca^{2+} are changed (Vincenzi and Hinds 1980). Preliminary data show that in contrast to animals, in plant cells extracellular signals affect the active transport systems. Dieter and Marmé (1981b) showed that the active accumulation of Ca^{2+} into mitochondria as well as the calmodulin-dependent microsomal Ca^{2+}-transport activity are inhibited when the intact plant seedlings were irradiated with far-red light. Incubation of plant segments with plant hormones like auxin, zeatin and kinetin also alter the activity of the microsomal plant Ca^{2+}-transport system (Kubowicz et al. 1982). Recently Oláh et al.

(1983) demonstrated that the affinity of a Ca^{2+} ATPase towards Ca^{2+} and calmodulin increases when the plant seedlings were treated with the synthetic cytokinin benzylaminopurine. These findings indicate that in plant cells, signals like light and hormones exert their action primarily by changing the properties of the active Ca^{2+}-transport systems.

In this paper we present evidence which indicates that the Ca^{2+}-transport ATPase, probably located in the plasma membrane, is mainly responsible for the maintenance of the low cytoplasmic Ca^{2+} concentration in the plant cell. We report further on the effect of calmodulin and far-red light on the kinetic properties of the mitochondrial and the microsomal Ca^{2+}-transport systems prepared from coleoptiles of either dark- or far-red-light-grown corn seedlings.

Material and methods

Chemicals. Na_2ATP was obtained from Pharma Waldhof, Düsseldorf, FRG; 2-(N-morpholino)propane sulfonic acid (Mops) and 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) were purchased from Roth, Karlsruhe, FRG; pyruvate, succinate, bovine serum albumin and sodium azide were obtained from Serva, Heidelberg, FRG; ethylene diaminetetraacetic acid (EDTA), potassium phosphate and $MgCl_2$ were purchased from Merck, Darmstadt, FRG; $^{45}CaCl_2$ was obtained from the Radiochemical Centre, Amersham, UK.

Preparation of mitochondrial and microsomal fractions. Corn (*Zea mays* L., Inracorn Hybrid, Categorie 5A, 3070, from Hambrecht, Freiburg, FRG) was grown on vermiculite at 25° C in total darkness or in far-red light (Schäfer 1977) for 5.5 d. All manipulations were carried out at 4° C. Coleoptiles were removed from the plants and suspended at a ratio of 0.5 g fresh weight per ml in buffer A (25 mM Mops titrated with 25 mM Tris to pH 7.5, containing 5 mM EDTA and 10% (w/w) sucrose). The material was chopped with razor blades, ground with mortar and pestle and filtered through a single layer of nylon cloth. The obtained homogenate was centrifuged 1,500 *g* for 15 min, the pellet was discarded and the supernatant centrifuged for another 15 min at 6,000 *g*. The pellet was resuspended in buffer B (buffer A without EDTA) and called the mitochondrial fraction. The remaining supernatant was centrifuged for 20 min at 50,000 *g*, the resulting pellet resuspended in buffer B and called the microsomal fraction.

Ca^{2+}-transport experiments. The incubation medium for the mitochondrial transport assay consisted of buffer B with 5 mM potassium phosphate, 4 mM succinate and 4 mM pyruvate as energy sources, 250 Bq of $^{45}CaCl_2$ per ml, various amounts of $CaCl_2$ (12.5 µM–400 µM) and, per ml, 50 µg protein of the mitochondrial fractions. For the microsomal transport assay, the incubation medium contained 5 mM $MgCl_2$, 3 mM NaN_3, 1 mM ATP as energy source, 250 Bq of $^{45}CaCl_2$ per ml, various amounts of $CaCl_2$ (1 µM–200 µM) and, per ml, 200 µg protein of the microsomal fraction. For the determination of the calmodulin-dependent microsomal Ca^{2+} uptake, 1 µM of bovine brain calmodulin was added. The uptake assays were carried out at 25° C. Uptake was started by adding succinate and pyruvate for the mitochondrial assay medium and ATP for the microsomal Ca^{2+}-transport medium to samples which have been preincubated for 2 min at 25° C. At different times, aliquots of 1 ml were filtered through cellulose-nitrate filters (0.45 µm pore size, Sartorius SM 11306, Göttingen, FRG) and washed with 5 ml of buffer B. The radioactivity retained on the filters was determined in 10 ml Bray's solution in a liquid scintillation counter. All experiments were verified by repetitions. Variations within experiments were less than 10%.

The concentration of the free calcium in the Ca^{2+}-transport assays was calculated by a computer program using the dissociation constants for the complexes Ca·ATP, Mg·ATP, Ca·phosphate, Ca·pyruvic acid, Ca·succinic acid and Ca·calmodulin, Mg·calmodulin according to Sullivan (1969) and Scharff (1981).

Other methods. Proteins were determined according to Spector (1978) with bovine serum albumin as a standard. Calmodulin from bovine brain was purified to homogeneity as described by Maruta et al. (1983). The amount of calmodulin was determined by weight.

Results and discussion

It has been shown recently that the Ca^{2+} uptake into a plasma-membrane-enriched fraction isolated from dark-grown zucchini hypocotyls is stimulated by the addition of calmodulin from either plants or animals (Dieter and Marmé 1980b). This stimulation could be totally overcome by the antipsychotic drug fluphenazine which is known to be an inhibitor of calmodulin-dependent reactions (Levin and Weiss 1979). It has also been shown that the Ca^{2+} accumulation into mitochondria from dark-grown zucchini hypocotyls is not affected by calmodulin (Dieter and Marmé 1980b). Table 1 shows that calmodulin and fluphenazine have comparable effects on the Ca^{2+} uptake into microsomes isolated from coleoptiles of dark-grown corn seedlings. The Ca^{2+} accumulation into mito-

Table 1. Effect of calmodulin and fluphenazine on the active Ca^{2+} uptake into mitochondria and microsomal vesicles prepared from coleoptiles of dark-grown corn seedlings

Addition	Ca^{2+} net uptake, nmol mg^{-1} protein[a]	
	Mitochondria	Microsomes
None	480	7.9
Calmodulin (1 µM)	473	18.2
Fluphenazine (20 µM)	560	7.2
Calmodulin (1 µM) + Fluphenazine (20 µM)	570	7.9

[a] Ca^{2+} net uptake was determined after 15 min and 30 min of incubation for mitochondria and microsomes, respectively. The free Ca^{2+} concentration was 107 µM for mitochondria and 9.5 µM for microsomes

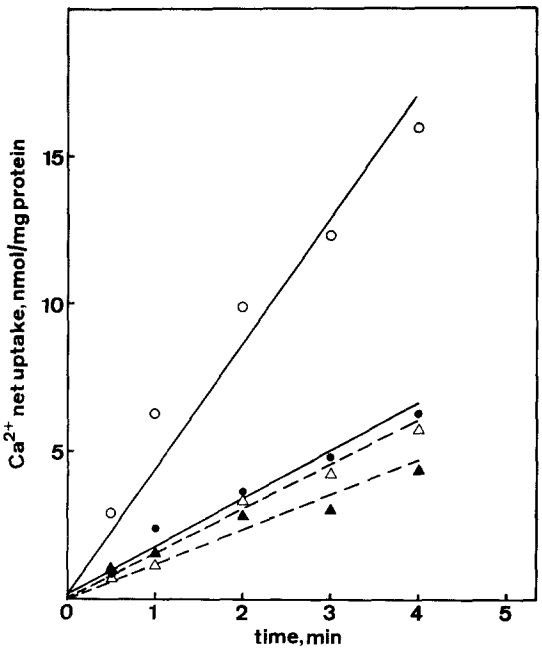

Fig. 1. Kinetics of Ca^{2+} uptake in the absence (*closed symbols*) and presence of 1 μM calmodulin (*open symbols*) into microsomal vesicles isolated from coleoptiles of dark- (*circles, solid lines*) and far-red-light-grown corn seedlings (*triangles, dotted lines*). The Ca^{2+} uptake was determined at a Ca^{2+} concentration of 1 μM

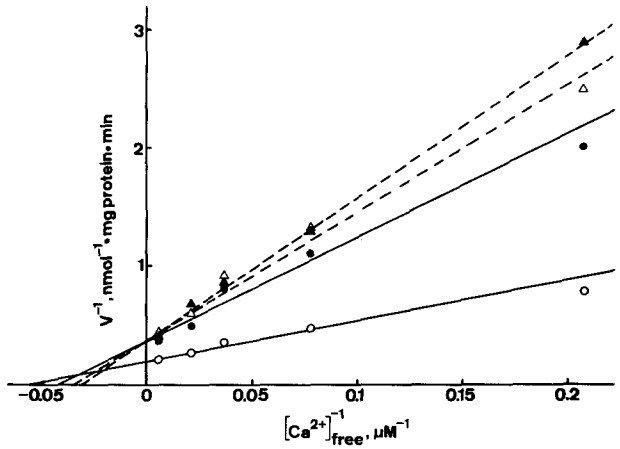

Fig. 2. Lineweaver-Burk representation of the Ca^{2+}-uptake rates into microsomal vesicles in the absence (*closed symbols*) and presence of 1 μM calmodulin (*open symbols*) from coleoptiles of dark- (*circles, solid lines*) and dark-red-light-grown corn seedlings (*triangles, dotted lines*)

chondria is not affected by calmodulin. Addition of fluphenazine slightly increases the mitochondrial Ca^{2+} uptake in the absence and presence of calmodulin. This indicates that this effect of fluphenazine is not the result of an interaction with calmodulin. As already demonstrated (Dieter and Marmé 1980a), it can be seen also in Table 1 that mitochondria possess a much higher specific Ca^{2+}-uptake capacity than microsomes. In contrast to the mitochondrial Ca^{2+} uptake the Ca^{2+} uptake in the microsomal fraction is appreciably increased by calmodulin. Fluphenazine completely inhibits the calmodulin-dependent Ca^{2+} uptake whereas the antipsychotic drug has no effect on the microsomal Ca^{2+} uptake in the absence of calmodulin. It has been shown by Dieter and Marmé (1981b) that irradiation of intact corn seedlings with far-red light leads to a loss of the mitochondrial and of the calmodulin-dependent microsomal Ca^{2+} uptake. The microsomal Ca^{2+} uptake in the absence of calmodulin was not appreciably altered.

In order to investigate the effect of calmodulin and light on the kinetic parameters K_m and V_{max} of the microsomal Ca^{2+}-transport system, microsomal vesicles isolated from coleoptiles of dark- and far-red-light-grown corn seedlings were incubated at different Ca^{2+} concentrations in the absence and presence of calmodulin. Figure 1 shows

Table 2. Effect of calmodulin on the kinetic parameters of the microsomal Ca^{2+} transport system from coleoptiles of dark- and far-red-light-grown corn seedlings. CaM, calmodulin

	K_m (μM)		V_{max} (nmol mg^{-1} protein min^{-1})	
	−CaM	+CaM	−CaM	+CaM
Dark grown	22	15	2.7	5.2
Far-red-light grown	27	26	2.3	2.8

the corresponding Ca^{2+}-uptake kinetics at a Ca^{2+} concentration of 1 μM. The Ca^{2+} uptake increases linearly with time for 4 min in the absence as well as in the presence of calmodulin. The Ca^{2+}-uptake kinetics were also linear when the microsomes were incubated at Ca^{2+} concentrations of 5, 12.5, 25, 50, 200 μM Ca^{2+} (data not shown). For Lineweaver-Burk analysis, the uptake rates at these Ca^{2+} concentrations were determined by the slope of the calculated regression lines. As is shown in Fig. 2, the double-reciprocal plots of the Ca^{2+}-uptake rates versus the concentration of free Ca^{2+} are linear for the calmodulin-dependent and calmodulin-independent uptake into microsomal vesicles isolated from coleoptiles of dark- as well as from the far-red-light-grown corn seedlings. The apparent values for K_m and V_{max} calculated from the corresponding regression lines are shown in Table 2. The apparent affinity for Ca^{2+} and the maximum transport velocity of the microsomal transport system from coleoptiles of dark-grown corn seedlings are both increased by calmodulin

(Table 2, first line). This result is consistent with the effect of calmodulin on the kinetic parameters of the Ca^{2+}-transport system in red blood cells where also an increase of V_{max} and the affinity for Ca^{2+} has been observed (Vincenzi and Hinds 1980). Far-red-light irradiation causes a slight decrease of the affinity for Ca^{2+} and of the maximum transport velocity of the plant microsomal transport system (Table 2, second line). However, the effect of calmodulin on the K_m and V_{max} is strongly reduced by the light irradiation. As it has been discussed elsewhere (Dieter and Marmé 1980a), the Ca^{2+}-accumulating vesicles in the microsomal fraction are probably inside-out vesicles derived from the plasma membrane. The far-red-light-induced decrease of the Ca^{2+}-transport rate as well as of the Ca^{2+} affinity of this transport system could therefore cause an increase of the cytoplasmic Ca^{2+} concentration and thus a modulation of Ca^{2+}, calmodulin-dependent enzymes as has been suggested for the NAD kinase (Marmé and Dieter 1983).

It has been reported that mitochondrial Ca^{2+} uptake exceeds by far that found with microsomal membrane vesicles obtained by plants or animals (Table 1; Bygrave 1978a; Dieter and Marmé 1980a). Based on the high Ca^{2+}-uptake capacity of mitochondria, a central role had been proposed for this organelle in cellular Ca^{2+} metabolism (Bygrave 1978b). However, the investigation of the affinity towards Ca^{2+} of the mitochondrial transport system from animals revealed that mitochondria probably play no crucial role in the maintenance of the low cytoplasmic Ca^{2+} concentration of an unstimulated animal cell (Rasmussen and Gustin 1978). The role of mitochondria in plant cellular Ca^{2+} metabolism is even more unclear. As shown in Table 1, mitochondria isolated from coleoptiles of dark-grown corn seedlings accumulate high amounts of Ca^{2+}. In order to investigate the kinetic parameters K_m and V_{max} of the plant mitochondrial Ca^{2+}-transport system, the Ca^{2+}-uptake rate of mitochondria isolated from coleoptiles of dark-grown corn seedlings was determined at different Ca^{2+} concentrations. In Fig. 3, the double-reciprocal plot of the mitochondrial Ca^{2+}-uptake rate versus the concentration of free Ca^{2+} is shown. The calculated values for K_m and V_{max} are 250 µM and 63 nmol mg^{-1} protein $\cdot min^{-1}$, respectively. The comparison with the respective kinetic constants of the microsomal Ca^{2+}-transport system (Table 2) reveals that the Ca^{2+}-transport system of mitochondria has at least a tenfold lower affinity for Ca^{2+} than that of microsomes. However, the maximum transport velocity of mitochondria is about 10- to 20-fold higher. This corresponds to the data which were obtained from animals (Rasmussen and Gustin 1978).

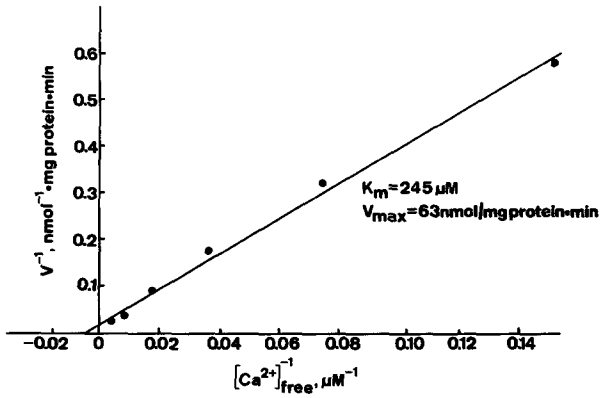

Fig. 3. Lineweaver-Burk representation of the Ca^{2+}-uptake rates of mitochondria from coleptiles of dark-grown corn seedlings

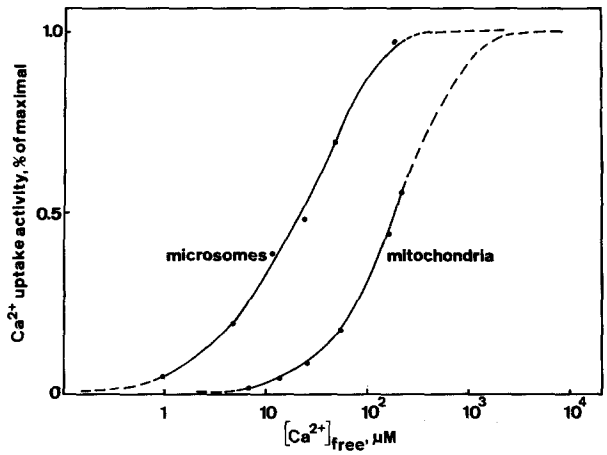

Fig. 4. Mitochondrial and microsomal Ca^{2+}-transport activity as a function of the free Ca^{2+} concentration. The values of the Ca^{2+}-uptake activity were determined by the ratio $V_{[Ca^{2+}]_{free}}/V_{max}$ for each free Ca^{2+} concentration

When the transport activities of mitochondria and microsomes are plotted as a function of the free Ca^{2+} concentration, it becomes obvious that the mitochondrial Ca^{2+} pump only operates at much higher free Ca^{2+} concentrations compared with the microsomal Ca^{2+} pump (Fig. 4). Therefore, one may assume that, as in animal cells, the low Ca^{2+} concentration in the unstimulated plant cell is maintained by the microsomal Ca^{2+} pump which has been proposed to be located probably in the plasma membrane (Dieter and Marmé 1980a). Conversely, at higher cytoplasmic Ca^{2+} concentrations the mitochondrial Ca^{2+} pump may become the major determinant of the cellular Ca^{2+} concentration. From the data presented in Fig. 4, it can be estimated that the free cytoplasmic calci-

um of an unstimulated plant cell which is controlled by the microsomal Ca^{2+} pump is in the range of about 1 to 20 μM. This is one order of magnitude higher than the corresponding estimated control range in animal cells (Rasmussen and Gustin 1978). The reason for this discrepancy may be the different experimental conditions used for Ca^{2+}-uptake measurements. We have performed our experiments with an unbuffered calcium solution whereas the kinetic parameters of the animal transport systems were determined using an ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA)-buffer system and it is reported by Waisman et al. (1981) that the apparent K_m is increased about 30-fold when an unbuffered Ca^{2+} solution is used instead of a EGTA-Ca^{2+} buffer. The determination of the kinetic constants for the mitochondrial and microsomal Ca^{2+}-transport systems from plants using an EGTA-Ca^{2+} buffer will provide more information on this question.

The data presented in this paper indicate that the concentration of free Ca^{2+} in the cytoplasm of a plant cell can be maintained at a low level by a calmodulin-dependent Ca^{2+} pump, probably located in the plasma membrane. Extracellular signals (e.g. far-red light) are able to enhance the cytoplasmic free Ca^{2+} concentration by changing the kinetic properties of this calmodulin-dependent Ca^{2+}-transport system. The free Ca^{2+} binds to calmodulin and thus activates Ca^{2+}, calmodulin-dependent enzymes like NAD kinase (Marmé 1982; Marmé and Dieter 1983). From these data it becomes apparent that Ca^{2+} fulfills all the criteria for being a second messenger in plants. The molecular mechanism of the effect of far-red light on the microsomal Ca^{2+}-transport system is not yet clear. Further research will determine whether light is effective at the transcriptional or translational level.

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