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Characteristics of Ca^{2+} transport by corn mitochondria

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Mitochondria isolated from corn (*Zea mays* L.) coleoptiles by an improved procedure which yields functionally intact preparations are much more active in respiration-coupled Ca^{2+} accumulation than those employed in most earlier studies. Ca^{2+} uptake by these mitochondria is phosphate-dependent and is accompanied by decrease in $\Delta\psi$, H^+ extrusion and increase in the rate of respiration. A sigmoidal plot with a Hill coefficient of 2.22 was obtained when the rates of Ca^{2+} uptake were plotted as a function of free Ca^{2+} concentration. The $K_{0.5}$ for Ca^{2+} influx was about 31 μM and a V_{max} of 140 nmol Ca^{2+} per min per mg was attained at a free-Ca^{2+} concentration of about 120 μM. Ca^{2+} uptake is sensitive to inhibition by ruthenium red and Mg^{2+}. The external free-Ca^{2+} concentration maintained at steady state was about 2 μM and was independent of the respiratory substrate and of external Na^+, but was increased by exogenous Mg^{2+}. In addition, this preparation of corn mitochondria has shown a much higher ability for Ca^{2+} retention in the presence of phosphate and NAD(P)H oxidants than liver mitochondria.

Introduction

Most of the animal mitochondria possess a very active Ca^{2+}-transport system which is believed to participate in the regulation of Ca^{2+} distribution within the cell by regulating either the cytosolic or the matrix Ca^{2+} concentration (cf. Refs. 1–4). The Ca^{2+} distribution between the matrix and extramitochondrial compartments in steady state is kinetically regulated by the simultaneous operation of two distinct pathways for Ca^{2+} influx and efflux (cf. Refs. 1–4). Ca^{2+} enters energized mitochondria by an electrophoretic uniport in a mechanism associated to a stoichiometric increase in oxygen consumption, proton extrusion and depolarization of the membrane potential [5,6]. Ca^{2+} efflux appears to occur via a $Ca^{2+}/2Na^+$ exchange in excitable tissues and a $Ca^{2+}/2H^+$ exchange in liver and other non-excitable tissues (cf. Refs. 1–4).

It has been shown that Ca^{2+} transport by plant mitochondria varies in many aspects between different plant species [7,8] and between different tissues and ages in the same species [8]. The data available also show that Ca^{2+} transport in plant mitochondria operates differently from that of animal mitochondria (cf. Ref. 9). For instance, it is claimed that Ca^{2+} uptake by plant mitochondria presents the following characteristics: (a) it is not associated to respiratory stimulation and depolari-

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; TPP^+, tetraphenylphosphonium; $\Delta\psi$, transmembrane electrical potential; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; P_i, phosphate; *t*-BuOOH, *t*-butylhydroperoxide.

zation of membrane potential; (b) it is substrate-dependent; (c) it has an absolute requirement for inorganic phosphate and; (d) it is insensitive to ruthenium red and Mg^{2+}, in at least some species.

In addition, the high K_m [10,11] and the low initial rate [10,12] of Ca^{2+} uptake has led to the conclusion that it is unlikely that mitochondria play an important role in the regulation of cytosolic Ca^{2+} in higher plants [9,10].

Recently, we have developed a procedure which allowed the isolation of coupled preparations of corn mitochondria showing the ability to accumulate and buffer external free Ca^{2+} at a concentration of about 2 μM [13]. This paper describes further properties of Ca^{2+} transport by this preparation of corn mitochondria.

A preliminary report of some of these findings has been presented [14].

Materials and Methods

Corn (*Zea mays* var. Maya/Normal from the corn germplasm bank of the Department of Genetics, I.B., UNICAMP) mitochondria were isolated from 3-days-old coleoptiles including primary leaves from plants germinated on moist filter paper at 28°C in the dark as described previously [13]. Rat liver mitochondria were prepared as in Ref. 15. The protein was determined according to Kies and Murphy [16]. The mitochondrial suspension was incubated at 30°C in the following basic medium: 300 mM mannitol/20 mM KCl/0.1% bovine serum albumin/2 mM Hepes buffer (pH 7.2). Other additions are specified in the legends of the figures. Changes in H^+ and oxygen concentrations in the suspending medium were followed using a pH sensitive combination glass electrode and a Clark oxygen electrode (Yellow Springs Instruments Co.), respectively. The outputs of the oxygen and H^+ electrodes were registered with a dual-channel recorder (Linear, model 1202). Changes in free-Ca^{2+} concentration in the suspending medium were followed using a Ca^{2+}-selective electrode (Radiometer, F2112 Calcium electrode), calibrated by the addition of Ca^{2+}/EGTA buffers to the reaction medium alone [17]. The transmembrane electrical potential ($\Delta\psi$) was measured with a TPP^+-electrode prepared in our laboratory [18,19].

Results

Stimulation of succinate oxidation in corn mitochondria by ADP and Ca^{2+}

In a previous communication we have shown that respiration-coupled Ca^{2+} uptake by corn mitochondria was parallelled by a decrease in the membrane potential [13], indicating that in spite of the phosphate dependence for Ca^{2+} uptake [12,20] the cation enters electrophoretically carrying at least one positive charge. Other events, associated to electrophoretic Ca^{2+} accumulation by corn mitochondria, such as stimulation of oxygen consumption and proton extrusion, were analysed in the experiment shown in Fig. 1. The traces from the O_2, H^+ and Ca^{2+} electrodes are

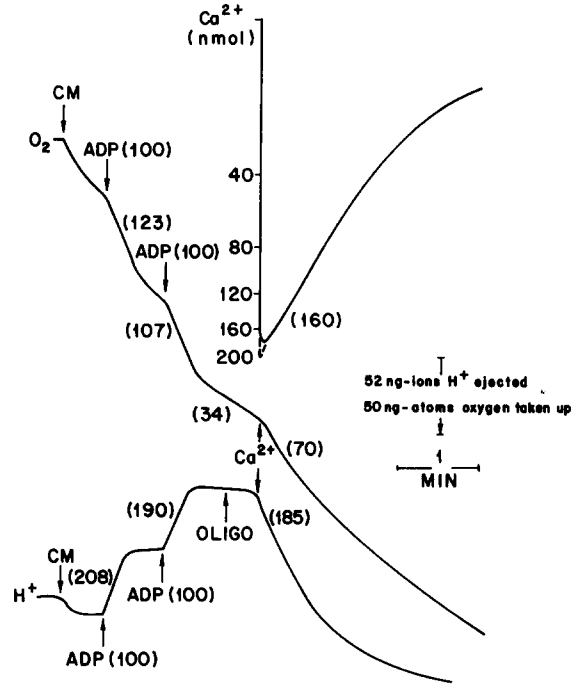

Fig. 1. Alterations in the rates of O_2 consumption and pH of the reaction medium caused by the additions of ADP and Ca^{2+}. Corn mitochondria (0.5 mg) were added to 1 ml of reaction medium containing the basic components (described in Materials and Methods) plus 5 mM succinate, 5 mM phosphate and 5 μM rotenone. Oligomycin (2 μg per mg protein), ADP (100 nmol) and Ca^{2+} (200 nmol) were added where indicated. The numbers in parenthesis refer to the velocities of O_2 consumption (ng atom O per min per mg), H^+ uptake or extrusion (nmol H^+ per min per mg) or Ca^{2+} uptake (nmol Ca^{2+} per min per mg). CM, corn mitochondria, OLIGO, oligomycin.

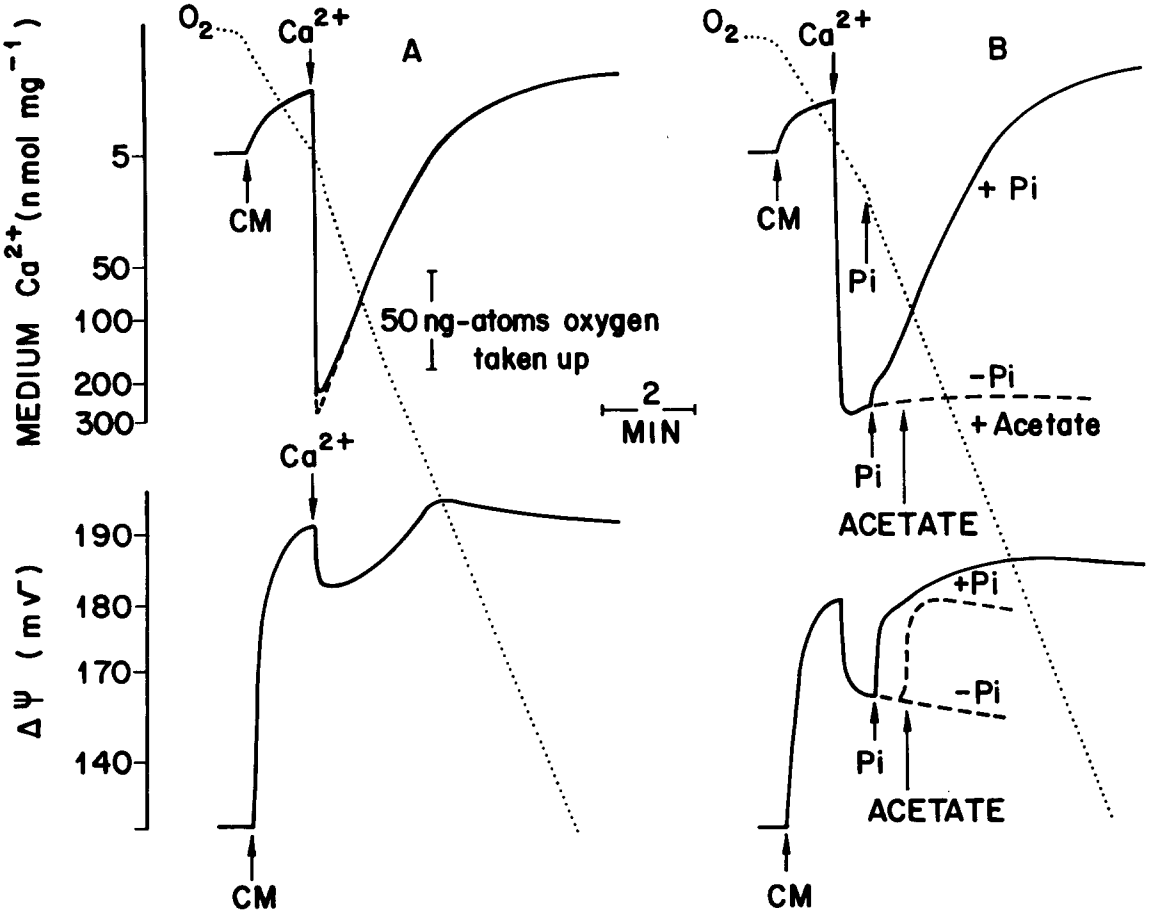

Fig. 2. Alteration in $\Delta\psi$ during Ca^{2+} uptake by corn mitochondria (CM): dependence of phosphate. Mitochondria (1 mg) were added to 1 ml of the basic medium containing 5 mM succinate, 5 μM rotenone and 3 μM TPP^+. (A) Phosphate (1 mM) was present in the reaction medium. (B) Phosphate (1 mM), Ca^{2+} (300 nmol) and acetate (10 mM) were added where indicated.

displayed together. It can be seen that addition of ADP induced an about 3-fold increase in the rate of oxygen consumption accompanied by uptake of H^+. The ADP/O ratio (nmol of ADP added/ng atom of oxygen consumed) was about 1.6 calculated from total oxygen uptake. Subsequent addition of Ca^{2+} evoked another cycle of respiratory stimulation with a respiratory control of 2; during respiratory stimulation by Ca^{2+} a proton efflux was also observed. From the initial steady-state rates of the three respiration-coupled processes an H^+/Ca^{2+} and a Ca^{2+}/O ratio of 1.1 and 2.3, respectively, were observed.

Correlation between Ca^{2+} uptake and $\Delta\psi$ in corn mitochondria: effect of phosphate and acetate

It has been proposed that there is an absolute requirement for phosphate in the mechanism of Ca^{2+} uptake by plant mitochondria [12,20]. This was studied in our preparation of corn mitochondria (Fig. 2) in connection with the alterations in $\Delta\psi$ and respiration caused by Ca^{2+} uptake in presence (A) and absence of phosphate (B). The traces from the oxygen, Ca^{2+} and TPP^+ electrodes are displayed together in Fig. 2. Panel A shows that introduction of mitochondria in the reaction medium containing 1 mM phosphate was followed by the development of a membrane potential of about 190 mV and the uptake of the contaminant Ca^{2+} in the medium. Addition of external Ca^{2+} (300 nmol $\cdot$ mg^{-1}) resulted in its complete uptake parallelled by a small decrease in $\Delta\psi$ and an increase in the rate of respiration. In the absence of exogenous phosphate (Fig. 2B) the $\Delta\psi$ attained

was slightly smaller (180 mV). The addition of Ca^{2+} (300 nmol · mg^{-1}) caused a decrease in $\Delta\psi$, but Ca^{2+} was not taken up as in the presence of phosphate. When phosphate was added later a biphasic increase in $\Delta\psi$ occurred, followed by an increase in the rate of respiration and Ca^{2+} accumulation. The first phase of the $\Delta\psi$ increase was very rapid and preceded the accumulation of Ca^{2+}, and certainly reflected a small decrease in ΔpH caused by phosphate entry. When acetate instead of phosphate was added a similar increase in $\Delta\psi$ corresponding to the first phase obtained when phosphate was used occurred, but no Ca^{2+} uptake was observed. Ca^{2+} accumulation also did not take place when thiosulfate or β-hydroxybutyrate were used as permeant anions or when phosphate transport was inhibited by mersalyl (not shown).

Dependence of the rate of Ca^{2+} uptake on the extramitochondrial Ca^{2+} concentration

The use of a Ca^{2+}-selective electrode has permitted direct measurements of the kinetics of Ca^{2+} uptake by corn mitochondria. Fig. 3A shows that the plot of initial rates of Ca^{2+} uptake vs. free Ca^{2+} concentrations in the medium had a sigmoidal character. A maximal rate of about 140 nmol Ca^{2+} per min per mg was attained at 120 μM free Ca^{2+}. From the Hill plot a straight line with a slope of 2.22 was obtained, giving an intercept ($K_{0.5}$) at approx. 31 μM Ca^{2+}.

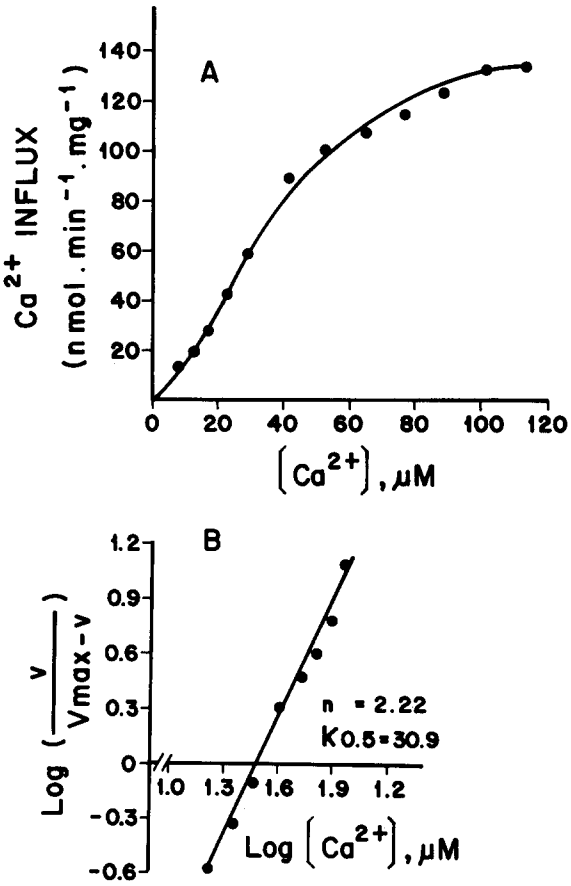

Fig. 3. Effect of free-Ca^{2+} concentration on the initial rate of Ca^{2+} uptake (A) and Hill plot of initial rate of Ca^{2+} uptake by corn mitochondria (B). Mitochondria (1 mg·ml^{-1}) were added to the basic medium containing 5 mM succinate/ 1 mM phosphate/5 μM rotenone.

Effect of Mg^{2+} on the rate of Ca^{2+} uptake

It has been found that Mg^{2+}, which inhibits Ca^{2+} uptake in animal mitochondria [21], does not significantly affect Ca^{2+} uptake in plant mitochondria [7,12]. Since Mg^{2+} is also present in the cytosol of plant cells we have studied its effect on Ca^{2+} uptake by corn mitochondria. Fig. 4 shows that Mg^{2+} does inhibit Ca^{2+} uptake in corn mitochondria contrary to the results observed in mung bean mitochondria [12]. A concentration of about 1.5 mM Mg^{2+} gave half-maximal inhibition of Ca^{2+} uptake when free Ca^{2+} was at 80 μM.

Effect of Mg^{2+} on extramitochondrial steady-state Ca^{2+} concentration

Fig. 5 shows that addition of mitochondria to the medium was followed by decrease in extramitochondrial Ca^{2+} concentration which reached a steady-state equilibrium at about 2.0 μM ('set point'). As reported previously for liver [22] and corn mitochondria [13] pulse additions of either Ca^{2+} or EGTA were followed by mitochondrial Ca^{2+} uptake or release, respectively, tending to restore the original Ca^{2+} steady state. Occasionally, EGTA produced a steady state concentration higher than that one in the beginning. The presence of 1 mM Mg^{2+} (trace B) raised the values of steady-state Ca^{2+} concentration to 3.5 μM and facilitated the return of Ca^{2+} to the original steady state value after EGTA addition. Similar effects of Mg^{2+} were previously reported for liver mitochondria [17].

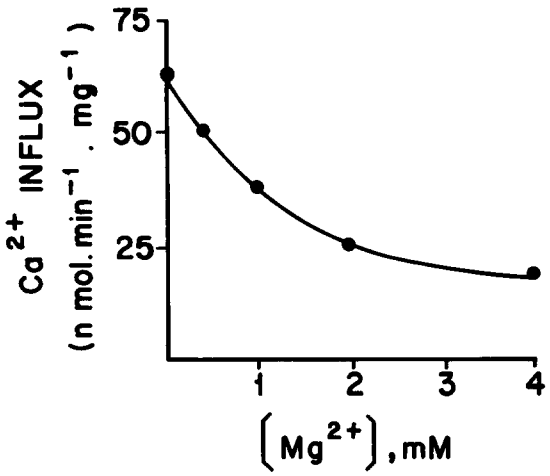

Fig. 4. Effect of Mg^{2+} on the rate of Ca^{2+} uptake. Corn mitochondria were incubated in the same conditions of Fig. 3, in the presence of 80 μM free Ca^{2+} and different concentrations of Mg^{2+}.

The Ca^{2+} 'set point' is independent of the respiratory substrate and is not affected by Na^+

It has been claimed that Ca^{2+} transport in plant mitochondria is substrate-dependent, insensitive to ruthenium red, and requires very high exogenous Ca^{2+} concentrations [9]. Fig. 6 shows two experiments in which Ca^{2+} flux was studied in corn mitochondria energized by malate plus glutamate (trace A) or succinate (trace B) in a medium containing moderate Ca^{2+} concentrations. Under these experimental conditions both the initial rates of Ca^{2+} accumulation and the Ca^{2+} 'set point' were similar in mitochondria energized with either substrate and not influenced by the presence of 10 mM Na^+ in the reaction medium (trace A). Ruthenium red induced Ca^{2+} efflux from these mitochondria indicating inhibition of the influx pathway (trace B). In addition Ca^{2+} efflux was not stimulated by external Na^+. Similar results were observed when Ca^{2+} transport was energized by ATP hydrolysis or by the system ascorbate plus TMPD (not shown).

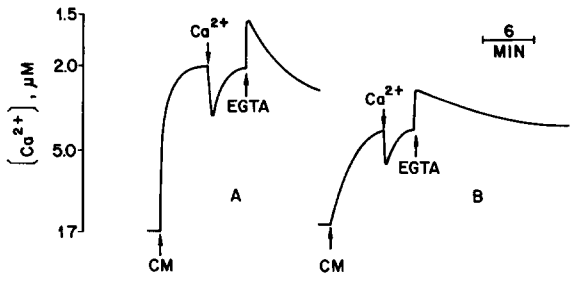

Fig. 5. Effect of Mg^{2+} on steady-state extramitochondrial Ca^{2+} concentration. Corn mitochondria (CM) (1 mg·ml^{-1}) were incubated in the reaction medium similar to Fig. 3 with 17 μM free Ca^{2+}, in the absence (A) or presence (B) of 1 mM Mg^{2+}. Ca^{2+} (10 nmol·mg^{-1}) or EGTA (8 nmol·ml^{-1}) were added where indicated.

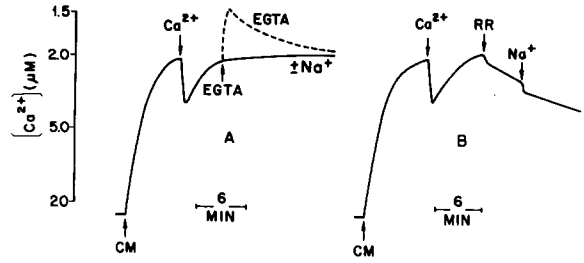

Fig. 6. Ca^{2+} flux in corn mitochondria (CM) energized with different substrates. Corn mitochondria (0.75 mg) were incubated in 1 ml of the basic reaction medium containing 5 mM phosphate. (A) Malate (1 mM) plus glutamate (20 mM). (B) Succinate (2 mM) plus rotenone (5 μM). Additions of Ca^{2+} (10 nmol), EGTA (15 nmol), ruthenium red (RR) (7.5 μM) and Na^+ (10 mM) were made where indicated.

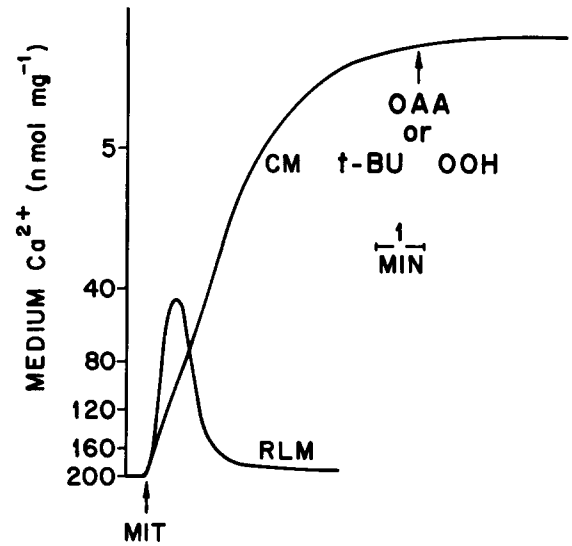

Fig. 7. Ability of corn mitochondria to retain Ca^{2+} in the presence of phosphate and NAD(P)H oxidants. Corn (CM) or rat liver mitochondria (RLM) were incubated in the reaction medium similar to Fig. 3 containing 200 nmol of Ca^{2+}. Oxaloacetate (OAA) (1 mM) or *t*-butylhydroperoxide (1 mM) were added where indicated.

Retention of Ca^{2+} by corn mitochondria in the presence of phosphate and NAD(P)H oxidants

Mitochondria isolated from animal tissues, particularly those from liver [23–25], undergo large amplitude swelling and gross alterations in membrane permeability following the uptake of Ca^{2+} in the presence of exogenous phosphate when certain membrane stabilizers, such as exogenous Mg^{2+} and adenine nucleotides are omitted in the reaction medium [26]. This process is accelerated by NAD(P)H oxidants and is accompanied by a precipitous loss of the accumulated Ca^{2+} and other matrix components [26]. Contrary to liver mitochondria corn mitochondria are capable of retaining previously accumulated Ca^{2+} (about 200 nmol per mg protein) even in the absence of membrane stabilizing agents. Ca^{2+} is also not released after addition of NAD(P)H oxidants, such as oxaloacetate and *t*-butylhydroperoxide.

Discussion

The present study shows that corn mitochondria prepared by an improved procedure are much more active in respiration-coupled Ca^{2+} accumulation than those employed in earlier studies with other plant mitochondria.

Addition of Ca^{2+} to this preparation of corn mitochondria respiring on succinate induced a stimulation of respiration accompanied by Ca^{2+} uptake and H^+ extrusion with a H^+/Ca^{2+} ratio close to 1.1 and a Ca^{2+}/O ratio of about 2.3. The low ADP/O and Ca^{2+}/O ratios observed with this preparation are readily explained by cyanide-insensitive, uncoupled respiration [27] which accounts for about 20% of state-3 respiration in these mitochondria. The H^+/Ca^{2+} ratio of 1.1 is similar to that obtained in liver mitochondria incubated in the presence of phosphate [28,29]. However in these animal mitochondria the H^+/Ca^{2+} ratio increases to 2 when phosphate influx is prevented [30–32]. This was taken as evidence for an electrophoretic Ca^{2+} uniporter carrying two positive charges [30–32]. In corn mitochondria we found that in the absence of phosphate or when phosphate influx is inhibited there is no Ca^{2+} uptake confirming that Ca^{2+} influx in these mitochondria has an absolute requirement for phosphate [12,20] and seems to be at least partially electroneutral. In fact, the experiment of Fig. 2B shows that acetate does not substitute phosphate as a permeant anion for Ca^{2+} uptake. We have also observed that other weak acid anions such as β-hydroxybutyrate and thiosulfate were unable to support Ca^{2+} uptake by corn mitochondria.

Regarding the changes in $\Delta\psi$ caused by Ca^{2+} uptake we have already shown [13] that in corn mitochondria Ca^{2+} uptake in the presence of phosphate caused a much smaller decrease in $\Delta\psi$ than in liver mitochondria. This indicates that the small drop in $\Delta\psi$ observed in corn mitochondria cannot be attributed only to the collapse of ΔpH due to phosphate entry. This small decrease in $\Delta\psi$ which accompanied the accumulation of Ca^{2+} and the low activity of the carrier (V_{max} of 140 nmol Ca^{2+} per mg per min) suggest that the carrier itself rather than the H^+ pump is rate-limiting at saturating Ca^{2+} concentrations. The small decrease in $\Delta\psi$ during Ca^{2+} accumulation may also be explained, at least in part, on the basis of the phosphate requirement for Ca^{2+} uptake. This may suggest that Ca^{2+} enters in the form of a Ca^{2+} phosphate complex carrying less than two positive charges as proposed by Day et al. [20]. This is corroborated by the results in Fig. 2B indicating that in the absence of added phosphate $\Delta\psi$ itself is not limiting the Ca^{2+} entry, because $\Delta\psi$ is maintained at a relatively high value (about 160 mV) even after Ca^{2+} addition. Moreover, the addition of acetate caused an increase in $\Delta\psi$ similar to that induced by phosphate addition, although no Ca^{2+} uptake occurred.

From the kinetic experiment we found that the respiration-coupled Ca^{2+} uptake may display sigmoidal kinetics with respect to the extramitochondrial free Ca^{2+} concentration. If we assume that the Ca^{2+} carrier itself is the limiting step at saturating Ca^{2+} concentration, such sigmoidicity would not be artifactual [4] and the Hill coefficient of 2.22 would indicate the existence of two Ca^{2+}-binding sites per carrier molecule. The $K_{0.5}$ of 31 μM for Ca^{2+} uptake found here is much lower than the data published by other authors [10,11]. The discrepancy might be due to differences in experimental conditions, methodology and possibly the quality of the mitochondrial preparation itself.

The experiments on steady-state extramitochondrial Ca^{2+} indicated that Ca^{2+} fluxes in corn mitochondria are independent of the different respiratory substrates. The presence of 10 mM Na^{+} did not alter either the Ca^{2+} 'set point' or the rate of ruthenium-red-induced Ca^{2+} efflux at variance with what is observed in mitochondria of excitable tissues [33,34], and to a lesser extent in liver mitochondria [35]. Mg^{2+}, on the other hand, decreases the rate of Ca^{2+} influx in corn mitochondria and as a consequence, significantly increases the concentration of steady-state extramitochondrial Ca^{2+}. In addition, Mg^{2+} has shown to be an important factor for the maintenance of a stable steady-state level of external Ca^{2+} after the addition of EGTA, similarly to what is observed in liver mitochondria [17].

An interesting observation in this work concerns the high capacity of Ca^{2+} retention by corn mitochondria incubated in the presence of phosphate and other Ca^{2+} releasing agents, such as oxaloacetate [36,37] and *t*-butylhydroperoxide [38]. Under the same conditions liver mitochondria undergo severe alterations in structure and functions causing the release of Ca^{2+} and other matrix components [24,25]. Comparative studies on these differences between corn and liver mitochondria may bring some light on the mechanism by which Ca^{2+} accumulation in the presence of phosphate or other agents like oxaloacetate and hydroperoxides causes such alterations in liver mitochondria.

In conclusion, these results, together with previous work from this laboratory [13], indicate that corn mitochondria, despite some differences to vertebrate mitochondria, also possess an efficient system for Ca^{2+} transport. They are able to provide a precise regulation of external Ca^{2+} concentration and may have some role in the regulation of Ca^{2+} distribution in the plant cell.

Acknowledgements

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