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Calcium Transport by Corn Mitochondria[1]

Evaluation of the Role of Phosphate

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ABSTRACT

Mitochondria from some plant tissues possess the ability to take up Ca^{2+} by a phosphate-dependent mechanism associated with a decrease in membrane potential, H^+ extrusion, and increase in the rate of respiration (AE Vercesi, L Pereira da Silva, IS Martins, CF Bernardes, EGS Carnieri, MM Fagian [1989] *In* G Fiskum, ed, Cell Calcium Metabolism. Plenum Press, New York, pp 103–111). The present study reexamined the nature of the phosphate requirement in this process. The main observations are: (a) Respiration-coupled Ca^{2+} uptake by isolated corn (*Zea mays* var Maya Normal) mitochondria or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone-induced efflux of the cation from such mitochondria are sensitive to mersalyl and cannot be dissociated from the silmultaneous movement of phosphate in the same direction. (b) Ruthenium red-induced efflux is not affected by mersalyl and can occur in the absence of phosphate movement. (c) In Ca^{2+}-loaded corn mitochondria, mersalyl causes net Ca^{2+} release unrelated to a decrease in membrane potential, probably due to an inhibition of Ca^{2+} cycling at the level of the influx pathway. It is concluded that corn mitochondria (and probably other plant mitochondria) do possess an electrophoretic influx pathway that appears to be a mersalyl-sensitive Ca^{2+}/inorganic phosphate-symporter and a phosphate-independent efflux pathway possibly similar to the Na^{2+}-independent Ca^{2+} efflux mechanism of vertebrate mitochondria, because it is not stimulated by Na^+.

In energized mitochondria from vertebrate tissues, Ca^{2+} distribution between the matrix and extramitochondrial compartments, under steady-state conditions, is determined by the simultaneous operation of two thiol-insensitive Ca^{2+} carriers: the electrophoretic Ca^{2+} influx uniporter and the electroneutral antiporter that exchanges matrix Ca^{2+} for external Na^+ or H^+ (9, 13, 14, 26). The simultaneous operation of these two pathways in isolated mitochondria establishes a "set point" for extramitochondrial free Ca^{2+} in the range of 0.5 to 1.0 μM (25). However, most of the recent data indicate that the kinetic characteristics of these calcium influx-efflux pathways is incompatible with the role of mitochondria as cytosolic calcium buffers (14). Instead, these data indicate that under *in situ* conditions, these combined pathways are responsible for setting the matrix free Ca^{2+} concentration in a range thought to regulate the activities of three Ca^{2+}-sensitive dehydrogenases that catalyze rate-limiting reactions of the Krebs cycle (2-oxoglutarate, NAD^+-linked isocitrate, and pyruvate dehydrogenases) and, therefore, the oxidative metabolism and ATP production (14, 22).

Considering the importance of this Ca^{2+} transport system in the regulation of oxidative metabolism in vertebrate tissues, we decided to reexamine the data indicating that only vertebrate mitochondria possess such a Ca^{2+} transporting system (7, 22).

Initial studies with plant and protozoa indicated that, contrary to the above-mentioned data (7, 22), both types of mitochondria showed the ability to accumulate external Ca^{2+} by an electrophoretic mechanism sensitive to ruthenium red (4, 6, 20, 21, 31, 32).

With regard to plant mitochondria, we have observed in previous studies using the hypocotyls of corn (*Zea mays*), soybean (*Glycine max*), bean (*Phaseolus vulgaris*), coffee (*Coffea arabica*), the white leaves of cabbage (*Brassica oleracea*), the tubers of potato (*Solanum tuberosum*), and the roots of red beet (*Beta vulgaris*), that, with the exception of cabbage, potato, and beet mitochondria, all others were able to take up Ca^{2+} by a thiol-sensitive phosphate-dependent mechanism, associated with a parallel increase in the rate of respiration, H^+ ejection, and decrease in $\Delta\psi$[3] (4, 20, 30).

The objective of the present study was to reexamine the phosphate dependence for Ca^{2+} transport in corn mitochondria. Therefore, the temporal sequence of Ca^{2+} and Pi movements in and out of mitochondria were determined in the presence or absence of either mersalyl (a thiol reagent) or rutheniun red to inhibit the transport of Pi or Ca^{2+}, respectively, in an attempt to dissociate the movements of both. The results indicated that the electrophoretic flux of Ca^{2+}

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[3] Abbreviations: $\Delta\psi$, transmembrane electrical potential; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TPP^+, tetraphenylphosphonium.

cannot be dissociated from phosphate, whereas ruthenium red-induced efflux is phosphate-independent.

MATERIALS AND METHODS

Preparation of Mitochondria

Rat liver mitochondria were isolated by homogenization in a medium containing 250 mM sucrose and 0.5 mM EGTA, followed by conventional differential centrifugation (29). Corn (*Zea mays* var Maya Normal) mitochondria were isolated from 3-d-old coleoptiles from seedlings germinated on moist filter paper at 28°C, kept in the dark as previously described (20). The mitochondrial protein was determined by a modified biuret method (17).

Standard Incubation Procedure

The plant mitochondrial suspension was incubated at 30°C in a basic reaction medium containing 0.3 M mannitol, 20 mM KCl, 2 mM Hepes buffer (pH 7.2), 0.1% BSA, 2 mM succinate, 1 mM phosphate, and 5 μM rotenone. In the case of liver mitochondria, the mannitol concentration was 0.24 M. Other additions and special conditions are specified in the figure legends.

Calcium and Phosphate Measurements

Changes in free Ca^{2+} concentrations were followed with a Ca^{2+}-selective electrode (Radiometer, F2112 calcium selectrode), calibrated by the additions of Ca^{2+}/EGTA buffers. For the determination of intramitochondrial Ca^{2+} and Pi contents, samples were withdrawn during the experiments. Each sample was placed in an Eppendorf tube and centrifuged for 2 min. The supernatants were discarded and the pellets washed twice with Ca^{2+}- and Pi-free media. The pellets were suspended in 0.1 mL of deionized water followed by the addition of 0.3 mL of 3.75% perchloric acid and left standing for 15 min at 4°C. Precipitated protein was removed by a 2-min centrifugation and the supernatant analyzed for Pi according to Grindey and Nichol (11), and for calcium using a Perkin Elmer Atomic Absorption Spectrophotometer.

Measurements of Mitochondrial Transmembrane Electrical Potential

Mitochondria were incubated in the reaction medium containing 3 μM TPP^+. The concentration of TPP^+ in the extramitochondrial medium was continuously monitored with a TPP^+-selective electrode prepared in our laboratory according to Kamo *et al.* (16). The mitochondrial membrane potential was then calculated assuming that the TPP^+ distribution between mitochondria and medium follows the Nernst equation (24). Due to the significant binding of TPP^+ to mitochondrial membranes, $\Delta\psi$ values were corrected according to Jensen *et al.* (15).

RESULTS AND DISCUSSION

We have previously shown (4, 20, 21) that mitochondria isolated from corn coleoptiles by a procedure that yields functionally intact preparations (respiratory control ratio between 3.0 and 4.0) are active in respiration-coupled Ca^{2+} accumulation. We used this mitochondrial preparation to reexamine the phosphate requirement of this mechanism (1, 5, 21, 23). In vertebrate mitochondria, the presence of phosphate or other weak acid anions such as acetate, for example, increase the capacity of Ca^{2+} accumulation because they move into mitochondria in parallel with Ca^{2+} and prevent the collapse of membrane potential as they move either in symport with H^+ or in exchange with OH^- (10, 18). Our previous data (21) indicated that this is not the case for corn mitochondria, in which the addition of acetate is followed by regeneration of the membrane potential but not by Ca^{2+} uptake. In this regard, Figure 1 permits the comparison of the effects of mersalyl and acetate on Ca^{2+} transport by liver (A) and corn (B) mitochondria. Both types of mitochondria were preincubated in media containing $CaCl_2$, rotenone, and phosphate. The solid lines show that the addition of succinate was followed by Ca^{2+} uptake by both types of mitochondria. The dotted lines indicate that the presence of mersalyl during the preincubation period limited the uptake of Ca^{2+} to a few nmol/mg in both types of mitochondria. Under these conditions, the fast deflection after succinate addition was caused, at least in part, by Ca^{2+} complexation by this anion. The inhibition of Ca^{2+} uptake by mersalyl was overcome by acetate in liver but not in corn mitochondria (dashed lines). The low amount of Ca^{2+} taken up by corn mitochondria prior to the addition of succinate is explained by the incomplete inhibition of respiration of these mitochondria by rotenone (7) and because the reaction of mersalyl with the phosphate carriers normally depends on a period of preincubation (3).

In the experiments depicted in Figure 2, the parallel movements of calcium and phosphate were determined during the uptake energized by succinate. At zero time (just prior to the succinate addition), in the absence of mersalyl, corn mitochondria contained 27.7 ± 2.8 nmol/mg calcium and 22.5 ± 0.7 nmol/mg phosphate. After the addition of succinate, there was a parallel uptake of both species, with a Ca^{2+}/Pi ratio close to 1.5, in agreement with previous data (5, 8, 21). When

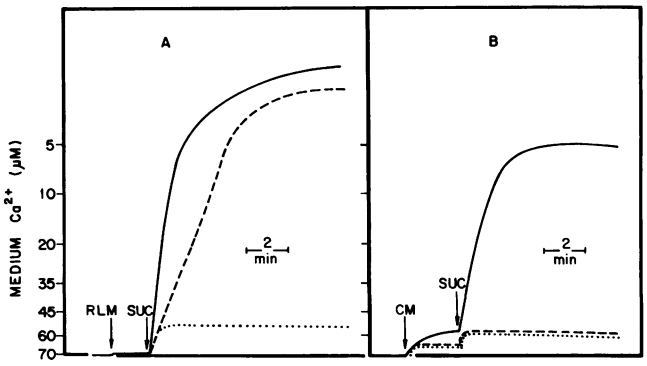

Figure 1. Effect of mersalyl on Ca^{2+} uptake by rat liver (RLM) (A) and corn mitochondria (CM) (B). Mitochondria (1 mg/mL) were added, where indicated, to the basic reaction medium containing 70 μM $CaCl_2$. After 2 min of preincubation, 2.0 mM succinate (SUC) was added to the medium in the absence of mersalyl (solid lines), or in the presence of 10 μM mersalyl (dotted lines), or 10 μM mersalyl plus 10 mM acetate (dashed lines).

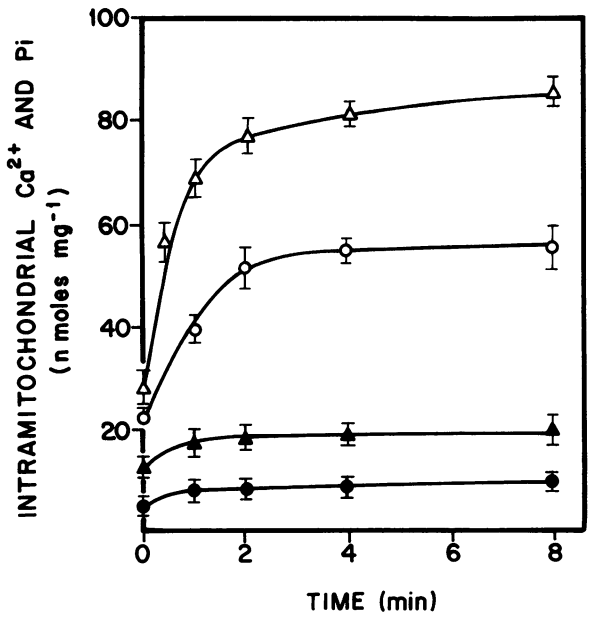

Figure 2. Ca^{2+} and phosphate uptake by corn mitochondria. The reaction was initiated by the addition of succinate after mitochondria were preincubated during 4 min in the basic reaction medium containing 82 μM $CaCl_2$ in the presence (filled symbols) or absence (open symbols) of 10 μM mersalyl. (△, ▲) Matrix calcium, (○, ●) matrix phosphate. Data are means ± SD (n = 5).

mersalyl was present during the preincubation period, the mitochondrial contents of calcium and phosphate were 12.4 ± 1.5 and 5.2 ± 0.5 nmol/mg, respectively. Only a few nmol/mg of Ca^{2+} and Pi were accumulated under these conditions. Although these results agree with previous data (5, 21), suggesting that Ca^{2+} enters in the form of a Ca^{2+} phosphate complex carrying less than two positive charges, they do not exclude the possibility that phosphate is moving separately through the Pi/H^+ carrier in response to a transmembrane pH gradient generated by an ejection of H^+ during Ca^{2+} influx. In this case, the role of phosphate could be not just to collapse ΔpH and regenerate $\Delta\psi$, but also to promote the retention of Ca^{2+} in the form of calcium phosphate precipitates.

To resolve this problem, we decided to study the FCCP or ruthenium red-induced release of mitochondrial Ca^{2+}. The first collapses $\Delta\psi$ and thus causes Ca^{2+} release via the reverse of the influx pathway (26). The second selectively inhibits the influx pathway and permits for the determination of Ca^{2+} efflux at high $\Delta\psi$, presumably, via an electroneutral pathway. With regard to this, Figure 3A shows that the additions of both ruthenium red or FCCP, under steady-state conditions, caused the release of mitochondrial Ca^{2+}. The efflux in the presence of FCCP was much faster (5.1 nmol/mg·min) than in the presence of ruthenium red (1.0 nmol/mg·min). Interestingly, the further addition of FCCP on top of ruthenium red failed to stimulate Ca^{2+} efflux. This indicates that the Ca^{2+} release via reverse of the influx pathway is inhibited by the dye and that the release induced by ruthenium red occurs through an independent efflux pathway. On the other hand, it can be observed in Figure 3B that the addition of mersalyl under the conditions of Figure 3A caused Ca^{2+} release after a lag of about 1 to 2 min. This Ca^{2+} efflux occurs at a rate similar to that induced by ruthenium red and was not modified by this dye. Because mersalyl, like ruthenium red, inhibits Ca^{2+} influx in these mitochondria, it might be possible that the net Ca^{2+} efflux caused by mersalyl is due to the inhibition of Ca^{2+} cycling across the inner mitochondrial membrane. The lag period that precedes Ca^{2+} efflux may be related to the time required for the interaction of mersalyl with the putative Ca^{2+}/Pi carrier.

A recent study with mitochondria isolated from Jerusalem artichoke (27) indicated that Ca^{2+} efflux induced by mersalyl takes place via reversal of the influx pathway due to the collapse of $\Delta\psi$ caused by this compound. These results prompted us to perform experiments to examine this mechanism.

Contrary to the above interpretation, Figure 4 shows that, although the addition of mersalyl (10–40 μM) caused an instantaneous dose-related, time-dependent decrease in $\Delta\psi$ from 10 to 33 mV (A), the rate of Ca^{2+} release was independent of mersalyl concentration, in this range, and occurred at a rate close to that induced by ruthenium red. This indicates that in corn mitochondria, mersalyl, like ruthenium red, inhibits Ca^{2+} transport at the level of the influx pathway and leads to net Ca^{2+} efflux through an independent pathway. This is confirmed by the experiments in Figure 5, showing that Ca^{2+} efflux induced by FCCP is strongly inhibited by the addition of mersalyl, but the efflux induced by ruthenium red is not (dashed lines). These results may indicate that, in the case of FCCP, Ca^{2+} release, like its uptake, is taking place via a mersalyl-sensitive calcium/phosphate porter. In the case of

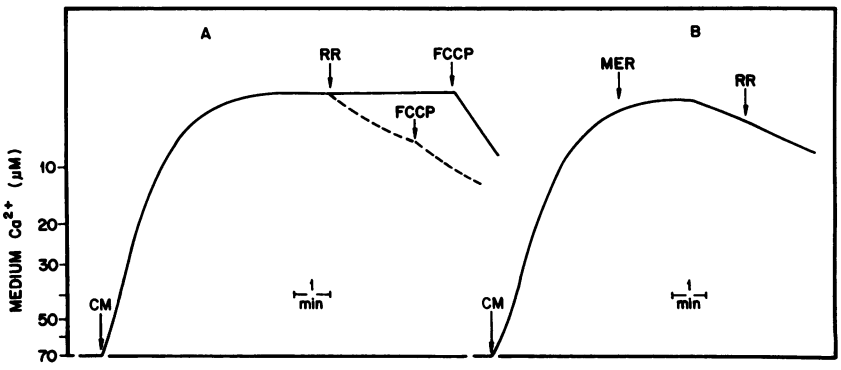

Figure 3. Ca^{2+} release from corn mitochondria (CM) induced by FCCP, ruthenium red, or mersalyl. Where indicated, 5.0 μM ruthenium red (RR), 1.0 μM FCCP, or 40 μM mersalyl (MER) were added to Ca^{2+}-loaded mitochondria under the same experimental conditions of Figure 1.

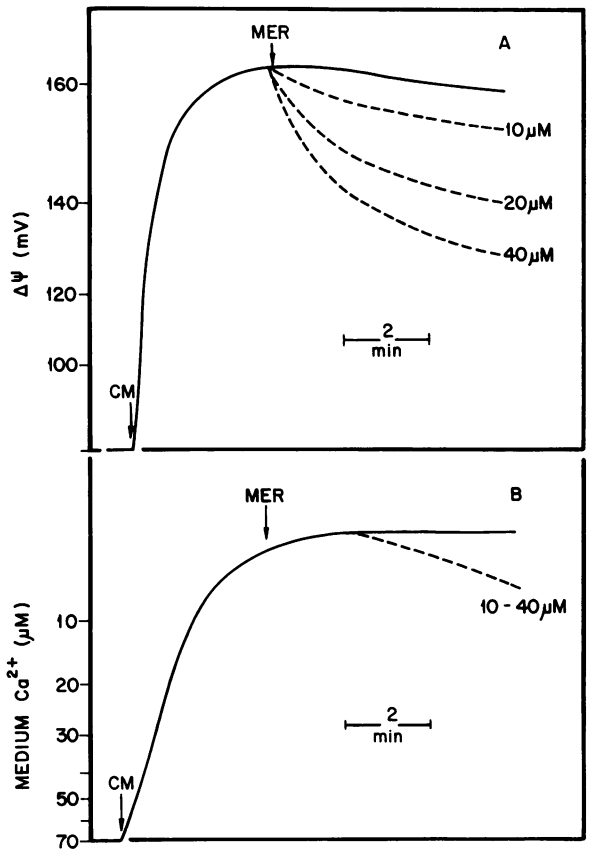

Figure 4. Effect of mersalyl concentration on membrane potential (A) and Ca^{2+} flux (B) in corn mitochondria (CM). Where indicated, mersalyl (MER) (10, 20, and 40 μM) was added to Ca^{2+}-loaded mitochondria under the same experimental conditions of Figure 1.

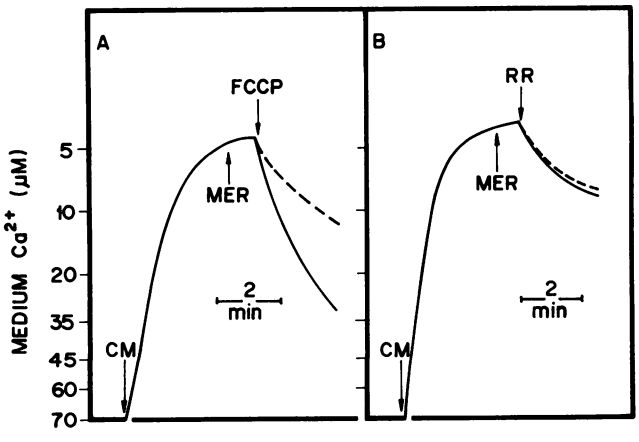

Figure 5. Effect of mersalyl on mitochondrial Ca^{2+} efflux induced by FCCP (A) or ruthenium red (B). FCCP (1 μM) or ruthenium red (RR) (5 μM) were added where shown to the reaction medium under the conditions of Figure 1 with (dashed lines) or without (solid lines) 40 μM mersalyl (MER).

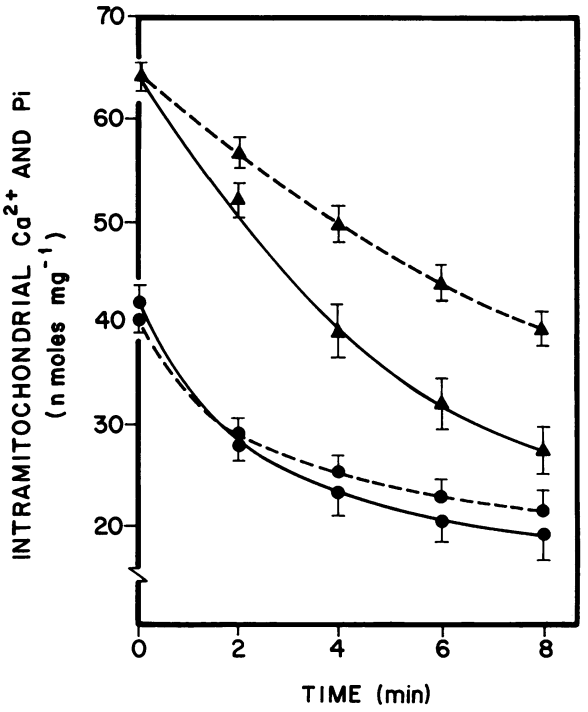

Figure 6. Effect of mersalyl on FCCP-induced Ca^{2+} and phosphate release from corn mitochondria at pH 7.2. The experimental conditions were exactly as in Figure 1. The samples for intramitochondrial calcium and phosphate determination were taken every 2 min as described in "Materials and Methods." FCCP was added at time zero. Solid lines (absence of mersalyl), dashed lines (presence of 40 μM mersalyl). ▲, Matrix calcium; ●, matrix phosphate. Data are means ± SD (n = 9).

ruthenium red, Ca^{2+} efflux may occur via a pathway insensitive to mersalyl, presumably a $Ca^{2+}/2H^+$ antiporter, as appears to be the case in vertebrate mitochondria (9, but see also refs. 2, 12, 13, 28).

Certainly, the temporal sequence of Ca^{2+} and phosphate release under the conditions of Figure 5 would allow for a better analysis of the results presented above. In this regard, Figure 6 shows the efflux of calcium and phosphate caused by the addition of FCCP to respiring corn mitochondria that contained 64.1 ± 4.4 nmol/mg calcium and 42 ± 2.9 nmol/mg phosphate. The solid lines (minus mersalyl) show the parallel movements of Ca^{2+} and phosphate with a Ca^{2+}/Pi ratio increasing from 0.9 to 1.6 during the period of 8 min. This increase in the Ca^{2+}/Pi ratio is due to a more accentuated decrease in the rate of phosphate rather than Ca^{2+} release with time. When mersalyl was added 1 min before FCCP (dashed lines), the inhibition of the rate of calcium efflux was higher than the inhibition of phosphate efflux. Both the change of the Ca^{2+}/Pi ratio, with the time, and the different sensitivities of Ca^{2+} and phosphate release to mersalyl appear to indicate that Ca^{2+} and phosphate are moving, at least in part, via different routes. Because inhibition of the Pi/H^+ carrier by −SH reagents increases with increasing matrix pH (19), we repeated the last experiment at pH 7.6 (0.4 pH units higher than in the experiment of Fig. 6). In the absence of mersalyl

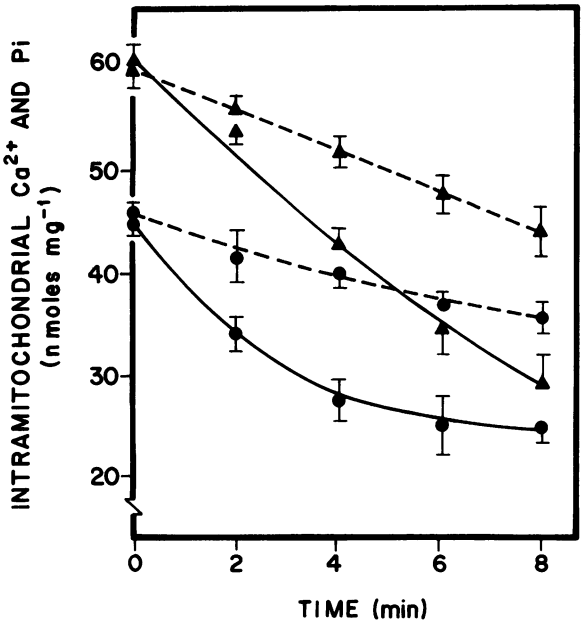

Figure 7. Effect of mersalyl on FCCP-induced Ca^{2+} and phosphate release from corn mitochondria at pH 7.6. The experimental conditions were exactly as in Figure 6, except that medium pH was 7.6 and the concentration of Hepes was 10 mM. Data are means ± SD (n = 9).

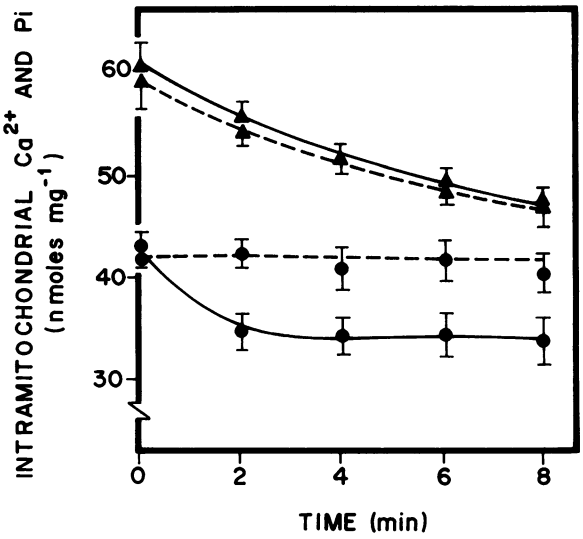

Figure 8. Effect of mersalyl on calcium and phosphate efflux induced by rutheniun red. The experimental conditions were exactly as in Figure 6, except that 5 μM ruthenium red, instead of FCCP, was added at time zero. Data are means ± SD (n = 9).

(Fig. 7, solid lines) the effluxes of Ca^{2+} and Pi were similar to those observed in the preceding experiments. However, in the presence of mersalyl (dashed lines), a much greater inhibition of both rates occurred. Under these conditions, the observed Ca^{2+} release may be, in part, coupled to phosphate release via reverse of the influx pathway due to its incomplete inhibition by mersalyl and, in part, via the putative Ca^{2+} efflux pathway, which is insensitive to mersalyl (Figs. 3 and 5).

The possible involvement of phosphate in this later component of Ca^{2+} efflux was studied in the experiments in which the influx pathway was blocked by ruthenium red and calcium efflux was allowed to take place at high membrane potential (Fig. 8). Interestingly, under these conditions, calcium release was followed by phosphate only during the initial 2 min (solid lines). In addition, the dashed lines show that, although mersalyl totally inhibits phosphate release under these conditions (in the absence of FCCP, the internal pH was not equilibrated with medium pH and $\Delta\psi$ was not collapsed as in Fig. 6), the onset of calcium efflux was similar to that in the absence of mersalyl. This indicates that the ruthenium red-induced Ca^{2+} efflux is phosphate-independent, and, in agreement with the results observed in Figure 5B, it is insensitive to mersalyl.

CONCLUSIONS

The results described in this paper are consistent with our hypothesis that calcium translocation in plant mitochondria, when it occurs, is mediated by a mersalyl-sensitive Ca^{2+}/Pi-symporter (see proposed scheme in Fig. 9), as suggested previously (5). The results showing that ruthenium red-induced Ca^{2+} release is phosphate-independent are also consist-

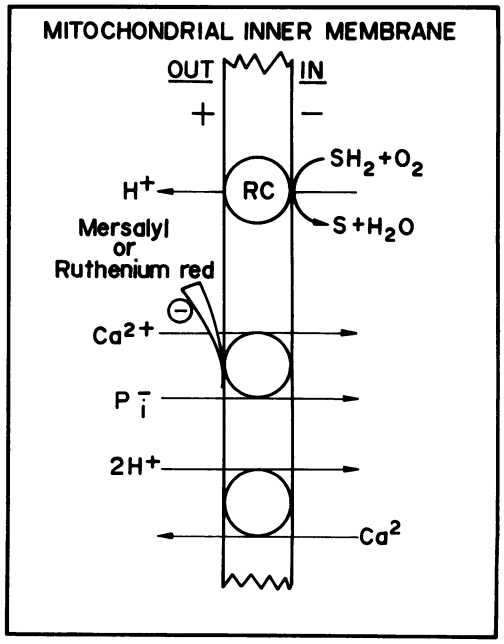

Figure 9. Proposed scheme of Ca^{2+} transport in corn mitochondria. The proton pumps of the respiratory chain (RC) generate an electrochemical proton gradient. The membrane potential drives the electrophoretic influx of Ca^{2+} and phosphate mediated by a ruthenium red- and mersalyl-sensitive Ca^{2+}/Pi symporter. The exact Ca^{2+} to Pi stoichiometry is still not defined. Ca^{2+} efflux is Na^+- and phosphate-independent and takes place through a mersalyl-insensitive pathway, which like vertebrate mitochondria may be directly or indirectly coupled to the influx of H^+ (13).

ent with our previous data (21), indicating that plant mitochondria, like vertebrate mitochondria, possess an independent pathway for Ca^{2+} efflux. Because this pathway operates at high membrane potential and is not stimulated by Na^+ (21), it might be similar to the Na^+-independent efflux mechanism of vertebrate mitochondria.

Because this Ca^{2+} transporting system is not ubiquitously distributed in plants (4, 20, 30), it might have a physiological role in the plant tissue in which it occurs. Unpublished data (I.S. Martins, 1987) from our laboratory indicate that the mitochondrial dehydrogenases, which in vertebrates are regulated by Ca^{2+}, do not respond to this cation in corn mitochondria. A possible role of this transport system in the distribution of phosphate between the mitochondrial matrix and the cytosol should be investigated.

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