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Transient induction of the mitochondrial permeability transition by uncoupler plus a Ca^{2+}-specific chelator

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Determinations of aqueous space volumes, swelling and Mg^{2+} release experiments demonstrate that EGTA plus uncoupler causes the permeability transition in Ca^{2+}-loaded mitochondria. Extramitochondrial Mg^{2+} is required to obtain this effect. Changes in transition-dependent parameters are smaller and more varied when induced by EGTA plus uncoupler than when induced by Ruthenium red plus uncoupler, although inhibitor-sensitive experiments show that the same basic mechanism is involved in both cases. Measurements of sucrose trapping and sucrose or inulin accessible space, after changes in transition-dependent parameters are complete, indicate that rapid reversal occurs when the transition is induced by EGTA plus uncoupler, explaining why limited responses are obtained. Data support the hypothesis that an external divalent cation binding site regulates activity of the mitochondrial Ca^{2+} uniporter.

Introduction

The mitochondrial inner membrane can be rendered freely permeable to molecules and ions of molecular weight less than about 1200 through a phenomenon referred to as the permeability transition (see Ref. 1 for review). Development of the high permeability state is reversible and is inhibited by cyclosporin A through a high-affinity interaction with a mitochondrial component present at 75–100 pmol/mg protein (see Ref. 1 for review). Recent evidence suggests that the cyclosporin A target may be a matrix space protein having peptide bond isomerase activity [2–4]. A physiological role for the transition has not been established, although it is suspected that loss of regulation under conditions of cell injury contributes to loss of mitochondrial function and the mechanism of cell death (see Ref. 5–7 for review).

In vivo, the transition is usually generated by Ca^{2+} loading, followed by addition of an inducing agent such as inorganic phosphate or *t*-butylhydroperoxide. The known inducing agents (see Ref. 1) include the combination of reagents Ruthenium red plus uncoupler, neither reagent being able to induce the transition alone [8–10]. These findings have been taken to indicate that uncoupling Ca^{2+}-loaded mitochondria will produce the transition unless Ca^{2+} can be rapidly released by reverse activity of the Ca^{2+} uniporter [10].

Recently, we reported that uncoupler plus EGTA can induce the transition, whereas uncoupler plus EDTA cannot [10]. From this and related findings it was proposed that a cation binding site exists on the cytoplasmic side of the inner membrane which exerts a strong regulatory influence on Ca^{2+} uniporter activity. According to this hypothesis, the uniporter is active when the site is occupied by Ca^{2+} or no divalent cation, while it is inactive when the site is occupied by Mg^{2+}. These findings have implications beyond description of conditions which induce the transition because they bear upon the important questions of uniporter regulation and the extent to which mitochondria respond to rapid Ca^{2+} transients in vivo. Accordingly, the data supporting the hypothesis must be fully scrutinized. In this regard, there are two areas of uncertainty which allow alternative interpretations of the previous study. First of all, the activity of Ruthenium red as an inhibitor of reverse uniport has been controversial [9,11–15], allowing for the possibility that mitochondria retain this pathway for Ca^{2+} efflux in the presence of

Abbreviations: CCP, carbonyl cyanide *p*-chlorophenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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Ruthenium red plus uncoupler. Secondly, the analogies between Ruthenium red and EGTA in producing transition-dependent phenomena are incomplete. Specifically, with Ruthenium red plus uncoupler, mitochondria become fully swollen, release essentially all endogenous Mg^{2+}, and display ultrastructural changes which are characteristic of those seen when the transition is fully expressed [10]. With EGTA plus uncoupler, swelling is limited to about 25% of the full response, Mg^{2+} release to 85%, while ultrastructural changes halt at an intermediary point (the orthodox form). These findings cast doubt on the mechanism of EGTA action because the transition normally occurs as an all or nothing process [1], while the measured variables change more nearly in parallel (e.g., Ref. 16).

To explain the smaller responses obtained with EGTA, we proposed that the normal permeability characteristics of the inner membrane are recovered soon after Ca^{2+} release, because of Ca^{2+} chelation, whereas with Ruthenium red the membrane remains permeable, allowing all responses to reach completion. This interpretation was based on work by Al-Nasser and Crompton, who showed that reducing the extramitochondrial Ca^{2+} concentration to the 10^{-6} M range reverses the permeability transition when it has been induced by Ca^{2+} plus inorganic phosphate [17]. Work presented here tests the assumption of rapid reversibility and further examines properties of the transition induced by uncoupler plus ruthenium red or EGTA. Aspects of this work have been presented in abstract form [18].

Materials and Methods

Reagents

Common chemicals were obtained from commercial sources and were reagent grade or better. Silicone oil, $d = 1.05$ g/ml, was from Aldrich. Li^+ salts of succinate, Hepes, EGTA, and EDTA were obtained by neutralizing the free acids with LiOH. Ruthenium red was used as provided by Sigma. Stock solutions of mannitol-sucrose and succinate were deionized by passage over amberlite MB-3 and stored subsequently in polyethylene containers. The following radioactive reagents were obtained from Amersham: ^3H_2O (specific activity 100 mCi/ml), [^{3}H]inulin (spec. act. 3.8 mCi/mmol), [^{3}H]dextran (spec. act. 386 mCi/g, average M_r = 70 000), [^{14}C]inulin (spec. act. 2.60 mCi/mmol), and [^{14}C]sucrose (spec. act. 540 mCi/mmol).

Preparation and incubation of mitochondria

Liver mitochondria were prepared from male Sprague-Dawley rats which weighed approx. 250 g, using procedures described previously [19]. EGTA (0.5 mM) and bovine serum albumin (2 mg/ml) were present in the homogenizing medium but were omitted from the washing medium, which contained 230 mM mannitol, 70 mM sucrose and 3 mM Hepes (Li^+) (pH 7.4). The final pellets were suspended at approx. 60 mg protein/ml in washing media and were maintained on ice until use. Protein concentration was determined by the Biuret reaction in the presence of 1% deoxycholate (Na^+).

Divalent cation-depleted mitochondria were prepared by one of two methods. For the depletion of Ca^{2+} only, the method of Wingrove and Gunter was employed [20]. The combined mitochondrial pellets ($\approx$ 150 mg protein), obtained from the first wash of the preparation procedure, were suspended in 100 ml of a medium containing 124 mM KCl, 10 mM NaCl, 4 mM succinate (K^+), 0.1 mM KP_i (pH 7.4) and 0.1 mM $MgCl_2$. The suspension was incubated with gentle stirring at 25 °C for 15 min, then placed on ice for an additional 5 min. Subsequently, the mitochondria were sedimented by centrifuging at $10\,000 \times g$ for 10 min and washed twice the mannitol, sucrose, Hepes washing medium described above. This procedure depletes Ca^{2+} by action of the $Ca^{2+}/2Na^+$ antiporter. Deenergization is specifically avoided to prevent the depleted preparation from becoming more sensitive to the permeability transition [20]. Atomic absorption measurements [16] showed that the final preparations contained Ca^{2+} and Mg^{2+} at 1.35 ± 0.45 and 19.42 ± 0.60 nmol/mg protein, respectively ($n = 4$). The initial content of these cations were 8.25 ± 1.75 nmol/mg protein (Ca^{2+}) and 28.6 ± 3.4 nmol/mg protein (Mg^{2+}).

For depletion of both Ca^{2+} and Mg^{2+}, the combined mitochondrial pellets from the first sedimentation in the preparation procedure were suspended in 20 ml of washing medium, and 5 mM EGTA (Li^+) plus about 1 nmol/mg protein of ionophore A23187 were added. The suspension was incubated briefly at room temperature and then for 5 min on ice, prior to sedimenting the mitochondria as described above for the Ca^{2+} depletion procedure. The pellet was washed three times using the same centrifugation conditions and 30 ml of media. For the first wash, 4 mg/ml of BSA and 5 mM EDTA (Li^+) were present in addition to the normal mannitol, sucrose, and Hepes. EDTA was omitted for the second wash, while EDTA plus BSA was omitted for the third wash and the final resuspensions. Mitochondria so treated contained residual Ca^{2+} and Mg^{2+} at < 0.05 and 1–2 nmol/mg protein, respectively. We could not detect residual A23187 by fluorescence measurements on the organic phase of Folch extracts prepared from the final suspension [19]. With the conditions employed, less than 10 pmol/mg protein would have been seen.

Most incubations were conducted at 25 °C in a medium which contained 10 mM succinate (Li^+) plus rotenone (0.5 nmol/mg protein), 207 mM mannitol, 63 mM sucrose and 3 mM Hepes (Li^+) (pH 7.4). The total

osmotic pressure was 300 mosM. For measurements of cation release or swelling, the protein concentration was 1 mg/ml. For the determination of mitochondrial volumes, this value was increased to 10 mg/ml. The latter incubations (initial volume up to 21 ml) were conducted with gentle stirring in 50-ml Erlenmeyer flasks to provide a large medium surface area. Enough O_2 diffused into the suspensions under these conditions to prevent deenergization for 10 min or longer, as judged by the absence of Ca^{2+} release. Further additions to media or deviations from the above conditions are described in the legends to figures and tables.

Determination of cation release and mitochondrial swelling

Duplicate 1.5-ml aliquots were taken periodically from large incubations and the mitochondria were sedimented within 15–20 s using an Eppendorf microcentrifuge [16]. Total centrifugation time was 1.5 min. The supernatants and/or pellets were analyzed for divalent cations by atomic absorption [16] and the results were expressed as nmol of cation released/mg protein and as percentage of intramitochondrial content. Swelling was monitored by apparent absorbance measurements at 540 nm in an Aminco DW2a spectrophotometer operated in split beam mode.

Measurement of mitochondrial volumes

These procedures were similar to earlier methods (e.g., Refs. 21,22). They assume that ^3H_2O readily equilibrates with all mitochondrial water compartments, that dextran (average $M_r = 70\,000$) does not permeate the outer or inner mitochondrial membranes, and that sucrose ($M_r = 359$) and inulin ($M_r = 5000$) permeate the outer but not the inner membrane of normal mitochondria. Following the permeability transition, sucrose but not inulin can enter the matrix space (see Refs. 17, 23, 24 and data shown below). Incubations were conducted under the normal conditions described above except that the protein concentration was 10 mg/ml and pairs of the compartment specific compounds were present, one labeled with tritium (approx. 3000 dpm/μl) and the other with ^{14}C (approx. 300 dpm/μl). Periodically, duplicate 0.5 ml aliquots were taken and layered over 0.3 ml of silicone oil ($d = 1.050$) which had previously been layered over 0.1 ml of 14% perchloric acid. The samples were prepared in 1.5-ml Eppendorf microcentrifuge tubes and were subsequently centrifuged for 1.5 min. These conditions were sufficient for the mitochondria to pass through the silicone oil and precipitate in the perchloric acid layer. The medium layer obtained on top of the silicone was removed, the silicone layer surface and tube walls were washed twice with 0.7 ml water, and the walls of the tube above the silicone were wiped dry. Subsequently, 0.5 ml water was added and the entire sample was sonicated briefly with a probe instrument to disperse the precipitated mitochondria. The resultant emulsion was again centrifuged for 1.5 min, whereupon the emulsion was broken; the silicone oil layer was obtained at the bottom of the tube and the combined H_2O-perchloric acid layer was on top. An aliquot of the aqueous phase was transferred to a vial containing 12 ml of 'Ecolite' scintillation mixture (West Chem, San Diego, CA), and radioactivity was determined in a Beckan LS3801 liquid scintillation counter.

For experiments in which mitochondria underwent complete swelling (use of uncoupler plus Ruthenium red alone, see Results), a modified procedure to separate them from media was required because the less dense organelles no longer passed through the silicone oil layer. In these cases, the mitochondria were sedimented by a 1.5 min centrifugation in the Eppendorf instrument and the supernatant removed. Samples were then centrifuged a second time, for 2 min, and the remaining supernatant was removed by careful blotting. The pellets were dispersed in 0.5 ml of 5% deoxycholate (Na^+), transferred to scintillation vials, and counted as described for the other procedure.

Using the initial amounts of radioactivity per volume of incubation and the amount of radioactivity recovered from the media separation procedures, solute accessible volumes and the volumes of specific mitochondrial compartments were calculated. The ^3H_2O space minus the dextran space was taken as the total mitochondrial water volume. The sucrose or inulin space minus the dextran space could be taken as the intermembrane volume when mitochondria were not subjected to the permeability transition (control experiments). When the transition occurs, sucrose, but not inulin, can enter the matrix. Therefore, with swollen mitochondria, inulin minus dextran was used to determine this value. Likewise, the water space minus the sucrose space or the inulin space is the matrix volume of normal mitochondria. When the transition occurs, water space minus inulin space is still the matrix volume, whereas the increase in sucrose accessible space describes the same quantity. We made use of both approaches to determine this value as required by different aspects of the work (see below).

Trapping of [^{14}C]sucrose within the matrix space was used as one method to demonstrate that the inner membrane had become permeable and then recovered its normal permeability characteristics. The procedure employed was similar to that of Al-Nasser and Crompton [17]. After incubation under conditions of interest, duplicate 0.5-ml aliquots containing 5 mg protein were diluted into 10 ml of ice-cold mitochondrial washing medium which also contained 1 mM EGTA (Li^+) and 1 mM $MgCl_2$. The mitochondria were sedimented by centrifugation at $10\,000 \times g$ for 10 min, resuspended in 3 ml of the same medium, and sedimented again. The

final pellet was solubilized with deoxycholate and counted as described above. Labeled sucrose remaining after the washing procedure was assumed to be trapped in the matrix space because the washing procedure removed all detectable label from suspensions of unaltered mitochondria.

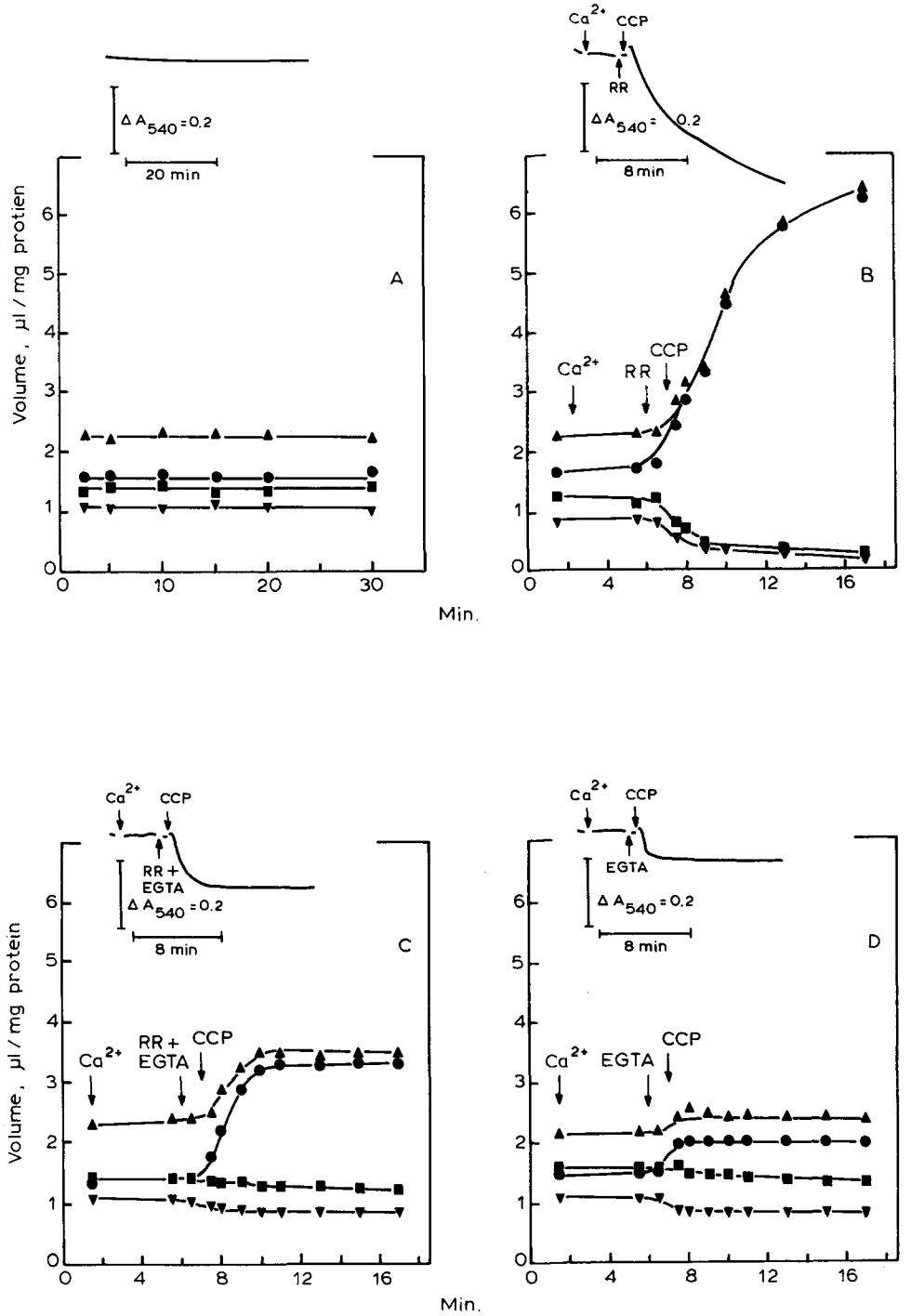

Fig. 1. Conditions affecting solute accessible mitochondrial volumes. Mitochondria were incubated with ^3H_2O plus [^{14}C]sucrose or [^{3}H]inulin plus [^{14}C]dextran as described under Materials and Methods. At the times indicated, samples were taken, the mitochondria were separated from media, and the volumes accessible to the labeled compounds were determined by scintillation counting (see Materials and Methods). Panel A, there were no medium additions following the addition of mitochondria. Panel B, $CaCl_2$ (60 nmol/mg protein), Ruthenium red (RR, 1 nmol/mg protein), and carbonyl cyanide *p*-chlorophenylhydrazone (CCP, 3 nmol/mg protein) were added where indicated. Panel C, same as Panel B except 0.5 mM EGTA was added together with Ruthenium red. Panel D, same as Panel B except that 0.5 mM EGTA was added instead of ruthenium red. For all panels, ▲, total water space; ●, sucrose accessible space; ■, inulin accessible space; ▼, dextran accessible space. The inset in each panel shows the swelling response (determined as described under Materials and Methods) which is referable to the volume changes observed.

Results

Induction and reversal of the inner membrane permeability transition by uncoupler plus EGTA

To examine further the properties of the transition induced by uncoupler plus EGTA and to test the reversibility explanation for the small effects seen with the normal indicators, we determined matrix space volumes and looked for trapping of solutes in the matrix space under various conditions. The data contained in Fig. 1, A and B, show that swelling of Ca^{2+}-loaded mitochondria induced by Ruthenium red plus uncoupler is accompanied by a large increase in the total water content of the organelle. Soon after uncoupler addition, sucrose has access to the entire water volume, indicating that the inner membrane has become permeable to that solute. The inulin and dextran accessible spaces become smaller simultaneously. In the case of inulin, this finding indicates that the permeability change which allows sucrose access to the matrix space does not affect the permeability of the larger inulin molecule over this time-course, and that swelling and the total water volume increase are due to expansion of the matrix which reduces the space available between the inner and outer membranes. * Reduction of the dextran space can be interpreted as a tighter packing of individual mitochondria during centrifugation, reducing the total volume which is not membrane bounded.

When EGTA is present together with Ruthenium red, uncoupler addition again produces rapid swelling; however, the extent is limited to approximately half of that seen with Ruthenium red alone (compare the inset in Fig. 1C with the one in 1B). The total water and sucrose accessible volumes also increase, but the extent is smaller than with Ruthenium red alone, in proportion to the relative extents of swelling. The reductions of inulin and dextran accessible spaces are also diminished by the presence of EGTA.

When EGTA is used in place of Ruthenium red, swelling and the total volume increase are reduced even further, but still occur (Fig. 1D). These small effects are seen more clearly when the marker accessible volumes are converted to specific mitochondrial volumes as in Fig. 2. This form of presentation reveals that the increase in sucrose accessible space (0.7–0.8 μl/mg protein) includes most but not all of the original matrix space volume, since the latter parameter increased from about 0.8 to about 1.05 μl/mg protein

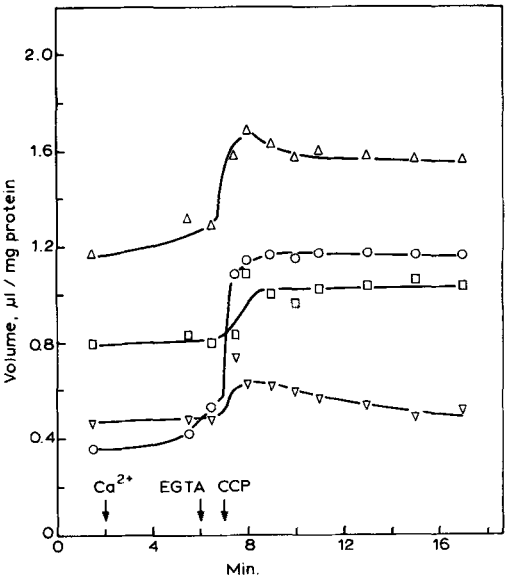

Fig. 2. Specific mitochondrial volume changes during induction of the transition by uncoupler plus EGTA. Experiments are analogous to the one shown in Fig. 1D except that the values presented are specific mitochondrial volumes as opposed to pellet space accessible to a particular solute. △, total mitochondrial water volume (water space minus the dextran space); ○, mitochondrial sucrose accessible space (sucrose space minus the dextran space); □, mitochondrial matrix space (water space minus the inulin space); ▽, the intermembrane space (inulin space minus the dextran space).

following addition of uncoupler. Both the results with Ruthenium red plus EGTA and those with EGTA alone are consistent with an interpretation whereby for a period of time following uncoupler addition, the inner membrane is permeable, allowing sucrose to enter the matrix and some swelling to occur. However, as free Ca^{2+} is chelated, reversal of the permeability change occurs before swelling is complete. Possible reasons for sucrose not equilibrating with the entire matrix space volume are considered in Discussion.

The reversibility explanation requires that sucrose taken up during swelling be ultimately trapped within the matrix space and that the increase in this volume be not accessible to sucrose or inulin added after the period of swelling. Fig. 3 addresses the first requirement by showing that sucrose trapping is observed and that the trapped sucrose volume approximates the increase in matrix space volume determined by an alternative method. Other experiments showed that sucrose was also trapped when EGTA and Ruthenium red were used together, but not when Ruthenium red was used alone (data not shown).

The changes in matrix space volume, measured as water space minus the sucrose or inulin accessible spaces, after the period of swelling is completed, are given in Table I. Both in the case of Ruthenium red plus EGTA and EGTA alone, an increased volume is measured, and the values obtained are independent of whether the sucrose space or the inulin space is used.

* In some preparations, control or decreasing values for the inulin and dextran accessible spaces are obtained initially, but these quantities increase substantially after a period of time (data not shown). We attribute this behavior to eventual breakage of the membranes, allowing inulin and dextran to enter all compartments.

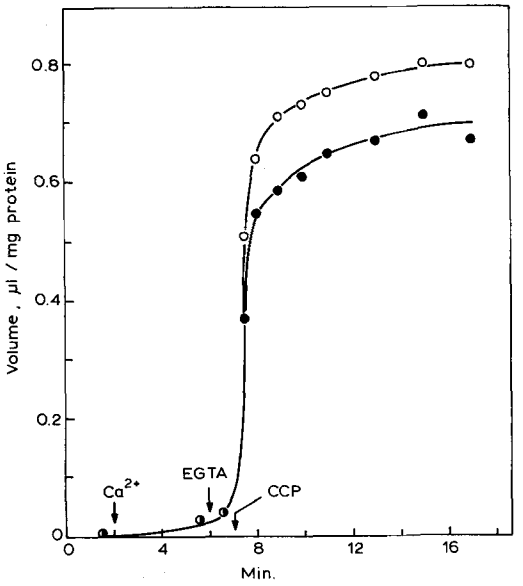

Fig. 3. Sucrose trappling in the matrix space during induction of the transition by uncoupler plus EGTA. •, Trapped sucrose volume. Mitochondria were incubated with ^3H_2O plus [^{14}C]sucrose as described in Materials and Methods. Additions of Ca^{2+}, EGTA and CCP were as described in the legend to Fig. 1D. Where indicated, samples were taken for the determination of trapped sucrose using the washing and sedimentation procedure described in Materials and Methods. ^3H_2O was essentially absent from the final pellet as expected. The values presented were calculated from the [^{14}C]sucrose which remained. ○, Change in matrix space volume. The experiment was run parallel to the one represented by closed circles using the same mitochondrial preparation. The incubation contained ^3H_2O plus [^{14}C]inulin, and the samples were sedimented through silicone oil as described in Materials and Methods. For each value, the initial matrix space value was subtracted to obtain the change in this parameter.

These findings show that the increase in matrix space volume is not accessible to either solute after swelling is complete, providing EGTA was present during the period of swelling (the second requirement described above). It is therefore clear that the permeability transition occurs, but is transient, when the medium contains that chelator.

Effects of Mg^{2+} on specific mitochondrial volume changes induced by uncoupler plus EGTA

EDTA cannot substitute for EGTA to induce the transition [10]. That finding and related data were taken to indicate that low concentrations of extramitochondrial Mg^{2+} are required to obtain inhibition of reverse uniport by Ca^{2+} chelation [10]. This interpretation implies that a pathway exists which can release several μM Mg^{2+} from the matrix space, relatively quickly, because the differing behavior obtained with the two chelators is seen even when EDTA is used in place of EGTA during mitochondrial preparation [10]. While the explanation is consistent with the data, it is argumentative because the known pathway for Mg^{2+} release is quite slow ($\sim$ 0.2 nmol/min per mg protein) and proceeds best when mitochondria are energized (see Ref. 25 for review).

To test the previous explanation for the ineffectiveness of EDTA, we investigated submitochondrial volume changes induced by chelators plus uncoupler using Mg^{2+} and Ca^{2+} depleted preparations. Depletion of Ca^{2+} alone gave results like those shown in Fig. 2 (not shown), while Fig. 4 shows that when endogenous Mg^{2+} has also been removed, EGTA plus uncoupler no longer produces swelling, matrix space expansion, or access of sucrose to this volume. Adding extramitochondrial Mg^{2+} results in the depleted preparations responding in the same way as the normal organelles (compare Figs. 2 and 4). Furthermore, when Mg^{2+} has been added back, EDTA again fails to produce the permeability response seen with EGTA (data not shown). Thus, a role for extramitochondrial Mg^{2+} in

TABLE I

Changes in the mitochondrial matrix space volume during uncoupler associated swelling

Mitochondria were incubated as described under Materials and Methods with $CaCl_2$ (60 nmol/mg protein) added at 2 min, followed 4 min later by Ruthenium red (1 nmol/mg protein) plus EGTA (0.5 mM), or EGTA alone. For the values designated 'before uncoupler', ^3H_2O plus [^{14}C]sucrose or ^3H_2O plus [^{14}C]inulin were then added and samples were taken for the determination of the matrix volume (see Materials and Methods) as the water space minus the sucrose space or the water space minus the inulin space. For the values designated 'after uncoupler', the addition of CCP (3 nmol/mg protein) followed Ruthenium red plus EGTA or EGTA alone and an additional 5 min were allowed for the swelling to reach completion. The labeled compounds were then added and samples were taken as described for the before uncoupler values. All values are means ± S.D. for four replicate determinations.

Condition	Matrix space volume (μl/mg protein)			
	water minus sucrose space		water minus inulin space	
	before uncoupler	after uncoupler	before uncoupler	after uncoupler
Ruthenium red plus EGTA	0.82 ± 0.06	2.31 ± 0.16	0.84 ± 0.04	2.40 ± 0.12
EGTA alone	0.78 ± 0.08	1.22 ± 0.05	0.80 ± 0.07	1.30 ± 0.16

generation of the transition by uncoupler plus EGTA is confirmed.

Effects of Sr^{2+} loading on the permeability transition induced by uncoupler plus EGTA

If the permeability transition induced by uncoupler plus EGTA and uncoupler plus Ruthenium red occurs through the same basic mechanism which is shared by the other known inducing agents, one would expect sensitivity to known inhibitors of the phenomenon. The most effective ways to antagonize the transition so far identified are substituting Sr^{2+}-loaded for Ca^{2+}-loaded mitochondria and employing the high activity inhibitor cyclosporin A [1]. Fig. 5 shows that Sr^{2+}-loaded mitochondria treated with EGTA plus uncoupler or Ruthenium red plus uncoupler do not swell and do not show an increase in water or matrix space volumes. They also fail to release a substantial fraction of endogenous Mg^{2+} (Fig. 6), which is further evidence that under these conditions the permeability transition does not occur. Sensitivity of the transition induced by uncoupler plus Ruthenium red to cyclosporin A has been shown before [26], and subsequent experiments have

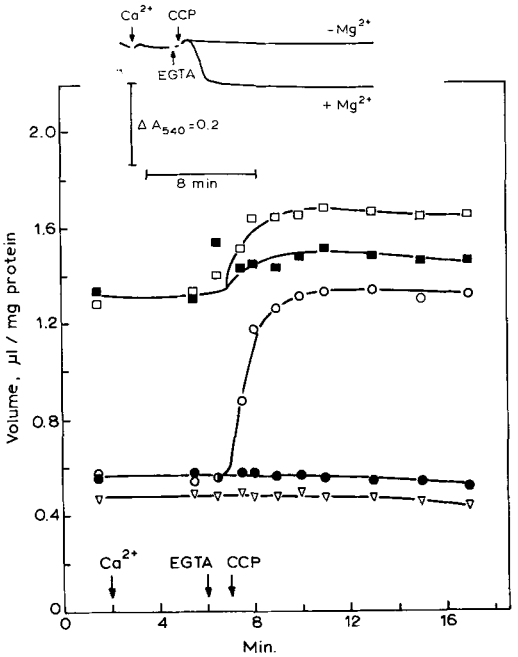

Fig. 4. Requirement for extramitochondrial Mg^{2+} to produce a transient permeable state. Experiments were conducted as described in the legend to Fig. 2 except Ca^{2+}, Mg^{2+} depleted mitochondria were used (see Materials and Methods). $CaCl_2$ (60 nmol/mg protein), EGTA (0.5 mM), and CCP (3 nmol/mg protein) were added where indicated, whereas when employed, $MgCl_2$ was present from the beginning of the incubations. ■ and □, total mitochondrial water volume with 1.0 mM $MgCl_2$ absent or present, respectively; ● and ○, sucrose accessible space with 1.0 mM $MgCl_2$ absent or present, respectively; ▽, intermembrane space 1.0 mM $MgCl_2$ absent or present. The inset shows the swelling response which is referable to the volume changes observed.

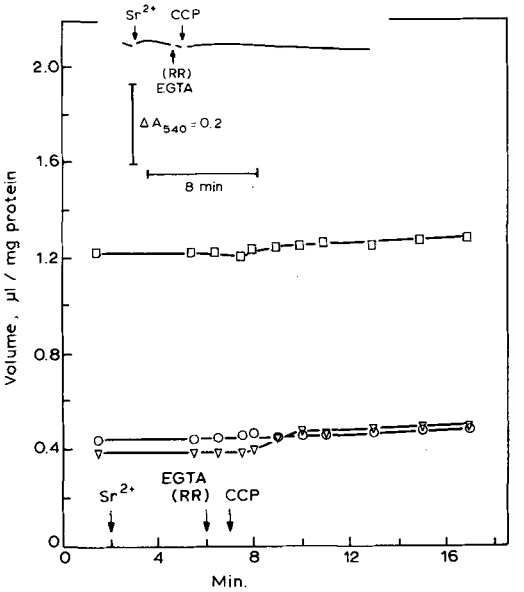

Fig. 5. The effect of reverse uniport inhibition and uncoupler on the volumes of Sr^{2+}-loaded mitochondria. Experiments were conducted as described under Materials and Methods and the legends to Figs. 1 and 2, except that mitochondria were loaded with 60 nmol/mg protein of $SrCl_2$ instead of $CaCl_2$. □, total mitochondrial water volume; ○, sucrose accessible space; ▽, intermembrane space. Results were essentially not affected by substituting ruthenium red for EGTA as indicated in the figure. The inset shows the swelling response (determined as described under Materials and Methods) which is referable to the volume changes observed.

produced analogous data using uncoupler plus EGTA (not shown).

Discussion

Swelling, Mg^{2+} release, and access of sucrose or other nontransported solutes to the matrix space are the most reliable indicators of the permeability transition. Parameters such as membrane potential or Ca^{2+} release, which depend directly upon the energetic state, are not applicable when uncoupler is present and are less useful in any case because they yield a distorted time-course, due to the coexistence of permeable and normal mitochondria as the phenomenon develops across a population [1]. By all three of the reliable criteria, the transition is generated by uncoupling Ca^{2+}-loaded, Ruthenium red-inhibited mitochondria (Figs. 1 and 6). Induction of the transition by EGTA plus uncoupler is demonstrated most clearly by Fig. 2, which shows that sucrose becomes largely equilibrated with the matrix space. The extent to which sucrose equilibrates can be ascertained from the data in that figure. The increase in sucrose accessible space upon addition of uncoupler is about 0.8 μl/mg protein, whereas the matrix space volume which is ultimately attained is about 1 μl/mg protein. This 80% equilibration corresponds closely to the extent of Mg^{2+} release

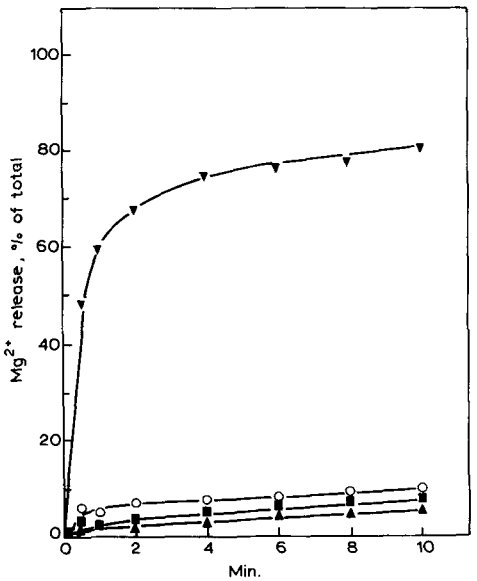

Fig. 6. The absence of Mg^{2+} release from Sr^{2+}-loaded mitochondria. Mitochondria were incubated (1.0 mg protein/ml) and samples were taken periodically for the determination of Mg^{2+} release as described under Materials and Methods. For all symbols except the inverted triangles (▼), $SrCl_2$ (60 nmol/mg protein) was added 2 min and CCP (3 nmol/mg protein) was added 7 min after the beginning of the incubation. For the inverted triangles (▼), $CaCl_2$ replaced $SrCl_2$ at the same concentration. In the figure, 0 min is the time of uncoupler addition. Additions made 1 min before uncoupler were as follows: ▲, Ruthenium red (1 nmol/mg protein); ■, EDTA (0.5 mM); ○, EGTA (0.5 mM); (▼) EGTA (0.5 mM). The amount of Mg^{2+} located in the extramitochondrial medium before Sr^{2+} addition has been subtracted from the experimental values and the total Mg^{2+} present before calculation of % release.

(Fig. 6) and could indicate that individual mitochondria are in the permeable state for a very short time, such that sucrose entry and Mg^{2+} release cannot be completed. Alternatively, 20% of the mitochondria could remain impermeable throughout the experiment and produce the same result. Because the diffusion constants of sucrose and Mg^{2+} differ substantially and because ultrastructural data show that some mitochondria retain the aggregated conformation long after the other responses are completed [10], it is probable that the second explanation is the correct one. It is not clear at present why some mitochondria would be more stable to the transition than others.

In addition to substituting for Ruthenium red, EGTA can limit the extent of swelling and matrix space expansion when it is used together with Ruthenium red to induce the transition (Fig. 1). This result is consistent with the known requirement for free Ca^{2+} to maintain the permeable state [17,23,27–30] and is a finding required by our interpretation of the EGTA effects. The smaller swelling and volume responses obtained with EGTA alone probably indicate that reversal of the transition is more rapid in the absence of Ruthenium red, although it is not clear why this would be true. Possible explanations include a more rapid depletion of Ca^{2+} following uncoupler addition when Ruthenium red is absent or a minor interaction between excess Ruthenium red and components involved in generating and reversing the permeable state. The first explanation is supported by recent work which shows that the rate of free Ca^{2+} depletion from the matrix space limits the rate of reversal [31]. Regardless of why the response magnitudes differ, the sucrose trapping experiments show clearly that in both cases the transition occurs but is quickly reversed. The demonstration that the transition can be generated and reversed on a time-scale which is fast enough to substantially avoid swelling and the resulting disruption of mitochondrial structure can be added to other arguments which support a possible physiological role for the transition [1].

The present data show unambiguously that EGTA does not induce the transition in conjunction with uncoupler in the absence of extramitochondrial Mg^{2+} (Fig. 4). This is in contrast to Ruthenium red plus uncoupler, which produce the transition in the normal way when Ca^{2+}- and Mg^{2+}-depleted mitochondria are employed (not shown). It was further determined that Ca^{2+}- and Mg^{2+}-depleted mitochondria take up and retain Ca^{2+} in the same fashion as normal mitochondria and do not lose Ca^{2+} rapidly upon the addition of EGTA alone (not shown). Thus, when interpreted within the context of reverse uniport inhibition by EGTA as the explanation for why the transition occurs, these findings indicate that the putative external cation binding site must be occupied by Mg^{2+} for inhibition to occur. Studies of EGTA effects on reverse uniport activity in Sr^{2+}-loaded mitochondria are strong support for this conclusion [32]. In contrast, Mg^{2+} appears not to be required to obtain reverse uniporter inhibition by Ruthenium red.

In summary, the present findings show that both Ruthenium red plus uncoupler and EGTA plus uncoupler induce the permeability transition in Ca^{2+}-loaded mitochondria. The differing extents to which mitochondrial volume changes and swelling, Mg^{2+} release and ultrastructural changes accompany the transition induced by the two conditions is a consequence of rapid restoration of the impermeable state which occurs with EGTA plus uncoupler. Pending resolution of controversy on the effectiveness of Ruthenium red as in inhibitor of reverse uniport (see Introduction), these findings indicate that EGTA (a low extramitochondrial Ca^{2+} concentration) inhibits reverse activity of the Ca^{2+} uniporter when the medium contains external Mg^{2+} (see also Ref. 32). Further work will be required to determine why uncoupling induces the transition when the uniporter is not active. The absence of a rapid Ca^{2+} efflux pathway is one factor involved as shown previously by use of Ca^{2+} ionophores [8]. De-

creased membrane potential, pyridine nucleotide and sulfhydryl oxidation, ATP hydrolysis and related effects associated with induction of the transition (1) are also produced by uncoupling. Presumably, it is these effects, coupled with a persistent high Ca^{2+} content which are responsible.

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