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# THE ALTERATIONS IN THE ENERGY LINKED PROPERTIES INDUCED IN RAT LIVER MITOCHONDRIA BY ACETYLSALYCILATE ARE PREVENTED BY CYCLOSPORIN A OR $Mg^{2+}$

CARLA BIBAN, VINICIO TASSANI, ANTONIO TONINELLO,*
DAGMAR SILIPRANDI and NORIS SILIPRANDI

Dipartimento di Chimica Biologica dell'Universita' di Padova e Centro Studio Fisiologia Mitocondriale del C.N.R., via Trieste 75, 35121 Padova, Italy

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**Abstract**—The alterations in rat liver mitochondria induced by acetylsalicylate in the presence of low concentrations of $Ca^{2+}$ (large amplitude swelling, permeability to [$^{14}$C]sucrose, collapse of transmembrane potential and effluxes of endogenous $Mg^{2+}$ and accumulated $Ca^{2+}$) were fully prevented by either cyclosporin A or $Mg^{2+}$. Cyclosporin A and $Mg^{2+}$ were also capable of restoring transmembrane potential upon its decrease induced by acetylsalicylate. The loss of endogenous $Mg^{2+}$ was the primary effect promoted by acetylsalicylate; the other noxious effects followed. These results indicate that $Mg^{2+}$ are fundamental components of the mitochondrial permeability barrier and that their loss might be responsible for the membrane transition induced by acetylsalicylate.

*Key words:* acetylsalicylate; mitochondria; permeability transition; $Ca^{2+}$; cyclosporin A; $Mg^{2+}$

Salicylates induce mitochondria dysfunctions analogous, or identical, to those found in Reye's syndrome [1–4]. Since the alterations of mitochondria permeability properties promoted by salicylates occur only in the presence of $Ca^{2+}$, Gunter and Pfeiffer [5] included these compounds on the list of "the inducers of permeability transition", i.e. the agents of permeability transition in $Ca^{2+}$-containing mitochondria. According to these authors, the release of endogenous $Mg^{2+}$ and the sensitivity to cyclosporin A may be considered unambiguous indicators of permeability transition. Until now these two parameters have not been studied in relationship with ASA† action. The present study sought to determine whether the alterations induced by ASA in liver mitochondria are ascribable to the permeability transition of mitochondrial membrane. The results reported herein show that ASA induces a prompt release of endogenous $Mg^{2+}$ from liver mitochondria and a concomitant large amplitude swelling. Either cyclosporin A or added $Mg^{2+}$ fully prevents these alterations as well as the decrease in $\Delta\Psi$ and the concomitant efflux of accumulated $Ca^{2+}$.

## MATERIALS AND METHODS

Rat liver mitochondria were isolated in 250 mM sucrose and 5 mM HEPES (pH 7.4) by conventional differential centrifugation [6]. Mitochondrial protein concentration was assayed by a biuret method with bovine serum albumin as standard.

$\Delta\Psi$ was measured by monitoring the distribution of the lipophilic cation, $TPP^+$, across the mitochondrial membrane with a selective electrode prepared in our laboratory according to published procedure [7, 8] and an Ag/AgCl reference electrode. The membrane potential measured in an open, thermostatically controlled and stirred vessel with the $TPP^+$ selective electrode was calibrated using the equation $\Delta\Psi = (\Delta\Psi_{electrode} - 66.16\ mV)/0.92$ as proposed by Jensen *et al.* [9]. Swelling was estimated by changes in absorbance at 540 nm using a Perkin-Elmer Lambda 5 spectrophotometer equipped with thermostatic control. $Ca^{2+}$ and $Mg^{2+}$ content either in mitochondrial pellets or in the supernatant was estimated by atomic absorption spectroscopy [10, 11]. [$^{14}$C]-Sucrose permeation was determined according to the method of Crompton and Costi [12].

Rat liver mitochondria (1 mg/mL) were incubated for 30 min at 20° in a standard medium containing 200 mM sucrose, 10 mM HEPES (pH 7.4), 5 mM succinate, 1 mM phosphate and 1.25 $\mu$M rotenone. Sodium salts were used.

## RESULTS

### *Mitochondria swelling and sucrose permeation*

As shown in Fig. 1 ASA induced a large amplitude swelling of rat liver mitochondria [3]. Omission of $Ca^{2+}$ or inhibition of their uptake by ruthenium red prevented the swelling, indicating that $Ca^{2+}$ uptake is a necessary condition for ASA action. It has also been observed that in the absence of ASA $Ca^{2+}$ had no effect *per se* (results not reported). That abnormal mitochondria permeability induced by ASA was further proven by the acquired permeability to sucrose [12] as shown by the numbers in the rectangles reported in Fig.1. These numbers stand for the amount of [$^{14}$C]sucrose taken up in the presence of ASA [12]. Either cyclosporin A or $Mg^{2+}$

* Corresponding author: Tel. +39-49-8286448; FAX +39-49-8073310.

† Abbreviations: ASA, acetylsalicylate; $\Delta\Psi$, electrical transmembrane potential; $TPP^+$, tetraphenylphosphonium.

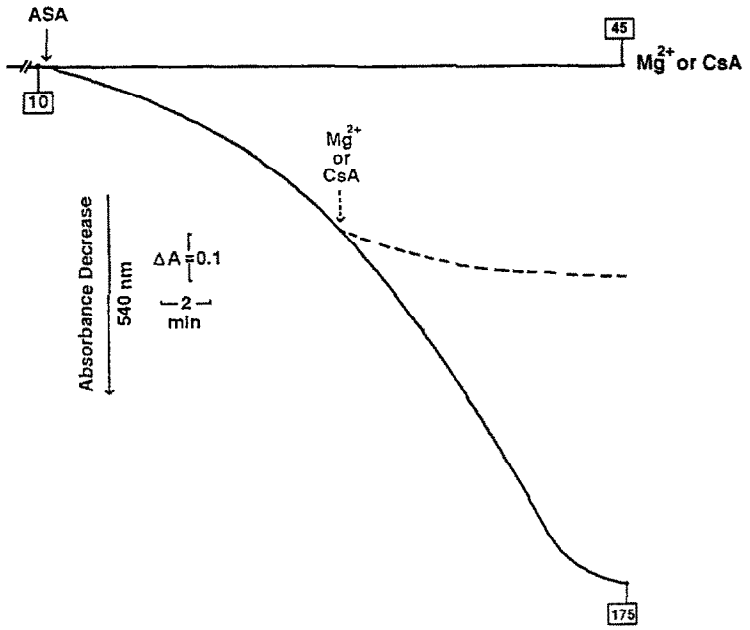


Fig. 1. Effect of cyclosporin A and $Mg^{2+}$ on large-amplitude swelling and sucrose permeation in rat liver mitochondria treated with ASA. Mitochondria (1 mg/mL) were incubated in the standard medium containing 10 $\mu$M $Ca^{2+}$. Where indicated 0.5 mM ASA, 0.5 $\mu$M cyclosporin A or 1 mM $Mg^{2+}$ were added. Numbers in rectangles indicate the amount (nmols/mg protein) of [$^{14}$C]sucrose (specific activity 0.5 $\mu$Ci/mmol) taken up.

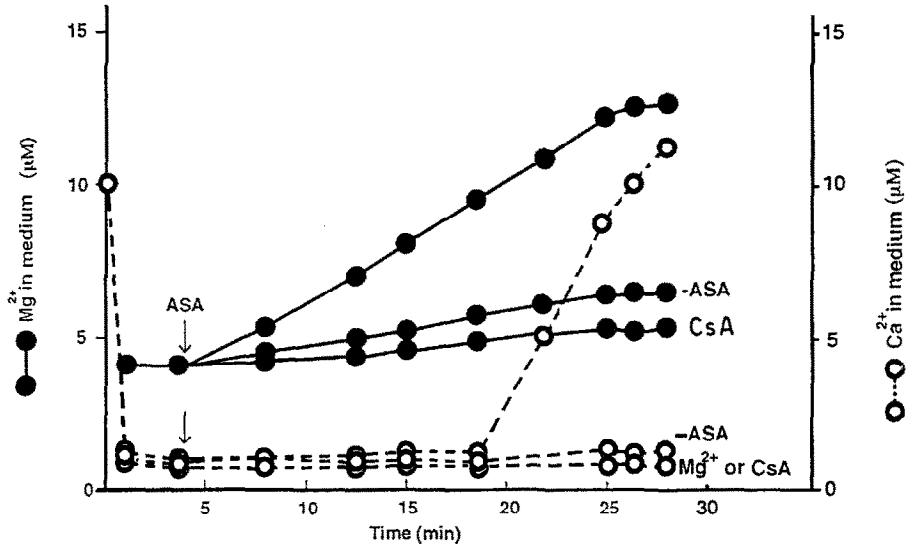


Fig. 2. Effect of cyclosporin A or $Mg^{2+}$ on the efflux of endogenous $Mg^{2+}$ and accumulated $Ca^{2+}$ induced by ASA. Experimental conditions as in Fig. 1. Where indicated 0.5 mM ASA, 0.5 $\mu$M cyclosporin A or 1 mM $Mg^{2+}$ were added. Endogenous cations content: $Mg^{2+}$ 16.6 nmol/mg protein, $Ca^{2+}$ 13 nmol/mg protein.

fully prevented both mitochondrial swelling and abnormal sucrose diffusion across mitochondrial membrane. Furthermore, addition of cyclosporin A or $Mg^{2+}$ after the swelling had begun strongly dampened the rate of swelling, which eventually came to a halt.

*Release of endogenous $Mg^{2+}$ and accumulated $Ca^{2+}$*

The appearance of $Mg^{2+}$ in the external medium (Fig. 2) immediately after the liver mitochondria were suspended indicates that $Mg^{2+}$ had been released from mitochondria, presumably from external sites (outer membrane and intermembrane

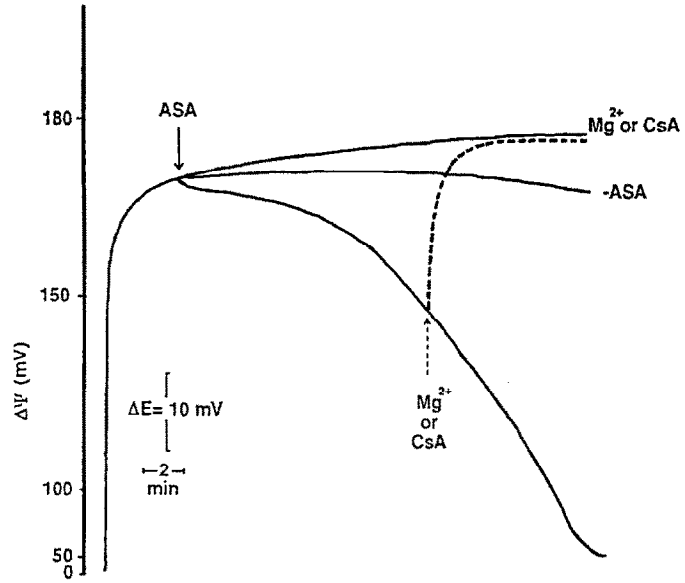


Fig. 3. Effect of cyclosporin A or $Mg^{2+}$ on $\Delta\Psi$ fall induced by ASA. Experimental conditions as in Fig. 1 with 2 $\mu$M $TPP^+$ present. Where indicated 0.5 mM ASA, 0.5 $\mu$M cyclosporin A or 1 mM $Mg^{2+}$ were added.

space). Thereafter the concentration of external $Mg^{2+}$ remained constant for at least 30 min. During this time the release of $Mg^{2+}$ increased very slowly in the presence of 10 $\mu$M $Ca^{2+}$. Such an increase was strongly enhanced by ASA, and prevented by cyclosporin A. Figure 2 also shows that ASA also induced the release of accumulated $Ca^{2+}$, otherwise retained until the completion of anaerobiosis. Unlike $Mg^{2+}$, $Ca^{2+}$ were not released immediately, but about after 18 min of incubation with ASA. At this time (see Fig. 3) the transmembrane potential has a value below 130 mV. Once again either cyclosporin A or $Mg^{2+}$ prevented $Ca^{2+}$ efflux.

Figure 3 shows that in the presence of ASA $\Delta\Psi$ of liver mitochondria, energized with succinate, decreased until collapse. Such a fall was prevented by either cyclosporin A or $Mg^{2+}$ when added from the beginning and fully restored when added later.

It should be outlined that the results relative to sucrose uptake, mitochondrial swelling and endogenous $Mg^{2+}$ and accumulated $Ca^{2+}$ effluxes have been obtained in simultaneous measurements. In another set of experiments, using the same mitochondria, $\Delta\Psi$, $Mg^{2+}$ and $Ca^{2+}$ effluxes have been measured concomitantly. The rate and the extent of $Ca^{2+}$ and $Mg^{2+}$ effluxes were identical in the two sets of experiments thus allowing the comparison of all the reported parameters.

The results obtained by applying the method of data analysis proposed by Riley and Pfeiffer [13] for the comparison of the time course of the considered parameters (large amplitude swelling, $\Delta\Psi$ variations and $Mg^{2+}$ release) are reported in Fig. 4. They seem to indicate that the release of endogenous $Mg^{2+}$ is the primary event promoted by ASA and that mitochondrial swelling and decay of $\Delta\Psi$, as well as $Ca^{2+}$ efflux, are secondary effects.

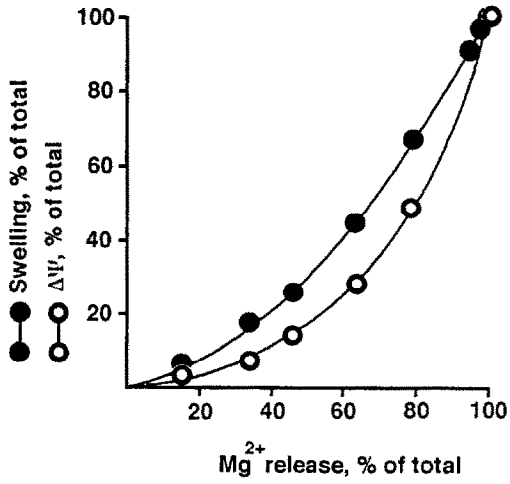


Fig. 4. Dependence of swelling and $\Delta\Psi$ on $Mg^{2+}$ efflux. Experiments were conducted as described in the legends to Figs 1–3. Data were converted to per cent of the maximal response on the basis of the total amount of $Mg^{2+}$ release (nmol/mg protein), swelling ($\Delta A_{540\,nm}$) and $\Delta\Psi$ fall (mV). The amounts of $Mg^{2+}$ which were found in the supernatant at 4 min (prior to the addition of ASA) were subtracted from the values observed at the latter times, as indicated in Fig. 2. The values presented for $\Delta A_{540\,nm}$ and $\Delta\Psi$ were extrapolated from Figs 1 and 3 at the same times.

## DISCUSSION

The results reported in the present paper confirm previous results [3, 4] and provide new insight into the problem of ASA potentiation of $Ca^{2+}$ harmful

action. The noxious action of ASA in the presence of low concentrations of $Ca^{2+}$ reported here is virtually identical to that induced by high concentrations of $Ca^{2+}$ [3, 14, 15]. Moreover, two novel results have been brought to light: the full prevention of ASA damage by either cyclosporin A or $Mg^{2+}$ and the restorative effects of these agents after induction of these alterations. The sensitivity to cyclosporin A indicates that the described effects of ASA are consistent with the induction of "permeability transition" previously described by Hunter and Haworth [16] and that ASA is an agent capable of opening in the inner mitochondrial membrane aspecific pores permeable to sucrose (Fig. 1) and susceptible of being resealed either by cyclosporin A or by removal of $Ca^{2+}$ [12]. As recently demonstrated by Schweizer *et al.* [17] cyclosporin A can also prevent the operation of the specific $Ca^{2+}$ release pathway, which does not engage the opening of the aspecific pore. However preliminary results, not reported in the present paper would indicate that ASA does not affect the specific $Ca^{2+}$ release. Therefore, also on the basis or previous results [2, 3] we propose that ASA might act as an activator of Ca and $P_i$ in promoting the permeability transition. Both cyclosporin A and $Mg^{2+}$ prevent this adverse effect expressed by large amplitude swelling, collapse of $\Delta\Psi$ and loss of endogenous $Mg^{2+}$. The importance of $Mg^{2+}$ in the described mechanism emerges from two observations: (1) addition of $Mg^{2+}$ to ASA-damaged mitochondria fully restores $\Delta\Psi$ (Fig. 3), and (2) according to the method proposed by Riley and Pfeiffer, based on the comparison of the percentage of the calculated data [13], it would appear that the release of endogenous $Mg^{2+}$ preceeds the other effects triggered by ASA (large amplitude swelling, collapse of $\Delta\Psi$ (Fig. 4)) and the dependent efflux of accumulated $Ca^{2+}$ (Fig. 2). The irreversibility of mitochondrial swelling may be accounted for by the trapping of sucrose and other external solutes in mitochondria upon the resealing of the ASA-produced pores. It is surprising, however, that despite massive swelling (Fig. 1) mitochondrial transmembrane potential is fully restored by $Mg^{2+}$. One explanation is that the impermeability of the membrane to protons could have been restored in the less injured mitochondria of the whole population. Evidently the proton permeability of less damaged mitochondria is restored by these cations, which should be considered fundamental components of the membrane permeability barrier [18]. Taken together the reported results indicate that $Mg^{2+}$ loss from mitochondria may be the first and main cause of the damaging effects of salicylates.

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