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Ca^{2+}-mediated action of long-chain acyl-CoA on liver mitochondria energy-linked processes

Fabio Di Lisa [1], Roberta Menabò [1], Gianni Miotto [1], Valentina Bobyleva-Guarriero [2] and Noris Siliprandi [1]

[1] *Centro Studio Fisiologia Mitocondriale, CNR and Dipartimento di Chimica Biologica, Università di Padova, Padova and* [2] *Istituto di Patologia Generale, Modena (Italy)*

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The decrease of steady-state transmembrane potential ($\Delta\Psi$) and loss of accumulated Ca^{2+} are magnified if palmitoyl-CoA is added to rat liver mitochondria exposed to Ca^{2+} and phosphate. The extent of this damage increases with increasing concentration of long-chain acyl-CoA. Addition of L-carnitine with or without the addition of palmitoyl-CoA considerably delays the deenergization. In the latter case, there is a substantial decrease in the assayed endogenous long-chain acyl-CoA content. This protective action of L-carnitine is abolished by L-aminocarnitine, a powerful inhibitor of carnitine palmitoyl transferase (palmitoyl-CoA: L-carnitine *O*-palmitoyltransferase, EC 2.3.1.21.). The removal of Ca^{2+} by EGTA, or the inhibition of its uptake by Ruthenium red or Mg^{2+} further enhances the degree of protection.

Introduction

Rat liver mitochondria submitted to stresses, such as exposure to hydroperoxides, or high concentration of Ca^{2+} and phosphate (P_i), undergo a rapid fall in their transmembrane potential ($\Delta\Psi$) with a concomitant release of endogenous Mg^{2+} and accumulated Ca^{2+} [1]. Addition of L-carnitine to the incubation medium considerably delays the onset of these effects [2].

The main function of carnitine is to interact with long-chain acyl-CoA (LCACoA) forming the corresponding acyl carnitine in a reaction catalysed by the membrane bound carnitine palmitoyltransferase (CPT) [3]. Hence the protective action of carnitine on mitochondrial function could be due to the removal of accumulated LCACoA. The latter, though probably harmless to mitochondria in physiological condition unless they accumulate above a critical concentration, may become toxic if the particles are stressed, particularly if this is done with high concentrations of Ca^{2+}.

In the present paper evidence is provided that addition of palmitoyl-CoA to rat liver mitochondria affects (in a concentration-dependent manner) such energy-linked functions as transmembrane potential and the capability to retain accumulated Ca^{2+}. This deenergizing action of LCACoA is largely prevented by added L-carnitine and entirely suppressed by removal of Ca^{2+} from the suspending medium by EGTA, or by inhibition of its uptake by Ruthenium red.

Methods

Mitochondria from livers of 24 h fasted rats were isolated by centrifugation in 0.25 M sucrose and 5 mM Hepes (pH 7.4) [4]. Membrane potential ($\Delta\Psi$) was measured by monitoring the distribution of tetraphenylphosphonium (TPP^+) across the mitochondrial membrane with a TPP^+-selective electrode prepared in our laboratory according to Kamo et al. [5], using a calomel electrode (radiometer K401) as the response electrode. The values obtained with the electrode potential ($\Delta\Psi_{electrode}$) are linear with respect to the logarithm of the TPP^+ concentration and have been corrected by the equation

$$\Delta\Psi = (\Delta\Psi_{electrode} - 66.16\ mV)/0.92$$

according to Jensen et al. [6]. The Ca^{2+} electrode signal was standardized against known values of free Ca^{2+} concentrations using Ca^{2+}/nitrilotriacetate buffers [7].

Abbreviations: EGTA, ehylene glycolbis(β-aminoethylether)-*N,N,N',N'*-tetracetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LCACoA, long-chain acyl-CoA; P_i, inorganic phosphate; CPT, carnitine palmitoyltransferase; TPP^+, tetraphenylphosphonium;

Correspondence: F. Di Lisa, Centro Studio Fisiologia Mitocondriale, CNR, Via Marzolo 3, 35131 Padova, Italy.

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Following a 5 min preincubation of a mitochondrial suspension (approx. 30 mg/ml) at 4°C with or without 2 mM L-carnitine present the mitochondrial LCACoA content was measured as free CoA after alkaline hydrolysis of the pellet resulting from perchloric acid deproteinization [8].

CoA was then determined both by the enzymatic cycling method of Veloso and Veech [9] and reversed-phase HPLC [10]. No major differences were found between the two methods. HPLC was performed by means of an octadecyl silane column (Altex Ultraphere-ODS, 4.6 × 250 mm, 5 μM) and a mobile phase containing 50 mM potassium phosphate (pH 5.5) and 12% methanol. The flow rate was 1 ml/min and CoA was detected at 259 nm.

Mitochondrial incubations were carried out at 20°C with 1 mg mitochondrial protein per ml in the following standard medium: 200 mM sucrose, 10 mM Hepes (pH 6.8), 5 mM succinate, 1.25 μM rotenone, 3 mM P_i and 30 μM Ca^{2+}. More precisely final [Ca^{2+}] was brought to 30 μM after having determined total calcium content in media by atomic absorption spectroscopy. Alternatively, mitochondria were incubated in an isosmotic KCl medium. Other additions are indicated in the figure legends.

Results

Addition of an increasing amount of palmitoyl-CoA to liver mitochondria suspended in a sucrose medium containing 30 μM Ca^{2+} and 3 mM P_i caused a progressive fall in their steady-state $\Delta\Psi$. Indeed at concentrations above 2 μM palmitoyl-CoA prevented the normal values (approx. 165 mV) being reached at all (Fig. 1).

This deleterious effect of added palmitoyl-CoA did not occur either if Ca^{2+} was removed from the medium with EGTA, or if its uptake was prevented by Ruthenium red. The same protective action was achieved by addition of 2 mM Mg^{2+}, which is known to abolish the calcium cycling in liver mitochondria [11] and the effects of long-chain fatty acids on potassium uptake by liver mitochondria [12]. Ca^{2+} flux across the mitochondrial membrane seems therefore to be a necessary condition for the deenergizing action of palmitoyl-CoA. The same action of palmitoyl-CoA has been observed by replacing the sucrose medium with isosmotic KCl (see dashed lines in Figs. 2 and 4). The steady-state $\Delta\Psi$ values obtained in KCl medium are probably due to the diffusion of potassium across the mitochondrial membrane.

As it may be deduced from the relative delays of $\Delta\Psi$ collapse, at a given long-chain acyl-CoA addition, 2 mM L-carnitine also had a protective effect, but quantitatively lower than that exhibited either by EGTA or by Ruthenium red or by Mg^{2+} (Fig. 2). However, unlike Ca^{2+} removal, L-carnitine addition did not prevent the

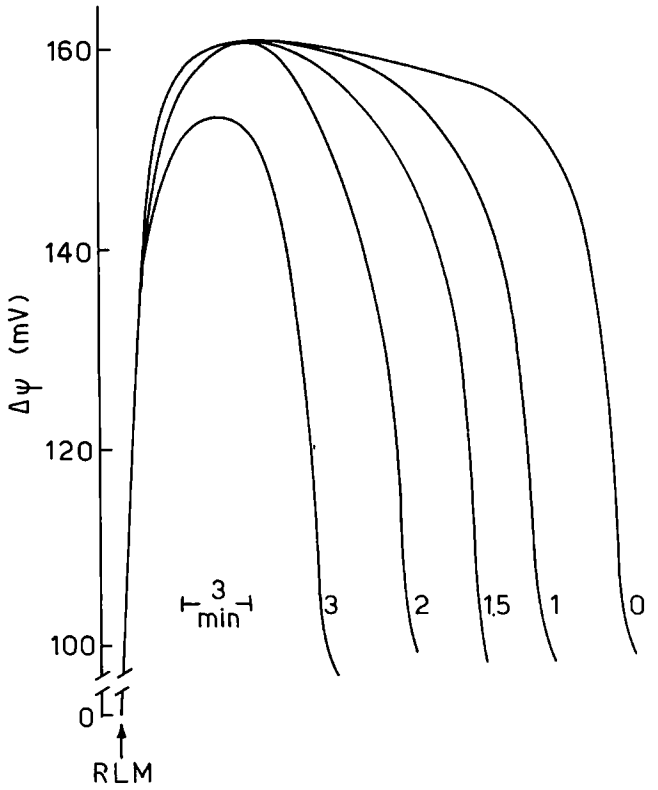

Fig. 1. Action of increasing amount of long-chain acyl-CoA on the maintenance of mitochondrial $\Delta\Psi$. Rat liver mitochondrial (RLM) were incubated in the standard medium described in Methods. Inserted numbers indicate the concentrations of added palmitoyl-CoA (nmol per mg mitochondrial protein) in the suspending medium.

decrease of steady-state $\Delta\Psi$ induced by Ca^{2+} and P_i addition. The addition of L-aminocarnitine, a potent inhibitor of CPT [13], abolished the effect of L-carnitine on mitochondrial $\Delta\Psi$, indicating that, as expected, the conversion of LCACoA into the corresponding long-chain acylcarnitine is involved in the protective process.

Added L-carnitine also retarded the collapse of $\Delta\Psi$ of mitochondria incubated in the presence of Ca^{2+} and P_i, but in the absence of palmitoyl-CoA (Fig. 3), suggesting that endogenous LCACoA could also be deleterious to mitochondria. However, whereas the removal of Ca^{2+} from the medium, or the inhibition of its uptake, allowed the maintenance of the steady-state level of $\Delta\Psi$, the partial removal of LCACoA by L-carnitine can account only for the significant but still transient delay in the collapse of $\Delta\Psi$ caused by Ca^{2+}.

This protective action of L-carnitine was also shown using another recognized criterion of mitochondria integrity, i.e., the ability to retain accumulated Ca^{2+}. As shown in Fig. 4, addition of palmitoyl-CoA to mitochondria incubated in a Ca^{2+}- and P_i-containing medium accelerated Ca^{2+} release, whereas in the presence of L-carnitine the efflux was so substantially delayed that it was now slower than for mitochondria not subjected to palmitoyl-CoA. This effect was observed not only when palmitoyl-CoA and L-carnitine were

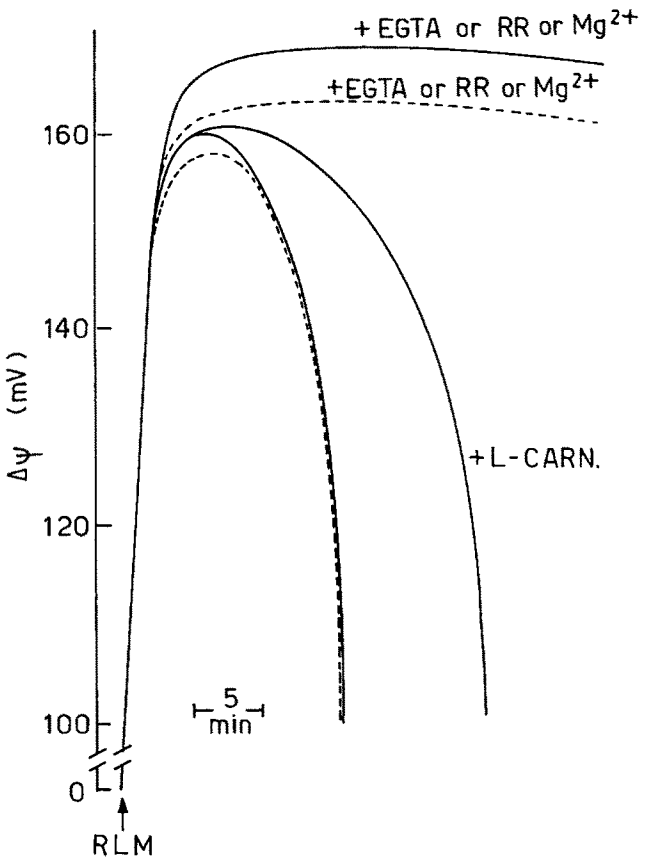

Fig. 2. Protective action of EGTA, Ruthenium red. (RR), Mg^{2+} and L-carnitine (L-CARN) on $\Delta\Psi$ of mitochondria incubated in the presence of 1.5 μM palmitoyl-CoA. Incubation conditions as in Methods (sucrose medium, solid lines) or, alternatively, mitochondria were incubated in an isoosmotic KCl medium (dashed lines). 0.5 mM EGTA, or 0.5 μM Ruthenium red, or 2 mM Mg^{2+}, or 2 mM L-CARN were added to the suspending medium before rat liver mitochondria (RLM).

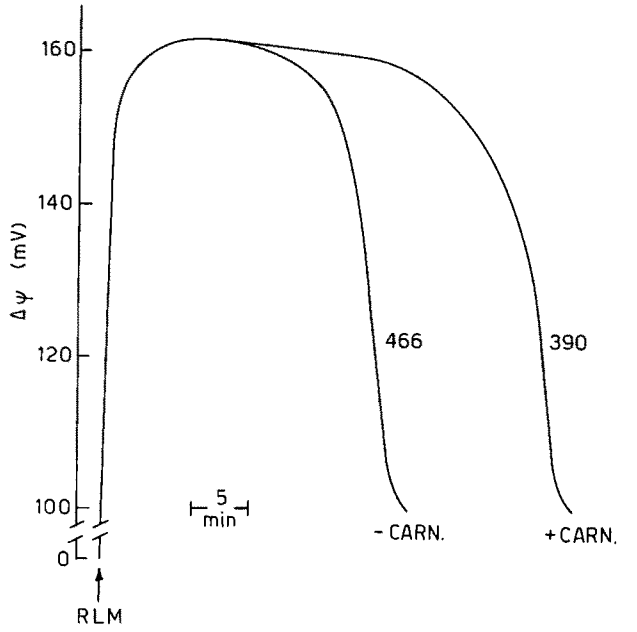

Fig. 3. Protective action of L-carnitine on $\Delta\Psi$. After a 5 min preincubation of rat liver mitochondrial (RLM) suspension (approx. 30 mg per ml) at 4° C in the presence (+CARN) or in the absence (−CARN) of 2 mM L-carnitine, rat liver mitochondria were assayed for their endogenous LCACoA content or added to the standard medium described in Methods for $\Delta\Psi$ monitoring. Inserted numbers indicate the amounts (pmol per mg mitochondrial protein) of long-chain acyl-CoA in mitochondria.

added together, but also if the latter was added later, when $\Delta\Psi$ was about to decrease and accumulated Ca^{2+} to be released, suggesting that removal of LCACoA by carnitine may induce reversal of deenergization induced by these deleterious compounds.

Discussion

The interaction of LCACoA with the protein and phospholipid constituents of biological membranes has been the object of several studies (for a review, see Ref. 14). Among others, both long-chain fatty acids and palmitoyl-CoA considerably increase the permeability of mitochondrial membranes to monovalent cations, thus facilitating the mitochondrial movements of potassium [12]. Therefore, LCACoA interactions with isolated mitochondria may result in an impairment of oxidative phosphorylation [15] and an alteration of Ca^{2+} flux across the inner mitochondrial membrane such as to inhibit the uptake and promote the release of this cation [16].

The results here reported show that even at submicellar concentration [17] LCACoA considerably impairs both transmembrane potential and the ability of

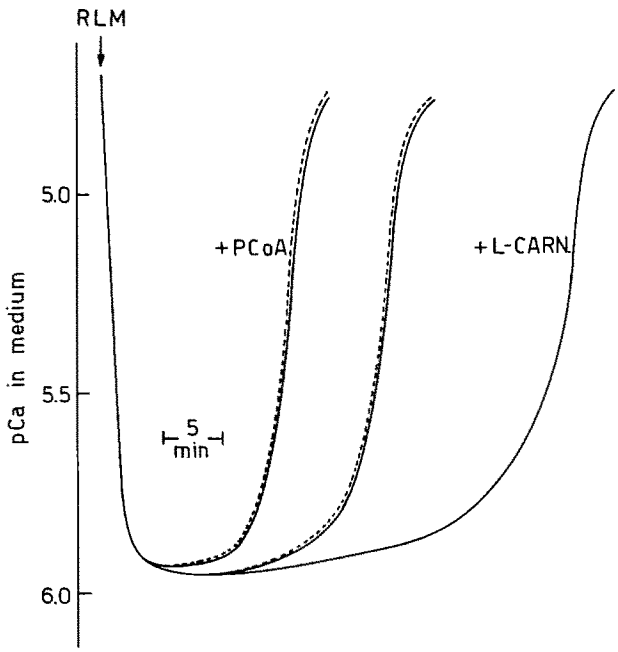

Fig. 4. Effects of L-carnitine (L-CARN) and palmitoyl-CoA (PCoA) on the release of accumulated Ca^{2+}. Mitochondria were incubated in a sucrose medium as described in Methods (solid lines), or in an isoosmotic KCl solution (dashed lines). Alternatively, 2 mM L-CARN or 1.5 μM palmitoyl-CoA were added to the suspending medium before mitochondria (RLM).

mitochondria to retain accumulated Ca^{2+}. These effects are mediated by Ca^{2+} flux across the inner mitochondrial membrane. Indeed, either the removal of Ca^{2+} from the suspending medium or the inhibition of Ca^{2+} uptake completely neutralised the action of LCACoA. Conceivably, LCACoA, by interpolation into the lipid bilayer, alters the membrane flux of Ca^{2+} perhaps by facilitating the access of this cation to phospholipase A_2. The consequent activation of the enzyme may induce the permeability alterations of the membrane responsibile for both $\Delta\Psi$ decay and the release of mitochondrial Ca^{2+} [18].

The involvement of an accelerated Ca^{2+} cycling is strongly supported by the synergistic effect of P_i which has been shown to favour both Ca^{2+} uptake and release [18]. The concurrent action of LCACoA and Ca^{2+} in the mitochondrial deenergization is established (a) by the full protection obtained by the inhibition of Ca^{2+} uptake with EGTA, or Ruthenium red, or Mg^{2+}; and (b) by the significant delay of $\Delta\Psi$ decay, induced by removing LCACoA by added L-carnitine. Even when impairment induced by endogenous or exogenous LCACoA has initiated (decay of $\Delta\Psi$ and Ca^{2+} release), membrane integrity can perhaps still be regenerated by LCACoA removal brought about by added carnitine. It has to be pointed out that L-carnitine concentrations below 2 mM still had a protective, although lower, effect.

The protective action of L-carnitine is suppressed by L-aminocarnitine, an inhibitor of CPT [13] and this is a direct evidence that LCACoA are in fact converted into the corresponding acylcarnitine. Evidently, the newly formed acylcarnitine exert, unlike the corresponding LCACoA, a much lower damaging action.

From a physiological point of view it seems possible that the acyl-CoA/acylcarnitine ratio may be an important factor in the regulation of Ca^{2+} homeostasis by mitochondria. Finally, the synergistic action of Ca^{2+} and LCACoA may be relevant to the mechanisms of cellular damage induced, e.g., by anoxic conditions, since these produce alterations of Ca^{2+} homeostasis and also the accumulation of LCACoA [14].

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