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Long-chain fatty acid-promoted swelling of mitochondria: further evidence for the protonophoric effect of fatty acids in the inner mitochondrial membrane

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Abstract Swelling of non-respiring rat liver mitochondria suspended in isotonic potassium acetate at pH 6.5–7.4 in the presence of valinomycin was promoted by long-chain fatty acids, such as myristate, indicating a protonophoric mechanism. This swelling was partly inhibited by inhibitors or substrates of mitochondrial anion carriers. The results show that the fatty acid cycling mechanism responsible for uncoupling of oxidative phosphorylation can also operate in the direction opposite to that originally proposed [Skulachev, V.P. (1991) FEBS Lett. 294, 158–162], i.e. the inwardly directed transfer of the fatty acid anion accompanied by outwardly directed free passage of undissociated fatty acid. They also extend the list of mitochondrial anion carriers, that are involved in this process, over the mono- and tricarboxylate transporters. At pH 8, myristate, but not the synthetic protonophore, *p*-trifluoromethoxycarbonylcyanide phenylhydrazone, induced mitochondrial swelling in both potassium acetate and KCl media, that did not require the presence of valinomycin. This indicates that, at alkaline pH, myristate facilitates permeation of the inner mitochondrial membrane to monovalent cations and, possibly, activates the inner membrane anion channel.

Key words: Fatty acid; Protonophoric effect; Anion carrier; Uncoupling; Swelling; Mitochondrion

1. Introduction

Non-esterified ('free') long-chain fatty acids (FFA) are known since about four decades as uncouplers of oxidative phosphorylation, but their protonophoric effect has not been completely resolved yet (for reviews see [1,2]). The protonophoric activity of FFA [3,4] is limited by a low flip-flop ability of FFA anions in artificial [5] and mitochondrial membranes [6]. It has been first reported by the Skulachev's group [3,7] that carboxyatractyloside, a specific inhibitor of the ADP/ATP carrier, partially reversed the protonophoric activity of FFA in mitochondria. Mainly on this basis Skulachev [8] postulated that the undissociated FFA permeate from the external side of the inner membrane to the matrix side and that the reverse transport of FFA anion is mediated by the ADP/ATP carrier (fatty acid cycling model) [8]. Later on, this view was extended by involving other anion carrier proteins of the inner membrane, such as the aspartate/glutamate carrier [9] and the dicarboxylate carrier [10] in the back-transport of FFA anions (for review see also [11]).

However, especially in liver mitochondria, the protonophoric activity of FFA can be attributed only partly to these anion carriers [12,13]. For example, the ADP/ATP carrier and the aspartate/glutamate carrier account for only about 25% of the protonophoric activity of the branched chain phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) [13]. Therefore, it seems likely that other up to now unrecognized inner membrane proteins may contribute to the FFA-mediated transmembrane proton transfer. In the present report we applied the swelling approach to look for the involvement of various anion carriers in the protonophoric activity of FFA.

Non-respiring rat liver mitochondria incubated in buffered isotonic potassium acetate in the presence of valinomycin do not swell, because acetate can cross the membrane only as undissociated acetic acid, and the transmembrane passage of potassium in form of the K^+-valinomycin complex generates a charge imbalance, preventing further permeation of K^+. Intramitochondrial accumulation of potassium acetate becomes only possible if H^+ can be exported from the inner compartment, thus enabling the inflow of K^+. This process can be mediated for example by synthetic protonophores [14]. Such swelling can be, therefore, used to check protonophoric ability of various compounds, among them fatty acids. In that case FFA would export protons from mitochondria by entering the inner compartment in the anionic form and flip-flopping in the reverse direction as undissociated acid (Fig. 1). The 'swelling system' has the advantage over the 'respiratory system' in searching for the mechanisms of protonophoric action of FFA because it eliminates the effects of inhibitors of anion carriers on the mitochondrial respiratory chain and primary dehydrogenases. In addition, in the 'swelling system', fatty acid anions are expected to be transported by the anion carriers from the external side of the membrane to its internal side, i.e. in the direction opposite to that postulated for FFA-induced uncoupling in energized mitochondria [8,11].

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Abbreviations: BCECF, 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein; CAT, carboxyatractyloside; DCCD, dicyclohexylcarbodiimide; FFA, non-esterified long-chain fatty acids; FCCP, carbonyl-cyanide *p*-trifluoromethoxyphenylhydrazone; IMAC, inner membrane anion carrier; Val, valinomycin

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2. Materials and methods

2.1. Mitochondria

Liver mitochondria were prepared from adult female Wistar rats (average weight 150–180 g) according to our standard protocol [4]. Protein content in the mitochondrial stock suspension was determined by a modified biuret method.

2.2. Swelling measurement

Swelling of deenergized rat liver mitochondria was recorded as the decrease of turbidity of the mitochondrial suspension. In short, an aliquot of mitochondria (1 mg mitochondrial protein) was added to 1 ml of the 'swelling medium' consisting of 120 mM K-acetate, 10 mM Tris–HCl, 0.5 mM EGTA, 2 μM rotenone and 0.5 μM valinomycin, adjusted to the required pH. It was found in preliminary experiments (not shown) that FFA can initiate the permeability transition even in the absence of externally added Ca^{2+} and in phosphate-free medium. Therefore, in order to eliminate possible contribution of the permeability transition [15] to mitochondrial swelling, the potassium acetate medium was additionally supplemented with 1 μM cyclosporin A, a potent blocker of the permeability transition pore [16]. Mitochondria were preincubated for 2 min in this swelling medium without or with the inhibitors to be examined and then the swelling was initiated by the addition of myristate or palmitate in form of 10 mM sodium salts in water or ethanol. Swelling was measured with a Cary 3E UV-visible spectrophotometer at 22°C and was quantified by determination of the initial rate of the decrease of light absorbance at 540 nm using the photometer software.

2.3. Mitochondrial membrane potential, respiration and other procedures

Oxygen consumption by mitochondria was measured polarographically using a Clark-type electrode in a chamber thermostated at 25°C. The 'respiration medium' was composed of 110 mM mannitol, 60 mM KCl, 60 mM Tris–HCl, 10 mM K_2HPO_4, 0.5 mM Na_2EDTA and 5 mM glutamate plus 5 mM malate as respiratory substrates (pH 7.4).

The mitochondrial membrane potential ($\Delta\psi$) was measured at 25°C in a medium containing 120 mM KCl, 10 mM Tris–HCl and 0.5 mM EGTA using the TPP^+-selective electrode [17] or by safranin O fluorescence [18]. pH gradient between mitochondrial inner compartment and the external medium (ΔpH) was measured using the fluorescent pH probe BCECF [19].

Rupture of the mitochondrial inner membrane by lytic activity of the applied FFA was assessed by measuring the release of the matrix enzyme malate dehydrogenase that was determined according to [20].

2.4. Chemicals

Myristic acid ($C_{14:0}$), FCCP, *n*-butylmalonate, phenylsuccinate, 1,2,3-benzene tricarboxylate, carboxyatractyloside, glutamate, malate, α-cyano-3-hydroxycinnamate, dicyclohexylcarbodiimide, safranin O and cyclosporin A were from Sigma (St. Louis, MO, USA). Acetoxymethyl ester of BCECF was from Molecular Probes (Eugen, OR, USA).

3. Results

Rat liver mitochondria swelled only slightly when suspended in K-acetate medium buffered at pH 7.4. Neither valinomycin, FCCP, nor myristate added alone accelerated the swelling (Fig. 2, traces A, B and C). As it is well known [14], valinomycin and the protonophore (FCCP in this case), when present together, produced a rapid swelling (Fig. 2, trace D). Similarly, the swelling was accelerated by myristate, or palmitate or oleate (not shown), added in the presence of valinomycin (Fig. 2, trace E). When the rate of initial swelling and the stimulation of resting state respiration were plotted against the amount of myristate added, a similar dependence was found (Fig. 3). This result strengthens the view that the swelling depends on the protonophoric action of myristate, as does the uncoupling of respiration.

Mitochondrial swelling in K-acetate medium was partly inhibited by well known inhibitors or substrates of various mitochondrial anion carrier proteins (Fig. 4): CAT (inhibitor of the ADP/ATP carrier), glutamate (substrate of the glutamate/aspartate carrier), α-cyano-3-hydroxycinnamate (inhibitor of the monocarboxylate carrier), phenylsuccinate and *n*-butylmalonate (inhibitors of the dicarboxylate carrier) and 1,2,3-benzenetricarboxylate (inhibitor of the tricarboxylate carrier). Measurements of the membrane potential ($\Delta\Psi$) in mitochondria energized with *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (plus ascorbate) revealed that these inhibitors (with the exception of α-cyano-3-hydroxycinnamate) as well as citrate (substrate of the tricarboxylate carrier) partly restored $\Delta\Psi$ decreased by the addition of myristate (not shown). It has to be noted that in this system energization of mitochondria was independent of NAD-linked substrates (for experimental details see [10]).

In non-respiring mitochondria suspended in the K-acetate medium addition of valinomycin did not elicit a potassium diffusion membrane. This has to be expected since the external concentration of potassium ions was approximately equal to the internal one estimated to be about 120 mM [18]. In contrast, mitochondria suspended in potassium acetate exhibited a pH gradient, acidic inside, due to the permeability of the

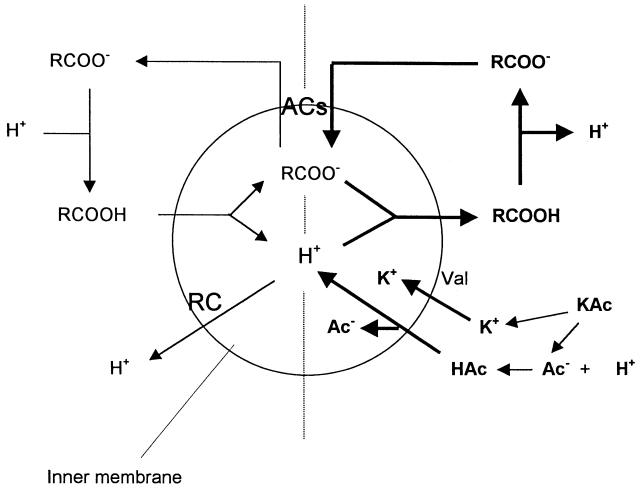

Fig. 1. Two experimental approaches to study the protonophoric mechanism of fatty acids in mitochondria. In mitochondria energized by operation of the respiratory chain ('respiratory system'), the protonophoric function of FFA results in a decrease of the membrane potential and, as consequence, an increase of the non-phosphorylating respiration. In the 'swelling system', non-energized mitochondria are suspended in isotonic potassium acetate. Because the inner mitochondrial membrane is permeable to undissociated acetic acid but not to the acetate anion, such mitochondria do not accumulate potassium acetate, even in the presence of K^+ ionophore, valinomycin, unless the protonophoric activity of FFA enables the export of protons. This is followed by osmotic swelling and manifested by a decrease of light scattering. In both systems the transmembrane proton passage is controlled by FFA anion capability to cross the inner membrane: in the 'respiratory system' from the inner side of the membrane to its external side, in the 'swelling system' in the reverse direction. For further explanation see text. Indications and abbreviations: RCOOH and $RCOO^-$, long-chain fatty acid and its anion, respectively; KAc, HAc and Ac^-, potassium acetate, acetic acid and acetate anion, respectively; ACs, mitochondrial anion carriers; RC, respiratory chain.

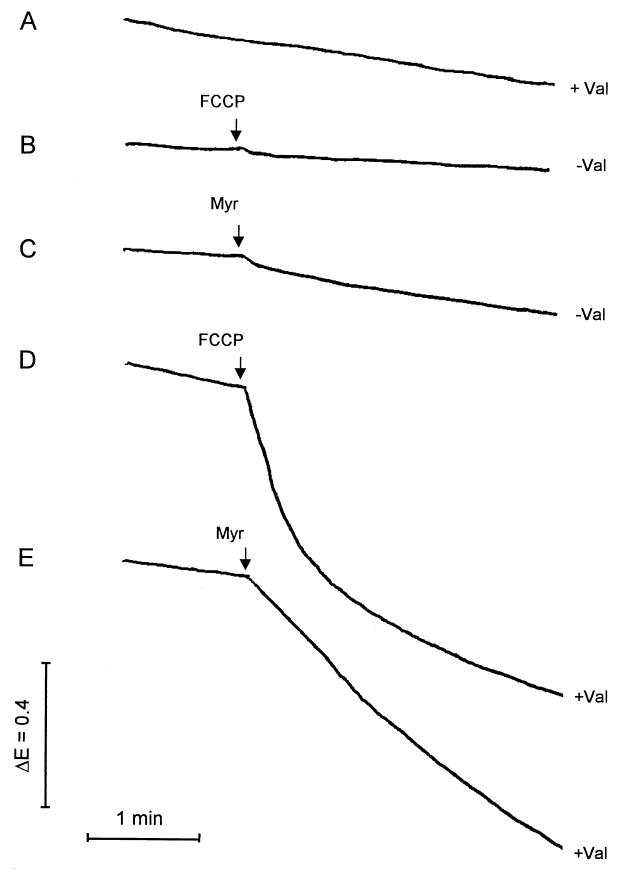

Fig. 2. Myristate- and FCCP-supported swelling of rat liver mitochondria in potassium acetate medium. Experimental conditions were as in Section 2. The experiments were started by the addition of mitochondria (1 mg protein; not shown) to the 'swelling medium' with valinomycin (A, D, E) or without valinomycin (B, C). Final concentrations of the additions (arrows) were: 2 μM FCCP (B, D), 80 μM myristate (C, E).

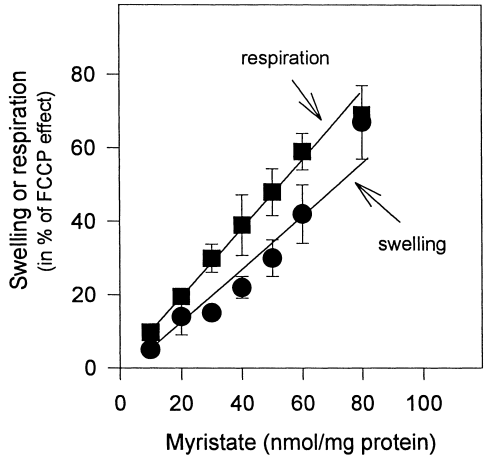

Fig. 3. Dependence of mitochondrial swelling and oxygen uptake on myristate concentration. Swelling was measured as in Fig. 2. The respiration was measured by incubation of mitochondria (1 mg protein/ml) in the 'respiration medium'. The non-phosphorylating respiration of mitochondria was titrated with FCCP to the maximum. Then, the respiration and swelling were stimulated by various concentrations of myristate and were expressed in percentage of that stimulated by FCCP. The data are mean values ± S.D. for three mitochondrial preparations, corrected for basic swelling or respiration (without fatty acid), respectively.

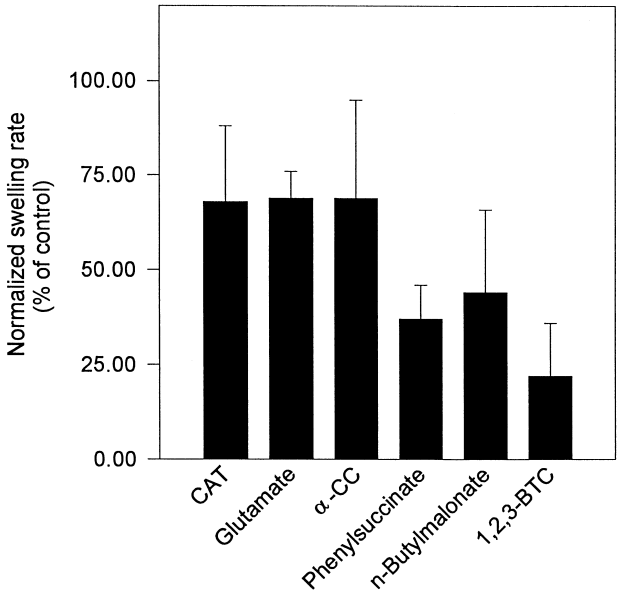

Fig. 4. The effect substrates or inhibitors of mitochondrial anion carriers on the myristate-induced swelling. Mitochondria were preincubated in the complete 'swelling medium' (pH 7.4) with the effectors of respective anion carriers: 5 μM CAT, 10 mM glutamate, 100 μM α-cyano-3-hydroxycinnamate (α-CC), 10 mM phenylsuccinate, 10 mM *n*-butylmalonate or 10 mM 1,2,3-benzenetricarboxylate (1,2,3-BTC). The normalized swelling rate (SR) was calculated from the rate of the decrease in light scattering before (DLS^E) and after (DLS^E_{Myr}) addition of 80 nmol myristate/mg mitochondrial protein: $SR = 100 \times (DLS^E_{Myr} - DLS^E)/(DLS_{Myr} - DLS)$, where DLS_{Myr} and DLS are rates of the decrease of light scattering with and without myristate, respectively, without the effector. The data shown (means ± S.D.) were calculated from experiments performed with five to seven mitochondrial preparations.

inner membrane to undissociated acetic acid but not to acetate anion. This pH gradient amounted to about 0.3 pH unit and slowly decreased to 0.1 pH unit after addition of valinomycin (not shown). Thus, in non-respiring mitochondria sus-

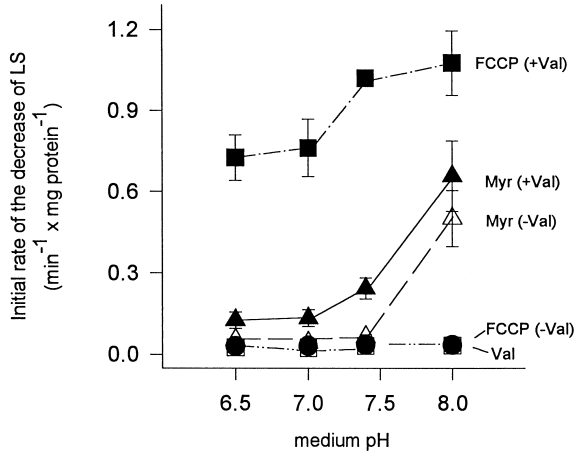

Fig. 5. Effect of pH on myristate- or FCCP-induced mitochondrial swelling. Mitochondria were incubated without or with 0.5 μM valinomycin in the 'swelling medium' adjusted to various pH values. Initial rates of swelling stimulated by addition of 80 nmol myristate/mg mitochondrial protein or by 2 μM FCCP were plotted against pH of the medium. The data are mean values ± S.D. for six mitochondrial preparations.

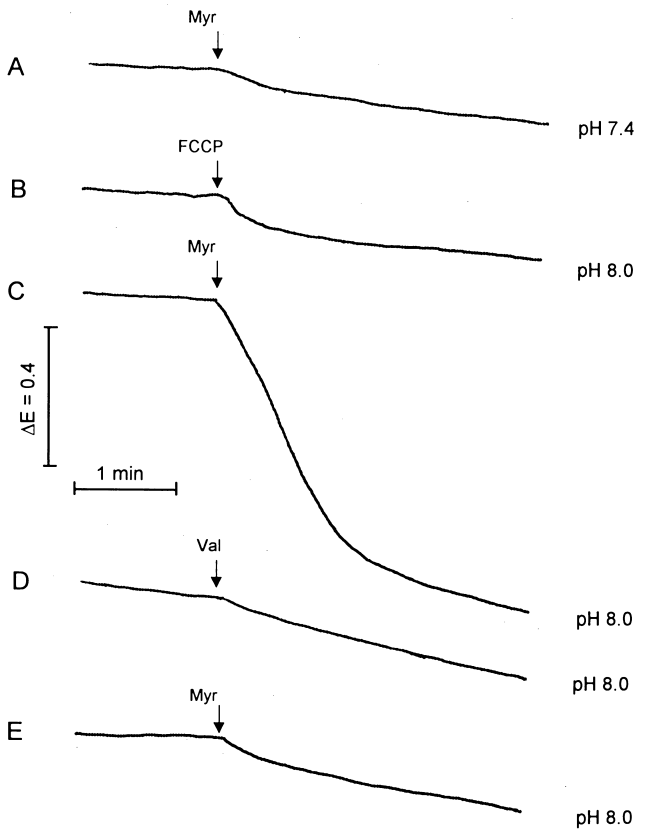

Fig. 6. Myristate-induced swelling of rat liver mitochondria in the KCl medium. Mitochondria (1 mg protein/ml) were incubated in the K^+-chloride medium: 120 mM KCl, 10 mM Tris–HCl, 0.5 mM EGTA, 1 μM rotenone, 1 μM cyclosporin A; pH was adjusted to 7.4 (A) or 8.0 (B–E). Final concentrations of the additions (arrows) were: 80 μM myristate (A, C, E), 2 μM FCCP (B) and 0.5 μM valinomycin (D). In one experiment mitochondria were preincubated for 2 min with 0.1 μM DCCD (E).

pended in isotonic K-acetate the driving force for the circulation of fatty acid was ΔpH rather than $\Delta\psi$.

The characteristics of mitochondrial swelling changed drastically with increasing pH of the medium. As shown in Fig. 5, the initial rate of swelling with myristate alone, myristate plus valinomycin, and FCCP plus valinomycin was strongly increased when pH was increased from 7.4 to 8.0. The swelling in the presence of FCCP or valinomycin alone was very low and was independent of pH in the range of 6.5–8.0.

As expected, the mitochondria did not swell at pH 7.4 when suspended in KCl medium in the presence of myristate (Fig. 6, trace A) or valinomycin (not shown, see [21]). However at pH 8, in contrast to what had been observed in the K-acetate medium (see Fig. 2), myristate alone initiated swelling in the KCl medium (Fig. 6, trace C). At that alkaline pH, the swelling was only slightly induced by valinomycin alone (Fig. 5, trace D; see also [21]). Myristate-induced swelling was inhibited by dicyclocarbodiimide (Fig. 5, traces E). FCCP did not induce mitochondrial swelling at pH 8.0 (Fig. 6, trace B). Leakage of malate dehydrogenase from mitochondria to the external medium (K-acetate or KCl medium) amounted to 2.5–3.5% at pH range of 7.4–8.0 and increased to only 5.5% at pH 8.0 in the presence of myristate. This indicates that the observed increase of the rate of light scattering decrease at more alkaline pH could not be due to an increase in the lysis of mitochondria but points to a real increase of swelling at pH 8.0.

4. Discussion

In the present study the protonophoric effect of FFA was investigated using the protonophore-mediated swelling of non-respiring mitochondria suspended in K-acetate medium supplemented with valinomycin [14], i.e. under conditions when FFA mediate a net transfer of protons from the inside to the outside of the inner membrane, that is, in the opposite direction than during FFA-induced uncoupling [8]. These results clearly show the fatty acid cycling mechanism can operate in both directions. In addition, they also indicate that the FFA-mediated protonophoric mechanism can function in non-energized mitochondria, when the driving force for cycling of the protonated form of fatty acid versus its anionic form is greatly reduced. Moreover, they provide a final support to the concept that the uncoupling effect of FFA is due to the protonophoric mechanism rather than to an interaction with the respiratory chain or the energy-generating machinery (for discussion of this point see [1]).

The present study also extends the list of mitochondrial anion carriers, that can be involved in the fatty acid anion transfer, over two more members of this carrier protein family: the tricarboxylate carrier and, most likely, the monocarboxylate carrier. It seems, however, likely that the mitochondrial anion carriers can transport fatty acid anions in both directions with different rates. This is indicated by the observation that blocking of a particular carrier may exert different effects on the recoupling ($\Delta\psi$ increase) and the FFA-mediated swelling. For example, we were unable to see any effect of α-cyano-3-hydroxycinnamate on the myristate-induced decrease of $\Delta\psi$, whereas the swelling was decreased by some 30% (Fig. 4). Similarly, blocking of the glutamate carrier had a smaller effect on the swelling than blocking of the dicarboxylate carrier (Fig. 4). In contrast, blocking of the glutamate carrier had a higher recoupling effect than blocking of the dicarboxylate carrier [10].

Furthermore, we report – to our knowledge for the first time – on a FFA-induced very pronounced mitochondrial swelling at alkaline pH in K-acetate, which is not attributable to protonophoric action (Fig. 5). This swelling also occurred in KCl medium (Fig. 6) and was independent of valinomycin, therefore pointing to a different mechanism, namely permeabilization of the mitochondrial inner membrane to both K^+ and Cl^-. In the absence of FFA, an increase of mitochondrial permeability to chloride as well as an increase in swelling of rat liver mitochondria in KCl medium (plus valinomycin) at alkaline pH have been previously described [21,22]. Both phenomena might be explained by operation of the inner membrane anion channel (IMAC) [23]. This suggests that myristate can act as activator of IMAC at alkaline pH and, in addition, increases permeability of the inner membrane to K^+. The latter phenomenon could be due to the capability of FFA to transport monovalent cations as fatty acid complexes or soap ('valinomycin-like effect') [24–26].

In liver mitochondria, IMAC is activated by increasing the pH or by lowering matrix Mg^{2+} [27]. Thus, it could be that FFA extract Mg^{2+} from the matrix by formation of a stable fatty acid anion–Mg^{2+} complex inside mitochondria and its

subsequent outward transport [28]. In addition, the finding that dicyclohexylcarbodiimide, a known inhibitor of IMAC [27], inhibits myristate-supported swelling, further strengthens our proposal of IMAC activation by fatty acids at alkaline pH.

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