Fatty acid-induced uncoupling of oxidative phosphorylation is partly due to opening of the mitochondrial permeability transition pore

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Received 22 December 1997; revised version received 23 January 1998

Abstract Addition of myristate at low concentration (30–60 nmol/mg protein) to energized rat liver mitochondria resulted in dissipation of the electric membrane potential which, in Ca^{2+}-free media, could be partly reversed by carboxyatractyloside but not by cyclosporin A. In contrast, in mitochondria preloaded with Ca^{2+} this energy-dissipating effect of fatty acid was partly prevented or reversed by cyclosporin A or ADP. In sucrose media, myristate, but not the protonophore carbonyl cyanide *m*-chlorophenylhydrazone, induced swelling of Ca^{2+}-loaded mitochondria which was inhibited by cyclosporin A and ADP. We conclude that long-chain fatty acids may induce opening of the mitochondrial permeability transition pore not only because of their protonophoric effect mediated by mitochondrial anion carriers [Skulachev, V.P., FEBS Lett. 294 (1991) 158–162; Więckowski, M.R. and Wojtczak, L., Biochem. Biophys. Res. Commun. (1997) 232, 414–417] but also by a direct interacton with the pore assembly.

Key words: Fatty acid; Uncoupling; Membrane potential; Swelling; Permeability transition pore

1. Introduction

The well known uncoupling of oxidative phosphorylation by long-chain fatty acids has been ascribed to their protonophoric action (for review see [1]). However, a simple transfer of H^+ due to the flip-flop of undissociated vs. dissociated (anionic) molecule of fatty acid is hindered by a very slow transbilayer movement of the latter [2,3]. Skulachev and coworkers [4,5] have proposed involvement of the adenine nucleotide translocase in mediating the transfer of fatty acid, thus providing a rationale for the fatty-acid cycling mechanism [6], i.e. spontaneous movement of the undissociated (protonated) form of fatty acid from the external leaflet of the membrane to the internal one and carrier-mediated transfer of the anionic form in the opposite direction. Involvement of the adenine nucleotide carrier in fatty acid-mediated proton transfer has been confirmed by reconstitution studies [7], photolabelling of the carrier with azido derivatives of fatty acids [8], studies on carrier-enriched mitochondria from hyperthyroid rats [9] and on carrier-deficient yeast mutants [10]. Participation of two other mitochondrial carriers, the glutamate/aspartate carrier [11,12] and the dicarboxylate carrier [12] in fatty acid-induced proton permeability has also been shown.

Non-esterified long-chain fatty acids also belong to compounds that promote opening of mitochondrial permeability transition pore (PTP) [13,14], a large non-selective channel that opens under specific conditions as a result of Ca^{2+} accumulation in the mitochondrial matrix compartment [15] (for reviews see [16,17]). Fatty acid-induced large-amplitude mitochondrial swelling, concomitant with uncoupling of oxidative phosphorylation, has been observed already in the late fifties and early sixties (see e.g. [18]). However, Hunter et al. [15] were probably the first to correlate it with the opening of an unspecific permeability pore. Its closure by cyclosporin A (CsA) [19] is now widely accepted as a characteristic feature [16,17].

In studies on the Ca^{2+}-dependent uncoupling effect of palmitate (for review see [20]), supposed to be due to the opening of PTP [15], Skulachev and his colleagues [21] proposed that the adenine nucleotide translocase can also be involved in this kind of uncoupling. They found that in incubation media supplemented with Ca^{2+} and/or phosphate (but without EGTA) (i) CsA and adenosine diphosphate (ADP) suppressed and carboxyatractyloside potentiated the uncoupling effect of fatty acid [5,21–23]; (ii) the potentiating effect of carboxyatractyloside did not occur when palmitate was substituted by a chemical protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) [22]; (iii) in liver mitochondria of ground squirrels arousing from hibernation, when elevated levels of non-esterified fatty acids could be expected [24], CsA produced a coupling effect whereas carboxyatractyloside potentiated $\Delta\psi$ dissipation [21,25].

Recently, Schönfeld and Bohnensack [26] have shown that fatty acids may de-energize mitochondria due to their pore-opening activity. Since PTP senses the transmembrane potential and its opening probability increases as the potential decreases [27] these authors propose that the main mechanism by which fatty acids affect the pore-opening is initiated by their protonophoric action described above, although they do not exclude a direct effect of fatty acid on PTP.

In the present work, we further document the pore-opening effect of long-chain fatty acids and its participation in the fatty acid-induced uncoupling and show that its mechanism consists of a direct action on the pore and not only via lowering of the mitochondrial membrane potential.

2. Materials and methods

Mitochondria were isolated from livers of male albino rats as described previously [12]. Due to the presence of EGTA and serum albumin in the homogenization medium, but not in the washing and suspending media, the content of mitochondrial calcium was reduced

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CsA, cyclosporin A; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; PTP, mitochondrial permeability transition pore; $\delta\psi$, mitochondrial transmembrane electric potential

PII S0014-5793(98)00118-5

and that of endogenous fatty acids was minimized. Protein content was estimated by the biuret method with serum albumin as standard.

Mitochondrial membrane potential was measured fluorimetrically with safranine O [28] in Shimadzu model RF 5000 spectrofluorimeter at 495 nm and 586 nm excitation and emission wavelengths, respectively. The system was not calibrated and therefore the results reflect changes of $\Delta\psi$ but not their absolute values. The incubation medium contained 200 mM sucrose, 10 mM KCl, 10 mM Tris-HCl (pH 7.2), 5 mM glutamate, 5 mM malate and 8 μM safranine O. The temperature was 25°C. The reaction was started by addition of 1.0 mg mitochondrial protein to 3.0 ml of the medium.

Mitochondrial swelling was followed by measuring light scattering at 520 nm in the same instrument and in the same medium. Mitochondrial concentration was 0.5 mg protein per 3.0 ml medium.

Myristate (Na salt, from Applied Sciences Laboratories, State College, PA, USA) was used as a 1-mM solution in ethanol/water (1:9, v/v).

Carboxyatractyloside, safranine O, oligomycin, ADP and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were from Sigma (St. Louis, MO, USA) and cyclosporin A from Sandoz (Basel, Switzerland).

3. Results

In Ca^{2+}-free medium (EGTA present) myristate at low concentration exerted a moderate uncoupling effect as measured by decrease of the mitochondrial membrane potential. This uncoupling was largely reversed by carboxyatractyloside (Fig. 1), as previously observed by Andreyev et al. [4,5] and ourselves [8,9,12]. In contrast, CsA neither prevented (trace B) nor reversed (traces A and C) the uncoupling produced by myristate. A different picture was obtained when mitochondria were preloaded with Ca^{2+} (Fig. 2). In this case, dissipation of $\Delta\psi$ produced by the same amount of myristate was much larger and was partly reversed by CsA (trace A). CsA added before myristate significantly protected against $\Delta\psi$ dissipation which was, in this case, of a similar magnitude as that in the absence of Ca^{2+} (Fig. 2, trace B, compare with Fig. 1, trace A). Carboxyatractyloside had an additional recoupling effect. It has to be noted that Ca^{2+} load alone had no deteriorating effect on mitochondrial energy coupling, as $\Delta\psi$ returned to its initial value after a transitory decrease due to energy-dependent Ca^{2+} uptake.

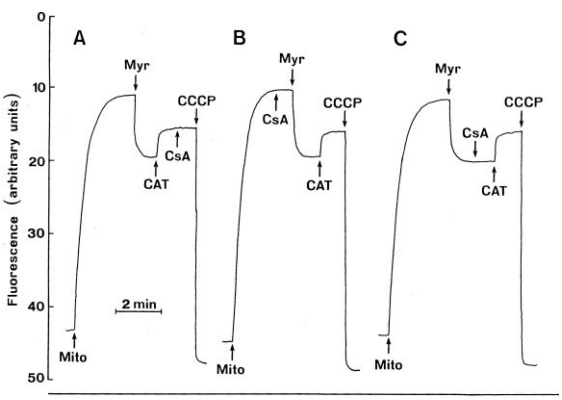

Fig. 1. Effect of myristate on the mitochondrial membrane potential in Ca^{2+}-free medium. The incubation medium for $\Delta\psi$ measurements (see Section 2) was supplemented with 0.5 mM EGTA. Additions: Mitochondria (Mito), 1 mg protein; myristate (Myr), 40 nmol/mg protein; carboxyatractyloside (CAT), 1.7 μM; CsA, 1.7 μM; CCCP, 1.7 μM. Increase of $\Delta\psi$ is depicted by quenching of safranine fluorescence [28].

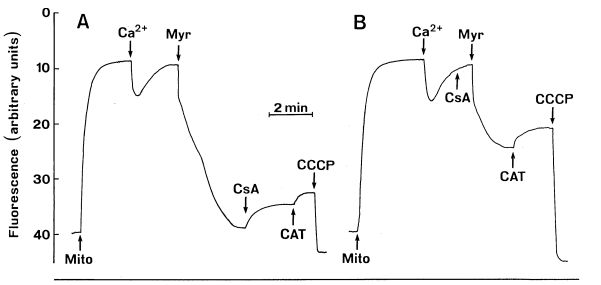

Fig. 2. Effect of myristate on $\Delta\psi$ in mitochondria preloaded with Ca^{2+}. The incubation medium did not contain EGTA. Ca^{2+} was added in the amount of 30 nmol/mg protein, as indicated by the arrow. Other additions as in Fig. 1.

These results can be interpreted as opening of PTP resulting from myristate addition in te presence of Ca^{2+} load. This was further substantiated by swelling experiments. In Ca^{2+}-loaded mitochondria addition of myristate induced a rapid swelling (Fig. 3, trace A) which could be inhibited (trace B) or prevented (trace C) by CsA. No such swelling resulted after addition of CCCP taken in the amount which produced a comparable decrease of $\Delta\psi$ decrease (about 70%) as the fatty acid (trace D). Only after addition of myristate a fast swelling was initiated. Moreover, the swelling-inducing effect of myristate could be prevented by ADP (trace E), known to promote the pore closure [13,29–33]. The swelling was, however, elicited by subsequent addition of carboxyatractyloside, known to antag-

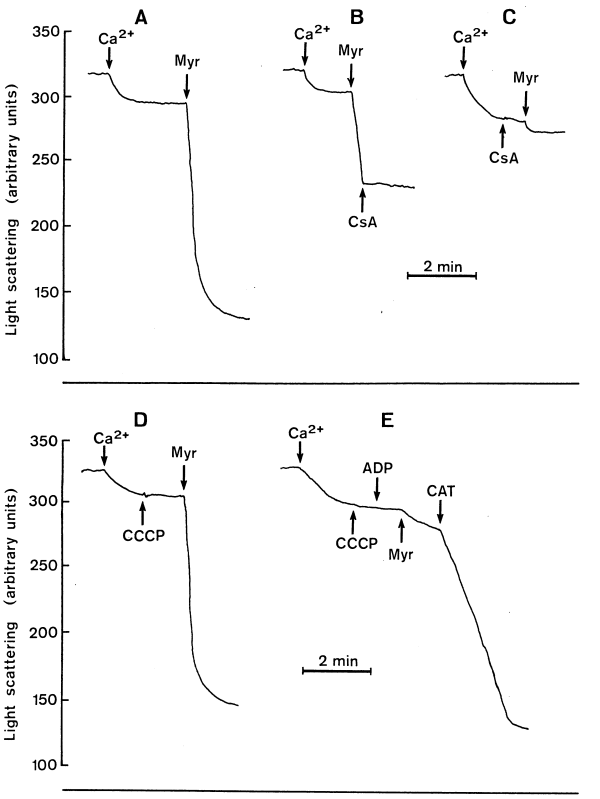

Fig. 3. Myristate-induced mitochondrial swelling. The incubation medium (see Section 2) was supplemented with oligomycin, 5 μg/mg mitochondrial protein. Additions: Ca^{2+}, 30 nmol/mg protein; myristate (Myr), 40 nmol/mg protein; CsA, 1.7 μM; ADP, 1 mM; CCCP, 1.7 μM.

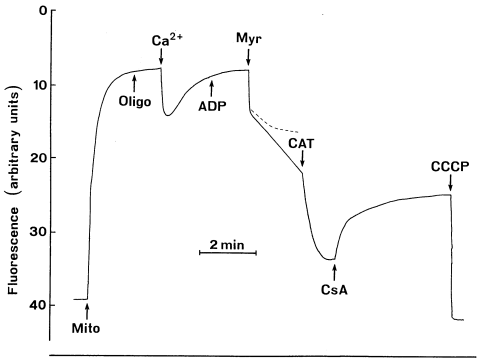

Fig. 4. Effect of ADP on fatty acid-induced dissipation of Δψ. Additions: Oligomycin (Oligo), 5 μg/mg protein; ADP, 1 mM (solid line) or 2 mM (dashed line); other additions as in Figs. 1 and 2.

onize the pore-closing effect of ADP [29,32,33]. This experiment shows that opening of PTP by fatty acid was not due to the decrease of Δψ.

This conclusion was further substantiated by measuring Δψ. In the presence of ADP the uncoupling effect of myristate was much less expressed than in its absence (Fig. 4, compare with trace A in Fig. 2) and only subsequent addition of carboxyatractyloside potentiated Δψ decrease in a CsA-sensitive manner. It has to be remembered that in the absence of Ca^{2+} load, carboxyatractyloside partly reverses, and not potentiates, fatty acid-induced uncoupling (Fig. 1) [4–6,8,9,12].

4. Discussion

Since CsA is known as a potent blocker of PTP [19], its inhibitory effects on both mitochondrial swelling and Δψ decrease after addition of myristate (Figs. 1–4) clearly point to opening of PTP as result of the action of fatty acid on mitochondria. This confirms recent observations by Schönfeld and Bohnensack [26] and forms a basis for a novel mechanism by which fatty acids uncouple oxidative phosphorylation. According to this concept fatty acids not only function as protonophores (with participation of mitochondrial carriers) but also promote opening of a channel for a direct flux of protons (and other ions) across the inner mitochondrial membrane.

Broekemeier and Pfeiffer [14] have proposed that pore opening by fatty acids may be due to an increase of the negative membrane surface potential. This possibility has been ruled out by Schönfeld and Bohnensack [26] who were unable to reproduce the pore opening effect using dodecylsulphate, another surface-active compound. These authors conclude that the main mechanism by which fatty acids open PTP is by decreasing Δψ, thus promoting the pore to open [27]. According to this mechanism fatty acid first partly decreases Δψ due to its protonophoric effect and then PTP opens, thus facilitating a massive influx of protons and a further decrease of the membrane potential. However, Schönfeld and Bohnensack [26] also found that mitochondrial swelling induced by phytanic acid was somewhat more sensitive to the decrease of Δψ than the swelling induced by a typical protonophore FCCP. On that basis, these authors propose that fatty acids may additionally influence PTP by interacting with the adenine nucleotide translocase which participates in the PTP assembly.

Results of the present investigation clearly indicate that the major mechanism is a direct action of fatty acid on PTP and not only via decreasing of Δψ. Under conditions of the present work, i.e. in the sucrose medium and in the absence of external phosphate, decrease of Δψ by a chemical protonophore CCCP did not induce pore opening (Fig. 3, traces D and E) most likely because of acidification of the matrix compartment [27,34]. This property allowed us to differentiate between an indirect effect of fatty acids as protonophores and a direct one on PTP. Moreover, ADP, known to decrease opening probability of PTP [13,29–33], prevented both myristate-induced swelling (Fig. 3, trace E) and collapse of Δψ (Fig. 4). In contrast, carboxyatractyloside, which per se reduces the protonophoric action of fatty acids [4–6,8,9,12], potentiated Δψ dissipation in a CsA-sensitive manner (Fig. 4).

The assembly of PTP is assumed to include the adenine nucleotide translocase [35–37]. Since long-chain fatty acids are known to exhibit a high affinity to this carrier protein [8,12] (see also review [1]), it can be expected that they modify the permeability pore in a similar way as, for example, carboxyatractyloside, another pore-opening [32,33] adenine nucleotide translocase ligand.

Acknowledgements: Skillful technical assistance of Maria Buszkowska is gratefully acknowledged. We thank Peter Schönfeld and Ralf Bohnensack (Institute of Biochemistry, Otto-von-Guericke University, Magdeburg, Germany) for making available their manuscript [26] prior to publication and Dr. Schönfeld for valuable discussions. We also thank Sandoz Pharma Ltd. (Basel) for generously offering us cyclosporin A. This work was supported in part by the Committee for Scientific Research under Grant 6 P04A 057 09.

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