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On the Nature of the Uncoupling Effect of Fatty Acids

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Abstract

The effect of palmitic acid on the electrical potential difference $\Delta\psi$ across the inner mitochondrial membrane appears to depend on the medium in which mitochondria are incubated. In medium A (cf. Luvisetto *et al.* (1987), *Biochemistry*, **26**, 7332–7338) $\Delta\psi$ decreases much more than in medium B (cf. Rottenberg and Hashimoto (1986), *Biochemistry*, **25**, 1747–1755) at concentrations of fatty acid which equally stimulate the rate of respiration in state 4. Valinomycin and NaCl were both present in medium B and absent in medium A. However, in both media the pattern of the P/O ratio as a function of antimycin in the presence of a constant amount of palmitic acid or of FCCP shows similar behaviour. We conclude that in both media palmitic acid increases the membrane conductance to protons, but for unclear reasons the $\Delta\psi$ assay fails to measure the decline of $\Delta\psi$ in medium B. However, the increase in membrane conductance induced by palmitic acid does not quantitatively account for the stimulation of the rate of respiration.

Key Words: Fatty acid; oxidative phosphorylation; rat liver; mitochondria

Introduction

Recent work from Rottenberg's and our laboratory has established that the uncoupling of oxidative phosphorylation by fatty acids in rat liver mitochondria is not quantitatively accounted for by their protonophoric or ionophoretic action (Rottenberg and Hashimoto, 1986; Luvisetto *et al.*, 1987; Pietrobon *et al.*, 1987; Rottenberg and Steiner-Mordoch, 1986). Rottenberg

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and coworkers found that[4] $\Delta\psi$ in both static head and state 3 was only very slightly depressed by concentrations of oleic acid or palmitic acid which almost completely inhibited the rate of ATP synthesis (Rottenberg and Hashimoto, 1986), and that in submitochondrial particles ATP synthesis driven by an artificially imposed $\Delta\psi$ was not inhibited by fatty acids (Rottenberg and Steiner-Mordoch, 1986). They have considered their results as evidence against the delocalized chemiosmotic hypothesis and proposed that fatty acids act as "decouplers" of intramembrane processes that mediated direct energy transfer between the electron transfer complexes and the ATPase.

We have found that oleic acid in the range of concentrations which inhibit ATP synthesis does increase the passive conductance of the mitochondrial inner membrane and decreases $\Delta\psi$ in static head (Luvisetto *et al.*, 1987). However, a quantitative comparison between the dissipative proton influx and the rate of either electron transfer or ATP hydrolysis in static head at the same $\Delta\psi$ showed that the increase in membrane conductance induced by oleic acid accounts for the stimulation of the rate of ATP synthesis but not for that of the rate of electron transfer. Oleic acid was found to have also an inhibitory effect on the maximal rate of respiration and ATP hydrolysis, suggesting inhibition of some redox enzyme as well as of the ATPase or the adenine nucleotide translocator (Andreyev *et al.*, 1988). Once these additional effects were taken into account, all our results, including the near constancy of $\Delta\psi$ in state 3 and the lack of stimulation of the rate of respiration in state 3, could be simulated with a delocalized chemiosmotic model assuming that oleic acid acts also as an intrinsic uncoupler of the redox pumps (Pietrobon *et al.*, 1987). Note that the quantitative analysis indicates that also a classical protonophore, such as FCCP, act as intrinsic uncoupler of the redox pumps.

The origin of the discrepancy between our laboratory and Rottenberg's concerning the effect of oleic acid on $\Delta\psi$ in static head has remained obscure. To clarify the issue and also to eliminate the possibility (Rottenberg, personal communication) that the inhibitory effects on the energy-coupling enzymes observed by us were due to secondary peroxidation products of the unsaturated oleic acid, we have measured the effect of a saturated fatty acid, palmitic acid, on different parameters of oxidative phosphorylation. Using palmitic acid, we have confirmed the results previously obtained with oleic

[4] Abbreviations: J_O, rate of oxygen consumption in static head (st. 4), in state 3 (st. 3), and in completely uncoupled state (unc.); J_p, rate of ATP synthesis; J_{ATP}, rate of ATP hydrolysis; J_K, rate of K^+ efflux; $\Delta\psi$, transmembrane electrical potential in static head (st. 4) and in state 3 (st. 3); Pi, inorganic phosphate; $TPMP^+$, triphenylmethylphosphonium ion; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone.

acid (Luvisetto *et al.*, 1987; Pietrobon *et al.*, 1987), and found that the origin of the discrepancy on the measurements of $\Delta\psi$ resides in the different incubation media. However, in both media, when measured on parameters which are independent of the $\Delta\psi$ assay, the effect of palmitic acid is similar to that of classical protonophores.

Materials and Methods

Rat liver mitochondria were prepared according to standard procedures (Massari *et al.*, 1972). The mitochondrial protein was assayed with the biuret method using serum albumin as a standard. Two solutions were used as incubation medium: solution A same as in Luvisetto *et al.* (1987) and Pietrobon *et al.* (1987), and solution B as in Rottenberg and Hashimoto (1986). Solution A: 0.2 M sucrose, 30 mM Tris/MOPS, 5 mM Pi/Tris, 10 mM succinate/Tris, 1 mM EDTA/Tris, 5 μM rotenone; pH 7.4, T 25°C. Solution B: 0.2 M sucrose, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM Pi/Tris, 5 mM succinate/Tris, 0.1 μM valinomycin, 5 mM HEPES, 2 mM EGTA/Tris, 1 μM rotenone; pH 7.4, T 25°C. Palmitic acid was freshly prepared in dilute ethanol solution (3 mM). In order to avoid formation of micelles, aliquots of mitochondria were pretreated with increasing concentrations of palmitic acid and after vigorous stirring were mixed with incubation medium. The measurement of the rates of respiration, of ATP synthesis and hydrolysis, and of K^+ efflux and the measure of $\Delta\psi$ were performed as described in Luvisetto *et al.* (1987). All reagents were of maximal purity commercial grade. Enzymes, nucleotides, inhibitors, and valinomycin were obtained from Sigma, FCCP was supplied by Dr. G. Heitler of Du Pont, and palmitic acid by Sigma.

Results

Figure 1 shows the effect of increasing concentrations of palmitate on the transmembrane electrical potentials, on the rates of respiration, on the rate of ATP hydrolysis, and on the rate of phosphorylation of mitochondria incubated in our usual medium (solution A). The effect of palmitic acid on the different parameters in Fig. 1 is similar to that of oleic acid previously published (Luvisetto *et al.*, 1987). In particular, note the decrease of $\Delta\psi$ in state 4, and the relative insensitivity of $\Delta\psi$ in state 3 at the beginning of the titration, together with the lack of stimulation of the rate of respiration state 3 and the inhibition of the maximal rate of respiration. Also note that the range of concentrations at which palmitic acid exerts these effect is almost one order of magnitude lower than of oleic acid in Luvisetto *et al.* (1987).

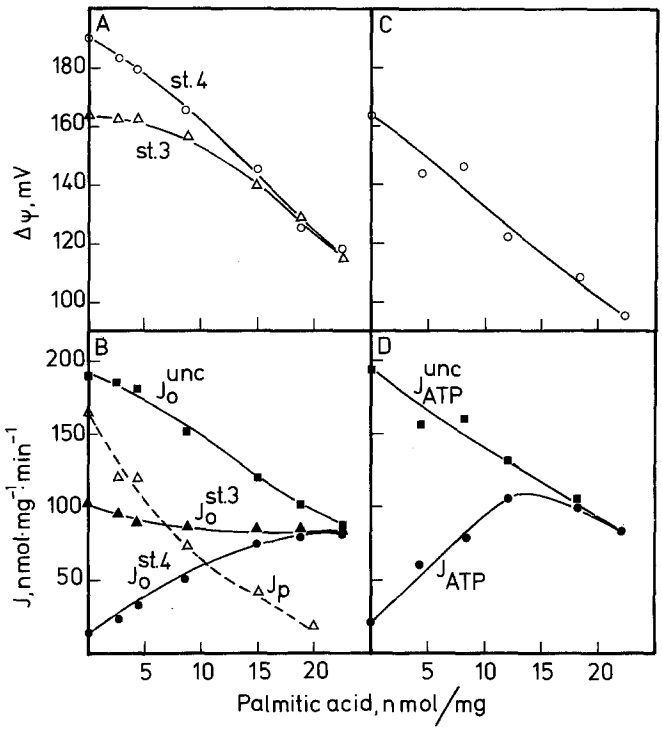

Fig. 1. Effect of palmitic acid on respiration, phosphorylation, ATPase activity, and membrane potentials. Panel A: transmembrane electrical potential difference in state 4 (○) and in state 3 (△). Panel B: rate of respiration in state 4 (●), in state 3 (▲), and maximal rate of respiration (■), and rate of ATP synthesis (△). Medium composition in panels A–B: solution A. After 2 min of incubation of rat liver mitochondria (1 mg/ml) with palmitic acid, succinate (10 mM) was added, followed after 2 min by addition of either ADP (1 mM) or excess of FCCP (0.2 μM). Panel C: transmembrane electrical potential difference in static head generated by ATP hydrolysis (○). Panel D: rate of ATP hydrolysis in static head (●) and in the presence of a constant amount of chloroform (■). In panels C–D medium composition was 0.2 M sucrose, 30 mM MOPS Tris, 0.2 mM EGTA, 2 mM $MgCl_2$, 5 mM Pi/Tris, 1 mM phosphoenolpyruvate, 0.1 mM NADH, and excess of pyruvate kinase and lactate dehydrogenase, pH 7.4, *T* 25°C. Same procedure as described for panels A and B with ATP (3 mM) instead of succinate and chloroform (25 mM) instead of excess FCCP.

The decrease of $\Delta\psi$ in static head at increasing concentrations of palmitate remains in sharp contrast with the almost constant $\Delta\psi$ reported by Rottenberg and colleagues in the same range of concentrations. To clarify the origin of the discrepancy, we have measured $\Delta\psi$ and the rate of respiration in state 4 of mitochondria incubated either in our usual medium (solution A) or in Rottenberg's medium (solution B). Figure 2 shows that increasing concentrations of palmitate caused a similar stimulation of the respiration in the two media but a very different effect on $\Delta\psi$. In medium A, $\Delta\psi$ in the

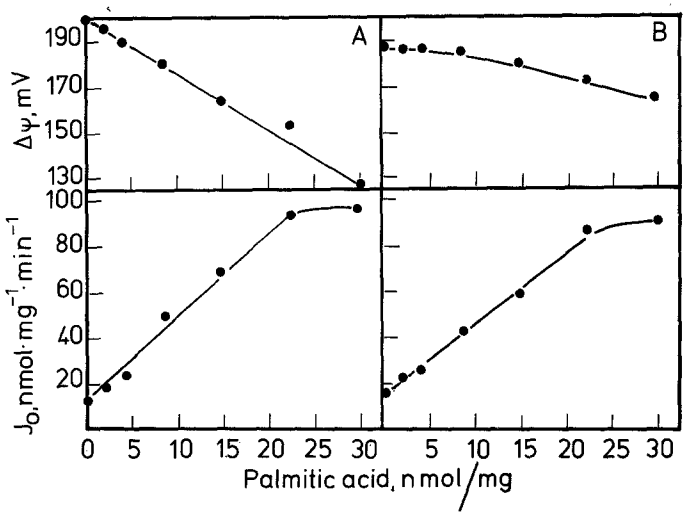

Fig 2. Rate of respiration and transmembrane electrical potential difference as a function of increasing concentration of palmitic acid in different incubation media. In panels A: solution A; in panels B: solution B. Experimental procedure was as in Fig. 1.

absence of palmitate was slightly higher than in medium B. At increasing palmitate concentrations $\Delta\psi$ decreases in medium A (cf also Fig. 1) but remains almost constant in medium B, as previously reported by Rottenberg and Hashimoto (1986). The constancy of $\Delta\psi$ in Rottenberg's medium was observed only when both valinomycin and sodium were present. If valinomycin were omitted, a decrease of $\Delta\psi$ was observed upon additon of palmitic acid (results not shown).

Figure 3 shows a comparison between the rate of oxygen consumption in static head, multiplied by the H^+/O stoichiometry, and the dissipative ionic current across the membrane, measured as initial rate of K^+ efflux upon addition of valinomycin to inhibited mitochondria, in the presence of increasing concentration of palmitic acid. As already reported for oleic acid (Luvisetto *et al.*, 1987), palmitic acid also increases the passive conductance of the membrane, as indicated by the increase of the dissipative ionic current. However, in this case also, this increase does not completely account for the stimulation of the rate of respiration.

The question arises as to the reason for the different responses of mitochondria incubated in the two media. The different behaviour of $\Delta\psi$ can reflect either a different physical effect of fatty acids in the two media or, alternatively, an interference of the medium with the $\Delta\psi$ assay. To decide between these two possibilities, we have performed an experiment which, as discussed in Pietrobon *et al.* (1987), give information on the type of

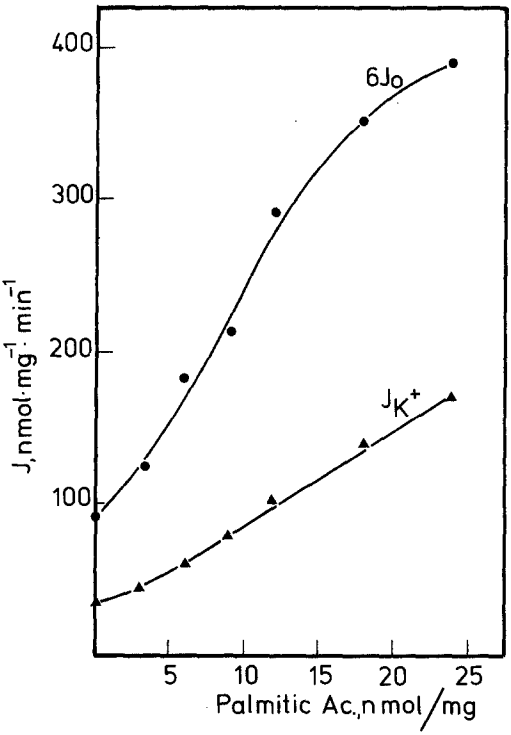

Fig. 3. Rate of respiration in static head multiplied by the H^+/O stoichiometry and initial rate of K^+ efflux after addition of valinomycin to respiratory-inhibited mitochondria. Medium composition: solution A. After 2 min of preincubation of RLM (1 mg/ml) with palmitic acid, succinate (10 mM) was added, and the rate of respiration and, in a parallel sample, the initial rate of K^+ efflux immediately after addition of excess antimycin (50 ng/mg) and valinomycin (150 ng/mg) were measured.

uncoupling that a specific agent exerts, without the need to measure $\Delta\psi$. The experiments consist in measuring the P/O ratio as the ratio between the initial rate of ATP synthesis and of oxygen consumption upon addition of ADP to mitochondria incubated in state 4, at increasing concentrations of antimycin in the presence of a certain concentration of uncoupler. Figure 4 (panel A) show that in mitochondria incubated with palmitate or FCCP, during a titration with antimycin in our usual incubation medium, there was a larger decline of the P/O ratio than in the control. In the absence of external agents the P/O ratio is initially rather insensitive to the inhibition of respiration;i.e., addition of antimycin causes an almost proportional decrease of the rates of respiration and phosphorylation in an extended range of inhibition. The condition of stationary state requires that in state 3 the rate of proton efflux through the redox pumps be equal to the sum of the rates of proton influxes the leaks and through the ATP synthetases. Thus, in redox titration as in

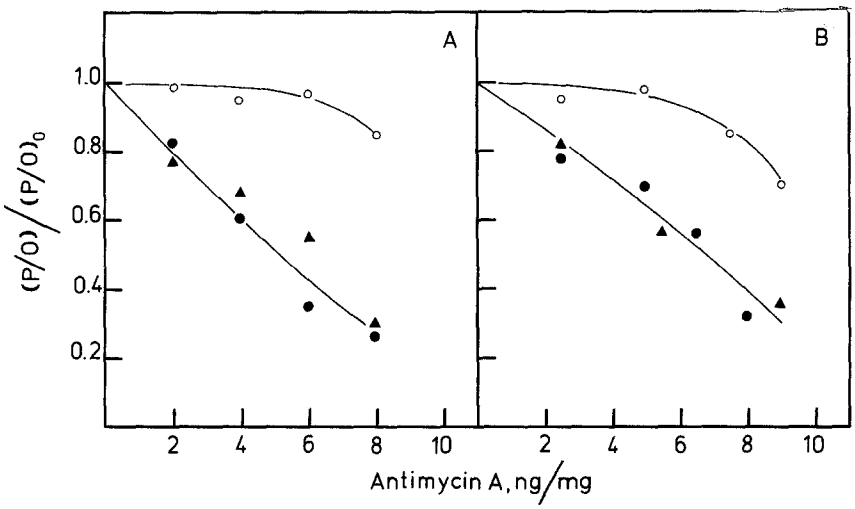

Fig. 4. Normalized P/O ratio as a function of antimycin in the presence of a constant amount of palmitic acid (4.5 nmol/mg, ▲), and FCCP (10 pmol/mg, ●) and in the absence of uncouplers (○). Media compositions as described in Fig. 2. RLM were preincubated for 3 min in the presence of palmitic acid and of increasing concentration of antimycin (0–10 ng/mg); succinate (10 mM) was then added followed after 2 min by ADP (1 mM). $(P/O)_0$ is the value of J_p/J_O in the absence of antimycin and was, respectively: Panel A, control: $(P/O)_0$ = 1.51, palmitic acid: $(P/O)_0$ = 1.21, FCCP: $(P/O)_0$ = 1.22; Panel B, control: $(P/O)_0$ = 1.72, palmitic acid: $(P/O)_0$ = 1.23, FCCP: $(P/O)_0$ = 1.15.

Fig. 4, the lower the membrane proton conductance, the lower the dissipative proton influxes via leaks, and the more extended the range of respiration inhibition within which the P/O ratio is expected to remain relatively constant. Alternatively, the higher the dissipative proton influxed, the more marked the depression of P/O ratio expected upon redox pump inhibition. The results reported in Fig. 4 (panel A) indicate a protonophoric effect of FCCP and oleic acid in our usual medium. Replacement of our incubation medium with Rottenberg's (panel B) resulted in a slightly lower P/O ratio decline. However, again the extent of decline was practically identical with FCCP or with palmitate. If the measured constancy of $\Delta\psi$ reflects a nonprotonophoric action of fatty acids in medium B, one should have found a behavior of the P/O ratio in Fig. 4 (panel B) similar to that of the control (Pietrobon *et al.*, 1987).

Discussion

The present study clarifies the origin of the discrepancy between the results of Rottenberg's group and ours concerning the effect of fatty acids on

the electrical potential difference $\Delta\psi$ across the inner mitochondrial membrane in state 4. Figure 2 shows that while the stimulation of the respiration in state 4 by palmitic acid is similar in the different incubation media used by the two laboratories, the extent of depression of $\Delta\psi$ is quite different. The lower depression of $\Delta\psi$ in Rottenberg's medium (solution B) is not due to a decreased protonophoric effect of the fatty acid in that medium since, when measured on tests independent of the $\Delta\psi$ assay, the uncoupling effect of palmitic acid is indistinguishable from that of FCCP in both media (cf Fig. 4). As discussed in detail elsewhere (Pietrobon *et al.*, 1987; Petronilli *et al.*, 1988), the pattern of the antimycin titration of the P/O ratio is a particularly sensitive test to decide whether the uncoupling occurs via induction of proton leaks or through some other mechanism. The sensitivity of the P/O ratio to redox inhibition is a reliable indication of the extent of leaks since it depends on the ratio between the dissipative proton flow and the rate of proton extrusion via the redox pump (Pietrobon *et al.*, 1987). On the basis of the results in Fig. 4, compared also with the results in Fig. 3, we then conclude that in both media palmitic acid increases the membrane conductance to protons. The reason why the $TPMP^+$ distribution in the presence of valinomycin and Na^+ fails to measure the decrease of $\Delta\psi$ in medium B is unclear.

Conclusion

Palmitic acid has essentially the same effects as oleic acid on the different parameters of oxidative phosphorylation (Luvisetto *et al.*, 1987; Pietrobon *et al.*, 1987). It appears therefore that: (1) the protonophoric effects of fatty acids account only partially for uncoupling of oxidative phosphorylation, and (2) the results are consistent with a mixed action of fatty acids as protonophores and intrinsic uncouplers of the redox pumps and cannot be considered as independent evidence in favor of local coupling mechanisms. The conclusion on the protonophoric effect of fatty acids, however, cannot reconcile with the observation of Rottenberg and Steiner-Mordoch (1986) that fatty acids do not inhibit ATP synthesis driven by artificially imposed $\Delta\psi$ in submitochondrial particles.

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