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A threshold membrane potential accounts for controversial effects of fatty acids on mitochondrial oxidative phosphorylation

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The uncoupling effect of free fatty acids on oxidative phosphorylation in mitochondria has been known for more than 35 years. The mechanism of action, however, remains controversial. In this report the physicochemical basis of uncoupling was elucidated by studying the effect of free fatty acids on the proton permeability and membrane potential of proteoliposomes containing reconstituted cytochrome *c* oxidase (COX). A threshold membrane potential of about 125 mV was identified for fatty acid-induced proton permeability. Only above this potential do free fatty acids translocate protons across the biological membrane. The data explain the controversial effects of long-chain fatty acids on oxidative phosphorylation as well as their role on non-shivering thermogenesis in larger mammals.

Fatty acid; Oxidative phosphorylation; Proton permeability; Cytochrome *c* oxidase; Membrane potential

1. INTRODUCTION

Free fatty acids uncouple oxidative phosphorylation in mitochondria, as already described in 1956 [1–3], but up until today the mechanism of action has remained obscure [4–6] (for review see [7]). Some investigators assume that free fatty acids uncouple oxidative phosphorylation in a manner indistinguishable from that of classic uncouplers [8], while others assume an interaction of fatty acids with the ADP carrier [9,10], cytochrome *c* oxidase (COX) [11,12] or ATP synthase [13]. Rottenberg and Hashimoto suggested a new principle of uncoupling, termed 'decoupling' [13], based on the uncoupling of rat liver mitochondria by oleate and palmitate without any significant decrease in the proton chemical gradient ($\Delta\bar{\mu}_{H^+}$). Azzone and co-workers concluded from their results that oleic acid acts on mitochondria in two ways, as a protonophore as well as an intrinsic uncoupler of redox proton pumps [14,15].

On the other hand, the role of free fatty acids on thermogenesis in brown fat tissue is more generally accepted [16–18]. The uncoupling protein, thermogenin, occurs in mitochondria only from brown fat tissue of mostly small mammals [19,20]. It is assumed that binding of free fatty acids to this protein opens a proton channel in the inner mitochondrial membrane [16,21,22].

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2. EXPERIMENTAL

COX was isolated from bovine heart and brown fat tissue of cold-adapted rats by the use of cholate and deoxycholate [23]. The two-subunits COX of *Paracoccus denitrificans* was isolated by Triton X-100 [24], and the three-subunits COX with dodecylmaltoside [25].

The COX isolated from different sources was reconstituted in liposomes using the cholate dialysis method [26]. Purified asolectin was sonicated to clarity in 2.3% sodium cholate, 20 mM HEPES, 40 mM KCl, pH 7.4, and after the addition of 3 μM COX the lipid concentration was adjusted to 40 mg/ml. The solution was dialysed for 40 h with four buffer changes against an 800-fold vol. of 20 mM HEPES, 40 mM KCl, 90 μM albumin (essentially fatty acid free), pH 7.4.

The oxygen uptake of proteoliposomes was measured polarographically with a Clark-type electrode [27] in the presence or absence of uncoupler (3 μM CCCP (carbonylcyanide-*m*-chlorophenylhydrazone) and 1 μg/ml valinomycin). Proton conductivity was measured as previously described [28].

For the simultaneous measurement of membrane potential and oxygen consumption [29]) a triphenylmethylphosphonium bromide (Ph_3MeP^+)-sensitive electrode [30] was used in addition to a Clark-type oxygen electrode. The experiments were performed with 5 μM Ph_3MeP^+, 0.1 μg/ml nigericin, 0.05% ethanol and increasing concentrations of Tris-ascorbate (6, 15 mM) and cytochrome *c* (0.1, 0.5, 2.5, 12, 31, 69 and 107 μM) in the absence or presence of 50 μM oleic acid or 100 nM CCCP.

The encapsulated volume of the vesicles was determined by the calcein cobalt method [31] and was the same for all vesicle preparations (1.07 ± 0.11 μl/mg lipid). The membrane potential was calculated from the Nernst equation:

$$\Delta\Psi = 59.5 \times \log [Ph_3MeP^+]_{intraliposomal}/[Ph_3Me^+]_{extraliposomal}$$

3. RESULTS

Since the mechanism of action of free fatty acids on oxidative phosphorylation is difficult to investigate in isolated total mitochondria, we chose purified and well-

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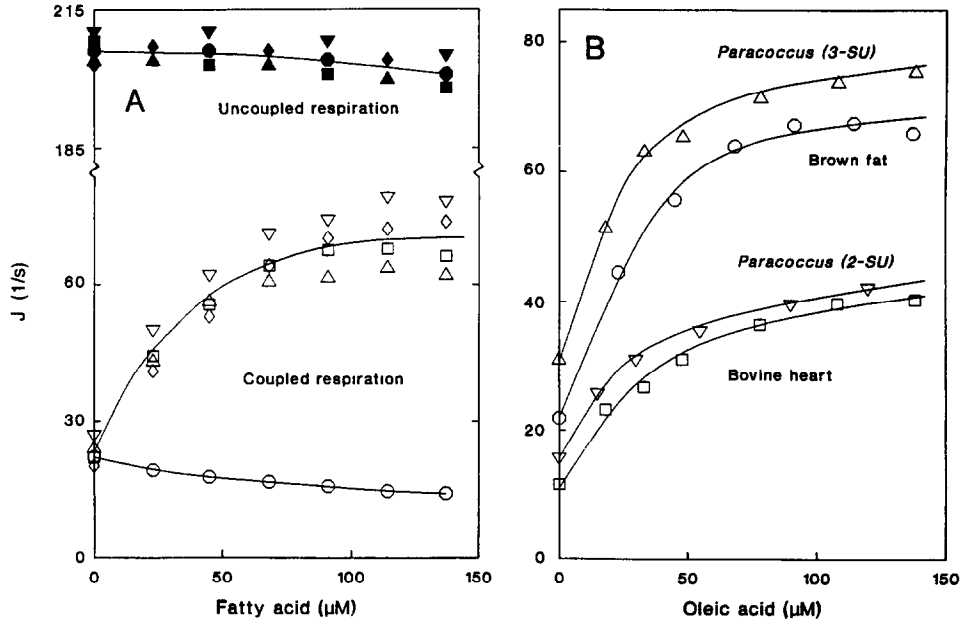

Fig. 1. Effect of fatty acids on the coupled and uncoupled respiration of COX vesicles. (A) The oxygen uptake of reconstituted COX from rat brown adipose tissue was measured polarographically in 20 mM HEPES, 40 mM KCl, 25 mM potassium ascorbate, 40 μM cytochrome *c*, pH 7.4, with increasing amounts of oleic acid (□), palmitic acid (◇), linoleic acid (△) and arachidonic acid (▽) in the presence (closed symbols) and absence (open symbols) of 1 μg/ml valinomycin and 3 μM CCCP. The fatty acids were added as 52.5 mM ethanolic stock solutions and pure ethanol was added in the same amounts up to 0.26% as a control (●,○). The enzyme concentration was adjusted to 20 nM. *J* indicates the turnover number of COX in s^{-1}. (B) The effects of oleic acid on the controlled respiration of reconstituted COX from rat brown fat (○), *Paracoccus denitrificans* (2-subunit, ▽; 3-subunit, △) and bovine heart (□), under the same experimental conditions as in A. Without fatty acids the uncoupled respiratory rates were found to be 206, 218, 334 and 186 ± 10 s^{-1}, respectively, and up to a fatty acid concentration of 138 μM or an ethanol content of 0.26% the rates decreased to 95 ± 2% of these values. Each data point represents the average of two independent assays. The percentage COX orientated right-side-out was determined according the method of Casey et al. [29] and was found to be 76 ± 3% for all vesicle preparations.

defined reconstituted cytochrome *c* oxidase (COX) to study their effect on respiration membrane potential and proton conductivity. The stimulation of oxygen uptake of reconstituted COX from rat brown adipose tissue by various fatty acids is presented in Fig. 1A. With increasing concentrations up to about 100 μM of the fatty acids studied, i.e. oleic, palmitic, linoleic and arachidonic acid, the controlled respiration is stimulated 3- to 4-fold. This rate of respiration, however, is still less than 1/3 of the rate of respiration obtained in the presence of the uncoupler, valinomycin + CCCP. The same

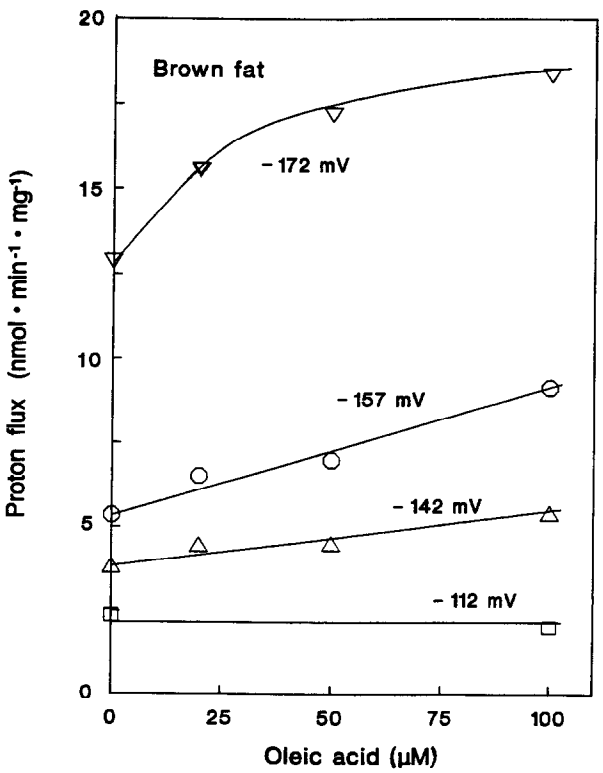

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Fig. 2. Effect of oleic acid on the passive proton conductivity of COX vesicles at varying membrane potentials. COX from rat brown adipose tissue was reconstituted as described in section 2 except that sonification was performed in 2.3% sodium cholate, 257 mM NaCl, 1 mM Na-HEPES, 0.3 mM KCl, pH 7.0, and the solution was dialysed against the same buffer without cholate. For measurements, the vesicles were diluted in 1 mM Na-HEPES, 0.25% ethanol, 0–100 μM oleic acid, pH 7.0, and 22.5–257 mM potassium chloride at varying concentrations of NaCl (234.5–0 mM) to an enzyme concentration of 0.4 μM. The rate of acidification after addition of 1 μg/ml valinomycin was recorded using a pH microcombination electrode. Atomic absorption spectroscopy was used to determine the potassium content of lipids and buffers. The data represent means for four independent experiments with an average standard deviation of ± 8%. Proton flux refers to mg lipid.

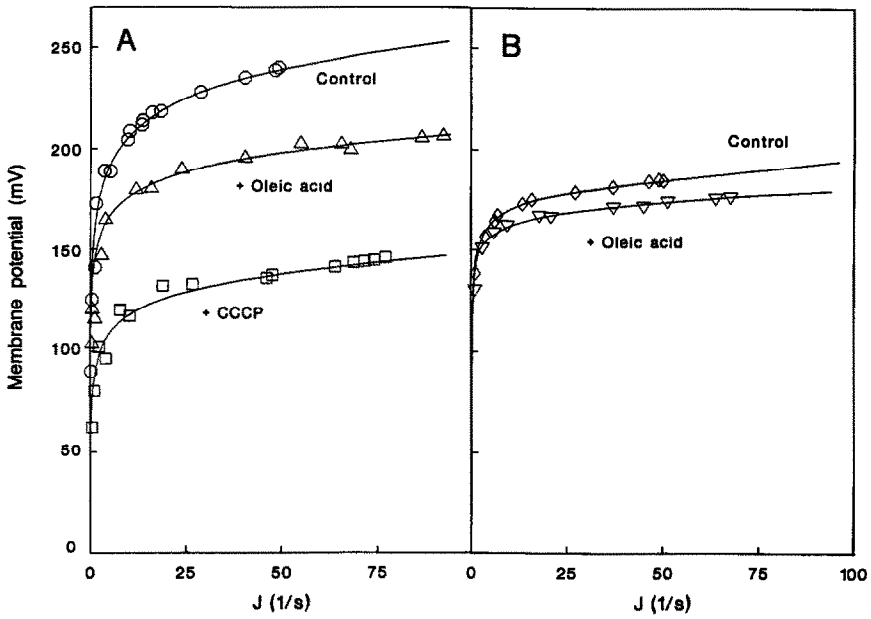
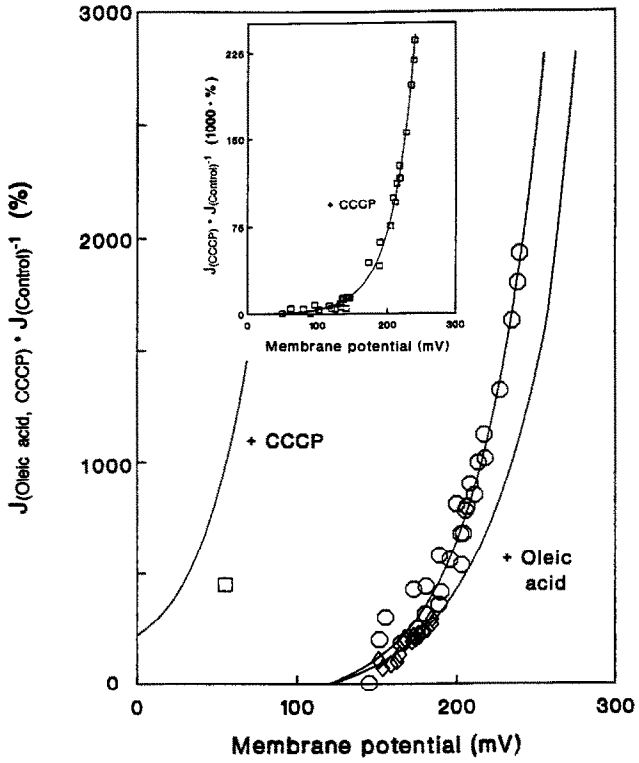


Fig. 3. The relationship between respiratory rate and membrane potential of COX vesicles in the presence and absence of oleic acid and CCCP. COX of *Paracoccus denitrificans* (two subunits, A) and rat brown adipose tissue (B) was reconstituted in the presence of 100 mM HEPES, 40 mM KCl, pH 7.2, and dialysed against the same buffer containing 90 μM albumin (fatty acid free). For measurements, the vesicles were diluted in albumin-free dialysis buffer to an enzyme concentration of 50 nM. The experiments were performed in the absence (○, ◇) or presence of 50 μM oleic acid (△, ▽) or 100 nM CCCP (□). Each point represents a single value and from the data the curves were calculated using the following exponential fit function (J = turnover number): $J = k_1 \times (e^{\Delta\Psi \times k_2} - 1$ [32]. With the enzyme of *Paracoccus* the correlation coefficients are 0.9984, 0.9951 and 0.9991 for the control, oleic acid and CCCP curves, respectively. Vesicles containing COX from brown fat tissue revealed correlation coefficients of 0.9998 and 0.9997 for the experiments performed with or without 50 μM oleic acid.



picture is obtained if the stimulation of respiration by oleate is measured with COX preparations from different species (bovine, rat, *Paracoccus*) or tissues (heart, brown adipose tissue of cold-adapted rats) (Fig. 1B). With all COX preparations oleic acid stimulates the respiration about 3-fold.

In a further approach we investigated the influence of oleic acid on the proton conductivity of COX vesicles containing the enzyme from rat brown fat tissue, at increasing membrane potentials, as shown in Fig. 2. The different membrane potentials were induced by valinomycin in vesicles with 0.3 mM potassium ions inside and varying concentrations outside, as previously described [28]. Apparently the stimulation of proton conductivity by oleate is dependent on the membrane potential. Stimulation by oleate was only obtained at membrane potentials above 112 mV.

The influence of oleic acid on the membrane potential

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Fig. 4. Percent stimulation of respiration by oleic acid or CCCP with respect to membrane potential. The data and calculated fit functions of the experiments performed with oleic acid or CCCP from Fig. 3 are divided by the data of the control experiments at membrane potentials between 1 and 300 mV. These ratios are expressed as percent stimulation of respiration by either CCCP (□) or oleic acid (*Paracoccus*, ○; brown fat, ◇) compared to the control. The single points are calculated by dividing a measured value of one curve by the corresponding fitted value of the other curve. The inset represents the CCCP data on a larger scale.

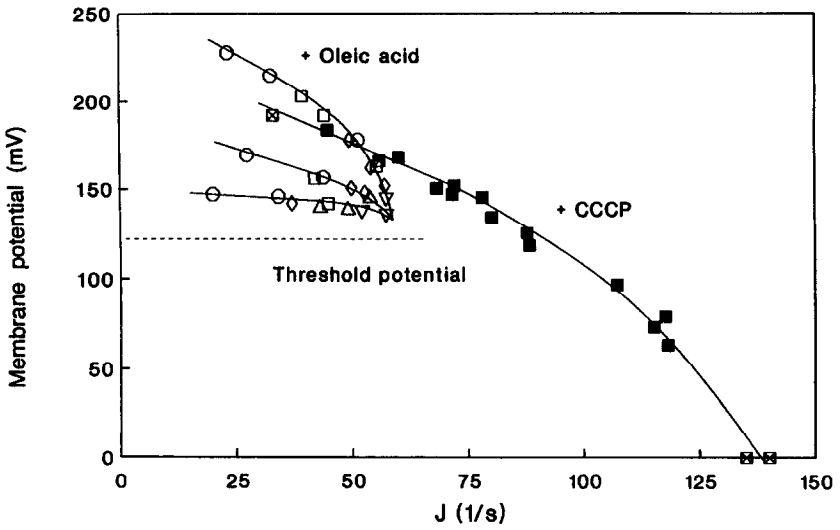

Fig. 5. Relationship between respiratory rate and membrane potential of COX vesicles at varying concentrations of oleic acid and CCCP. Vesicles containing the 2-subunit COX of *Paracoccus* were reconstituted as described in the legend to Fig. 3. Different membrane potentials and electron flux rates at the beginning of each experiment (○, ⊠) were adjusted by using different concentrations of Tris-ascorbate (15, 25 mM), cytochrome *c* (40, 60, 80 μM) and valinomycin in suboptimal amounts (0 up to approximately 20 ng/ml). Addition of oleic acid as a 175 mM ethanolic stock solution led to end concentrations of 50 (□), 100 (◇), 150 (△) and 200 μM (▽). CCCP was added as a 6.3, 2.1 or 0.42 mM ethanolic stock solution and concentrations of 0.1, 0.2, 0.34, 0.58, 0.86, 1.6, 2.1, 3.6 and 4.8 μM (all ■) were prepared. Control experiments performed with pure ethanol in the same amounts (maximal 0.11%) showed that 8 ± 3% of $\Delta\Psi$ dissipation caused by oleic acid or CCCP is due to the solvent. The data represent single values.

of reconstituted two-subunits COX from *Paracoccus* at various rates of oxygen uptake is presented in Fig. 3A, and of COX from brown fat tissue in Fig. 3B. The membrane potential was measured with a $TPMP^+$ electrode at increasing concentrations of cytochrome *c* and ascorbate, as previously described [28,29]. A non-linear dependence of membrane potential on oxygen uptake is obtained. The non-linear relationship is found with oleic acid, however, at diminished membrane potentials. Titration of the membrane potential with cytochrome *c* in the presence of low concentrations of the classical uncoupler, CCCP, appears to result in a similar titration curve but at lower membrane potentials (Fig. 3A). A mathematical analysis of the curves, however, reveals significant differences between the effect of fatty acid and that of the classical uncoupler. The stimulation of respiration by oleic acid and CCCP at various membrane potentials, calculated from the measurements of Fig. 3, as well as their fit functions, are presented in Fig. 4. Clearly, below a membrane potential of about 125 mV, no stimulation of respiration is obtained with oleic acid. In contrast, with the uncoupler the respiratory rate is increased at all membrane potentials. It should be pointed out that this result is not due to inaccurate data points of the membrane potentials at low rates of respiration. Omission of the data at turnover numbers below 3 s^{-1} changes the constants, k_1 and k_2, of the fit functions only negligibly. These results indicate that in contrast to classical uncouplers, fatty acids require a threshold membrane potential to cross the membrane as the charged anion, and thus to act as uncouplers of oxidative phosphorylation [8,33].

The difference between the classical uncoupler CCCP, and fatty acids becomes more evident from the results of Fig. 5, which shows the reconstituted two-subunit COX of *Paracoccus* titrated with increasing concentrations of either CCCP or oleic acid, beginning at various membrane potentials. With oleic acid, at any concentration, the membrane potential does decrease below the threshold potential, and the uncoupled respiration remains below the value obtained with the classical uncoupler. In contrast, with CCCP, the membrane potential decreases to zero at increasing concentrations. Starting the oleic acid titration at potentials next to the threshold potential leads to stimulation of respiration with no significant dissipation of $\Delta\Psi$. Apparently the proton-pumping capacity of COX is strong enough to overcome the increased backflow of protons via fatty acids by increasing the electron flow, while maintaining the membrane potential just above the threshold potential. At higher potentials or in the presence of the more effective classical uncoupler, CCCP, the H^+-backflow overcomes the H^+-pumping capacity and the potential strongly decreases. This effect may account for the controversy between the 'decoupling' obtained by Rottenberg and Hashimoto [13] and 'uncoupling' obtained by Schönfeld et al. [8].

4. DISCUSSION

The above data demonstrate a membrane potential dependence of the uncoupling effect of free fatty acids, with a threshold of about 125 mV, and can explain most previous results. They do not explain the inhibition of

fatty acid uncoupling in mitochondria by carboxyatractylate which suggests the translocation of fatty acid anions through the ADP/ATP carrier [7,9,10,34]. Thus it appears that at least two mechanisms can account for the controversial effects of free fatty acids.

The uncoupling effect of free fatty acids appears to be independent of the type of long-chain fatty acid and of the membrane potential generator (proton pump). In fact, no indication for a specific interaction between fatty acids and the proton pump (COX), as suggested previously [11,12,14,15], was obtained.

Our results have implications on the mechanism of non-shivering thermogenesis. The threshold membrane potential of about 125 mV is sufficient for ATP synthesis by ATP synthase in the presence of a ΔpH of 1–2 [35]. Thus at low $\Delta\Psi$, free fatty acids should not impair ATP synthesis in mitochondria. In small mammals, non-shivering thermogenesis occurs mainly in brown adipose tissue via opening of a proton pore in the protein, thermogenin [19,20]. In larger mammals lacking brown adipose tissue, a different mechanism of heat production was postulated [36]. The above data suggest regulation of non-shivering thermogenesis via regulation of the mitochondrial membrane potential. This mechanism of thermogenesis will depend on both a membrane potential above 125 mV and a fatty acid concentration of 10–50 μM. Under normal conditions, fatty acids are complexed by intracellular fatty acid-binding proteins [37], which have a binding constant of less than 1 μM and occur in all mammalian tissues [38]. The relatively high amounts of fatty acids required for uncoupling of oxidative phosphorylation may be induced by noradrenalin, which also activates thermogenesis in brown adipose tissue [16–18].

A tissue-specific mechanism of thermogenesis was recently suggested in heart and skeletal muscle [39], based on the interaction of nucleotides with the heart isoform of COX subunit VIa (VIa-H) [40]. It was suggested that, at rest, the increased energy state (ATP:ADP ratio) of the mitochondrial matrix decreases the efficiency of COX (increased heat production) only in heart and skeletal muscle. Thus, various mechanisms are involved in mammalian thermogenesis.

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