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Long-chain fatty acids act as protonophoric uncouplers of oxidative phosphorylation in rat liver mitochondria

Peter Schönfeld, Lorenz Schild and Wolfgang Kunz

Institut für Biochemie der Medizinischen Akademie Magdeburg, Magdeburg (G.D.R.)

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The effect of long-chain fatty acids (LCFA) on respiration and transmembrane potential ($\Delta\psi$) in the resting state, and the rate of $\Delta\psi$ dissipation ($(d\Delta\psi/dt)_i$) was investigated with oligomycin-inhibited rat liver mitochondria using succinate (plus rotenone) as substrate. The results obtained were compared with those of classical protonophores such as 2,4-dinitrophenol (DNP) and 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB). The effects of oleate or palmitate and that of DNP or TTFB on respiration and $\Delta\psi$ can be described by a common force–flow relationship. These facts all in all are not compatible with a decoupler-type uncoupling mechanism of LCFA; still, they indicate that the latter are protonophores. Moreover, the oleate-induced increase in the rate of $\Delta\psi$ dissipation closely correlates with that in respiration, suggesting that the uncoupling activity and the protonophoric activity of LCFA are interrelated. Carboxyatractyloside (CAT) exerted only a small inhibitory effect on oleate-induced respiration and $\Delta\psi$ dissipation, indicating that the adenine nucleotide translocase contributes to the uncoupling effect of LCFA to a minor extent only. Proton transport through the lipid region of the membrane as mediated by permeation of the protonated and deprotonated forms of LCFA is interpreted as the main process of the uncoupling of LCFA.

Introduction

Being important hydrogen-supplying substrates to the primary dehydrogenases of the respiratory chain, long-chain fatty acids (LCFA) are also able to uncouple the oxidative phosphorylation (for review see Ref. 1). However, there is no general agreement on the uncoupler mechanism operating [1–5]. According to the chemiosmotic hypothesis, the action of a protonophoric uncoupler is based on its short-circuit effect on the proton flow through the mitochondrial inner membrane [6]. Consequently, addition of a protonophore to mitochondria incubated in the resting state should be expected to decrease $\Delta\tilde{\mu}_{H^+}$ and stimulate oxygen uptake due to diminished backpressure on the proton pumps of the respiratory chain. Rat liver mitochondria respond to artificial protonophores in this way [3]. Furthermore, a reciprocal relationship between the rate of oxygen uptake and $\Delta\tilde{\mu}_{H^+}$ was found with mitochondria from brown adipose tissue when they were incubated with low concentrations of LCFA [7,8]. This special type of mitochondrion contains an integral protein (thermogenin) operating as a proton-conducting channel [9]. In the basal state, proton conducting is prevented by tight binding of extramitochondrial purine nucleotides. Binding of fatty acid to this protein overcomes the inhibition by nucleotides [10,11].

For mitochondria from liver tissue, the uncoupler effect of LCFA was explained by two different hypotheses: (i) dissipation of intramembranal pools of occluded protons without reduction of $\Delta\tilde{\mu}_{H^+}$ (decoupling [3,4]); (ii) LCFA – mediated shuttling of protons through the mitochondrial inner membrane [5]. According to the latter mechanism, LCFA in their protonated form permeate the inner membrane and release the carboxylic group-linked proton on the matrix side. The anionic form of LCFA is transported back to the cytosolic side by means of adenine nucleotide translocase (ANT) [5]. In this case the proton shuttling must result in a reduction of $\Delta\tilde{\mu}_{H^+}$. In view of the high lipid solubility of

Abbreviations: LCFA, long-chain fatty acids; $\Delta\psi$, transmembrane potential; DNP, 2,4-dinitrophenol; DDA^+, dimethyldibenzylammonium; TPB^-, tetraphenylboron; CAT, carboxyatractyloside; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; ANT, adenine nucleotide translocase; V_{resp}, rate of respiration.

Correspondence:P. Schönfeld, Institut für Biochemie, Medizinische Akademie Magdeburg, Leipziger Str. 44, Magdeburg 3090, G.D.R.

LCFA, however, the deprotonated form of LCFA may shuttle protons through the inner membrane without ANT being involved. Thus, the effects of a variety of fatty acids on respiration and $\Delta\psi$, the main component of $\Delta\tilde{\mu}_{H^+}$, was studied to gain deeper insight into LCFA-linked uncoupling. The protonophoric activity of LCFA was estimated in view of their effect on the conductance of the inner membrane, from the $\Delta\psi$ dissipation measured after blockade of its generating process. The results obtained were compared with those for DNP and TTFB.

The findings presented in this paper clearly demonstrate that the uncoupling effect of LCFA is attributed to an increase in inner membrane conductance and is not due to decoupling.

Materials and Methods

Isolation of mitochondria

Mitochondria were isolated from liver of female albino rats (150–200 g body weight) by the standard procedure [12], suspended in 250 mM sucrose adjusted to pH 7.4 with Tris. The protein content of this stock suspension (25 ± 3 mg/ml) was measured by the biuret method. The functional integrity was determined by measuring the respiratory control ratio with ADP (0.4 mM), succinate (plus rotenone) being used as substrate. Only mitochondria showing respiratory control ratios greater than 5 were used.

Incubation conditions

The standard incubation medium for mitochondria contained in mM: 90 sucrose; 60 tricine, 60 Tris, 10 sodium phosphate, 5 $MgCl_2$, 15 glucose, 40 nicotinamide, 10 sodium succinate, 0.5 Na_2EDTA, 0.001 rotenone; adjusted to pH 7.4 by HCl and gassed by air. The incubation medium was additionally supplemented with F_0F_1-ATPase inhibitor oligomycin (2 μg/ml) to preclude effects caused by mitochondrial fatty acid activation. The incubations were performed at 25 °C.

Measurement of oxygen uptake and membrane potential

Oxygen uptake by mitochondria was measured polarographically and recording either the oxygen concentration trace or its first derivative ($d[O_2]/dt$). $\Delta\psi$ was measured by a DDA^+-sensitive electrode [13]. For simultaneous measurements of oxygen uptake and $\Delta\psi$, incubations were performed in a closed, temperature-controlled and magnetically stirred vessel equipped with a Clark-type electrode, a DDA^+-electrode and a calomel electrode. Calculation of the oxygen uptake was based on an O_2 concentration of 230 μM [14]. The DDA^+-electrode was calibrated before each incubation, $\Delta\psi$ being calculated as in Ref. 13 with a mitochondrial matrix volume of 1 μl/mg protein. The effect of LCFA on the proton conductance of the mitochondrial inner membrane was derived from the measurement of the rate of $\Delta\psi$ dissipation, the $\Delta\psi$-generating process being blocked by cyanide [15]. This approach was adopted to rule out side-effects of LCFA (e.g., partial inhibition of the respiratory chain) which might affect proton conductance data if determined by calculating the effective proton conductance (C_mH^+ [16]).

Materials

Tris, Tricine, succinic acid, FCCP, ATP, ADP and yeast hexokinase (EC 2.7.1.1) were obtained from Boehringer; CAT, oligomycin, rotenone and DCCD from Sigma; DDA^+ from Ferak; 1-palmitoylcarnitine and palmitoyl-CoA from Serva; fatty acids were purchased from Merck. TTFB was a kind gift by Dr. Beechey (Aberystwith, U.K.). All other chemicals were analytical grades produced in the G.D.R. Arachinate (20 : 0), oleate (18 : 1), stearate (18 : 0), palmitate (16 : 0) were used as 5 mM, myristate (14 : 0), laurate (12 : 0) and caprinate (10 : 0) as 100 mM solution in ethanol.

Results and Discussion

Uncoupling effect

Fig. 1 demonstrates how the uncoupling effect of the fatty acids was measured. Known to be one of the most potent uncoupling fatty acids [3], oleate was added to rat liver mitochondria incubated with succinate (plus rotenone) as hydrogen-supplying substrate and under

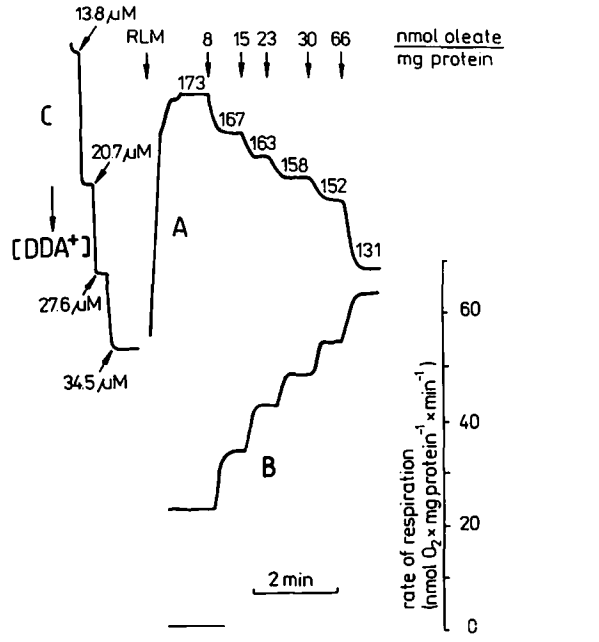

Fig. 1. Effect of oleate on e.m.f. of the DDA^+ electrode reflecting changes in $\Delta\psi$ (A) and on respiration (B). Oleate was added to an incubation mixture (4.28 ml) containing 0.65 mg mitochondrial protein per ml. Further additions: 36 μM DDA^+, 3.6 μM TPB^-. Trace C is the calibration curve of the DDA^+ electrode. The numbers on the trace of the DDA^+ electrode indicate $\Delta\psi$ in mV.

conditions where the F_0F_1-ATPase was blocked by oligomycin. It is seen that oleate exerted an uncoupling effect indicated by stimulation of respiration (B) and a decrease in $\Delta\psi$ (A). The increase in respiration produced by stepwise addition of oleate was paralleled by diminishing $\Delta\psi$. No effect was noted after addition of a volume of pure ethanol equivalent to the maximal amount of oleate solution added (36 μl).

These effects of oleate on the rate of respiration and $\Delta\psi$, the main component of $\Delta\tilde{\mu}_{H^+}$, were exactly those predicted by the chemiosmotic hypothesis of artificial protonophoric uncouplers [6]. This conclusion is in contradiction to another one presented in a recent paper [3], where no significant effect of LCFA addition was seen on the $\Delta\psi$ maintained by rat liver mitochondria in the resting state. Neither did the authors find a difference in $\Delta\psi$ between the active and the resting state. In this case, a 20–30 mV difference has been found generally [17–19].

While predicting that the uncoupler effect of LCFA is mediated by a protonophoric action, LCFA derivatives in which the carboxylic group is esterified cannot have an uncoupling activity. This hypothesis was checked for the derivatives of palmitic acid, 1-palmitoylcarnitine and palmitoyl-CoA. In Fig. 2 showing a typical experiment with respect to the free acid, it is seen that 1-palmitoylcarnitine stimulated the respiration only to a very small extent and palmitoyl CoA had no influence on respiration, indicating that the COOH group is essential to the uncoupling effect of LCFA. Furthermore, since the esterified derivatives (which are also lipophilic) of LCFA did not exert uncoupling, it may be assumed that the uncoupling action cannot have

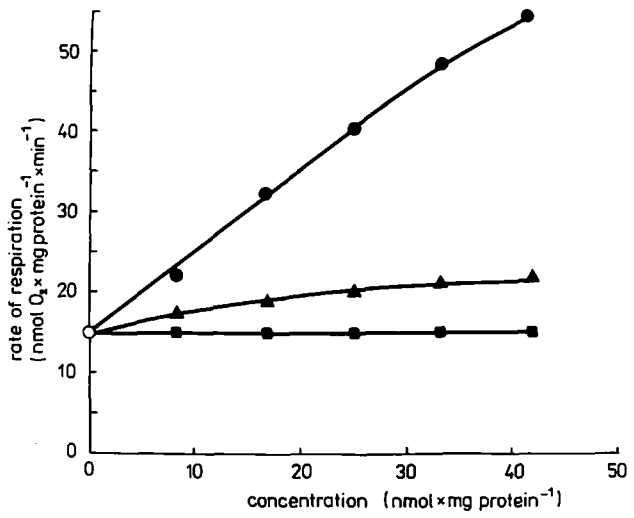

Fig. 2. Effect of stepwise increase in concentrations of palmitate, 1-palmitoylcarnitine and palmitoyl-CoA on resting-state respiration. Incubations were performed as described in the legend to Fig. 1. Values of the rate of respiration represent the mean of two experiments with 0.65 and 0.95 mg protein per ml. Palmitate (●), 1-palmitoylcarnitine (▲), palmitoyl-CoA (■).

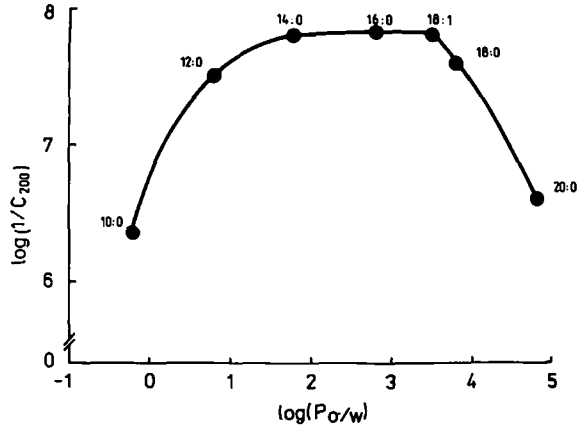

Fig. 3. Uncoupling activity vs. lipophilicity of various LCFA. Incubations were performed as described in the legend to Fig. 1. The concentration doubling the resting-state respiration (C_{200}) was determined from the obtained relationship between respiration and concentration of the fatty acid used. The C_{200} values represent the mean of two different experiments with 1.2 or 0.8 mg mitochondrial protein per ml. The partition coefficients for the octanol/water system, $P_{o/w}$, were taken from Ref. 23.

been caused by an alteration in the membrane structure as a result of incorporation of LCFA into the inner membrane.

Studies with substituted phenol-type uncouplers have revealed that their uncoupling activity can be attributed to two factors: acidity and hydrophobicity [20,21]. Comparing fatty acids of various chain lengths with each other, there is no remarkable difference in acidity [22]. In order to investigate the effect of an increase in the hydrophobicity of LCFA on their uncoupling activity, the concentration needed to double the rate of respiration was determined in the resting state (C_{200}) for a number of fatty acids characterized by different chain lengths. In Fig. 3 the uncoupling activity expressed as log $1/C_{200}$ was plotted vs. the partition coefficients in the octanol/water system ($P_{o/w}$) being a common measure of hydrophobicity. It can be seen that log $1/C_{200}$ increased with log $P_{o/w}$ from caprinate (10 : 0) to myristate (14 : 0). However, a further increase in hydrophobicity was not paralleled by an increase in uncoupling activity; in fact, the latter diminished, as was revealed by comparison of palmitate (16 : 0) with arachinate (20 : 0). This finding can be explained by a reduced motility of the fatty acids within the membrane, resulting in a decrease in protonophoric activity.

All these facts taken together, we conclude that LCFA are protonophores. This being true, artificial protonophores and LCFA should be expected to exhibit comparable effects on $\Delta\psi$ and respiration. To verify this prediction we determined the relationship between the rate of respiration and corresponding $\Delta\psi$ produced by increasing amounts of artificial protonophores or LCFA. Fig. 4 depicts the results of a typical experiment conducted with oleate, palmitate, DNP and TTFB. It is

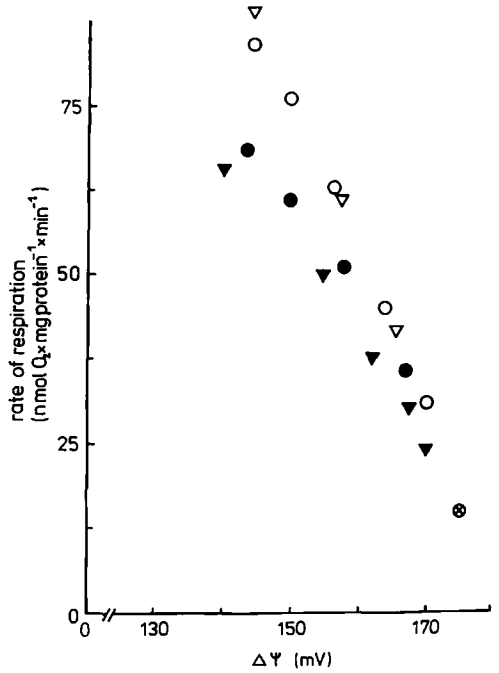

Fig. 4. Force–flow relationship of $\Delta\psi$ and respiration for oleate, palmitate, DNP and TTFB. Oleate (●), palmitate (▼) were added to the incubation mixture (0.7 mg protein/ml) to final concentrations of 4.8, 9.5, 14.3, 19.0 and 42.0 (except oleate) μM. Final concentrations of DNP (○) were 4.7, 9.4, 14.1, 20.9 and 27.2 μM; those of TTFB (▽) 0.1, 0.19 and 0.29 μM. ⊗ was obtained without addition of fatty acids or uncouplers.

evident that there exists one force–flow relationship for both types of uncoupler. The difference in $\Delta\psi$ at higher rates of respiration might be attributed to an inhibitory effect of LCFA on the $\Delta\psi$-generating process [1].

Protonophoric effect

To study the direct influence of LCFA on the conductance of mitochondrial inner membrane we measured the initial rate of $\Delta\psi$ dissipation $(d\Delta\psi/dt)_i$ with the $\Delta\psi$-generating process blocked after adjusting partial uncoupling. Under such conditions, $(d\Delta\psi/dt)_i$ can be considered as a measure of the proton current due to the proton transport through the mitochondrial inner membrane [15]. Fig. 5 shows the experimental protocols of experiments conducted to elucidate the effect of limiting amounts of oleate (trace B) and DNP (trace C) on the release of DDA^+ by RLM after inhibition of the respiratory chain by cyanide, indicating the dissipation of $\Delta\psi$. Since $(d\Delta\psi/dt)_i$ is a function of $\Delta\psi$, malonate known as an inhibitor of succinate dehydrogenase was added in a reference experiment (trace A) to adjust $\Delta\psi$ to nearly the same value seen with oleate and DNP. It can be seen that, in the presence of oleate and DNP, the $(d\Delta\psi/dt)_i$ increased when compared to the rate of $\Delta\psi$ dissipation linked to the endogenous proton leak (trace A). This result tends to indicate an increase in proton transport across the inner membrane by both types of protonophore.

Speculating that the oleate-induced increase in $\Delta\psi$ dissipation is caused by a shuttle-type mechanism, the rate of $\Delta\psi$ dissipation should be expected to be proportional to the number of oleate molecules. So, the dependency of $(d\Delta\psi/dt)_i$ on the oleate concentration was studied. To adjust experimental conditions where $(d\Delta\psi/dt)_i$ would be a function of the oleate concentration only, following the addition of limiting amounts of oleate $\Delta\psi$ was titrated by malonate to a constant value in all incubations before eventually cyanide was added. Fig. 6 demonstrates, that $(d\Delta\psi/dt)_i$ indeed was directly proportional to the oleate and the DNP concentration, thus favouring the suggestion that oleate acts as a proton-shuttling protonophore. That the data obtained with oleate and DNP are overlapping is explained by their comparable uncoupling potency (Ref. 26 and Fig. 3). With stronger uncouplers, e.g., FCCP, a steeper dependence of $(d\Delta\psi/dt)_i$ on the uncoupler concentration is expected.

The molar ratio of lipid/oleate or lipid/DNP at which the permeability of the membrane to protons (indicated as $(d\Delta\psi/dt)_i$) was doubled is about 30 (for calculation of the molar ratio using a lipid/protein ratio of 0.24 [24] and an average lipid M_r of 740). With proteoliposomes as model system [25], an increase of the LCFA-mediated permeability of the liposomal membrane to protons was seen, too, but at a lipid/LCFA ratio of about 1. This difference might be explained by the liposomal membrane being less sensitive to protonophores (for comparison see the effect of CCCP in Fig. 4 of Ref. 25 and Table I of Ref. 26).

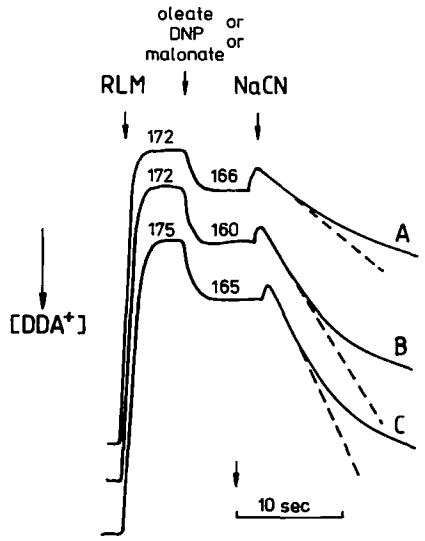

Fig. 5. Effect of oleate and DNP on the dissipation of $\Delta\psi$ after inhibition of its regeneration. The mitochondrial protein concentration was 0.45 mg/ml of the standard incubation medium in each experiment. Addition as indicated were: 3.6 μM oleate (B), 4.8 μM DNP (C) and 1.2 mM malonate (A). Regeneration of $\Delta\psi$ was blocked by addition of 2 mM NaCN. The numbers on the traces indicate $\Delta\psi$ in mV.

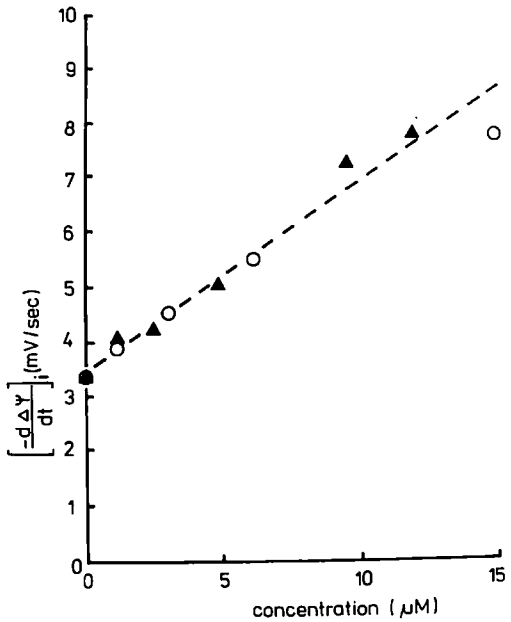

Fig. 6. Rate of $\Delta\psi$ dissipation as a function of oleate or DNP concentration. Incubations (0.8 mg protein/ml) were performed as described in the legend to Fig. 5. Final concentration of oleate (○) were 1.2, 3.0, 5.9, 14.8 μM and those of DNP (▲) 1.2, 2.4, 4.7, 9.4, 11.9 μM. NaCN was added after adjusting the $\Delta\psi$ in each incubation with malonate to approximately a value which corresponded to that maintained in the active state (adjusted with 298 mU hexokinase per mg protein plus 1 mM ATP).

For the transport of protons through the mitochondrial inner membrane by artificial protonophoric uncouplers, a shuttle-type mechanism has been postulated [20,21,27–32]. The comparable acidity of artificial protonophores (pK_a 4.1–6.8 [26]) and fatty acids (pK_a 4.7–5.8 [22]) as well as the ability of fatty acids to accumulate in the lipophilic phase [23] provokes the idea that a similar mechanism operates in the case of LCFA, too. Such a mechanism demands that the protonated fatty acid and its anion can permeate separately across the inner membrane. As for the protonated fatty acid, there is no doubt that it has the ability to permeate (allowing as short-chain type a fatty acid such as acetic acid to be used as pH probe). It might be argued, however, that the poor ability of the alkyl group to delocalize the negative charge from the COO^- group hinders the fatty acid anion in permeation. As the permeability of the fatty acid anion depending not only on the COO^- group but on the lipophilicity of the alkyl group as well, it can be reasonably speculated that an elongation of the chain length of the alkyl group may circumvent the resistance of the COO^- group against the permeation of the fatty acid anion across the inner membrane. In line with this view is the finding that the uncoupling concentration decreases from caprinate to palmitate (Fig. 3).

Role of the adenine nucleotide translocator

In view of recent reports it should be taken into consideration that ANT [5] and/or an anion channel [33,34] may be involved in transport of the deprotonated fatty acids through the inner membrane. Both ANT and the anion channel would allow a uniport transport of the fatty acid anion.

Our findings revealing that the rate of uncoupled respiration as well as the rate of $\Delta\psi$ dissipation induced by oleate were slightly decreased by CAT (which is known to be an irreversible inhibitor of ADP-ATP transport) are in line with this suggestion. The results of two experiments are given in Table I. The average inhibitory effect of CAT on uncoupled respiration and the rate of $\Delta\psi$ dissipation was found to be 16% and 13%, respectively. In view of this relatively small inhibitory effect of CAT, the involvement of ANT is concluded to be of minor importance in the total uncoupling effect of LCFA. Since CAT has a small inhibitory effect on the DNP-stimulated respiration (not shown), the involvement of ANT in the uncoupling effect of LCFA and artificial uncouplers seems to be by a rather nonspecific mechanism. Taking into account similarities in structure and composition observed for ANT and thermogenin [35], there is reason to speculate that ANT could operate somewhat in a manner as described for thermogenin. Furthermore, known as a blocking agent for the anion channel [33,34], DCCD did not prevent

TABLE I

Effects of CAT on the rate of respiration and rate of dissipation of partially uncoupled rat liver mitochondria

]The rate of respiration and the rate of $\Delta\psi$ dissipation were determined in incubations with partially oleate-uncoupled rat liver mitochondria (Expt. 1: 1.04 mg protein/ml; Expt. 2: 0.84 mg protein/ml) without and with CAT (1.12 μM). The increase in DDA^+ accumulation observed after addition of CAT was titrated with malonate back to that recorded before CAT was added. Values of respiration marked $^+$ were those without malonate.

Oleate (nmol/mg protein)		CAT	V_{resp} (nmol O_2/min per mg protein)	$-[\frac{d\Delta\psi}{dt}]_i$	
				mV	mV/s
Expt. 1	–	–	17.5	176.7	2.05
	8.6	–	33.1	161.4	4.59
		+	30.0 $^+$		4.08
	14.8	–	47.7	153.2	6.23
		+	41.8 $^+$		5.03
	29.7	–	58.9	145.2	7.73
		+	51.1		7.31
Expt. 2	–	–	17.6	176.0	2.70
	7.1	–	30.1	162.9	4.51
		+	25.7 $^+$		3.81
	14.2	–	41.9	151.4	6.27
		+	31.0 $^+$		4.99
	21.3	–	53.9	144.7	6.81
		+	43.2 $^+$		5.97

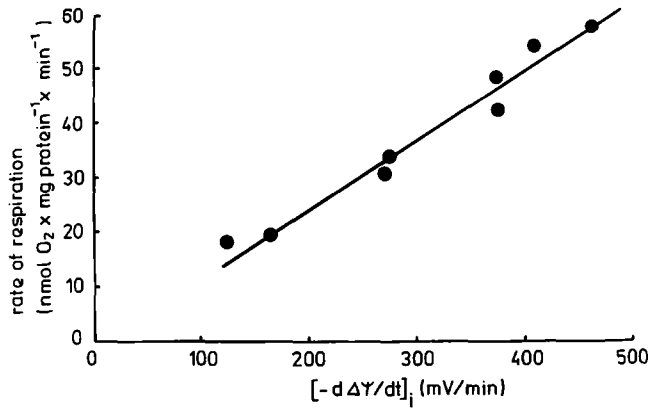

Fig. 7. Rate of uncoupled respiration vs. rate of $\Delta\psi$ dissipation. Values of respiration and $\Delta\psi$ dissipation determined in incubation experiments without CAT were taken from Table I. The relationship was obtained by linear regression, where $r = 0.983$. For the relationship $V_{resp} = a[d\Delta\psi/dt]_i + b$, a slope $(a) = -0.256$ nmol O_2/mg protein per mV and an intercept $(b) = 1.92$ nmol O_2/mg protein per min were found.

oleate-based uncoupled respiration (not shown). Moreover, if transport of the deprotonated form of fatty acids were accomplished by an anion channel as postulated for the transport of small hydrophilic anions [33], then the hydrophobicity of fatty acids should not affect their uncoupling activity so drastically, and therefore short-chain fatty acids, too, may be expected to act as uncouplers. Moreover, with the data compiled in Table I, the uncoupling activity (V_{resp}) and the protonophoric activity $(d\Delta\psi/dt)_i$) can be demonstrated to be closely interrelated. In Fig. 7, the data of the rate of respiration plotted against those of the rate of dissipation produced by different amounts of oleate (without CAT) are seen to yield a straight line. This finding is not consistent with a view that LCFA induce proton slipping during respiration-driven proton pumping [2].

The question as to whether or not LCFA are able to increase the proton conductance of biological membranes which do not contain thermogenin is of relevance to explaining the mechanism of how LCFA act as uncouplers. Our findings based on the influence of LCFA on the rate of $\Delta\psi$ dissipation evidenced for the first time that LCFA applied at suitable concentrations increase the proton conductance of inner membrane of rat liver mitochondria. Therefore, the present results convey the suggestion that a common uncoupling mechanism exists for artificial uncouplers and LCFA, but do not support the hypothesis that LCFA uncouple oxidative phosphorylation by a specific mechanism which is referred to as decoupling [3,4]. In line with this is the lack of decoupling effect of LCFA on the redox pump associated with cytochrome *c* oxidase, as was found in Ref. 25.

Finally, the mechanism of LCFA-stimulated respiration discussed in this paper is different from that found with short-chain and medium-chain fatty acids studied in the same concentration range. In a recent paper [36], we presented arguments favouring the conception that short-chain and medium-chain fatty acids induce an increase in phosphorylating respiration by an intramitochondrial futile cycle. This mode of action can be excluded in the case of LCFA, since these fatty acids are not activated within the mitochondrial matrix space (for review see Ref. 37).

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