Mitochondria uncoupling by fatty acids in vivo is still questionable, being confounded by their dual role as substrates for oxidation and as putative genuine uncouplers of oxidative phosphorylation. To dissociate between substrate and the uncoupling activity of fatty acids in oxidative phosphorylation, the uncoupling effect was studied here using a nonmetabolizable long chain fatty acyl analogue, ββ'-methyl-substituted hexadecane α,ω-dioic acid (MEDICA 16) is reported here to induce in freshly isolated liver cells a saturable oligomycin-insensitive decrease in mitochondrial proton motive force with a concomitant increase in cellular respiration. Similarly, MEDICA 16 induced a saturable decrease in membrane potential, proton gradient, and proton motive force in isolated liver and heart mitochondria accompanied by an increase in mitochondrial respiration. Uncoupling by MEDICA 16 in isolated mitochondria was partially suppressed by added atracyloside. Hence, fatty acids may act as genuine uncouplers of cellular oxidative phosphorylation by interacting with specific mitochondrial proteins, including the adenine nucleotide translocase.

Mitochondrial uncoupling by fatty acids has been well reported during the last 50 years (reviewed in Ref. 1). Uncoupling of isolated mitochondria by fatty acids interferes with mitochondrial ATP synthase activity and results in increase in mitochondrial state 4 respiration with a concomitant decrease in the P/O ratio. Uncoupling by fatty acids was ascribed to the protonophoric action of fatty acids resulting in dissipation of the mitochondrial inner membrane potential (1, 2), to interference by fatty acids with a putative localized coupling element interconnecting proton pumping and the ATP synthase (3, 4), or alternatively, to “slippage” of proton pumping and/or the ATP synthase machinery (5–7). The protonophoric action of fatty acids was proposed to be mediated by fatty acids crossing the mitochondrial membrane in their protonated form followed by efflux of the fatty acid anion through an anion channel or paired with a putative membrane-permeant cation (1, 8). Inhibition of fatty acid-induced uncoupling by atracyloside or ADP (9) has indicated that the adenine nucleotide translocase (ANT) could perhaps serve as anion channel for the dissociated fatty acid (10). ANT has indeed been recently reported to mediate proton transport by free fatty acids in reconstituted ANT-cytochrome c oxidase proteoliposomes (11). In contrast to the well reported uncoupling effect in isolated mitochondria, the uncoupling activity of fatty acids in vivo is still questionable. Fatty acids are well known to stimulate respiration of isolated hepatocytes, perfused liver, and heart. However, it remains disputed whether stimulation of respiration by fatty acids in these systems is accounted for by their availability as substrates for oxidation or may be further ascribed to their intrinsic mitochondrial uncoupling activity. Uncoupling of the perfused liver by fatty acids has been claimed by Soboll and co-workers (12, 13) based on resolving the relaxation kinetics of the increase in oxygen consumption induced by added fatty acids into a rapid “oxidative” component and a slower “uncoupling-like” component accompanied by acidification of mitochondrial matrix. These findings have been further corroborated in perfused hearts where added octanoate was observed to induce an increase in oxygen consumption, while ATP synthesis measured by 31P NMR magnetization transfer technique remained unaffected, thus resulting in a concomitant decline in P/O ratio (14).

These claims were, however, challenged by other observations made both in perfused systems as well as in cultured cells and employing inhibitors of ATP synthesis. Thus, increase in oxygen consumption induced by fatty acids in the perfused liver (15) or in isolated liver cells (16, 17) was reported to be essentially eliminated by added oligomycin or atracyloside, indicating that oxidative phosphorylation remained fully coupled in the presence of added fatty acids. Furthermore, mitochondrial membrane potential measured in situ in isolated hepatocytes (18) or phosphorylation potential measured in situ in the perfused heart (19) were found to be rather increased by added fatty acids, thus refuting a protonophoric effect exerted by fatty acids. Similarly, extra oxygen consumed upon oleate respiration in rat hepatocytes could be essentially accounted for by extra glucose formation (20). Hence, extramitochondrial ATP-consuming reactions stimulated by fatty acids were proposed to account for the increase in oxidation of the added fatty acid substrate in vivo (21). Residual respiration in the presence of oligomycin was ascribed to increase in conductance of the inner mitochondrial membrane (21) due to a non-ohmic proton leak induced by the highly oxidizable fatty acid substrate.

To dissociate between the substrate and modulatory effects of fatty acids on oxidative phosphorylation, the putative uncoupling effect induced by long chain fatty acids was studied here using a nonmetabolizable methyl-substituted α,ω-dicarboxylic acid (MEDICA 16) (HOOC-CH2-C(CH3)2-(CH2)10-C(CH3)2-CH2-COOH) consisting of a long chain fatty acid of 16 carbon atoms in length, carboxy-substituted at the ω-end to eliminate its ω-oxidation and methyl-substituted at the β,β’ positions to eliminate β-oxidation of the fatty acyl analogue (22). MEDICA 16 is reported here to uncouple oxidative phosphorylation in liver cells and in isolated liver and heart mitochondria.
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Rat hepatocytes were prepared as described in Ref. 23 with minor modifications. Viability of freshly isolated cells was evaluated by the exclusion of erythrosin B and amounted to >85%. Isolated cells were kept on ice and used within 4 h. Liver and heart mitochondria were prepared as described previously (24, 25).

Oxygen consumption of freshly isolated hepatocytes or isolated mitochondria was measured using an oxygen electrode in a 3-ml chamber under constant steering. Liver cell suspensions were adjusted to a density of 1×10^6 cells/ml and incubated at 35 °C in the presence of 10 mM Hepes (pH 7.4), 137 mM NaCl, 5.4 mM KCl, 4.2 mM NaHCO_3, 0.5 mM Na_2HPO_4, 10 mM glucose, 10 mM lactate, 1 mM pyruvate, 1 mM MgCl_2, 0.8 mM MgSO_4, 1.25 mM CaCl_2, and MEDICA 16 or palmitate as indicated. Global ATP content was measured after 20 min of incubation at 37 °C in a medium containing 0.188 mM succrose, 50 mM NaCl, 8 mM MgCl_2, 5 mM Na_2HPO_4 (pH 7.4), 2 mM EGTA, 5 mM succinate, 5 μM rotenone, and MEDICA 16 or palmitate as indicated.

Mitochondrial membrane potential of freshly isolated hepatocytes was evaluated by following the intracellular distribution of JC-1 (26) using FACScan flow cytometry (27). For JC-1 staining, cell suspension was adjusted to a density of 1×10^6 cells/ml in a medium containing 10 mM Hepes (pH 7.4), 137 mM NaCl, 5.4 mM KCl, 4.2 mM NaHCO_3, 0.5 mM Na_2HPO_4, 10 mM glucose, 10 mM lactate, 1 mM pyruvate, 1 mM MgCl_2, 0.8 mM MgSO_4, 1.25 mM CaCl_2, and MEDICA 16 or palmitate as indicated together with defatted albumin at a molar ratio of 1/41 or 6/1, respectively. Mitochondrial suspensions were adjusted to 0.2–0.6 mg of protein/ml and incubated at 30 °C in a medium containing 0.188 mM succrose, 50 mM NaCl, 8 mM MgCl_2, 5 mM Na_2HPO_4 (pH 7.4), 2 mM EGTA, 5 mM succinate, 5 μM rotenone, and MEDICA 16 or palmitate as indicated.

Mitochondrial membrane potential (∆Γ) and proton gradient (∆pH) of isolated mitochondria were measured as described previously (24, 28) using 3938 (in the presence of valinomycin) and 1^4Clacetate, respectively. Extramitochondrial spaces were evaluated by following the distribution of H_2O together with [1^4Cl]sucrose. For measuring succinate-generated mitochondrial ∆pH, 2–2.5 mg of mitochondrial protein were incubated at 30 °C in 1 ml of incubation medium containing 250 mM sucrose, 5 mM Hepes (pH 7.2), 1 mM succinate, 5 μM rotenone, 0.6 μg/ml oligomycin, 320 pmol of valinomycin-coupled protein, and MEDICA 16 as indicated. For measuring ATP-generated mitochondrial ∆ψ and ∆pH, 2–2.5 mg of mitochondrial protein were incubated in 1 ml of incubation medium containing 200 mM mannitol, 75 mM sucrose, 2 mM EDTA, 20 mM Hepes (pH 7.2), 3 mM ATP, 5 μM rotenone, 320 pmol of valinomycin/mg of protein, and MEDICA 16 as indicated. Proton motive force (pHm) was calculated as described in Ref. 28.

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Mitochondrial membrane potential (∆ψ) and proton gradient (∆pH) of isolated mitochondria were measured as described previously (24, 28) using 3938 (in the presence of valinomycin) and 1^4Clacetate, respectively. Extra- and intramitochondrial spaces were evaluated by following the distribution of H_2O together with [1^4Cl]sucrose. For measuring succinate-generated mitochondrial ∆ψ and ∆pH, 2–2.5 mg of mitochondrial protein were incubated at 30 °C in 1 ml of incubation medium containing 250 mM sucrose, 5 mM Hepes (pH 7.2), 1 mM succinate, 5 μM rotenone, 0.6 μg/ml oligomycin, 320 pmol of valinomycin-coupled protein, and MEDICA 16 as indicated. For measuring ATP-generated mitochondrial ∆ψ and ∆pH, 2–2.5 mg of mitochondrial protein were incubated in 1 ml of incubation medium containing 200 mM mannitol, 75 mM sucrose, 2 mM EDTA, 20 mM Hepes (pH 7.2), 3 mM ATP, 5 μM rotenone, 320 pmol of valinomycin/mg of protein, and MEDICA 16 as indicated. Proton motive force (pHm) was calculated as described in Ref. 28.

Statistical analysis was performed by one-way repeated measures analysis of variance, followed by multiple comparison analysis of means. When a significant value (p < 0.05) was obtained, differences between individual means were analyzed by Bonferroni test (45).

RESULTS

Respiration rate of freshly isolated hepatocytes was increased by MEDICA 16 approaching 1.6-fold activation of basal respiration at saturating MEDICA 16 concentrations (Fig. 1). Respiration induced by MEDICA 16 was as pronounced as that induced by palmitate (Fig. 1). MEDICA 16 concentrations required for half-maximal activation of basal respiration amounted to 0.06 mM as compared with 0.34 mM of palmitate, reflecting presumably the metabolic stability of MEDICA 16. In line with previous reports (15–17), palmitate respiration was effective (but not completely inhibited) by oligomycin, whereas activation of respiration by MEDICA 16 remained unaffected by oligomycin (not shown), indicating that activation by palmitate was partly due to coupled respiration of an oxidizable substrate.

Mitochondrial membrane potential of isolated liver cells incubated in the presence or absence of MEDICA 16 was evaluated by following the intracellular distribution of the lipophilic cation JC-1 (26). While the cytosolic monomeric dye emits at 530 nm (when excited at 488 nm), the fluorescence emission of the intramitochondrial aggregated dye shifts to 590 nm. The 530/590 fluorescence ratio thus reflects the cytosolic/mitochondrial distribution of the dye and the prevailing mitochondrial inner membrane potential of respective cells (27, 29). As shown in Fig. 2, incubating JC-1-stained hepatocytes in the presence of added valinomycin or MEDICA 16 resulted in an overall 590 to 530 nm shift in the fluorescence emission of the cells, thus reflecting a decrease in intramitochondrial JC-1 aggregates as a result of mitochondrial inner membrane depolarization. Decrease in mitochondrial membrane potential as a function of added MEDICA 16 was concentration-dependent and saturable having an EC_50 of 0.08 mM (Fig. 3). In contrast to MEDICA 16 and in line with previous reports (21), no substantial change in mitochondrial membrane potential was induced by added palmitate under conditions where respiration was significantly activated.

The apparent uncoupling effect induced by MEDICA 16 in liver cells was further analyzed in isolated liver mitochondria. State 4 respiration of succinate respiring mitochondria was similarly activated by MEDICA 16 or palmitate (Fig. 4). Activation of respiration by MEDICA 16 (or palmitate) was concentration-dependent, having an EC_50 of 14.0 nmol/mg of protein and reaching 3-fold activation at saturation.

The effect of MEDICA 16 on pmf, membrane potential, and proton gradient in isolated mitochondria was evaluated under conditions where mitochondrial pmf was either generated at the expense of succinate respiration or of ATP hydrolysis (Fig. 5). As shown in Fig. 5A, adding MEDICA 16 to mitochondria respiring on succinate resulted in a concentration-dependent saturable decrease of all three parameters. The maximal effect exerted by MEDICA 16 at saturation amounted to 87% decrease in proton gradient, membrane potential, or pmf. The EC_50 for the MEDICA 16 uncoupling effect amounted to 11.5 mM/mg of protein, being similar to the EC_50 value for activation of mitochondrial respiration of succinate by MEDICA 16 (Fig. 4). The three mitochondrial parameters were similarly affected by MEDICA 16 under conditions where mitochondrial pmf was maintained by ATP hydrolysis (Fig. 5B). Here again,
the uncoupling effect of MEDICA 16 was saturable, reaching 30% decrease in pmf at saturating MEDICA 16 and having an EC$_{50}$ of 9.6 nmol/mg of protein. However, in contrast to succinate generated pmf, membrane potential was the main pmf component affected by MEDICA 16 under conditions of ATP-generated mitochondrial pmf.

Mitochondrial uncoupling by MEDICA 16 was essentially eliminated upon washing out MEDICA 16 (Fig. 6). Similarly, proton gradient, mitochondrial membrane potential, and pmf of liver mitochondria isolated from rats treated with MEDICA 16 in vivo and studied in vitro in the absence of MEDICA 16 added to the incubation medium were all found to remain unaffected by the in vivo treatment with MEDICA 16 (not shown). Hence, the MEDICA 16 effect requires its direct interaction with mitochondria and may be washed out in the course of preparing liver mitochondria from MEDICA 16-treated animals.

The putative role played by ANT in the uncoupling effect of MEDICA 16 was verified by studying the effect exerted by MEDICA 16 and palmitate. In light of the enrichment of ANT in heart mitochondria as compared with liver mitochondria (30), the atracylate effect was studied in isolated heart mitochondria. As shown in Fig. 7 and in line with previous reports (9), added atracylate partially suppressed the increase in respiration induced by palmitate. MEDICA 16 uncoupling was similarly and significantly suppressed by atracylate, indicating that uncoupling by MEDICA 16 was partially mediated by ANT.
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FIG. 5. Mitochondrial depolarization by MEDICA 16. Mitochondrial $\Delta pH$ (A), $\Delta \Psi$ (B), and $\Delta \rho$ (C) were measured in the presence of added MEDICA 16 as indicated under conditions of succinate-generated pmf (A) or ATP-generated pmf (B) as described under “Materials and Methods.” Values are expressed as percent of the basal respective value (100%) measured in the absence of added effector. Basal values amounted to $69.3 \pm 5.6$ mV, $171.2 \pm 3.1$ mV, and $242.1 \pm 5.1$ mV for succinate-generated $\Delta pH$, $\Delta \Psi$, and $\Delta \rho$, respectively, and to $73.3 \pm 4.1$ mV, $123.7 \pm 9.1$ mV, and $197.0 \pm 12.9$ mV for ATP-generated $\Delta pH$, $\Delta \Psi$, and $\Delta \rho$, respectively. Each value is the mean $\pm$ S.E. of eight (A) and three (B) independent experiments.

FIG. 6. Reversibility of MEDICA 16-induced mitochondrial depolarization. Mitochondrial $\Delta pH$, $\Delta \Psi$, and $\Delta \rho$ were measured as described in the legend Fig. 5A in the absence (filled bars) or presence (empty bars) of 20 nmol of MEDICA 16/mg of protein (A). Similarly incubated mitochondria were subjected to three washings with incubation medium devoid of MEDICA 16 followed by further incubation in the absence of added MEDICA 16 for measuring again $\Delta pH$, $\Delta \Psi$, and $\Delta \rho$ (B). Values are expressed as percent of the basal respective value measured in the absence of added MEDICA 16. Basal values amounted to $72.0 \pm 4.0$ mV, $177.3 \pm 3.8$ mV, and $249.3 \pm 7.7$ mV for $\Delta pH$, $\Delta \Psi$, and $\Delta \rho$, respectively. Each value is the mean $\pm$ S.E. of three independent experiments. *: significant as compared with the respective value in the absence of added MEDICA 16.

DISCUSSION

Despite the established uncoupling effect of long chain fatty acids in isolated mitochondria (1), their uncoupling activity in vitro is still disputed. Thus, stimulation of respiration by fatty acids in cell cultures and perfusion systems was ascribed to their availability as substrates for mitochondrial oxidation (15–17), to stimulating extramitochondrial ATP-consuming reactions (20, 21), to their putative protonophoric activity (12–14), or to a non-Ohmic proton leak induced by an highly oxidizable substrate (21). Indeed, evaluating the extent of uncoupling during the course of fatty acid respiration in perfusion systems or cell cultures maintained at or near state 3 may be flawed by (a) the opposing effects on mitochondrial membrane potential of uncoupling (resulting in decreased pmf) and respiration of a highly oxidizable substrate (resulting in increased pmf), (b) masking the putative intrinsic uncoupling effect of fatty acids by possible extramitochondrial ATP-consuming processes or by a non-Ohmic proton leak induced by fatty acids or their oxidation products (18), (c) possible interference by oligomycin or atracyloside with mitochondrial components claimed to be involved in fatty acid-induced uncoupling in isolated mitochondria (e.g. ANT, ATPase, proton pumps) or with nonmitochondrial components (e.g. Na$^+$, K$^+$-ATPase (31), SOC-mediated calcium influx (32)), which could affect mitochondrial oxidative phosphorylation. To dissociate between substrate and modulatory effects of fatty acids on oxidative phosphorylation, the putative uncoupling effect of fatty acids was studied here using a fatty acyl analogue which does not serve as substrate for $\beta$- or $\omega$-oxidation, thus making it possible to analyze the intrinsic uncoupling cellular activity of fatty acids in the absence of inhibitors of oxidative phosphorylation and under conditions where uncoupling is not interfered by respiration of the putative uncoupler.

MEDICA 16 was found here to induce in liver cells a saturable decrease in mitochondrial pmf with a concomitant oligomycin-insensitive increase in cellular respiration. The strict correlation observed between increased respiration and decreased pmf seems to be consistent with uncoupling rather than “decoupling” (3, 4) of mitochondrial oxidative phosphorylation by MEDICA 16. This uncoupling activity of MEDICA 16 may therefore implicate an intrinsic uncoupling activity of long chain fatty acids independent of their availability as substrates for oxidation. However, and in contrast to MEDICA 16, uncoupling by fatty acids still allows for substrate availability, thus avoiding substrate limitation under conditions of enhanced uncoupled respiration. Hence, the apparent maintenance of mitochondrial membrane potential in the presence of added palmitate (Fig. 3) may reflect the resultant of decreased pmf due to uncoupling by the long chain fatty acid compromised by increased pmf due to respiration of a highly oxidizable substrate. This conclusion is in line with the limited sensitivity of palmitate respiration to oligomycin in liver cells (Fig. 1) and conforms with studies previously reported by Soboll and co-workers (12, 13) in the perfused liver.

Uncoupling of liver cells or isolated mitochondria by MEDICA 16 is saturable, thus implicating its interaction with a specific mitochondrial component involved in modulating mitochondrial membrane conductance. As the MEDICA 16 effect in mitochondria is observed at $\mu M$ concentrations of the compound and pH range of 7.4, the uncoupling activity of MEDICA 16 may not be accounted for by its transport through the dicarboxylate electroneutral transporter (33), which requires millimolar concentrations of the transported dicarboxylate and
is inhibited at neutral pH (33). Furthermore, partially suppressing the activity of MEDICA 16 by atracyloside (Fig. 7) may specifically implicate ANT in the uncoupling activity of MEDICA 16, in line with recent studies reporting proton transport mediated by free fatty acids in reconstituted ANT-cytochrome c oxidase proteoliposomes (11). In light of the homology between ANT and the fatty acid-responsive UCP1 protein (thermogenin) of brown adipose tissue (34), saturation by MEDICA 16 may be accounted for by ANT-catalyzed flip-flop of the MEDICA 16 anion followed by diffusion/flip-flop of the protonated acid (10, 11, 35). It is noteworthy that the four ββ′-methyl substitutions are obligatory for the cycling protonophoric activity of MEDICA 16, as nonsubstituted long chain dicarboxylic acids have been reported to be inactive as cycling protonophores (36, 37). ANT-mediated uncoupling by fatty acids or their analogues could perhaps be further complemented by similar ANT-related, fatty acid-responsive mitochondrial proteins controlling the conductance of the mitochondrial inner membrane in liver or muscle. The recently cloned UCP2 and UCP3 genes are of special interest in this context in light of their ubiquitous abundance (38, 39).

Substantial uncoupling by long chain fatty acids requires fatty acid concentrations in the range of 0.5–2 mM (Fig. 3). These concentrations are higher than those prevailing under normal physiological conditions but may be observed during starvation, diabetes, or thermogenesis. Uncoupling of liver cells by long chain fatty acids under ketogenic conditions may allow for liver production of exported ketone bodies at rates that are not limited by liver ATP consumption and requirements. Similarly, and in analogy with thermogenin-mediated uncoupling in brown adipose tissue, uncoupling of muscle and/or liver by long chain fatty acids may allow for heat production not limited by ATP requirements of the concerned organs. Loose mitochondrial coupling by long chain fatty acids within the normal concentration range could also be of importance in modulating the efficiency of body weight gain in mammals lacking brown adipose tissue.

Mitochondria uncoupling by nonmetabolizable fatty acyl analogues like MEDICA 16 may be pharmacologically exploited for controlling human obesity and obesity-related pathologies, e.g., non-insulin-dependent diabetes mellitus, dyslipoproteinemia, and others constituting the Metabolic Syndrome (40). Mitochondria uncoupling by MEDICA 16 may indeed account for the pronounced decrease in liver phosphate and redox potential (41) as well as the increase in liver2 and total body oxygen consumption (42) in MEDICA 16-treated animals. Direct uncoupling by MEDICA 16 may further complement the calorigenic activity of MEDICA 16 previously ascribed to transcriptional activation of liver thyroid hormone-dependent genes (43, 44). The contribution made by the nuclear and mitochondrial activities of MEDICA 16 to the overall calorigenic activity of the drug in vivo still remains to be investigated.

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