J Bioenerg Biomembr (2007) 39:109–115
DOI 10.1007/s10863-006-9067-5

ORIGINAL PAPER

Fatty acid efficiency profile in uncoupling of *Acanthamoeba castellanii* mitochondria

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Received: 13 October 2006 / Accepted: 13 November 2006 / Published online: 2 March 2007

Abstract A profile of free fatty acid (FFA) specificity in *Acanthamoeba castellanii* mitochondrial uncoupling is described. The FFA uncoupling specificity was observed as different abilities to stimulate resting respiration, to decrease resting membrane potential, and to decrease oxidative phosphorylation efficiency. Tested unsaturated FFA (C18–20) were more effective as uncouplers and protonophores when compared to tested saturated FFA (C8–18), with palmitic acid (C16:0) as the most active. As FFA efficiency in mitochondrial uncoupling is related to physiological changes of fatty acid composition (and thereby FFA availability) during growth of amoeba cells, it could be a way to regulate the activity of an uncoupling protein and thereby the efficiency of oxidative phosphorylation during a cell life of this unicellular organism.

Keywords Fatty acids · Uncoupling · Uncoupling protein · Mitochondria · *Acanthamoeba castellanii*

Abbreviations FFA: free fatty acids · UCP: uncoupling protein · AcUCP: uncoupling protein of *Acanthamoeba castellanii* · $\Delta\Psi$: mitochondrial membrane potential · $\Delta\mu H^+$: proton electrochemical gradient · FCCP: *p*-trifluoromethoxyphenylhydrazone

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Introduction

Most mitochondria possess free energy-dissipating systems that decrease the yield of oxidative phosphorylation. In in-vitro situations, uncoupling proteins (UCPs) dissipate the proton electrochemical gradient ($\Delta\mu H^+$) built up by the mitochondrial respiratory chain in animal, plant, some fungal and protist mitochondria (Ricquier and Bouillaud, 2000; Sluse and Jarmuszkiewicz, 2002; Jezek, 2002; Krauss et al., 2005; Nicholls, 2006; Sluse et al., 2006; Vercesi et al., 2006). Uncoupling proteins, forming a subfamily within the mitochondrial anion carrier protein family, have been proposed to fulfil a physiological function through a $\Delta\mu H^+$ dissipation by a free fatty acid (FFA)-activated purine nucleotide-inhibited H^+ cycling process driven by membrane potential ($\Delta\Psi$) and pH (both constituting $\Delta\mu H^+$) (Garlid et al., 1996). As a consequence, uncoupling respiration from phosphorylation leads to a decrease in the yield of oxidative phosphorylation, i.e. ATP production per oxygen consumed (Jarmuszkiewicz et al., 2000a). Indications that UCP is present in unicellulars are based on functional studies and cross-reactivity of around 32 kDa mitochondrial protein with antibodies developed against plant and mammalian UCP (Jarmuszkiewicz et al., 1999, 2000b; Uyemura et al., 2000; Tudella et al., 2003). In mitochondria of the amoeboid protozoan *Acanthamoeba castellanii*, the action of UCP (AcUCP) has been shown to mediate FFA-activated, purine nucleotide-inhibited H^+ re-uptake driven by $\Delta\mu H^+$ that in state 3 respiration can divert energy from oxidative phosphorylation in a fatty acid-dependent way (Jarmuszkiewicz et al., 1999; 2004a). The efficiency of inhibition by purine nucleotides depends on the redox state of endogenous ubiquinone (Jarmuszkiewicz et al., 2005). It has been shown that UCP could be a cold response protein in unicellulars as cold treatment of amoeba culture increases AcUCP activity and

protein level (Jarmuszkiewicz et al., 2004b). Moreover, we have shown that UCP may play a role in decreasing reactive oxygen species production in unicellular organisms, such as amoeba *A. castellanii* (Czarna and Jarmuszkiewicz, 2005).

Understanding the modulation of the proton gradient and respiration uncoupling by UCP is complicated since other members of the mitochondrial carrier family may mediate, at least in animal and plant mitochondria, FFA-induced mitochondrial uncoupling, mainly in a high energy state of mitochondria (at a high $\Delta\Psi$). The FFA-dependent H^+ recycling is a side function of these carriers and can be inhibited by their substrates or specific inhibitors. Thus, the FFA-induced uncoupling could be mediated, at least in part, by the ATP/ADP antiporter (Andreyev et al., 1989), the aspartate/glutamate antiporter (Samartsev et al., 1997), the dicarboxylate carrier (Wieckowski and Wojtczak, 1997), and phosphate carrier (Zackova et al., 2000), likely at high $\Delta\Psi$ but unlikely during phosphorylating respiration, when they are mainly employed in the import of ADP, glutamate, dicarboxylates, and phosphate, respectively. In *A. castellanii* mitochondria, the contribution of these carriers in FFA-induced mitochondrial uncoupling seems to be minor when compared to AcUCP (Jarmuszkiewicz et al., 1999, 2004b).

So far, only the effect of linoleic acid on *A. castellanii* mitochondrial uncoupling has been studied. The aim of the present work was to describe the effect of different non-esterified ("free") fatty acids on mitochondrial respiration and oxidative phosphorylation in unicellular organism, such as amoeba *A. castellanii*. This is a first attempt to compare the FFA-induced *A. castellanii* mitochondrial uncoupling with that observed in plant and mammalian mitochondria. A fatty acid-efficiency profile in uncoupling of *A. castellanii* mitochondria seems to be related to physiological changes of fatty acid composition (and thereby FFA availability) during growth of amoeba cells. The applied experimental conditions allow the studied FFA-induced mitochondrial uncoupling to be attributed to the operation of AcUCP.

Materials and methods

Cell culture and mitochondrial isolation

Soil amoeba *Acanthamoeba castellanii*, strain Neff, was cultured as described earlier (Jarmuszkiewicz et al., 1997). Trophozoites of amoeba were collected 60–64 h following inoculation at the stationary phase (at a density of about 8–10×10^6 cells/ml). Mitochondria were isolated and purified on a self-generating Percoll gradient (31%) (Jarmuszkiewicz et al., 1997). The presence of 0.4% bovine serum albumin (BSA) in isolation media allowed FFA to be chelated from the mitochondrial suspension and mitochondria fully depleted of FFA to be obtained. For each mitochondrial preparation, measuring the effect of BSA on the linoleic acid-induced state 4 respiration tested full depletion of FFA (Jarmuszkiewicz et al., 1998). Namely, similar oxygen uptake before the addition of linoleic acid and after the subsequent addition of BSA confirmed the absence of endogenous FFA in the mitochondrial suspension. This test was performed at the beginning and at the end of experiments with given mitochondrial preparation. Mitochondrial protein concentration was determined by the biuret method.

Oxygen uptake and membrane potential

Oxygen uptake was measured polarographically using a Rank Bros. (Cambridge UK) oxygen electrode in 2.8 ml of standard incubation medium (25°C) containing: 120 mM KCl, 20 mM Tris-HCl pH 7.4, 3 mM KH_2PO_4, and 2 mM $MgCl_2$, with 1–1.5 mg of mitochondrial protein. Membrane potential ($\Delta\Psi$) of mitochondria was measured simultaneously with oxygen uptake using a tetraphenylphosphonium-specific electrode (Kamo et al., 1979). For calculation of the $\Delta\Psi$ value the matrix volume of amoeba mitochondria was assumed as 2.0 μl mg^{-1} protein.

All measurements were made in the presence of 10 mM succinate, 3 mM glutamate, 1.5 mM benzohydroxamate (to inhibit an alternative oxidase), 170 μM ATP (to activate succinate dehydrogenase), and 4 μM rotenone. The ADP/O ratio was determined by an ADP pulse method with 400 nmoles of ADP. The total amount of oxygen consumed during state 3 (phosphorylating) respiration was used for calculation of the ratio. Measurements of $\Delta\Psi$ allowed fine control of the duration of state 3 respiration. State 4 (resting) respiration measurements were made in the presence of 1.5 μM carboxyatractyloside, 0.5 μg/ml oligomycin, and 10 μM glybenclamid acid. Values of O uptake are in nmol O/min per mg of protein.

All applied FFA were purchased from Sigma. They were solved in methanol. Control traces with a given amount of solvent were performed and taken into account in calculations. Throughout the text, we use starting concentrations of added FFA expressed per mg of mitochondrial protein. Free concentrations of applied FFA depend on their solubility and partitioning coefficient between the aqueous and membranous phases.

Results

To study the effect of FFA and their derivatives on AcUCP activation, all measurements were made in the presence of succinate and glutamate in order to exclude the potential dicarboxylate and glutamate/aspartate carrier-mediated FFA-linked mitochondrial uncoupling. State 4 measurements were made in the presence of carboxyatractyloside

Table 1 Stimulation of respiration and decrease in membrane potential in resting state

FFA (17 nmol/mg prot.)	Membrane potential Decrease in $\Delta\Psi 4$ (mV)	Respiration		
		% of state 4	% of maximal FFA-induced stimulation)	$S_{0.5}$ (nmol/mg prot.)
Linoleic, C18:2, *cis*-9,12	23 ± 0.5	230 ± 16	100 ± 7	8.7 ± 0.5
Oleic, C18:1, *cis*-9	19 ± 0.6	205 ± 18	81 ± 7	9.5 ± 0.5
Linolenic, C18:3, *cis*-9,12,15	17.5 ± 0.4	202 ± 16	78 ± 6	10.8 ± 0.7
Retinoic, C20:5, *all-trans*	16.5 ± 0.9	193 ± 13	72 ± 5	12.2 ± 0.5
Elaidic, C18:1, *trans*-9	14 ± 0.5	176 ± 14	61 ± 5	14.4 ± 0.7
Eicosadienoic, C20:2, *cis*-11,14	13 ± 0.3	173 ± 18	57 ± 6	16.6 ± 0.8
γ-Linolenic, C18:3, *cis*-6,9,12	10.6 ± 1	162 ± 14	47 ± 4	19.0 ± 0.8
Palmitic, C16:0	8.8 ± 0.6	148 ± 16	37 ± 4	24.3 ± 0.9
Myristic, C14:0	7.0 ± 0.6	143 ± 13	33 ± 3	28.5 ± 1.1
Lauric, C12:0	5.7 ± 0.6	130 ± 17	23 ± 3	51.8 ± 3.0
Stearic, C18:0	1.5 ± 0.5	110 ± 10	7.8 ± 1	193 ± 11
Capric, C10:0	1.0 ± 0.8	107 ± 6	4.9 ± 0.5	234 ± 18
Caprylic, C8:0	0.4 ± 0.3	103 ± 3	2.2 ± 1.2	um

Note. Fully uncoupled respiration (with 1 μM carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone, FCCP) amounted to 145 ± 11 nmol O/min per mg of mitochondrial protein ($n = 11$), while maximally FFA-uncoupled respiration (i.e. linoleic acid-uncoupled) was equal to 136 ± 13 nmol O/min per mg of mitochondrial protein ($n = 11$). State 4 respiration (no FFA) was equal to 60 ± 3 nmol O/min per mg of mitochondrial protein ($n = 11$). The presented percentage of maximal FFA-induced stimulation refers to FFA-stimulated respiration (i.e. the difference between respiration in the presence of FFA minus unstimulated resting respiration) measured in the presence of 17 nmol/ mg prot. of FFA. $S_{0.5}$ (concentration that produces 50% stimulation) was estimated from concentration-response curves performed for each FFA. Increasing concentrations of FFA were obtained by successive additions when steady-state respiration rate was achieved. Several oxygen traces were needed to cover the full investigated range of concentrations of given FFA. Resting state 4 membrane potential ($\Delta\Psi 4$) was 185 ± 5 mV ($n = 11$). Decrease in $\Delta\Psi 4$ by 17 nmol/mg prot. FFA is shown. Values represent the mean (±SD) for at least 3 experiments (different mitochondrial preparation). The number of carbon atoms and the number and localization and conformation of double bonds are shown. Unmeasurable: um.

(an inhibitor of ATP/ADP antiporter), and in the presence of glybenclamid acid (an inhibitor of the ATP-inhibited potassium channel). The above conditions secure the study of FFA-induced mitochondrial uncoupling mostly due to the operation of AcUCP.

In the study we tested the effect of 13 non-esterified FFA that differ in carbon chain length (8–20), number (0–5) and/or localization and/or conformation of double bounds (see Table 1). The choice of the employed FFA was made mainly on the basis of the fatty acid composition of *A. castellanii* cells and their enriched mitochondrial membrane fraction (Avery et al., 1994a). The effect of various exogenous FFA on mitochondrial respiration was measured in amoeba mitochondria depleted of endogenous FFA (see Material and Methods).

Effect of different free fatty acids on resting respiration

We carried out a study of the relative ability of 13 natural FFA to uncouple respiration and to decrease $\Delta\Psi$ under resting conditions in isolated mitochondria of *A. castellanii*. A decrease of mitochondrial $\Delta\Psi$ by FFA can be considered as the most sensitive indicator of the protonophoric effect. On the other hand, an increase in the resting respiration is another indication of increased proton leak. It turned out that in *A. castellanii* mitochondria, the potency of tested FFA (at least up to a concentration of 17 nmol/mg prot. that corresponds to 8.5 μM) to uncouple respiration and to decrease $\Delta\Psi$ was parallel (Table 1, Fig. 1). A higher protonophoric and uncoupling effect was observed with unsaturated than with saturated FFA (Table 1). The results given in Table 1 compare the effect of various C18 and C20 unsaturated FFA. Among unsaturated FFA, the highest effect on $\Delta\Psi$ and respiration (with the lowest $S_{0.5}$ value equal to 8.7 ± 0.5 nmol/mg prot.) was revealed by linoleic acid (C18:2), the lowest – by γ-linolenic acid (C18:3) with $S_{0.5}$ equal to 19 ± 0.8 nmol/mg protein. All tested unsaturated FFA (linoleic, oleic, linolenic, retinoic, elaidic, eicosadienoic, and γ-linolenic, in the order of efficiency) were better uncouplers and protonophores than tested saturated acids. One possible factor that could influence the specificity of uncoupling by FFA, i.e. the degree of unsaturation, could be rejected. Double (linoleic acid) and triple (linolenic acid) unsaturated FFA had about the same stimulatory effectivity as a single unsaturated acid (oleic acid). With saturated FFA under investigation, the potency to diminish $\Delta\Psi$ and to stimulate respiration decreased with its decreasing carbon chain length, so that the palmitic (C16:0) and myristic (C14:0) acids were quite active (with $S_{0.5}$ around 24–29 nmol/mg prot.) and the capric (10:0) and caprylic (8:0) acids were practically inactive. The exception was stearic acid (18:0) which was slightly active.

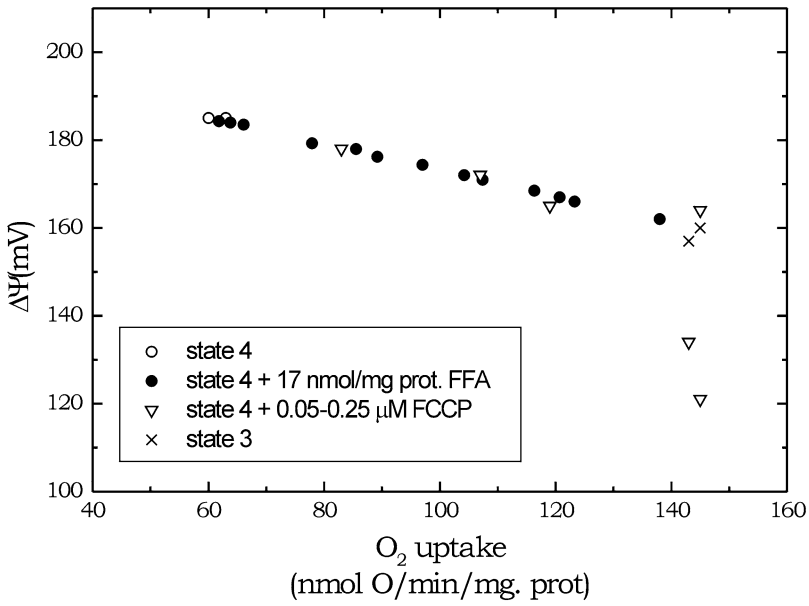

Fig. 1 The effect of FFA (17 nmol/mg prot.) on the relation between $\Delta\Psi$ and mitochondrial respiration. State 4 (+1.5 μM carboxyactractyloside, 0.5 μg/ml oligomycin, and 10 μM glybenclamid acid) was measured in the presence of 0.05–0.25 μM FCCP or studied FFA (17 nmol/mg prot.). For attribution of points obtained in the presence FFA (•) to a given FFA see Table 1 (state 4 $\Delta\Psi$ values) and Table 2 (state 4 oxygen uptake values). State 3 was measured in the absence of FFA with 400 nmoles of ADP

Thus, with saturated FFA, the maximum uncoupling effect was observed with C16:0 palmitic acid. A low apparent *Km* value ($S_{0.5}$) (8.7–51.8 nmol/mg prot. that corresponds to 4.3–25.9 μM) for most of the tested FFA (with the exception of stearic, capric and caprylic acids) indicates a high specificity of AcUCP for FFA. These quantitative functional measurements are in accordance with the proposal of a FFA cycling-linked H^+ reuptake catalyzed by AcUCP in *A. castellanii* mitochondria (Jarmuszkiewicz et al., 1999, 2004a,b, 2005).

If FFA-induced respiration is only due to a proton recycling by AcUCP, it must correspond to a pure protonophoric effect of FFA, indistinguishable from the effect of other well known protonophores like carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (Jarmuszkiewicz et al., 1999). Figure 1 reports the relation between couples of $\Delta\Psi$ and respiration rate measurements under four conditions: (1) in state 4 in the absence of protonophore; (2) in state 4 with 17 nmol/mg prot. of given tested FFA; (3) in state 4 with increasing concentrations of FCCP; and (4) in state 3 (phosphorylation state). A set of conditions constituted a single force-flow relationship, indicating that tested natural FFA did not react with the respiratory electron transport chain in amoeba mitochondria and had a pure protonophoric effect at least at employed concentration. Therefore, 17 nmol/mg prot. FFA could be safely used to study their specific effect in the activation of AcUCP in *A. castellanii* mitochondria.

Under the employed experimental conditions, i.e., in the presence of carboxyatractyloside, glybenclamid acid, the high phosphate, glutamate and dicarboxylate concentrations, and in the presence of a low FFA concentration (at 17 nmol/mg prot) that guarantee action mostly through AcUCP, the specificity of natural FFA to uncouple *A. castellanii* mitochondrial respiration, was carried out. The results show that unsaturated (C18–20) FFA, with leading linoleic acid, are the most active as uncouplers and protonophores.

Effect of different free fatty acids on phosphorylating respiration

In *A. castellanii* mitochondria, AcUCP as an energy-dissipating system leads to a decrease in oxidative phosphorylation efficiency (Jarmuszkiewicz et al., 1999, 2004a, 2005). In the present work, the ADP/O ratio, the best parameter for estimating oxidative phosphorylation efficiency was measured in the absence or presence of given tested FFA at a concentration of 17 nmol/mg prot. (Fig. 2, Table 2). Under control conditions, the ADP/O ratio measured with succinate (+glutamate) plus rotenone (1.41 ± 0.02, S.D., $n = 3$) was the same as that measured with the addition of 0.2% BSA (not shown), thereby in accordance with previous results observed in *A. castellanii* mitochondria (Jarmuszkiewicz et al., 1999, 2004a,b, 2005). When in the absence of BSA, 17 nmol/mg prot. FFA was added to mitochondria, that is when FFA-stimulated uncoupling (likely through AcUCP) was induced, the ADP/O ratio decreased dependently on the efficiency of given FFA to uncouple resting respiration (Fig. 2, Table 1). Figure 2 shows the effect of linoleic acid (the best unsaturated FFA uncoupler) and palmitic acid (the best saturated FFA uncoupler) on oxygen consumption and $\Delta\Psi$ during phosphorylating respiration. Both FFA caused uncoupling of oxidative phosporylation (much stronger in the case of linoleic acid) revealed by prolonged state 3 respiration, decreased respiratory control ratio (state 3 versus state 4) and ADP/O ratio. In both cases, state 3 respiratory rate did not increase in the

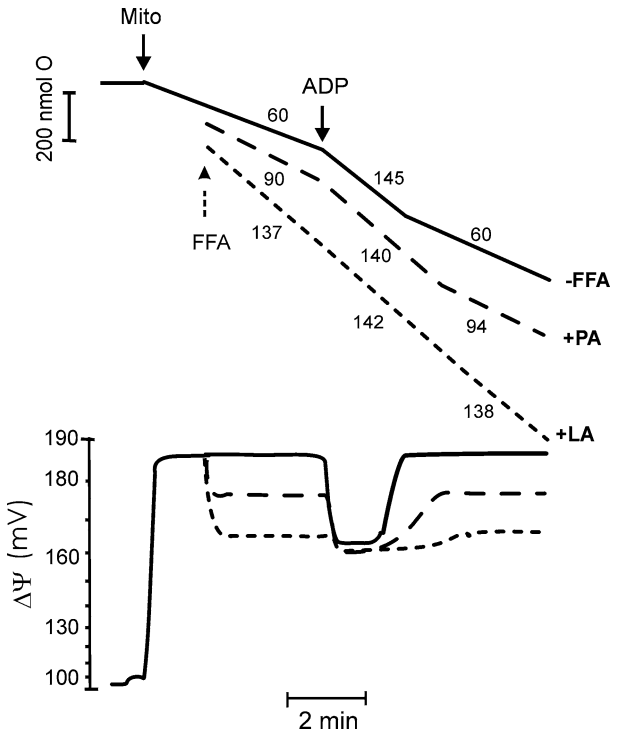

Fig. 2 The effect on coupling parameters. Measurements of oxygen uptake and $\Delta\Psi$ in the absence (*solid line*) or in the presence of linoleic acid (+LA, *dotted line*) or palmitic acid (+PA, *dashed line*) (as examples) are shown. Mitochondria (Mito) were incubated as described under Material and Methods. Additions (where indicated): 17 nmol/mg prot. of FFA, 400 nmoles of ADP. Measurements of $\Delta\Psi$ allowed a fine control of the duration of state 3 respiration and the ADP/O calculations. Numbers on the traces refer to oxygen consumption rates in nmol O/min per mg of mitochondrial protein

presence of FFA, thus indicating saturation of the electron transport respiratory chain in *A. castellanii* mitochondria. At the applied FFA concentration (17 nmol/mg prot.) state 3 $\Delta\Psi$ was also not significantly modified, even with linoleic acid, the most efficient uncoupler and protonophor. Table 2 illustrates the effects of tested unsaturated and saturated FFA on coupling parameters in isolated *A. castellanii* mitochondria at a concentration of 17 nmol/mg protein. As expected, decrease in oxidative phosphorylation efficiency is related to the protonophoric and uncoupling efficiency of FFA, i.e. on efficiency in the stimulation of respiration and decrease in $\Delta\Psi$ (Tables 1 and 2). Thus, the order of FFA efficiency obtained for resting respiration (Table 1) is the same as that obtained for phosphorylating respiration (Table 2). Similarly, the strongest uncoupling of oxidative phosphorylation was observed for unsaturated FFA (C18–20), with linoleic acid as the most efficient. Among saturated FFA, palmitic acid (C16:0) had the biggest uncoupling effect (at chosen concentration), while the stearic (C18:0), capric (C10:0) and caprylic (C8:0) acids had almost no effect.

The results presented in this work indicate that a low FFA concentration (17 nmol/mg prot)-induced uncoupling (likely through AcUCP) can divert energy from ATP synthesis in state 3 respiration, with a decrease in the ADP/O ratio. This energy-dissipating ability depends on the uncoupling and protonophoric efficiency of FFA, therefore on their character (carbon chain length, saturation).

Discussion

The results presented in this study show that in *A. castellanii* mitochondria the most active as uncouplers and protonophores are unsaturated (C18–20) FFA, with linoleic acid (C18:2) as the most effective. Among saturated FFA which all are weaker uncouplers (therefore AcUCP substrates) when compared to unsaturated FFA, the potency to diminish $\Delta\Psi$ and to stimulate respiration decreased with a decreasing carbon chain length (except for stearic acid C18:0, which was slightly active). The palmitic (C16:0) and myristic (C14:0) acids were quite effective as uncouplers, while the capric (10:0) and caprylic (8:0) acids were almost inactive. A very similar pattern of FFA specificity to

Table 2 The effect of FFA (17 nmol/mg prot., 8.5 μM) on coupling parameters: the ADP/O ratio and respiratory control ratio (RCR)

FFA (17 nmol/mg prot.)	State 4	RCR	ADP/O
no FFA	60 ± 4	2.42	1.410 ± 0.019
Linoleic, C18:2, *cis*-9,12	138 ± 11	1.05	0.612 ± 0.015
Oleic, C18:1, *cis*-9	123 ± 8	1.18	0.688 ± 0.013
Linolenic, C18:3, *cis*-9,12,15	121 ± 9	1.20	0.700 ± 0.017
Retinoic, C20:5, *all-trans*	116 ± 10	1.25	0.728 ± 0.012
Elaidic, C18:1, *trans*-9	107 ± 8	1.36	0.792 ± 0.017
Eicosadienoic, C20:2, *cis*-11,14	104 ± 9	1.39	0.810 ± 0.018
γ-Lomicron, C 18:3, *cis*-6,9,12	97 ± 8	1.50	0.874 ± 0.018
Palmitic, C16:0	89 ± 8	1.63	0.950 ± 0.019
Myristic, C14:0	86 ± 8	1.69	0.985 ± 0.011
Lauric, C12:0	78 ± 6	1.86	1.080 ± 0.021
Stearic, C18:0	66 ± 4	2.20	1.292 ± 0.016
Capric, C10:0	64 ± 4	2.27	1.323 ± 0.018
Caprylic, C8:0	62 ± 5	2.34	1.363 ± 0.026

Note. Assay conditions as in Fig. 1. Values represent the mean (±SD) for 3 experiments (different mitochondrial preparation).

uncouple mitochondrial respiration (to decrease $\Delta\Psi$) was found in plant (durum wheat) mitochondria (Pastore et al., 2000). In these mitochondria, in the presence of the ATP/ADP antiporter inhibitor, unsaturated FFA (with linoleic as the most active) were more effective in decreasing resting $\Delta\Psi$ than saturated FFA (with more active palmitic acid C16:0 and lauric acid C12:0 when compared to stearic acid (C18:0). This FFA-induced purine nucleotide-inhibited mitochondrial uncoupling was attributed to plant UCP activity. A different (compared to amoeba and plant UCPs) profile of specificity towards FFA is observed for UCP from brown adipose tissue (UCP1) described in UCP1 proteoliposomes (Winkler and Klingenberg, 1994; Klingenberg and Huang, 1999). Among saturated FFA, the maximum activity (H^+ uptake) of UCP1 is observed with C12:0 (lauric acid) and C14:0 (myristic acid) thus shifts to shorter carbon chain FFA when compared to AcUCP-mediated activity (C16:0, Table 1). Moreover, in the case of UCP1, the most active unsaturated FFA (linoleic C18:2 and oleic C18:1) reveal the same effectivity as the most active saturated FFA (lauric C12:0 and myristic C14:0) (Winkler and Klingenberg, 1994; Klingenberg and Huang, 1999). Similarly, in the case of other mammalian UCPs (UCP2, ubiquitous in all tissue; UCP3, muscle specific), studies with proteoliposomes indicate that naturally abundant FFA, both saturated and unsaturated (especially polyunsaturated) can induce a high turnover of these proteins (Zackova et al., 2003). In amoeba mitochondria, uncoupling activity with the best unsaturated FFA (linoleic C18:2) is 2.7 fold higher than with the best saturated FFA (palmitic C16:0) (Table 1). Interestingly, similarly to UCP1 and UCP2 (Chomiki et al., 2001; Tomas et al., 2004), in amoeba mitochondria, a high uncoupling and protonophoric effect (attributed to AcUCP-mediated activity) was observed with *all-trans* retinoic acid, that plays an important role (at least in mammals) in a variety of biological processes such as cell growth and differentiation or apoptosis.

It has been described previously that AcUCP activity diverts energy from ATP synthesis during state 3 respiration in a fatty acid concentration-dependent way (Jarmuszkiewicz et al., 2004a, 2005). This study indicates that this energy-dissipating ability depends on the uncoupling and protonophoric efficiency of FFA, therefore on their character (carbon chain length, saturation degree). Hence, a change in cellular FFA concentration and composition in *A. castellanii* cells could decrease the efficiency of oxidative phosphorylation mainly through an activation of AcUCP. A cellular FFA condition is related to a level of fatty acids incorporated in triglycerides and membrane phospholipids. Study of changes in the fatty acid composition of whole cells and purified membrane fractions of *A. castellanii* during growth in batch culture (Avery et al., 1994a) indicates that the linoleic (C18:2) and oleic (C18:1) acids, the most effective activators of AcUCP (this study) are very abundant in amoeba cells. Together they compose 34–54% (depending on the batch culture age) of total cellular fatty acids and 31–43% of total membrane fraction fatty acids (Avery et al., 1994a). It is very interesting that during the aging an *A. castellanii* batch culture, the greatest change was evident in the linoleate to oleate ratio (with increasing with age contribution of the latter) and in a concomitant decline in the total unsaturation of fatty acids. Moreover, the chilling of amoeba culture which leads to an increase in the overall degree of fatty acid unsaturation and the relative proportion of linoleic acid (Avery et al., 1994b) is related to an increase in AcUCP protein content and activity (linoleic acid-stimulated UCP-mediated carboxyatractiloside-resistant state 4 respiration (Jarmuszkiewicz et al., 2004b). Thus, in *A. castellanii* cells changes in the FFA composition (and their unsaturation) could be a way to regulate the activity of AcUCP and thereby the efficiency of oxidative phosphorylation during a cell life of this eukaryotic unicellular organism.

It has been shown previously, that in *A. castellanii* mitochondria, the contribution of carboxyatractyloside-sensitive (thus ATP/ADP antiporter-mediated) uncoupling to total FFA-induced uncoupling is quite low (no more than 8% with 17 nmol/mg prot. linoleic acid) (Jarmuszkiewicz et al., 2004b). Similarly, an insignificant contribution of glybenclamid acid-sensitive (thus potassium channel inhibited by ATP-mediated) mitochondrial uncoupling to total FFA-induced uncoupling was observed (3% with 17 nmol/mg prot. linoleic acid). Therefore, it seems that in mitochondria of unicellular *A. castellanii*, AcUCP is mainly involved in FFA-induced mitochondrial uncoupling (at low physiological concentration of FFA). Similarly, in other unicellular organisms, such the malaria parasite *Plasmodium berghei berghei* (Uyemura et al., 2000) and fungus *Aspergillus fumigatus* (Tudella et al., 2003), FFA-induced mitochondrial uncoupling was insensitive to the ATP/ADP antiporter inhibitors. In contrast, in plant and animal mitochondria under state 4 conditions, FFA-induced mitochondrial uncoupling could be mediated in a more significant range (despite UCPs) by other carriers, especially the ATP/ADP antiporter (Andreyev et al., 1989; Samartsev et al., 1997; Wieckowski and Wojtczak, 1997; Zackova et al., 2000; Wojtczak et al., 1998; Vianello et al., 1994; Popov et al., 2002; Casolo et al., 2000).

The attribution of studied FFA-induced mitochondrial uncoupling to the operation of AcUCP could be made, despite the weak inhibitory effect of purine nucleotides in state 4 conditions (around 10%) (Czarna and Jarmuszkiewicz, 2005). In the case of *A. castellanii* mitochondria, state 4 conditions (that are optimal, due to a high $\Delta\Psi$, to study the FFA-efficiency profile in mitochondrial uncoupling) are not convenient (due to a very high ubiquinone reduction level, around 80–90% with succinate as respiratory substrate) to study the inhibitory effect of purine nucleotides.

Namely, it has been shown previously that in *A. castellanii* mitochondria as in mammalian (with UCP2 and UCP3) and plant mitochondria, the efficiency of the inhibition of FFA-induced uncoupling under phosphorylating conditions depends on the redox state of ubiquinone (Jarmuszkiewicz et al., 2004c; 2005, Navet et al., 2005). Thus, in the conditions used in this work, the maximally FFA-induced uncoupling (with 17 nmol/mg prot. linoleic acid) is inhibited by 1 mM GTP when the ubiquinone reduction level is sufficiently decreased (Jarmuszkiewicz et al., 2005).

In conclusion, it is worth noting that for the first time in the case of an unicellular organism, such as *A. castellanii*, a profile of fatty acid specificity in mitochondrial uncoupling that can be attributed to UCP activity was described. As this profile is related to physiological changes of fatty acid composition (and thereby FFA availability) during growth of amoeba cells, it could be a way to regulate the activity of AcUCP and thereby the efficiency of oxidative phosphorylation that could play a role in the whole energy and metabolic balance during *A. castellanii* cell life.

Acknowledgements The State Committee of Scientific Research (KBN, 0290/P04/2003/25) supported this work. Malgorzata Czarna was supported by the Foundation for Polish Science.

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