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The effect of growth at low temperature on the activity and expression of the uncoupling protein in *Acanthamoeba castellanii* mitochondria

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Abstract Mitochondria of amoeba *Acanthamoeba castellanii*, a non-photosynthetic soil amoeboid protozoon, possess an uncoupling protein (AcUCP) that mediates free fatty acid-activated proton re-uptake dissipating the proton electrochemical gradient built up by respiration. The present study provides the first evidence that UCP could be a cold response protein in unicellulars. In mitochondria isolated from an amoeba batch culture grown temporarily at low temperature (6 °C), the content of AcUCP was increased and correlated with an increase in the linoleic acid (LA)-stimulated UCP-mediated carboxyatractyloside-resistant state 4 respiration, as compared to a control culture (routinely grown at 28 °C). Moreover, the cytochrome pathway activity was found to be insensitive to the cold exposure of amoeba cells, as indicated by respiration and membrane potential measurements as well as by an absence of change in the adenine nucleotide translocator and cytochrome oxidase expression levels. Furthermore, in mitochondria from the low-temperature-grown cells, at fixed LA concentration, the increased contribution of AcUCP activity to total mitochondrial phosphorylating respiration accompanied by lower coupling parameters was found, as was confirmed by calculation of this contribution using ADP/O measurements.

Keywords: Mitochondrion; Uncoupling protein; Low temperature adaptation; *Acanthamoeba castellanii*

1. Introduction

Uncoupling proteins (UCPs), forming a subfamily within the mitochondrial anion carrier protein family, dissipate an H^+ electrochemical gradient ($\Delta\mu H^+$) built up by a mitochondrial respiratory chain through a free fatty acid (FFA)-activated purine nucleotide-inhibited H^+ cycling process. As a consequence, uncoupling respiration from phosphorylation leads to a decrease in the yield of oxidative phosphorylation. Before 1995, the uncoupling protein of mammalian brown adipose tissue (UCP1) was believed to be a late evolutionary acquisition required for a non-shivering transient thermogenesis and restricted to newborn, cold-acclimated and hibernating mammals [1]. The discovery of plant UCP and several novel UCPs in various mammalian tissues showed that UCP is more widespread than previously believed and that could have various physiological roles [2–8]. Moreover, the discovery of UCP in protozoa, including *Acanthamoeba castellanii* [9,10], fungi [11,12], and mycetozoa [13] indicates that UCPs, as specialized proteins for the FFA-linked H^+ recycling, emerged very early during phylogenesis and could occur throughout the whole eukaryotic world. Indications that UCP is present in unicellulars are mainly based on functional studies and cross-reactivity of around 32 kDa mitochondrial protein with antibodies developed against plant UCP. In *A. castellanii* mitochondria, the action of UCP (AcUCP) has been shown to mediate FFA-activated, poorly purine nucleotide-inhibited H^+ re-uptake driven by $\Delta\mu H^+$ that in state 3 (phosphorylating respiration in the presence of ADP) respiration can divert energy from oxidative phosphorylation [9].

The amoeboid protozoan *A. castellanii* and higher plants share several common features at the level of the respiratory chain of the inner mitochondrial membrane such as the presence of an alternative ubiquinol cyanide-resistant oxidase and non-phosphorylating rotenone-insensitive internal (matricial face) and external (cytosolic face) NADH dehydrogenases [14,15]. Our previous results have shown that the activity and protein content of *A. castellanii* alternative oxidase is clearly increased by a growth of amoeba cells at low temperature [16]. Both, alternative oxidase and UCP, are free energy-dissipating systems which lead to the same final effect, i.e., a decrease in ATP synthesis. In mammalian and plant mitochondria, uncoupling through UCP is affected by numerous factors, including growth temperature. Cold temperatures can increase the activity and expression of mammalian UCP1-3 (for review, see [17–19]) and plant UCP [3,20,21]. So far, there has been no report on the effect of cold exposure on UCP in unicellulars.

The only obvious physiological function of UCP can be recognized in specialized thermogenic mammalian tissue (brown adipose tissue) as heat generation related to an increase in temperature [1]. In unicellular organisms, as well as in

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Abbreviations: AcUCP, uncoupling protein of *Acanthamoeba castellanii*; ANT, adenine nucleotide translocator; BSA, bovine serum albumin; COX, cytochrome oxidase; CAT, carboxyatractyloside; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; FFA, free fatty acids; LA, linoleic acid; RCR, respiratory control ratio; state 3, phosphorylating respiration in the presence of ADP; state 4, resting respiration in the absence of added ADP; UCP, uncoupling protein; $\Delta\mu H^+$, proton electrochemical gradient; $\Delta\Psi$, mitochondrial transmembrane electrical potential

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non-thermogenic plant and animal tissues, the physiological role of this energy-dissipating pathway remains unclear. UCP could play a central role in the maintenance of cell energy metabolism balance related to the regulation of ATP production, control of the $NADH/NAD^+$ ratio, and limitation of the production of mitochondrial reactive oxygen species [18,22–26].

The aim of this study was to determine the effect of low temperature of growth on AcUCP activity and protein content in mitochondria isolated from cultures temporarily grown at 6 °C in comparison with control cultures grown routinely at 28 °C. In cold-exposed cells, we observed an increased level of AcUCP protein. Therefore, our study was designed to check if this increase in protein content could be correlated with the increased fatty acid-induced mitochondrial uncoupling mediated by AcUCP. Moreover, nucleotide- and carboxyatractyloside (CAT)-induced recoupling in both types of mitochondria was studied. The present work may shed light on the possible role of UCP as a response protein to growth at low temperatures in unicellular organisms, such as amoeba *A. castellanii*.

2. Materials and methods

2.1. Cell culture and mitochondrial isolation

Control amoeba *A. castellanii* batch culture was routinely grown at 28 °C. In batch cultures grown at low temperature, 24 h after inoculation, amoeba cells were transferred for 24 h from 28 to 6 °C and then returned to 28 °C for 12 h. To isolate mitochondria, trophozoites of amoeba were harvested after 60 h following inoculation in both types of batch cultures reaching a similar density ($7–8 \times 10^6$ cells/ml) corresponding to the early stationary phase of growth [16].

2.2. Oxygen uptake and membrane potential

Oxygen uptake was measured polarographically with a Rank Brothers (Cambridge, UK) oxygen electrode in 3 ml of the medium (25 °C) containing 120 mM KCl, 10 mM Tris/Cl, pH 7.4, 3 mM KH_2PO_4, 0.1 mM ATP, 1.5 mM benzohydroxamate plus or minus 0.2% bovine serum albumin (BSA, to remove fatty acids), with 1–2 mg of mitochondrial protein. Benzohydroxymate was used to inhibit the alternative oxidase activity. In state 3 respiration measurements, 0.25 mM $MgCl_2$ was added to the incubation medium. The mitochondrial transmembrane electrical potential ($\Delta\Psi$) was measured simultaneously with oxygen uptake using a tetraphenylphosphonium-specific electrode according to Kamo et al. [27]. For calculation of the $\Delta\Psi$ value, the matrix volume of amoeba mitochondria was assumed as 2.0 $\mu l \times mg^{-1}$ protein. Succinate (10 mM) plus rotenone (5 μM) or succinate (5 mM) and glutamate (5 mM) were used as respiratory substrates for state 3 and state 4 (resting respiration in the absence of added ADP) measurements, respectively. Succinate dehydrogenase was activated by 0.1 mM ATP. To measure AcUCP activity, different concentrations of linoleic acid (LA) were used. To exclude the activity of ATP/ADP antiporter, 1 μM CAT was used in state 4 measurements. For ADP/O ratio calculation, the total amount of oxygen consumed during state 3 respiration was used. Values of O_2 uptake and $\Delta\Psi$ are presented in nmol $O \times min^{-1} \times mg^{-1}$ protein and mV, respectively.

2.3. SDS–PAGE and immunoblotting

160 μg of freshly isolated mitochondrial protein was solubilized in the sample buffer (1% (w/v) SDS, 60 mM Tris/Cl (pH.6.8), 10% glycerol, 0.004% (w/v) bromophenol blue, and 8% mercaptoethanol) and boiled for 4 min. Electrophoresis (SDS–PAGE) was carried out using a 5% polyacrylamide stacking gel and a 12% polyacrylamide resolving gel (for AcUCP detection additionally containing 4.5 M urea), as described earlier [9], followed by Western blotting. Bio-Rad prestained low molecular mass markers were used. Antibodies against UCP3 of human skeletal muscle (Gentaur, UCP34-A) were used at dilutions of 1:1000. Similar results were obtained with antibodies raised against *Arabidopsis thaliana* UCP (not shown). Antibodies against subunit III of yeast cytochrome oxidase (COX III, Biocorne, A6408) and antibodies against adenine nucleotide translocator (ANT) of *Neurospora crassa* (generously supplied by Dr. W. Neupert, Germany) were used at dilutions of 1:2000 and 1:500, respectively. Protein bands were visualized using the Amersham ECL system and quantitated digitally.

3. Results and discussion

As shown previously, in amoeba *A. castellanii* cells, mitochondrial respiratory activities including the cytochrome pathway-mediated respiration change with the age of the amoeba batch culture revealing a maximum at the exponential phase of growth [14]. Changes in membrane fatty acid composition during the growth of amoeba in the batch culture were also observed [28]. Therefore, to isolate mitochondria, amoeba trophozoites were harvested at 60 h following inoculation when the early stationary phase of growth was reached in both the control and cold-exposed cultures. Cold exposure at 6 °C for 24 h followed by 12 h growing at 28 °C was chosen in order to compare the respiratory activities of isolated mitochondria when the cold-treated culture reached a similar density to the control culture ($6–7 \times 10^6$ cells/ml) and thereby passed through a similar number of cell divisions.

3.1. Respiratory characteristics of mitochondria isolated from control and cold-exposed amoeba cells

With succinate (plus rotenone) as a respiratory substrate, in the presence of BSA that removes fatty acids, respiratory rates and coupling parameters were similar for mitochondria from the low-temperature-grown cells ($ADP/O = 1.40 \pm 0.04$, $RC = 2.3 \pm 0.3$, S.D., $n = 8$) and for the control mitochondria ($ADP/O = 1.41 \pm 0.03$, $RC = 2.4 \pm 0.2$, S.D., $n = 8$), indicating that the respiratory activity of the cytochrome pathway of *A. castellanii* was not sensitive to the low temperature of growth of amoeba culture (Table 1). To inhibit the alternative oxidase activity, the measurements were carried out in the presence of 1.5 mM benzohydroxamate. Furthermore, in the presence of BSA, there was no difference in state 4 and state 3 membrane potential values measured in mitochondria in either the control or low-temperature-grown amoeba cultures (Fig. 1A). The same values of state 4 respiratory rate and membrane potential in the absence and in the presence of BSA (Fig. 1A, before LA or ADP addition) indicate that after isolation endogenous FFA in mitochondria from the control and low-temperature-grown cells were not detectable. Therefore, as there was no recoupling effect of BSA (that binds FFA), i.e., no decrease in oxygen uptake and no increase in membrane potential, we can suppose that both types of mitochondria were fully depleted of FFA allowing us to study the effect of exogenous FFA in isolated respiring mitochondria.

3.2. Effect of low growth temperature on A. castellanii uncoupling protein (AcUCP) level

Immunodetection showed that the amount of approximately 32 kDa AcUCP protein increased markedly in the mitochondria of amoeba cells grown at 6 °C as compared with cells grown permanently at 28 °C (Fig. 1B). However, there was almost no significant change in the level of COX subunit III and ANT signals (both around 30 kDa), confirming respiratory activity measurements (Fig. 1B) indicating insensitivity of the cytochrome pathway of *A. castellanii* to cold exposure.

Table 1
The effect of growth temperature on respiration and coupling parameters in *A. castellanii* mitochondria in the presence or absence of BSA

	28 °C				6 °C			
	State 4	State 3	ADP/O	RCR	State 4	State 3	ADP/O	RCR
−BSA	62 ± 9	142 ± 11	1.38 ± 0.04	2.3 ± 0.3	64 ± 7	140 ± 10	1.39 ± 0.06	2.2 ± 0.4
+BSA	56 ± 5	133 ± 11	1.41 ± 0.03	2.4 ± 0.2	58 ± 7	135 ± 9	1.40 ± 0.04	2.3 ± 0.3

Mitochondria isolated from control cultures (28 °C) and from cultures temporarily grown at 6 °C were incubated in the presence of 10 mM succinate, 5 μM rotenone, and in the absence or presence of 0.2% BSA. State 3 respiration was measured in the presence of 400 nmol of ADP. Mean values (±S.D.) from four separate experiments ($n = 8$) are shown.

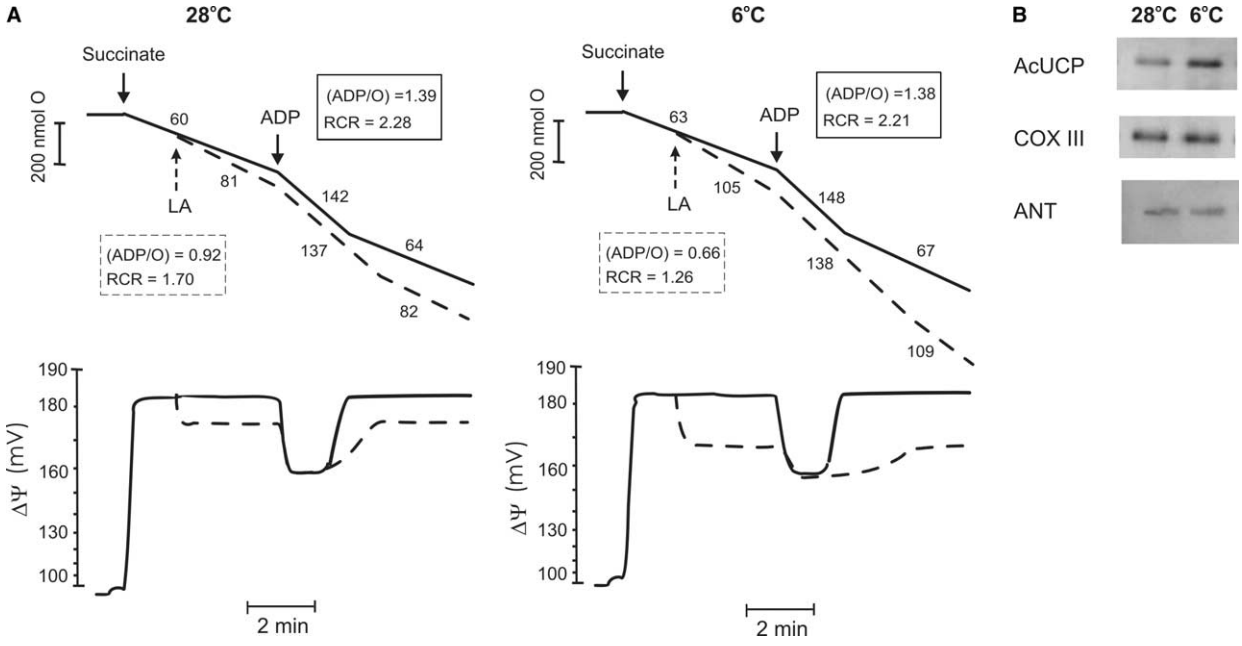

Fig. 1. (A) Influence of 3 μM LA on respiratory rates, coupling parameters and membrane potential. Examples of oxygen uptake and $\Delta\Psi$ measurements are shown. Mitochondria were incubated in the presence of 10 mM succinate, 5 μM rotenone, and in the absence (dashed lines) or presence (solid lines) of 0.2% BSA. LA (3 μM) was added where indicated into mitochondria with no BSA (dashed lines). State 3 respiration was measured in the presence of 400 nmol of ADP. (B) Immunoblots of the uncoupling protein (AcUCP), ANT and COX III in isolated *A. castellanii* mitochondria from control cultures grown at 28 °C and cultures temporarily grown at 6 °C. Mitochondrial protein equivalent to 160 μg was loaded in all lanes. An example of three immunodetections (using mitochondria from different preparations) is shown.

Photodensitometric comparison of immunodetectable signals of AcUCP and COX (as reference) in both types of mitochondria revealed a 2.1 ± 0.4 (S.D., $n = 3$) fold increase in the amount of AcUCP observed in mitochondria from low-temperature-grown cultures. Thus, in cold-grown amoeba cells, the level of AcUCP protein in mitochondria was clearly up-regulated. This could be correlated with the increase in the FFA-linked mitochondrial uncoupling and AcUCP activity.

3.3. Effect of low temperature of growth on AcUCP activity in phosphorylating respiration

It has been shown that in *A. castellanii* mitochondria, the LA-induced AcUCP activity is present during phosphorylating (state 3) respiration and is able to divert energy from oxidative phosphorylation [9]. Thus, a direct consequence of UCP activity is a decrease in ATP synthesis per oxygen consumed, i.e., the yield of oxidative phosphorylation. In the absence of BSA, uncoupling of *A. castellanii* mitochondria respiring in the presence of a low concentration of LA is revealed by lower coupling parameters, i.e., ADP/O and respiratory control ratio (RCR). When comparing mitochondria isolated from the control and cold-treated cultures, a higher sensitivity of state 4 respiratory rate and membrane potential to 3 μM LA accompanied by a more significant prolongation of phosphorylation resulted by a more significant decrease in coupling parameters was observed in mitochondria from low-temperature-grown cells (Fig. 1A). This observation could suggest that there is a higher contribution of respiration sustained by the dissipative UCP-mediated H^+ re-uptake to total state 3 respiration in the mitochondria from cold-exposed cells. Calculation of the respective contributions to overall steady-state 3 respiration of ATP synthesis, which consumes $\Delta\mu H^+$ with energy conservation, and of UCP activity, which consumes $\Delta\mu H^+$ with energy dissipation, can be calculated using the ADP/O measurements in the presence or absence of a given concentration of LA, as described previously [29]. Table 2 shows the contribution of AcUCP activity to state 3 respiration with different concentrations of LA in both types of mitochondria. For a given LA concentration, the calculated contribution of AcUCP is higher in mitochondria isolated from cold-treated amoeba culture. With 7.5 μM LA, the contribution of AcUCP represented 57% of state 3 respiration in the control mitochondria, while in mitochondria from the low-temperature-grown culture this concentration of LA led to full mitochondrial uncoupling.

Table 2
Contribution of AcUCP activity to overall state 3 respiration when activated by different concentrations of LA

Conditions	28 °C		6 °C	
	ADP/O	V_{UCP} (%)	ADP/O	V_{UCP} (%)
+BSA, −LA	1.408	0	1.400	0
−BSA, −LA	1.392	0.01	1.381	0.90
−BSA, +LA (μM)				
3	0.921	34.6	0.658	53.0
5	0.706	49.9	0.533	61.9
7.5	0.599	57.0	–	–

Assay conditions as in Fig. 1B. Different concentrations of LA were used (0, 3, 5, 7.5 μM). Contribution of AcUCP (V_{UCP}) to H^+ re-uptake at a given state 3 respiration ($V3$) is calculated from the ADP/O values in the absence or presence of LA: $V_{UCP} = V3 - V3 \times (ADP/O)_{+LA}/(ADP/O)_{-LA}$ as described in [28]. Mean values (±S.D.) from four separate experiments ($n = 4$) are shown.

In animal and plant mitochondria, FFA-linked uncoupling can be mediated, at least in part, by mitochondrial anion carriers structurally similar to UCP, namely ATP/ADP antiporter [30], aspartate/glutamate antiporter [31], dicarboxylate carrier [32] and phosphate carrier [33]. However, this uncoupling is exclusively observed in resting respiration of oligomycin-treated mitochondria, thus at high $\Delta\mu H^+$. Moreover, in our state 3 measurements, succinate, phosphate and nucleotide translocation, respectively, by dicarboxylate, phosphate and adenine nucleotide carriers should prevent potential uncoupling through these carriers. In the case of ANT, the protein level was not detectably different in either the control or the cold-treated mitochondria (Fig. 1B). Therefore, the results presented in Fig. 1B and Table 2 could indicate a higher activity of AcUCP in mitochondrial phosphorylating respiration in cold-exposed amoeba cells.

3.4. Effect of low temperature of growth on AcUCP activity in resting respiration

First, the effect of CAT on the LA-induced uncoupling in mitochondria respiring in state 4 with succinate and glutamate as respiratory substrates was checked (Fig. 2A). It was noticed that the addition of CAT partially recoupled both types of mitochondria increasing $\Delta\Psi$ and decreasing oxygen uptake. This indicates that in *A. castellanii* mitochondria ATP/ADP antiporter can partially mediate FFA-induced uncoupling. However, contribution of this CAT-sensitive uncoupling to total FFA-induced uncoupling was quite similar (around 10%) for mitochondria from the control and cold-treated amoeba cultures that is in accordance with no change in ANT content observed by immunodetection in these mitochondria (Fig. 1B).

To study the effect of low growth temperature on AcUCP activity in resting (state 4) respiration, oxygen uptake and membrane potential measurements were made in the presence of succinate and glutamate in order to exclude the potential dicarboxylate and glutamate/aspartate carrier-mediated FFA-linked uncouplings, and in the presence of CAT, an inhibitor of ATP/ADP antiporter. These conditions secure the study of FFA-induced mitochondrial uncoupling due to the operation of AcUCP only. The results obtained in the presence of CAT (Fig. 2A and B) show a higher sensitivity of state 4 respiration and membrane potential to a given concentration of LA, revealed as a greater stimulation of respiratory rate and a greater decrease in $\Delta\Psi$, in mitochondria isolated from the cold-exposed cultures when compared to the control mitochondria. This indicates that there is a higher level of LA-induced AcUCP activity in amoeba mitochondria from the cold-treated culture. In both types of mitochondria, the addition of BSA, which adsorbs FFA, completely reversed the respiratory stimulation and correspondingly restored $\Delta\Psi$ (Fig. 2A and B). However, it is difficult to estimate the recoupling effect of 1 mM GTP on the FFA-induced respiration and $\Delta\Psi$ as it is quite weak both when added before and after BSA. Moreover, a similarly weak sensitivity to 1 mM GTP was observed in mitochondria from the control and cold-treated cells. The other purine nucleotides (ATP, ADP, AMP, GDP, and GMP) were used (up to 3 mM) but no significant inhibitory effect on the FFA-induced AcUCP-mediated mitochondrial uncoupling was observed (not shown). Such a

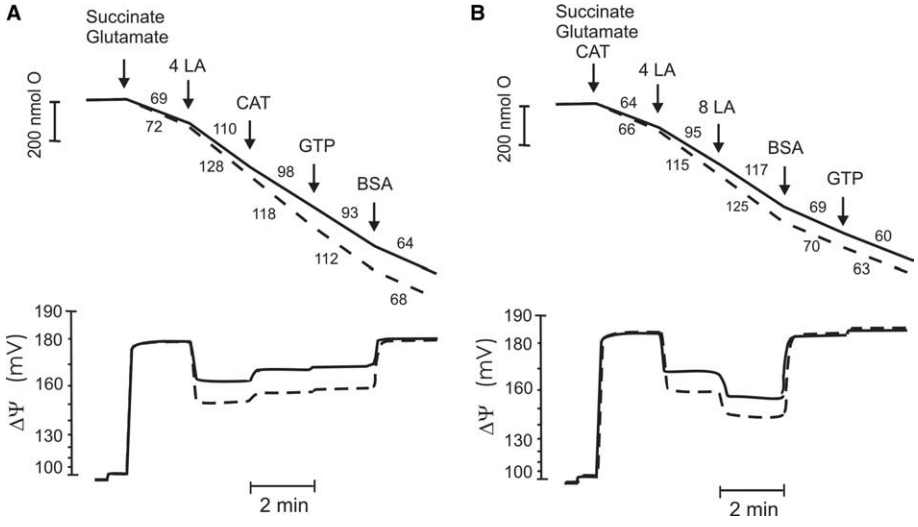

Fig. 2. The effect of LA on state 4 respiratory rate and membrane potential. Examples of oxygen uptake and $\Delta\Psi$ measurements in *A. castellanii* mitochondria isolated from control cultures grown at 28 °C (solid line) and cultures temporarily grown at 6 °C (dashed line). Additions (where indicated): 5 mM succinate plus 5 mM glutamate, 4 or 8 μM LA, 1 μM CAT, 1 mM GTP, and 0.2% BSA.

weak inhibitory effect of purine nucleotides on UCP activity was also observed in isolated respiring mitochondria of slime mold *Dictyostelium discoideum* [13], indicating the specific regulatory property of the protein in these eukaryotic unicellulars. The weak sensitivity of *A. castellanii* and *D. discoideum* UCPs to purine nucleotides (at least in state 4 respiration) could be related to a simultaneous functioning of the cyanide-resistant purine nucleotide-stimulated alternative oxidase in the mitochondria of these organisms. In *A. castellanii* and *D. discoideum*, the two energy-dissipating systems, UCP and alternative oxidase, may work together at their maximal capacities as, unlike plant and fungi mitochondria, alternative oxidase is not inhibited by FFA (UCP activators) and UCP is weakly inhibited by purine nucleotides (alternative oxidase activators) [9,13,26]. The co-regulation of UCP and alternative oxidase in non-plant and non-fungal mitochondria, that may involve the coenzyme Q or reactive oxygen species, has still to be elucidated.

Fig. 3 shows the concentration dependence of the LA-induced stimulation of state 4 respiration (i.e., the difference between respiratory rate in the presence of LA minus respiratory rate after the addition of BSA) representing the part of respiration sustained by AcUCP activity (i.e., FFA-linked H^+ re-uptake) in mitochondria isolated from control and low-temperature-grown cultures. As calculated from a double reciprocal plot (Fig. 3, inset), the concentration of LA that led to a half maximal stimulation of AcUCP-sustained state 4 respiration was the same ($S_{0.5}$ around 8 μM) for both types of mitochondria, while the apparent maximal LA-induced respiration was higher for mitochondria from the cold-treated cells ($V_{max} = 100 \pm 6$ and 158 ± 7 nmol $O \times min^{-1} \times mig^{-1}$ pr., for the control and cold-exposed cells, respectively). Thus, the same $S_{0.5}$ values, at a higher (around 60%) V_{max} value for mitochondria from the low-temperature-grown cells, indicate the higher activity of AcUCP in these mitochondria.

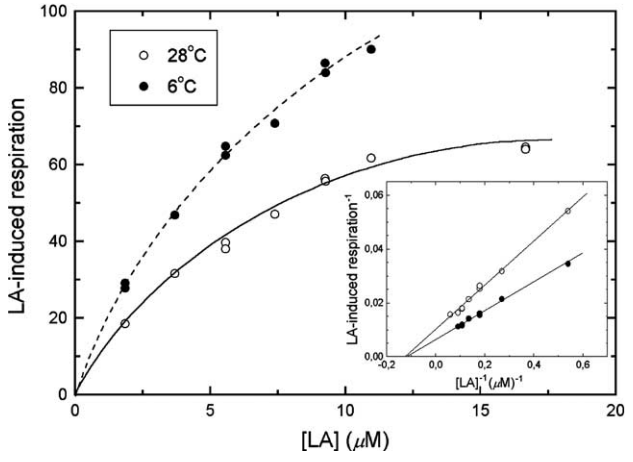

Fig. 3. LA-induced respiration versus LA concentration. Mitochondria isolated from the control and low-temperature-grown cultures were incubated with 5 mM succinate plus 5 mM glutamate and 1 μM CAT. The LA-induced respiration is calculated as a difference between respiratory rate in the presence of LA minus respiratory rate after the addition of BSA. Increasing concentrations of LA (1.8–16 μM) were obtained by successive additions when the steady-state respiration rate was achieved. Several oxygen traces were needed to cover the full investigated range of LA concentration. Inset: Double reciprocal plot. The linear regressions were made giving the apparent maximal LA-induced respiration, $V_{max} = 100 \pm 6$ and 158 ± 7 nmol $O \times min^{-1} \times mig^{-1}$ pr., and LA concentration that produces 50% stimulation, $S_{0.5}$ for LA-induced respiration $= 8.17 \pm 0.31$ and 8.11 ± 0.26 μM, for mitochondria from control and low-temperature-grown cultures, respectively.

3.5. *Voltage dependence of electron flux in the respiratory chain in* A. castellanii *mitochondria from control and cold-treated cells*

The voltage dependence of electron flux in mitochondria of *A. castellanii* shows that the LA-induced respiration is only due to a proton recycling by UCP as it corresponds to a pure protonophoretic effect of LA not distinguishable from the effect of a well known protonophore, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) [9]. For both, mitochondria from the control and cold treated cells, couples of membrane potential and respiratory rate measurements (Fig. 4) in resting state 4 respiration with increasing concentrations of FCCP or LA (in the presence of 1 μM CAT) and in phosphorylating state 3 respiration with increasing concentrations of CAT constituted a single force-flow relationship. In mitochondria from cold-exposed cells, the maximal total respiration was reached at a lower LA concentration when compared to control mitochondria. Results presented in Fig. 4 indicate that LA did not interact with the respiratory chain of *A. castellanii* mitochondria and that the growth-temperature of amoeba cells did not disturb the voltage dependence of electron flux in the respiratory chain of these mitochondria.

3.6. *AcUCP as a cold response protein in* A. castellanii *mitochondria*

To summarize, the present study shows how temporary growth of amoeba cells at low temperature (6 °C instead of 28 °C) increased AcUCP activity and the amount of the protein in the inner mitochondrial membrane. This is the first evidence to show that UCP could be a cold response protein in unicellu-

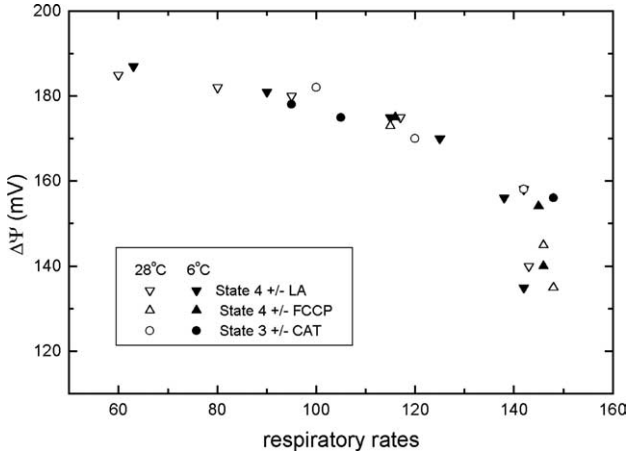

Fig. 4. The relation between membrane potential and mitochondrial respiration rate in mitochondria isolated from control and low-temperature-grown amoeba cells. All measurements were made in the presence of 5 mM succinate plus 5 mM glutamate. State 4 was measured in the presence of 1 μM CAT, and state 3 in the presence of 1.5 mM ADP. Mitochondria isolated from cells grown at 28 °C, open symbols; mitochondria isolated from cells grown temporarily at 6 °C, solid symbols. (▽, ▼) State 4 with increasing concentration of LA (0–16 μM); (△, ▲) state 4 with increasing concentration of FCCP (0–0.17 μM); (○, ●); state 3 with increasing concentration of CAT (0–1.2 μg/mg protein).

lars. In amoeba mitochondria isolated from low-temperature-grown cells, higher activity of AcUCP in state 4 respiration was revealed by the higher LA-induced UCP-mediated respiration and lower LA-induced membrane potential (for a given LA concentration) and as calculated from the linear regression, by the higher maximal rate of LA-induced respiration, at the same value of half maximal stimulation by LA. Moreover, in state 3 respiration, for a given concentration of LA, mitochondria from the cold-treated cells revealed the prolongated phosphorylation, thereby lower coupling parameters (ADP/O and RCR) and thus the increased contribution of AcUCP activity to total phosphorylating respiration as calculated using ADP/O measurements.

Free-living soil amoeba, *A. castellanii*, are quite tolerant to the low growth temperature [33,34]. However, their optimal growth temperature is around 28 °C. A question arises as to what could be the physiological role of AcUCP protein up-regulation as response to cold adaptation during the growth of amoeba cells. In physiological conditions, the accumulation of FFA caused by cold-exposure of amoeba cells [28] in addition to the increased level of the AcUCP protein (as observed in this study) could be a source of the higher AcUCP activity. The FFA-linked UCP-mediated mitochondrial uncoupling, dissipating $\Delta\mu H^+$ built up by respiration, present in animals, higher plants, some fungi and protozoa, leads to a decrease in ATP synthesis accompanied by heat production, and thermogenesis but only in specialized mammalian tissue, i.e., brown adipose tissue [1]. However, the concept of mitochondrial uncoupling as "thermal balance" that would be a response to environmental-thermal pressure cannot be extended to unicellulars because of their microscopic size which does not allow for increase in temperature [24,26]. Indeed, their surface/volume ratio is so high that it excludes the possibility of any steady-state local heating as fast heat diffusion in the surrounding medium occurs. Moreover, thermogenesis through UCP depends on the net increase in overall steady-state oxygen uptake [24,26]. Therefore, the increase in activity and expression of AcUCP (but also the cyanide-resistant alternative oxidase, [16]) after cold stress cannot be related to thermogenesis. In non-thermogenic plant tissues and in unicellular organisms, heat production by energy-dissipating systems (UCP and alternative oxidase) may only be a minor side event. In amoeba cells, AcUCP could be considered as a response protein to cold exposure (translational regulation) allowing increase in oxygen consumption (thereby increase of mild uncoupling) leading to some improvement of biosynthesis and growth at low external temperature but at the expense of oxidative phosphorylation yield. Amoeba UCP could have a subtle role (more pronounced during chilling) in energy metabolism control, working as safety valves when overloads in redox potential or/and in phosphate potential occur [24,26]. Operation of AcUCP, increased during low temperature exposure, could diminish the phosphate potential as well as reducing power by diverting energy from oxidative phosphorylation and by increasing the electron flux in the respiratory chain freed from $\Delta\mu H^+$ control, respectively. Thus, the increased AcUCP activity (resulting from increased protein level) could possibly play a more pronounced role during chilling, for instance in maintaining the metabolic and energy balance of the cell as well as in defense against the production of toxic reactive oxygen species. Indeed, conditions that decrease the reduction level of mitochondrial electron carriers lead to a decrease in damaging reactive oxygen species production [22] as shown for UCP operation in mammalian and plant mitochondria [35–37]. Therefore, further studies are necessary to elucidate the role of AcUCP in amoeba adaptation to coldness by measuring the reactive oxygen production in mitochondria from control and chilled cells. It should shed light on the possible role of UCP as the response protein to growth at a low temperature in unicellular organisms, such as amoeba *A. castellanii*, possibly as an antioxidant system preventing damage of the cell at the level of energy production but at the expense of oxidative phosphorylation yield.

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