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In Phosphorylating *Acanthamoeba castellanii* Mitochondria the Sensitivity of Uncoupling Protein Activity to GTP Depends on the Redox State of Quinone

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In isolated *Acanthamoeba castellanii* mitochondria respiring in state 3 with external NADH or succinate, the linoleic acid-induced purine nucleotide-sensitive uncoupling protein activity is able to uncouple oxidative phosphorylation. The linoleic acid-induced uncoupling can be inhibited by a purine nucleotide (GTP) when quinone (Q) is sufficiently oxidized, indicating that in *A. castellanii* mitochondria respiring in state 3, the sensitivity of uncoupling protein activity to GTP depends on the redox state of the membranous Q. Namely, the inhibition of the linoleic acid-induced uncoupling by GTP is not observed in uninhibited state 3 respiration as well as in state 3 respiration progressively inhibited by complex III inhibitors, i.e., when the rate of quinol (QH_2)-oxidizing pathway is decreased. On the contrary, the progressive decrease of state 3 respiration by declining respiratory substrate availability (by succinate uptake limitation or by decreasing external NADH concentration), i.e., when the rate of Q-reducing pathways is decreased, progressively leads to a full inhibitory effect of GTP. Moreover, in *A. castellanii* mitochondria isolated from cold-treated cells, where a higher uncoupling protein activity is observed, the inhibition of the linoleic acid-induced proton leak by GTP is revealed for the same low values of the Q reduction level.

KEY WORDS: Mitochondria; uncoupling protein; purine nucleotide inhibition; quinone redox state; *Acanthamoeba castellanii*.

INTRODUCTION

Uncoupling proteins (UCPs) form a subfamily within the mitochondrial anion carrier protein family. They dissipate an H^+ electrochemical gradient ($\Delta\mu H^+$) built up by the mitochondrial respiratory chain through a free fatty acid (FFA)-activated purine nucleotide-inhibited H^+ cycling process driven by membrane potential ($\Delta\Psi$) and ΔpH (both constituting $\Delta\mu H^+$) in animal, plant, some fungal and protist mitochondria (Ricquier and Bouillaud, 2000; Sluse and Jarmuszkiewicz, 2002; Golgia and Skulachev, 2003). As a consequence, uncoupling respiration from phosphorylation leads to a decrease in the yield of oxidative phosphorylation, i.e. ATP production per oxygen consumed. The discovery of UCP in protozoa, including *Acanthamoeba castellanii* (Jarmuszkiewicz *et al*., 1999; Uyemura *et al*., 2000), fungi (Jarmuszkiewicz

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Key to abbreviations: $\Delta\mu H^+$, proton electrochemical gradient; UCP, uncoupling protein; UCP1, uncoupling protein of brown adipose tissue mitochondria; FFA, free fatty acids; $\Delta\Psi$, mitochondrial membrane potential; LA, linoleic acid; BSA, bovine serum albumin; BHAM, benzohydroxamate; AcUCP, uncoupling protein of *Acanthamoeba castellanii* mitochondria; Q, quinone; Qox, oxidized quinone; Qred or QH_2 reduced quinone (quinol); Qtot, total endogenous pool of quinone in the inner mitochondrial membrane (Qox + Qred); Qred/Qtot, reduction level of quinone; *J*o, respiratory rate in phosphorylating state 3; *J*p, rate of ATP synthesis.

et al., 2000a; Tudella *et al*., 2003), and mycetozoa (Jarmuszkiewicz *et al*., 2003) indicates that UCPs, as specialized proteins for FFA-linked $\Delta\mu H^+$ dissipation, emerged very early during phylogenesis and could occur in the whole eukaryotic world having various physiological roles. 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 and mammalian UCP (Jarmuszkiewicz *et al*., 1999, 2004a; Uyemura *et al*., 2000). 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 respiration can divert energy from ATP synthesis in a fatty acid-dependent way (Jarmuszkiewicz *et al*., 1999, 2004c). Recently, we have 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*., 2004a).

The only obvious physiological function of UCP can be recognized in specialized thermogenic mammalian tissue (brown adipose tissue) as heat generation leading to increase in temperature (Klingenberg, 1990). In unicellular organisms, as well as in 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 the cell energy metabolism balance related to regulation of ATP production, control of the NADH/NAD^+ ratio, and limitation of the production of mitochondrial reactive oxygen species (Skulachev, 1998; Sluse and Jarmuszkiewicz, 2002; Ježek, 2002; Goglia and Skulachev, 2003).

As UCPs are specialized proteins for FFA-linked $\Delta\mu H^+$ dissipation, their activity must be finely regulated so as be switched on or off according to the physiological state of cells. As regards the sensitivity to purine nucleotides in isolated respiring mitochondria, in a high energy state, UCPs of amoeboid eukaryotes (*A. castellanii* and *D. discoideum*) differ from brown adipose tissue UCP (UCP1) in not revealing a significant inhibitory effect of 1–2 mM GTP or ATP (Jarmuszkiewicz *et al*., 1999, 2003). Similar insensitivity to purine nucleotides has been observed for plant UCPs and UCP2 (UCP ubiquitous in mammalian tissues) and UCP3 (UCP predominantly specific for skeletal muscle) in isolated mitochondria respiring in non-phosphorylating conditions in the absence of superoxide (Jarmuszkiewicz *et al*., 1998, 2004b; Echtay *et al*., 2002). However, inhibition by purine nucleotides has been observed for UCP2 and UCP3 in a reconstituted system (Jaburek *et al*., 1999; Žáčková *et al*., 2003) and for UCP in isolated mitochondria of *C. parapsilosis* (Jarmuszkiewicz *et al*., 2000a). In the case of mammalian UCPs, quinone (Q) has been shown to be an obligatory cofactor for their action (Echtay *et al*., 2000, 2001). Moreover, during non-phosphorylating respiration, stimulation by superoxide has been shown to be necessary to reveal sensitivity to purine nucleotides in isolated kidney and skeletal muscle mitochondria (Echtay *et al*., 2002; Tablot *et al*., 2004) as well as in plant mitochondria (Considine *et al*., 2003). However, in the reconstituted system with heterogously expressed mammalian UCPs, no superoxide activation has been required to demonstrate the FFA-activated purine nucleotide-sensitive H^+ translocation and Q has no significant activating effect nor any effect on the inhibition by purine nucleotides (Jaburek and Garlid, 2003; Žáčková *et al*., 2003). Moreover, taking into account the apparent affinity of reconstituted UCPs for purine nucleotides (Echtay *et al*., 2001; Žáčková *et al*., 2003) and the concentration of nucleotides in vivo (2–15 mM, depending on material), UCPs should be almost fully inhibited under in vivo conditions, even in the presence of FFA, unless an unknown regulatory factor or mechanism could modulate (lower) the inhibition by purine nucleotides (Jarmuszkiewicz *et al*., 2004b). Recently, it has been proposed that the membrane Q redox state could be a metabolic sensor that modulates the purine nucleotide inhibition of FFA-activated UCPs as observed in isolated muscle mitochondria respiring in phosphorylating conditions, in the absence of endogenous superoxide production (Jarmuszkiewicz *et al*., 2004b).

The ADP/O method has been applied to calculate the contributions of UCP activity and ATP synthesis in state 3 respiration of several isolated mitochondria (plant, mammalian, and protist), using pair measurements of ADP/O ratios in the absence or presence of various concentrations of linoleic acid (LA) (Jarmuszkiewicz *et al*., 2000b, 2004b,c). The method is based on the consideration that UCP and ATP synthase, both able to consume $\Delta\mu H^+$ built up by the mitochondrial respiratory chain, may be treated as two paths, i.e., UCP as the $\Delta\mu H^+$ energy-dissipating path, and ATP synthase as the $\Delta\mu H^+$ energy-conserving path. As it is highly unlikely that non-specific uncoupling through the other mitochondrial carriers (Andreyev *et al*., 1989; Samartsev *et al*., 1997; Wieckowski and Wojtczak, 1997; Žáčková *et al*., 2000) takes place in phosphorylating conditions (at a lower $\Delta\mu H^+$), where the particular carriers are employed in the import of ADP, respiratory substrates or phosphate, the LA-induced $\Delta\mu H^+$-dissipating activity has been attributed to the UCP activity only (Jarmuszkiewicz *et al*., 2000b, 2004b,c). Furthermore, a positive correlation has been found between UCP protein level and the amplitude

of the effects induced by LA in non-phosphorylating respiration as well as in phosphorylating respiration (effect on decrease in ADP/O ratio) in cold-treated *A. castellanii* cells (Jarmuszkiewicz *et al*., 2004a).

The aim of the present work is to elucidate the regulation of UCP activity in mitochondria of *A. castellanii*, evolutionarily one of "the oldest" known organisms possessing this protein. We check if the endogenous Q redox state can modulate the GTP inhibition of FFA-activated AcUCP, as observed in isolated muscle mitochondria respiring in phosphorylating conditions (Jarmuszkiewicz *et al*., 2004b). We determine the efficiency of FFA-induced AcUCP activity in the uncoupling of oxidative phosphorylation when state 3 respiration is gradually decreased by a lowering rate of the quinone (Q)-reducing (substrate oxidation) or quinol (QH_2)-oxidizing (complex III activity) pathways. Results show that in isolated, phosphorylating *A. castellanii* mitochondria, respiring with external NADH or succinate, the FFA-induced AcUCP activity can be clearly inhibited by GTP, when Q is sufficiently oxidized. It is also observed for *A. castellanii* mitochondria isolated from cold-treated cells with a higher AcUCP activity and protein level.

MATERIALS AND METHODS

Cell Culture and Mitochondrial Isolation

Soil amoeba *A. castellanii*, strain Neff, was cultured as described by Jarmuszkiewicz *et al*. (1997). A control amoeba *A. castellanii* batch culture was routinely grown at 28°C. For some experiments, amoeba cells were grown at low temperature. In batch cultures grown at low temperature, 12 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 48 h following inoculation in both types of batch cultures reaching a similar density (around 6×10^6 cells/ml) corresponding to the late exponential phase of growth (Jarmuszkiewicz *et al*., 2001).

Mitochondria were isolated and purified on a self-generating Percoll gradient (31%) as described earlier (Jarmuszkiewicz *et al*., 1997). The presence of 0.4% bovine serum albumin (BSA) in isolation media allowed chelating of FFA from the mitochondrial suspension and also allowed mitochondria fully depleted of FFA to be obtained. For each mitochondrial preparation, full depletion of FFA was tested by measuring the effect of BSA on LA-induced respiration as described by Jarmuszkiewicz *et al*. (1998a). Mitochondrial protein concentration was determined by the biuret method.

Assay Procedures

Oxygen uptake was measured polarographically using a Rank Bros. (Cambridge, UK) oxygen electrode or a Hansatech oxygen electrode in 1.4 or 2.7 ml (respectively) of standard incubation medium (25°C) containing: 120 mM KCl, 20 mM Tris–HCl pH 7.4, 3 mM KH_2PO_4, and 0.5 mM $MgCl_2$, with 1–2.5 mg of mitochondrial protein. The membrane potential of mitochondria was measured simultaneously with oxygen uptake using a tetraphenylphosphonium-specific electrode according to Kamo *et al*. (1979). For calculation of the $\Delta\Psi$ value (in mV) the matrix volume of amoeba mitochondria was assumed as 2.0 μl mg^{-1} protein.

The oxidizable substrates were succinate (7 mM) or external NADH (1 mM or 0.5–0.02 mM) in the presence of rotenone (4 μM) to block electron input from complex I. To decrease the rate of the Q-reducing pathway during state 3 respiration (thereby to decrease steady-state 3 respiration), titration of a dehydrogenase activity was made. To titrate succinate oxidation an increasing concentration of *n*-butyl malonate (0–30 mM), a competitive inhibitor of succinate uptake, was used. Titration of NADH oxidation was performed as described previously varying the NADH concentration (0.5–0.02 mM) in the presence of an enzymatic regenerating system (Hoefnagel and Wiskich, 1996). To decrease the rate of the QH_2-oxidizing pathway during state 3 respiration, the bc_1 complex (complex III) was inhibited with stigmatelin (up to 0.03 μM) or antimycin A (up to 0.057 μg/ml). All measurements were made in the presence of an oxidizable substrate (NADH or succinate), 1.5 mM benzohydroxamate (BHAM) (to inhibit an alternative oxidase), 4 μM rotenone, and 0–12.6 μM LA. The ADP/O ratio was determined by an ADP pulse method with 250–500 nmol of ADP. The total amount of oxygen consumed during state 3 respiration (Jo) was used for calculation of the ratio. The ADP/O ratio and Jo were used to calculate the rate of ADP phosphorylation ($Jp = Jo \times$ ADP/O). A prepulse of ADP (80 μM) was always applied before the main pulse to ensure that a true state 4 had been achieved and to activate succinate dehydrogenase by the produced ATP. Measurements of $\Delta\Psi$ allowed fine control of the duration of state 3 respiration. Values of O_2 uptake are in nmol $O_2 \times min^{-1}$ mg^{-1} protein.

The redox state of quinone (Q) in steady-state respiration was determined by an extraction technique followed by HPLC detection according to Van den Bergen *et al*.

(1994). As previously found, endogenous quinone in *A. castellanii* mitochondria is Q_9 (1998b). For calibration of the peaks commercial Q_9 (Sigma) was used. A completely oxidized extract was obtained during incubation in the absence of substrate using evaporation/ventilation step, a completely reduced extract was obtained upon anaerobiosis and in the presence of substrates (7 mM succinate or 1 mM NADH), 1.5 mM cyanide and 1.5 mM BHAM. An inactive Q pool contains quinol that can never be oxidized and quinone that can never be reduced.

RESULTS

The Effect of LA on Coupling Parameters of *A. castellanii* Mitochondria

State 3 respiratory rates and ADP/O ratios were measured during ADP pulses in the absence or presence of different concentrations of LA in isolated *A. castellanii* mitochondria depleted of endogenous FFA, with external NADH or succinate as respiratory substrates. Pulse duration was defined with the help of the $\Delta\Psi$ measurement. Figure 1 shows an example of the effect of 9.1 μM LA on coupling parameters with 1 mM NADH as an oxidizable substrate, during $\Delta\Psi$ and oxygen uptake measurements. In the resting state (state 4), LA increased the respiratory rate and decreased the $\Delta\Psi$. On the other hand, LA scarcely modified phosphorylating state 3 respiration (Jo) and membrane potential ($\Delta\Psi$). As a consequence, the ADP/O ratio and the respiratory control ratio were clearly lowered in the presence of LA. The same results with 1 mM NADH were obtained in the presence of 9.1 μM LA and 0.56 mM GTP (not shown). Decrease in the coupling parameters indicates activation of AcUCP, i.e., the increased, LA-induced, AcUCP-mediated H^+ re-uptake-sustained part of state 3 respiration. Therefore, the method based on the determination of oxidative yield can be used to investigate the LA-induced uncoupling in amoeba mitochondria during state 3 respiration.

The Linoleic Acid-Induced AcUCP Activity-Mediated Proton Leak During Titration of the Rate of the Q-Reducing and QH_2-Oxidizing Pathways in State 3 Respiration

The amplitude of proton leak induced by the addition of LA can be determined in state 3 respiration by the decrease of ADP/O when the rate of oxygen consumption is titrated with various inhibitors (Jarmuszkiewicz *et al.*, 2000b, 2004b). In order to describe how the LA-induced mitochondrial uncoupling (that can be attributed to AcUCP activity) changes with variations in state 3 respiration, the rate of the Q-reducing (respiratory substrate oxidation) and Q-oxidizing (cytochrome pathway activity) pathways was gradually decreased as described under "Materials and Methods." The ADP/O ratio, calculated from the total amount of oxygen consumed during state 3 respiration, induced by a pulse of ADP, and the corresponding rate of oxygen uptake (Jo) measured (in the same assay) during state 3 respiration were used to calculate the rate of ADP phosphorylation ($J\text{p} = J\text{o} \times \text{ADP/O}$) that can be plotted versus the state 3 respiratory rate. Pair measurements of ADP/O ratios and respiratory rates in the absence or presence of a given LA concentration were performed. In the absence of LA, the ADP/O ratio remained constant independent of Jo or respiratory control ratio, when both NADH or succinate oxidation-sustained respiration was decreased. The mean ADP/O value was 1.41 ± 0.03 (SD, $n = 29$) and 1.40 ± 0.02 (SD, $n = 14$), with NADH and succinate, respectively (Figs. 2 and 3). In the presence of LA, the ADP/O ratio was lower and declined with decreasing state 3 respiration (both during substrate availability titration and complex III activity titration). Thus, the lowering of electron flux upstream and

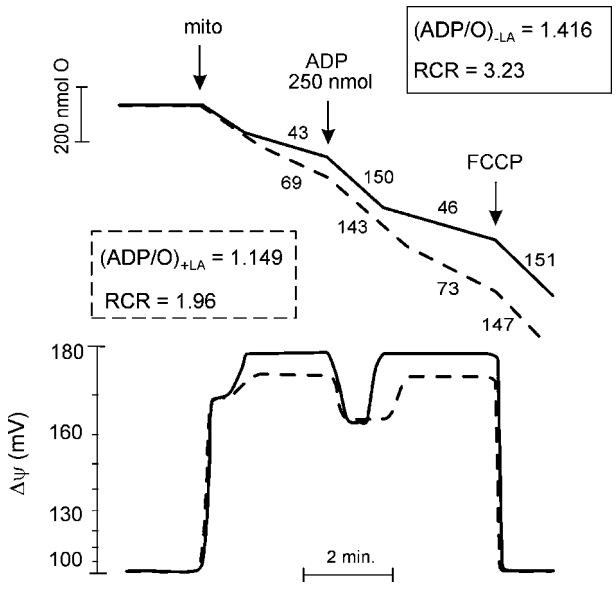

Fig. 1. The effect of LA on coupling parameters of *A. castellanii* mitochondria. Mitochondria (mito) were incubated in the presence of 1 mM NADH as oxidizable substrate, 4 μM rotenone, 1.5 mM BHAM, in the absence (solid line) or presence of 9.1 μM LA (dashed line). After the ADP pulse, respiration was uncoupled and membrane potential was collapsed by 1 μM carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone (FCCP). RCR, respiratory control ratio. Numbers on the traces refer to O_2 consumption rates in nmol $O_2 \times \text{min}^{-1}\ \text{mg}^{-1}$ protein.

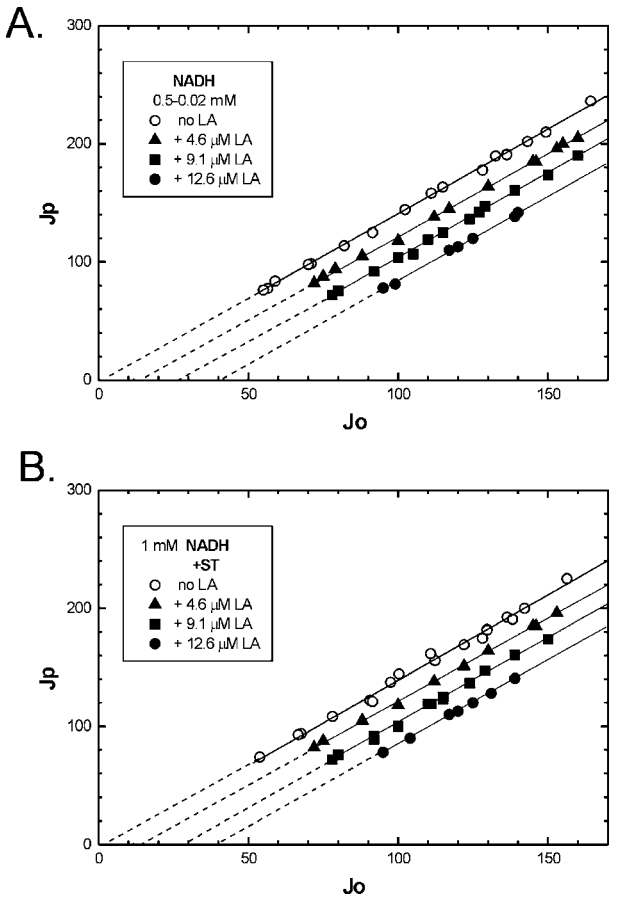

Fig. 2. The effect of different LA concentrations on the relationship between the rate of ADP phosphorylation (*J*p) and rate of state 3 respiration (*J*o) when the rate of the Q-reducing (A) and QH_2-oxidizing (B) pathways is decreased during state 3 respiration in mitochondria oxidizing external NADH. (A) Titration of NADH oxidation (Q-reducing pathway) was performed as described under "Materials and Methods." (B) To decrease the rate of the QH_2-oxidizing pathway during state 3 respiration with 1 mM NADH, the bc_1 complex (complex III) was inhibited with stigmatelin (ST) (up to 0.03 μM). ADP/O ratios used to calculate *J*o were determined in the presence of 0, 4.6, 9.1, and 12.6 μM LA. *J*o and *J*p are expressed in nmol $O_2 \times min^{-1}\ mg^{-1}$ protein. Data deal with six experiments. The mean value of the ADP/O ratio in the absence of LA is 1.413 ± 0.021 (SD, $n = 17$) for NADH oxidation titration (A) and 1.423 ± 0.033 (SD, $n = 18$) for QH_2-oxidizing pathway titration (B). In (A) and (B), straight lines are least square regression lines (dashed part refers to extrapolated range). The respiration sustained by the LA-induced leak (that is equal to the value of intercept with abscissa axis) is: 2 ± 1 (SD, $n = 17$), 14 ± 3 (SD, $n = 13$), 27 ± 5 (SD, $n = 14$), 41 ± 6 (SD, $n = 7$) (A) and 3 ± 1 (SD, $n = 18$), 14 ± 2 (SD, $n = 10$), 28 ± 5 (SD, $n = 14$), 40 ± 5 (SD, $n = 7$) (B) (all in nmol $O_2 \times min^{-1}\ mg^{-1}$ protein) for 0, 4.6, 9.1, and 12.6 μM LA, respectively. Slopes of the regression lines are: 1.438 ± 0.014, 1.398 ± 0.013, 1.430 ± 0.016, 1.424 ± 0.028 (A) and 1.434 ± 0.029, 1.400 ± 0.016, 1.433 ± 0.021, 1.416 ± 0.019 (B) for 0, and increasing concentrations of LA.

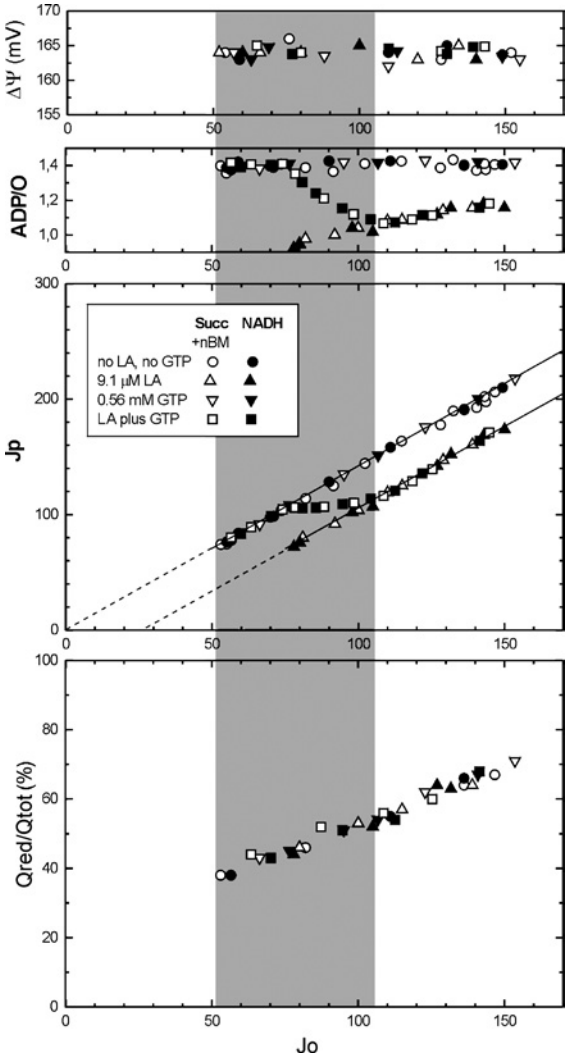

Fig. 3. Inhibitory effect of GTP on the respiration sustained by the LA-induced H^+ leak (AcUCP activity) when the rate of the Q-reducing pathway is decreased during state 3 respiration in mitochondria oxidizing external NADH or succinate. The relationships between $\Delta\Psi$, ADP/O, the rate of ADP phosphorylation (*J*p), as well as the Q reduction level (Qred/Qtot) and the rate of oxygen uptake in state 3 respiration (*J*o) are presented. Substrate oxidation was gradually decreased by increasing the concentration (0–30 mM) of *n*-butyl malonate (nBM) (with succinate) or by decreasing the concentration (0.5–0.02 mM) of NADH (with NADH) as described under "Materials and Methods." Measurements were made (where indicated) in the absence or presence of 9.1 μM LA, and/or in the absence or presence of 0.56 mM GTP. The mean value of theADP/O ratio in the absence of LA (and GTP) is 1.403 ± 0.022 (SD, $n = 14$) for succinate oxidation, and 1.410 ± 0.020 (SD, $n = 12$) for NADH oxidation. Data deal with eight mitochondrial preparations. Straight lines are least square regression lines (dashed part refers to extrapolated range). *J*o and *J*p are expressed in nmol $O_2 \times min^{-1}\ mg^{-1}$ protein. Values of the Q reduction level deal with the active Q pool for a given mitochondrial preparation. The range of respiration with the inhibitory effect of GTP is marked as a grey box.

downstream of the Q pool amplified the decrease in ADP/O induced by LA, suggesting an increase in AcUCP-mediated uncoupling.

Figure 2 shows that titration of state 3 respiration by decreasing the rate of the Q-reducing pathway (i.e. by decreasing NADH concentration) (Fig. 2(A)) and the rate of the QH_2-oxidizing pathway (i.e. by increasing concentration of stigmatelin, an inhibitor of complex III) (Fig. 2(B)) led to a linear relationship between the rate of ATP synthesis (Jp) and the rate of state 3 respiration (Jo) both in the absence or presence of a given concentration of LA. This parallelism, verified with 4.6, 9.1, and 12.6 μM LA, indicates that AcUCP activity (the LA-induced proton leak) remains constant when Jo is varied, independently of the way of Jo titration, i.e. both by decreasing the rate of Q-reducing and QH_2-oxidizing pathways. Similar results were obtained with FFA depleted muscle mitochondria (containing UCP2 and 3) oxidizing with succinate (Jarmuszkiewicz *et al*., 2004b). This is also in accordance with results obtained for kidney mitochondria (containing UCP2), where the basal proton conductance is unaffected by the redox state of endogenous Q (Echtay and Brand, 2001). In *A. castellanii* mitochondria, in the absence of LA, the straight line intersects the abscissa axis at a point close to the origin (Fig. 2), indicating no endogenous proton leak and constancy of the intrinsic ADP/O ratio in both ways of the state 3 rate lowering (as the slopes of the regression lines were 1.438 ± 0.014, SD, $n = 17$ and 1.434 ± 0.029 SD, $n = 18$, Fig. 2(A) and (B), respectively). Neither was any endogenous proton leak observed in tomato mitochondria (Jarmuszkiewicz *et al*., 2000b) though this was not the case in muscle mitochondria (Jarmuszkiewicz *et al*., 2004b). With increasing LA concentration, the abscissa axis intercept (the respiration sustained by the LA-induced AcUCP-mediated proton leak) was shifted more to the right although the slope (the intrinsic ADP/O ratio) was not modified. During Jo titration using the oxidizable substrate or complex III activity variations (within the studied range of state 3 respiration, till around 50 nmol $O_2 \times min^{-1}$ mg^{-1} protein), the LA-induced leak was constant and almost proportional at a low LA concentration (up to 12.6 μM) (Fig. 2(A) and (B)). Concentrations of LA higher than 12.6 μM have not been investigated because of the difficulty in safely determining ADP/O ratios using the ADP/O-pulse method when the respiratory control ratio decreases below 1.3. Thus, the set of parallel straight lines shown in Fig. 2 strongly suggests that LA has no effect on the stoichiometries of the H^+ translocating enzymes employed in the oxidative phosphorylation and that the H^+ reuptake rate via AcUCP does not change with respiratory rates. These results show how efficiently, in the FFA concentration-dependent manner, AcUCP activity can divert energy from oxidative phosphorylation, especially when state 3 respiration is progressively inhibited by decreasing either the rate of Q-reducing and QH_2-oxidizing pathways.

The Inhibitory Effect of GTP on the Respiration Sustained by the LA-Induced H^+ Leak (AcUCP Activity) When the Rate of the Q-Reducing Pathway Is Decreased During State 3 Respiration

In order to confirm that AcUCP activation is responsible for the LA-induced H^+ leak observed during state 3 respiration, we investigated whether this leak is sensitive to purine nucleotides. Figure 3 shows the effect of 0.56 mM GTP on the respiration sustained by the LA-induced H^+ leak (putative AcUCP activity) when the rate of the Q-reducing pathway is decreased in mitochondria oxidizing external NADH or succinate. Figure 3 presents the relationship between the $\Delta\Psi$, ADP/O ratio, the rate of ADP phosphorylation (Jp) as well as the Q reduction level (Qred/Qtot) versus the state 3 respiratory rate (Jo), when substrate oxidation was gradually decreased by increasing the concentration of *n*-butyl malonate (with succinate as respiratory substrates) or by decreasing the concentration of NADH. There was no difference between the results obtained with NADH and with succinate. In all investigated conditions, i.e., in the absence of LA and GTP, or in the presence of one or two of them, the $\Delta\Psi$ of decreasing state 3 respiration remained constant (around 164 mV) within the tested range of Jo (till around 50 nmol $O_2 \times$ min^{-1} mg^{-1} protein), for the two respiratory substrates (Fig. 3, upper part). Moreover, in the absence of LA (plus or minus GTP), the ADP/O ratio remained constant while in the presence of LA (minus GTP) it declined during progressive decreasing of state 3 respiration. The Q reduction level versus Jo gave a single relationship for the all sets of experimental conditions (Fig. 3, lower part). This single relationship, showing that the Q reduction level declined linearly from 70 to 38%, clearly demonstrates that LA and/or GTP do not affect the activity of the cytochrome pathway during substrate oxidation titration. Moreover, the same Jp/Jo relationship was observed in the absence or presence of GTP (without LA) (Fig. 3, middle part). A similar observation has been made for rat muscle mitochondria (Jarmuszkiewicz *et al*., 2004b). This means that GTP has no protonophoric effect and does not affect the intrinsic coupling ratio of the cytochrome pathway (H^+/O) and of the ATP synthase (ADP/H^+).

The Jp/Jo relationship and the ADP/O ratios obtained in the presence of 0.56 mM GTP and 9.1 μM LA revealed the inhibitory effect of GTP on the LA-induced

H^+ leak (Fig. 3). When *J*o is decreased, points obtained in the presence of LA and GTP shifted from points obtained in the presence only of LA to those obtained in the absence of LA (plus or minus GTP) (Fig. 3, middle part). Namely, for the two respiratory substrates, the GTP inhibition was observed at *J*o lower than 106 nmol O_2 $\times$ min^{-1} mg^{-1} protein (that corresponds to Q reduction level lower than 54%) (see the range of inhibition marked in Fig. 3). The full inhibition by GTP (observed when the *J*p/*J*o relationship and the ADP/O ratio in the presence of LA and GTP became identical with those in the absence of LA) was reached at *J*o less than 72 nmol O_2 $\times$ min^{-1} mg^{-1} protein that corresponds to a Q reduction level lower than 45%. The inhibitory effect of GTP is a strong indication that the LA-induced H^+ leak can be attributed to the activation of AcUCP by LA. The progressive inhibition of the LA-induced H^+ leak in the presence of GTP cannot be attributed to changes in the $\Delta\Psi$ (as it remains constant in the investigated *J*o range) (Fig. 3, upper part) or to a side effect of *n*-butyl malonate applied to titrate succinate oxidation (as the same effect was observed during NADH concentration titration). Thus, the results presented in Fig. 3 indicate that the inhibitory effect of GTP on the LA-induced AcUCP-mediated H^+ leak during phosphorylating respiration depends on the membranous Q redox state, being the most efficient when the Q reduction level decreases below 45%.

Figure 4 shows that for a sufficiently decreased state 3 respiration (with a low concentration of NADH), when Q is sufficiently oxidized (Fig. 3), the LA-induced mitochondrial uncoupling can be inhibited by GTP, as revealed by no change in the oxidative phosphorylation yield (ADP/O ratio) when compared with control conditions (no LA). This confirms that in phosphorylating mitochondria, the sensitivity of AcUCP activity to GTP depends on the Q redox state.

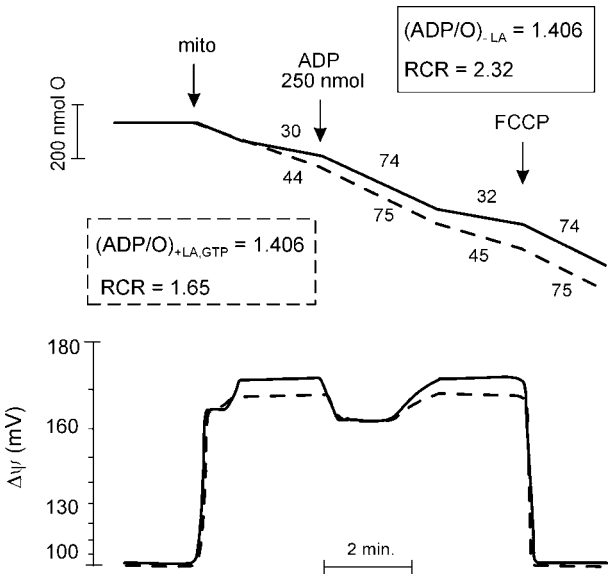

Fig. 4. Effect of GTP on the linoleic acid-induced mitochondrial uncoupling when state 3 respiration is sufficiently decreased. Mitochondria (mito) were incubated in the presence of 0.038 mM NADH as oxidizable substrate, 4 μM rotenone, 1.5 mM BHAM, in the absence of LA (solid line) and in the presence of 9.1 μM LA and 0.56 mM GTP (dashed line). After the ADP pulse, respiration was uncoupled and membrane potential was collapsed by 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). RCR, respiratory control ratio. Numbers on the traces refer to O_2 consumption rates in nmol O_2 $\times$ min^{-1} mg^{-1} protein.

The Lack of Inhibitory Effect of GTP on the Respiration Sustained by the LA-Induced H^+ Leak (AcUCP Activity) When the Rate of the QH_2-Oxidizing Pathway Is Decreased During State 3 Respiration

Figure 5 presents the relationship between $\Delta\Psi$, *J*p as well as the Q reduction level and the state 3 respiratory rate (*J*o), when the rate of the QH_2-oxidizing pathway (the cytochrome pathway activity) was gradually decreased by increasing the concentration of complex III inhibitors (i.e., antimycin A or stigmatelin) in mitochondria oxidizing NADH in the presence or absence of 0.56 mM GTP (plus or minus 9.1 μM LA). In all investigated conditions, the $\Delta\Psi$ of decreasing state 3 respiration remained constant (around 163 mV) within the tested range of *J*o (till around 60 nmol O_2 $\times$ min^{-1} mg^{-1} protein) (Fig. 5, upper part). Moreover, the Q reduction level versus *J*o gave a single relationship for all sets of experimental conditions, showing the increase in Q redox state from 73 to 78% during state 3 titration with both complex III inhibitors (Fig. 5, lower part). The lack of inhibitory effect of GTP on the LA-induced AcUCP-mediated H^+ leak observed when decrease in the state 3 respiration was accompanied by a slight increase in the Q reduction level proves that the inhibition by purine nucleotides can be revealed only for a low Q redox state.

The Linoleic Acid-Induced AcUCP Activity-Mediated H^+ Leak in Cold-Treated *A. castellanii* Mitochondria

Recently, it has been shown that a cold treatment of amoeba cell culture increases the AcUCP activity and protein level (Jarmuszkiewicz *et al.*, 2004a). Indeed, in cold-treated *A. castellanii* cells, a positive correlation

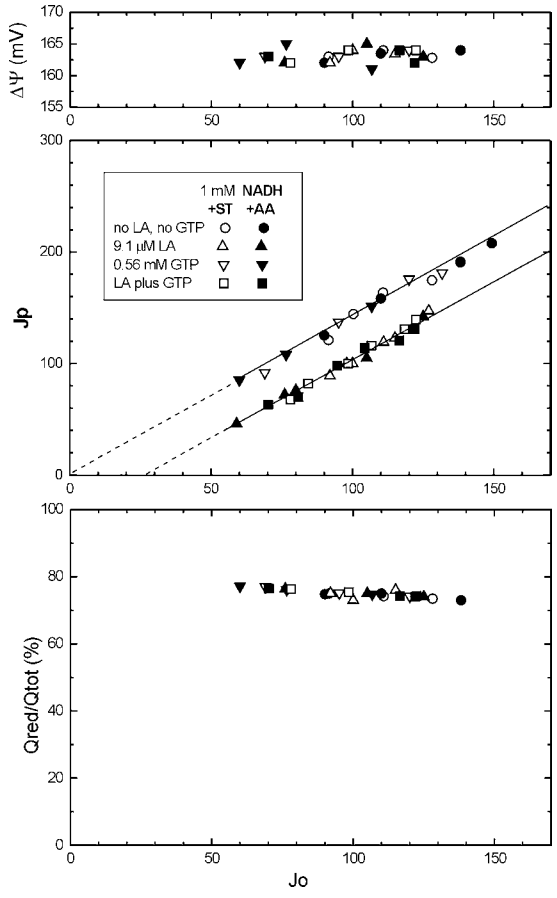

Fig. 5. No inhibitory effect of GTP on the respiration sustained by the LA-induced H^+ leak (AcUCP activity) when the rate of QH_2-oxidizing pathway is decreased during state 3 respiration in mitochondria oxidizing external NADH. The relationships between the membrane potential ($\Delta\Psi$) and the rate of oxygen uptake in state 3 respiration (Jo), the rate of ADP phosphorylation (Jp) and Jo, as well as the Q reduction level (Qred/Qtot) and Jo are presented. To decrease the rate of the QH_2-oxidizing pathway during state 3 respiration with 1 mM NADH, the bc_1 complex (complex III) was inhibited with stigmatelin (ST) (up to 0.03 μM) or antimycin A (AA) (up to 0.057 μg/ml). Measurements were made (where indicated) in the absence or presence of 9.1 μM LA, and/or in the absence or presence of 0.56 mM GTP. Data deal with 6 mitochondrial preparations. Straight lines are least square regression lines (dashed part refers to extrapolated range). Jo and Jp are expressed in nmol $O_2 \times min^{-1}$ mg^{-1} protein. Values of the Q reduction level deal with the active Q pool for a given mitochondrial preparation.

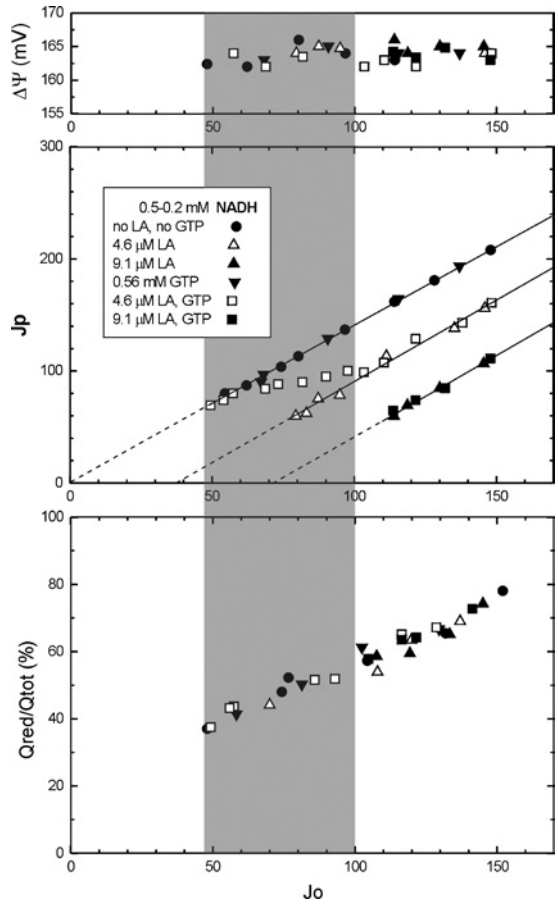

Fig. 6. Inhibitory effect of GTP in mitochondria isolated from cold-treated amoeba cultures, oxidizing external NADH. Experimental conditions and the way of data presentation as in Fig. 3, except that measurements were made in the presence (where indicated) of two concentrations of LA, i.e., 4.6 and 9.1 μM LA, and only NADH (in decreasing concentration, 0.5–0.02 mM) was used as the respiratory substrate. Data from four experiments are shown. The mean value of the ADP/O ratio in the absence of LA is 1.416 ± 0.022 (SD, $n = 9$). The respiration sustained by the LA-induced leak (that is equal to the value of intercept with abscissa axis) is: 1 ± 1 (SD, $n = 8$), 38 ± 5 (SD, $n = 7$), 72 ± 6 (SD, $n = 4$) for 0, 4.6, and 9.1 μM LA, respectively. Slopes of the regression lines are: 1.400 ± 0.015, 1.448 ± 0.068, 1.445 ± 0.057, for 0 and increasing concentrations of LA.

has been found between the AcUCP protein level and the amplitude of the effects induced by LA in non-phosphorylating respiration (on the $\Delta\Psi$ and respiratory rate) as well as in state 3 respiration (on the decrease in ADP/O ratio). Figure 6 presents the relationship between the $\Delta\Psi$, Jp as well as the Q reduction level and the state 3 respiratory rate (Jo) during NADH oxidation titration (by decreasing substrate concentration) in the absence of LA and GTP, or in the presence of one or two of them, in *A. castellanii* mitochondria isolated from cells temporarily grown at 6 °C. In the absence of LA (plus or minus GTP) (Fig. 6), the similar results (respiratory rates and ADP/O ratios) were obtained as for mitochondria from not cold-treated cells (Fig. 3), confirming that the oxidative phosphorylation is insensitive to cold exposure of amoeba cells as observed previously (Jarmuszkiewicz *et al.*, 2004a). However, in a presence of a given concentration of LA, the LA-induced H^+ leak was about 2.7 times higher in mitochondria from cold-treated cells compared to control

mitochondria (Figs. 6 and 3, middle parts), confirming a much higher activity of AcUCP in state 3 respiration of the former. In mitochondria from cold-treated cells, the inhibitory effect of GTP was only observed in the presence of 4.6 μM LA (Fig. 6). Indeed, the higher concentration of LA (9.1 μM) leaded to too enhanced respiratory rates in state 3 (*J*o) which were accompanied by too high Q redox state values. Because of difficulty in safely determining ADP/O ratios using the ADP/O method when the respiratory control ratio is too low, titration of NADH oxidation in these conditions could not be performed below *J*o of 110 nmol $O_2 \times min^{-1}$ mg^{-1} protein (thereby below 54% of the Q reduction level), thus below the range of the Q redox state where inhibition could be observed as it was in the case of non-treated amoeba cells (Fig. 3, middle part). Indeed, in mitochondria from cold-treated cells, the inhibition of the LA-induced H^+ leak was observed in the presence of lower LA concentration (4.6 μM) for Q reduction level below 57% (Fig. 6, middle part). Thus, the inhibitory effect of GTP revealed for almost the same Q reduction values in mitochondria isolated from cold-treated amoeba cells with the higher AcUCP protein and activity levels as in mitochondria from non-treated amoeba cells (Figs. 3 and 6). Thus, these results confirm that in *A. castellanii* mitochondria, FFA-induced uncoupling can be inhibited by purine nucleotides and this inhibition is modulated by the redox state of endogenous Q.

DISCUSSION

It may be concluded that in phosphorylating *A. castellanii* mitochondria depleted of FFA (i) LA (up to 12.6 μM) decreases the yield of oxidative phosphorylation by a pure protonophoric process (Fig. 2), and (ii) contribution sustained by the LA-induced protonophoric activity (in the absence of GTP) remains almost constant when the respiratory substrate availability (Figs. 2, 3, 6) or the cytochrome pathway (complex III) activity (Fig. 5) are decreased. This LA-induced contribution is independent of the Q redox state. Similar results have been obtained for tomato and rat muscle mitochondria (Jarmuszkiewicz *et al*., 2000b, 2004b). The inability of LA to increase state 3 respiration observed in amoeba mitochondria (Fig. 1) as well as in tomato and rat muscle mitochondria (Jarmuszkiewicz *et al*., 2000b, 2004b) suggests that in phosphorylating state oxygen consumption is likely at a maximum value limited by the electron supply to the cytochrome pathway (Jarmuszkiewicz *et al*., 1998a, 1999). Another aspect of interest is that no decrease in $\Delta\Psi$ (the main part of protonmotive force) is detected in state 3 in the presence of LA (Fig. 1), while it decreases the ATP synthesis yield (ADP/O ratio) due to the high sensitivity of ATP synthase to minute decreases of $\Delta\Psi$ (steep dependence) (Jarmuszkiewicz *et al*., 2004b). Furthermore, the LA-induced H^+ leak (which is constant) is not sensitive to the minute $\Delta\Psi$ decreases occurring when the state 3 respiratory rate is decreased by titration of substrate oxidation or titration of complex III activity within the investigated *J*o range (to around 50% of *J*o). Of course, the linear relationships shown in this work must fail at lower *J*o (not shown) because the protonmotive force and consequently the proton leak will decrease and ultimately vanish together with the phosphorylation rate for *J*o close to nil. Nevertheless, a linear extrapolation to the abscissa (Figs. 2, 3, 5, 6) provides valuable assessments of the LA-induced proton leak-sustained respiration (corresponding with the LA-induced AcUCP activity) at the almost constant protonmotive force prevailing in the applied experimental conditions (i.e. for the Jo range between around 150 to 50 nmol $O_2 \times min^{-1}$ mg^{-1} protein).

The inhibitory effect of GTP on the LA-induced H^+ leak in *A. castellanii* phosphorylating mitochondria is a strong indication that the latter results from the activation of AcUCP by LA (Fig. 3). Indeed, it is highly unlikely that a non-specific uncoupling through the other mitochondrial carriers (Andreyev *et al*., 1989; Samartsev *et al*., 1997; Wieckowski and Wojtczak, 1997; Žáčková *et al*., 2000) could occur in state 3 conditions (Jarmuszkiewicz *et al*., 2004b). An additional argument for the contribution of *A. castellanii* UCP in the LA-induced GTP-sensitive H^+ leak observed in state 3 respiration is provided by experiments performed with mitochondria isolated from cold-treated amoeba cells, where a higher LA-induced GTP-sensitive uncoupling (Fig. 6) corresponds with an increased AcUCP protein level (Jarmuszkiewicz *et al*., 2004a). Thus, we can conclude that the LA-induced GTP-sensitive proton leak observed in isolated amoeba mitochondria is most likely mediated by AcUCP.

We have shown that LA-induced mitochondrial uncoupling can be inhibited by the purine nucleotide only when Q is oxidized sufficiently, indicating that in *A. castellanii* mitochondria respiring in phosphorylating state, sensitivity of uncoupling protein activity to GTP depends on the reduced state of the membranous Q. Similar findings have been recently found for isolated rat skeletal muscle mitochondria (Jarmuszkiewicz *et al*., 2004b). Therefore, as in the molecular phylogenetic tree of eukaryotes, *A. castellanii*, the non-photo synthesizing amoeboid protozoan, appears on a branch basal to the divergence points of plants, animals, and fungi (Wainright *et al*., 1993; Gray *et al*., 1999), the evidence of Q redox state-dependent

sensitivity to purine nucleotides of AcUCP, suggests that this phenomenon could occur in UCPs in the whole eukaryotic world. Thus, the redox state of the membranous coenzyme Q may be a metabolic sensor that might release the inhibition by purine nucleotides to achieve a higher or complete activation of UCP-mediated mitochondrial uncoupling, at least in phosphorylating respiration.

The present work confirms the observation made for rat skeletal muscle mitochondria (Jarmuszkiewicz *et al*., 2004b) that the Q redox state has no effect at the level of FFA-induced UCP activity in the absence of GTP and could only regulate this activity indirectly through the efficiency of inhibition by GTP. Indeed, in *A. castellanii* mitochondria when the Q redox state decreases (when the rate of the Q-reducing pathways decreases accompanied by decrease in state 3 respiration) (Figs. 3 and 6), the efficiency of inhibition by GTP increases and the activity of AcUCP becomes sensitive to GTP. The full inhibitory effect of GTP on the LA-induced AcUCP-mediated H^+ leak in state 3 respiration is observed when the Q reduction level is below 45%, as observed both in mitochondria isolated from non-treated (Fig. 3) and cold-treated (Fig. 6) amoeba cells. It seems that range of the Q redox state when the inhibition by GTP occurs could be different for different mitochondria, as in rat muscle mitochondria the full sensitivity to GTP has been observed for a Q reduction level below 57% (Jarmuszkiewicz *et al*., 2004b).

The dependence of the sensitivity of the FFA-induced UCP activity to purine nucleotides helps to explain why, in some mitochondria, including *A. castellanii* mitochondria, no or weak inhibition by GTP of the LA-induced uncoupling has been observed in resting (state 4) respiration when the Q reduction level is high. However, during resting respiration, stimulation of UCP activity by superoxide is sensitive to purine nucleotides in isolated kidney and skeletal muscle mitochondria (Echtay and Brand 2001; Echtay *et al*., 2002; Tablot *et al*., 2004) as well as in plant mitochondria (Considine *et al*., 2003). Moreover, FFA are not required for UCP3 activation in resting respiration when superoxide is produced endogenously in the mitochondrial matrix (Tablot *et al*., 2004). Thus, UCP could be activated by either FFA or superoxide leading to two active forms differently regulated.

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