Journal of Bioenergetics and Biomembranes, Vol. 36, No. 5, October 2004 (© 2004)

Redox State of Endogenous Coenzyme Q Modulates the Inhibition of Linoleic Acid-Induced Uncoupling by Guanosine Triphosphate in Isolated Skeletal Muscle Mitochondria

Wieslawa Jarmuszkiewicz,[1,2] Rachel Navet,[2,3] Luciane C. Alberici,[4] Pierre Douette,[3] Claudine M. Sluse-Goffart,[3] Francis E. Sluse,[3,5] and Anibal E. Vercesi[4]

Received April 1, 2004; accepted July 12, 2004

The skeletal muscle mitochondria contain two isoforms of uncoupling protein, UCP2 and mainly UCP3, which had been shown to be activated by free fatty acids and inhibited by purine nucleotides in reconstituted systems. On the contrary in isolated mitochondria, the protonophoretic action of muscle UCPs had failed to be demonstrated in the absence of superoxide production. We showed here for the first time that muscle UCPs were activated in state 3 respiration by linoleic acid and dissipated energy from oxidative phosphorylation by decreasing the ADP/O ratio. The efficiency of UCPs in mitochondrial uncoupling increased when the state 3 respiratory rate decreased. The inhibition of the linoleic acid-induced uncoupling by a purine nucleotide (GTP), was not observed in state 4 respiration, in uninhibited state 3 respiration, as well as in state 3 respiration inhibited by complex III inhibitors. On the contrary, the progressive inhibition of state 3 respiration by *n*-butyl malonate, which inhibits the uptake of succinate, led to a full inhibitory effect of GTP. Therefore, as the inhibitory effect of GTP was observed only when the reduced state of coenzyme Q was decreased, we propose that the coenzyme Q redox state could be a metabolic sensor that modulates the purine nucleotide inhibition of FFA-activated UCPs in muscle mitochondria.

KEY WORDS: Muscle; mitochondria; uncoupling proteins; coenzyme Q; purine nucleotide inhibition.

INTRODUCTION

The function, regulation, and physiological role of the uncoupling protein of brown adipose tissue (UCP1, thermogenin) are well described: (i) UCP1 catalyzes the dissipation of the proton electrochemical gradient built up by the respiratory chain, (ii) its activity is stimulated by free fatty acids (FFA) and inhibited by purine nucleotides (PN) di- and triphosphates, and (iii) it is responsible for a nonshivering thermoregulatory thermogenesis in newborn, hibernating, and cold acclimated animals (Klingenberg and Echtay, 2001; Nicholls and Rial, 1999). Since 1995, novel mitochondrial UCPs have been discovered in plants (Laloi *et al.*, 1997; Vercesi *et al.*, 1995), in various mammalian tissues including ubiquitous UCP2 (Fleury *et al.*, 1997), predominantly skeletal muscle-specific UCP3 (Boss *et al.*, 1997; Vidal-Puig *et al.*, 1997) and brain-specific UCP4 and UCP5 (Mao *et al.*, 1999; Sanchis *et al.*, 1998), as well as in other eukaryotic organisms like soil amoeba *Acanthamoeba castellanii* (Jarmuszkiewicz *et al.*, 1999), nonfermentative yeast

[1] Laboratory of Bioenergetics, Adam Mickiewicz University, Poznan, Poland.
[2] W. Jarmuszkiewicz and R. Navet contributed equally to this work.
[3] Laboratory of Bioenergetics, Center of Oxygen Research and Development, Department of Life Sciences, Institute of Chemistry B6c, University of Liège, Sart Tilman, Liège, Belgium.
[4] Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, CP 6111, Campinas, SP, Brazil.
[5] To whom correspondence should be addressed; e-mail: F.Sluse@ulg.ac.be.

Key to abbreviations: AA, antimycin; BSA, bovine serum albumin; FFA, free fatty acids; GTP, guanosine triphosphate; J_o, respiratory rate in phosphorylating state 3; J_p, rate of ATP synthesis; LA, linoleic acid; MX, myxothiazol; PN, purine nucleotides; UCP, uncoupling protein; $\Delta\Psi$, mitochondrial membrane electrical potential; nBM, *n*-butyl malonate; CoQ, coenzyme Q.

Candida parapsilosis (Jarmuszkiewicz *et al.*, 2000b), and mycetozoan *Dictyostelium discoideum* (Jarmuszkiewicz *et al.*, 2002b). All these uncoupling proteins form a subfamily within the mitochondrial anion carrier protein family. The discovery of these "novel UCPs" raises the question of their physiological role in various nonthermogenic mammalian tissues, in plants, and in microorganisms (Jarmuszkiewicz *et al.*, 2001; Ježek, 2002; Sluse and Jarmuszkiewicz, 2002). In any case, three potential basic roles of all UCPs are the consequences of their protonophoretic action: (i) control of energy metabolism balance between reducing substrate supply and energy and carbon demand through an acceleration of the metabolism caused by an increase in the respiratory rate (Jarmuszkiewicz *et al.*, 2001); (ii) reduction in reactive oxygen species formation by decreasing the reduced state of the respiratory chain electron carriers (Skulachev, 1998), and (iii) mild thermogenesis related to an increase in the respiratory rate (Skulachev, 1998).

The novel mammalian UCPs, mainly UCP2–3 are suspected to be implicated in several physiological and physiopathological processes such as body weight regulation and obesity, adaptive thermogenesis, apoptotic processes, type II diabetes, and heart failure (for recent review see Argyropoulos and Harper, 2002; Ježek, 2002). Whatever could be the *in vivo* role, all UCPs represent a potential danger for the energy conservation and consequently they must be finetuned regulated. Besides up- and downregulation of their expression, biochemical regulations are involved. The first line of regulation is FFA activation of UCP1 and of plant (Jarmuszkiewicz *et al.*, 1998a, 2000a; Ježek *et al.*, 1997), protozoan (Jarmuszkiewicz *et al.*, 1999), yeast *C.parapsilosis* (Jarmuszkiewicz *et al.*, 2000b), mycetozoan (Jarmuszkiewicz *et al.*, 2002a) UCPs as well as UCP2–3 (Echtay *et al.*, 2001). Inhibition by PN has also been observed for UCP2–3 in reconstituted system (Jaburek *et al.*, 1999; Žácková *et al.*, 2003) and for UCP in isolated mitochondria of *C. parapsilosis* (Jarmuszkiewicz *et al.*, 2000b). UCP1 inhibition by PN is lowered by alkaline pH (Huang *et al.*, 1998), but pH modulation of UCP2–3 uncoupling remains uncertain (Žácková *et al.*, 2003). Cofactors like coenzyme Q10 (Echtay *et al.*, 2000, 2001) and superoxide (Echtay *et al.*, 2002) are claimed to be required in order to observe FFA-induced activation of UCP2–3 and only this "superoxide activated" state has been inhibited by PN di- and triphosphates in isolated kidney or muscle mitochondria (Echtay *et al.*, 2002). However, conflicting results have been obtained with heterologously expressed UCP1, UCP2, and UCP3 in reconstituted systems, where no superoxide activation is required to observe PN-sensitivity of FFA-activated H^+ translocation and where oxidized coenzyme Q has neither a significant activating effect nor an effect on PN regulation (Jaburek and Garlid, 2003; Žácková *et al.*, 2003). It must be pointed out that with regard to the apparent PN affinities of reconstituted UCPs (Echtay *et al.*, 2001; Žácková *et al.*, 2003) and to the *in vivo* PN concentrations (2–15 mM), UCPs should be almost fully inhibited under *in vivo* condition even in the presence of FFA and cellular magnesium, unless unknown regulatory factors or mechanisms modulate the inhibition by PN.

Since UCP and ATP synthase are able to consume $\Delta\mu H^+$ built up by the proton pumps of the respiratory chain, they may be considered as two branching pathways contributing to respiration. UCP is a $\Delta\mu H^+$ energy-dissipating path and ATP synthase is the $\Delta\mu H^+$ energy-conserving path. Preparation of mitochondria depleted of their FFA [from green tomato fruit (Jarmuszkiewicz *et al.*, 2000a), *A. castellanii* (Jarmuszkiewicz *et al.*, 1999), *C. parapsilosis* (Jarmuszkiewicz *et al.*, 2000b), and *D. discoideum* (Jarmuszkiewicz *et al.*, 2002a)] and subsequent observation of the decrease in ADP/O induced by linoleic acid (LA) addition have allowed calculation of the contribution of the LA-induced uncoupling and ATP synthesis in state 3 respiration using pair measurements of ADP/O ratios in the absence or presence of LA (the ADP/O method developed in Jarmuszkiewicz *et al.*, 2000a). The LA-induced $\Delta\mu H^+$-dissipating activity responsible for a decrease in ADP/O ratio was attributed to the UCP activity only. However, several members of the mitochondrial carrier family are known to mediate the protonophoretic action of FFA, which can be inhibited by their specific substrates and inhibitors: the ADP/ATP carrier (Andreyev *et al.*, 1989), the dicarboxylate carrier (Wieckowski and Wojtczak, 1997), the aspartate/glutamate carrier (Samartsev *et al.*, 2000), and the phosphate carrier (Žácková *et al.*, 2000). Thus, the observed LA-induced H^+ recycling could be mediated, at least in part, by these carriers likely at high membrane potential (state 4 respiration) but unlikely during phosphorylating respiration where the ADP/ATP, dicarboxylate, and phosphate carriers are employed in the import of ADP, succinate, and phosphate, respectively (Jarmuszkiewicz *et al.*, 2000a). Furthermore, in tomato fruit mitochondria, some pieces of evidence in favor of the main responsibility of UCP in the LA-induced H^+ recycling have been obtained during postharvest ripening (Almeida *et al.*, 1999). Indeed, positive correlation has been established between UCP protein concentration in the membrane and amplitude of the effects induced by LA in state 4 respiration. The same correlation has been observed in cold-stressed *Acanthamoeba castellanii* (Jarmuszkiewicz *et al.*, 2004) and in skeletal muscle after

starvation (Navet *et al.*, unpublished results) where UCP protein expression increases in parallel with the LA effect on state 4 respiration and ADP/O ratio.

As biochemical studies in the absence of superoxide production (Echtay *et al.*, 2002; Talbot *et al.*, 2004) have so far failed to reveal uncoupling by UCP2 and UCP3 in isolated mitochondria (Jaburek and Garlid, 2003), the aim of present work based on the ADP/O method is to determine the actual contribution of the LA-induced uncoupling protein (UCP2–3) activity in state 3 respiration and to demonstrate their efficiency to uncouple oxidative phosphorylation. For this purpose, we have isolated skeletal muscle mitochondria fully depleted of FFA and described how the LA-induced uncoupling contribution varied during titration of state 3 respiration (with succinate as oxidizable substrate) either when the rate of succinate dehydrogenase is decreased by succinate uptake limitation or when complex III activity is decreased by antimycin A or myxothiazol. On the basis of our results we propose that muscle FFA-activated UCPs can divert energy from oxidative phosphorylation *in vitro* (more efficiently with decreasing respiration as UCP contribution is constant at a given LA concentration) and that GTP inhibits the LA-induced UCP activity in a way that could be modulated by the redox state of membranous coenzyme Q (CoQ).

MATERIAL AND METHODS

Mitochondrial Isolation

Mitochondria were isolated from hind limb skeletal muscles of male Wistar rats (6–8 week-old) by homogenization in ice-cold medium containing 100-mM sucrose, 100-mM KCl, 50-mM Tris-HCl, 1-mM K_2HPO_4, 0.1-mM EGTA, and 0.2% BSA, pH 7.4, followed by differential centrifugation (Tonkonogi and Salhin, 1997). The final mitochondrial pellet was resuspended in ice-cold storage buffer containing 225-mM mannitol, 75-mM sucrose, 10-mM Tris-HCl, and 0.1-mM EDTA, pH 7.4. The protein concentration was usually around 20–30 mg mL^{-1}, as determined by a modified Biuret method (Gornall *et al.*, 1949). Depletion of endogenous FFA was ensured by the presence of 0.2% fatty acid-free BSA in isolation buffers. For each mitochondrial preparation, full depletion of FFA was verified by measuring the effect of BSA on the LA-induced respiration in the presence of oligomycin (2.5-μg mg^{-1} protein) as described in Fig. 1(A).

Assay Procedures

Mitochondrial respiration was measured using a Clark-type electrode (Hansatech) in 1.3 mL of standard incubation medium (25°C) containing: 225-mM mannitol, 75-mM sucrose, 10-mM Tris-HCl, 0.5-mM EDTA, 5-mM K_2HPO_4, and 10-mM KCl, pH 7.4, with 0.5 mg of mitochondrial protein. Succinate (5 mM) plus 5-μM rotenone was used as oxidizable substrate. ATP (80 μM) was needed to ensure the activation of succinate

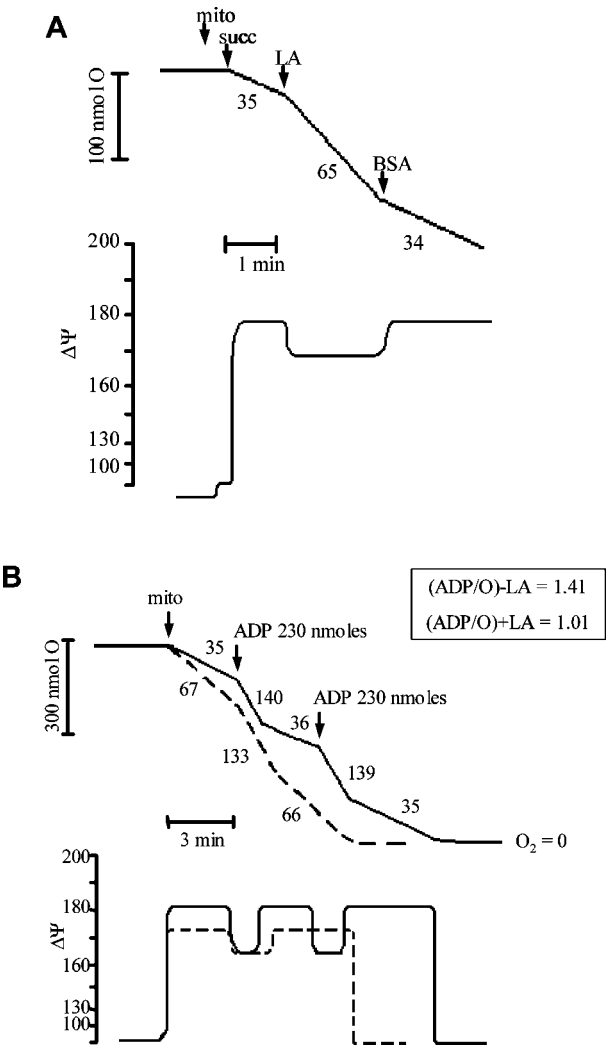

Fig. 1. Experimental setting: (A) Effect of LA on respiratory rates and membrane potential ($\Delta\Psi$) of skeletal muscle mitochondria depleted of FFA; (B) Effect of LA on coupling parameters. (A), Mitochondria are incubated as described in Material and Methods in the presence of 5-mM succinate (plus 5-μM rotenone and 80-μM ATP) as oxidizable substrate and oligomycin to inhibit ATP synthase. Mitochondria (mito), 10-μM LA, and 0.5% BSA are added where indicated. (B), Mitochondria respire in state 4 in the absence (solid line) or presence (dashed line) of 10-μM LA. For state 3 respiration, ADP pulse (twice for −LA condition, once for +LA condition) is added. After the ADP pulse(s), membrane potential is collapsed when zero oxygen is reached. Numbers on the traces refer to oxygen consumption rates. The membrane potential is given in mV.

dehydrogenase. The ADP/O ratio was calculated using the total amount of oxygen consumed during state 3 respiration (J_o) induced by a pulse of 230 nmoles of ADP. ADP/O ratio and J_o were used to calculate the rate of ADP phosphorylation ($J_p = J_o \times$ ADP/O).

Mitochondrial Membrane Electrical Potential Measurements

The mitochondrial membrane electrical potential ($\Delta\Psi$) was measured using a tetraphenyl phosphonium (TPP^+)-selective electrode (Kamo *et al.*, 1979), in a final volume of 3 mL in the presence of 5.8-μM TPP^+, under the same conditions as used for oxygen uptake. The membrane potential was calculated by assuming that the TPP^+ distribution between mitochondria and the medium followed the Nernst's equation. A calibration in the absence of mitochondria was obtained by three successive additions of TPP^+ up to a final concentration of 5.8 μM. For calculation of the $\Delta\Psi$ value, the matrix volume of skeletal muscle mitochondria was assumed to be 2-μL mg^{-1} protein.

The Redox State of Coenzyme Q Determination

The redox state of CoQ, i.e., the reduced quinone *versus* the total endogenous pool of quinone in the inner mitochondrial membrane was determined in mitochondrial steady-state 3 respiration by an extraction technique followed by HPLC measurements according to Van den Bergen *et al*. (1994). For calibration of the HPLC peaks, commercial Q_9 was used.

Chemicals

Q_9 was purchased from Sigma, and BSA and nucleotides from ICN Biochemicals. All chemicals were of the highest purity grade.

RESULTS

Effect of Linoleic Acid on Respiratory Rates and Membrane Potential

All experiments are performed with isolated muscle mitochondria depleted of endogenous FFA, in the presence of succinate (plus rotenone) as oxidizable substrate. Figure 1 presents an example of $\Delta\Psi$ and oxygen uptake measurements in the presence or absence of LA. Figure 1(A) shows an increase in state 4 respiration (plus oligomycin that inhibits ATP synthase) (from 35 to 65-nmol O/min/mg of mitochondrial protein) following the addition of 10-μM LA. This acceleration in the respiratory rate is accompanied by a drop in $\Delta\Psi$ from 180 to 169 mV. The initial respiratory rate and $\Delta\Psi$ are reestablished by subsequent addition of 0.5% BSA that chelates FFA. The similar respiratory rates before the addition of LA and after the addition of BSA demonstrate the absence of endogenous FFA in muscle mitochondria isolated by our procedure. This FFA depletion is required for all experiments reported here. The ADP/O ratio (the amount of phosphorylated ADP *versus* the amount of reduced oxygen atom) and state 3 respiration rate (J_o) are measured during ADP pulses in the absence or in the presence of LA [Fig. 1(B)]. In state 4 respiration (in the absence of oligomycin), the presence of 10-μM LA almost doubles the respiratory rate and clearly decreases $\Delta\Psi$ [as in the presence of oligomycin, Fig. 1(A)], while in state 3 respiration 10-μM LA scarcely modifies steady state respiratory rate (slight decrease) and has no effect on membrane potential and then seemingly does not uncouple the respiration in state 3. However, the ADP/O ratio is significantly lowered by LA suggesting that the fatty acid activates an uncoupling system able to decrease the phosphorylation yield. The effects of LA on $\Delta\Psi$ in state 4 and on ADP/O in state 3 are LA-concentration dependent (not shown). Thus, the method based on the determination of oxidative phosphorylation yield can be used to investigate the LA-induced uncoupling in isolated skeletal muscle mitochondria fully depleted of endogenous FFA during state 3 respiration.

Constancy of the Linoleic Acid–Induced Proton Leak During Respiration Titration in the Absence of GTP

The amplitude of proton leak induced by the addition of LA can be determined in state 3 respiration (Jarmuszkiewicz *et al.*, 2000a) by the decrease of ADP/O ratio when the rate of oxygen consumption is titrated with various inhibitors. Respiration in the presence of succinate as oxidizable substrate (plus rotenone to inhibit complex I) can be titrated with *n*-butyl malonate (nBM), a competitive inhibitor of succinate uptake. *n*-Butyl malonate inhibits respiration upstream the ubiquinone pool leading to a decrease in the CoQ reduction level. On the contrary, inhibitors of complex III like myxothiazol (MX) and antimycin A (AA) increase the CoQ reduction level by inhibiting the oxidation of ubiquinol ($CoQH_2$) (Jarmuszkiewicz *et al.*, 2002b; Van den Bergen *et al.*, 1994). The rate of ADP phosphorylation (J_p) can be

calculated by multiplying the measured ADP/O ratio by the corresponding (measured in the same assay) state 3 respiratory rate (J_o) and plotted versus the state 3 respiratory rates. The selected concentration of 10-μM LA allows titration of large range of J_o by the inhibitors that still enables the ADP/O ratio determinations.

In the absence of GTP and LA, titration of respiration with nBM reveals a linear relationship between J_p and J_o crossing the abscissa axis on the right of the origin [Fig. 2(A)]. This means that a part of state 3 respiration of FFA-depleted mitochondria is sustained by a constant endogenous H^+ leak (within the range of titration, see Discussion), assuming that the slope of the straight line (1.55) is the intrinsic ADP/O ratio, i.e., the product of the stoichiometric ratio of ATP synthase (ADP/H^+) and of the cytochrome pathway (H^+/O). Remarkably the same titration range of state 3 respiration in the presence of 10-μM LA indicates that LA induces an additional constant H^+ leak (shift of the straight line to the right) with no modification of the intrinsic stoichiometric ADP/O ratio [Fig. 2(A)] as the slope of the linear relationship in the presence of LA is not modified when compared to the slope of the linear relationship in the absence of LA.

In the absence and in the presence of LA, titrations of respiration with MX and AA [Fig. 2(A)] yield the same J_p *versus* J_o relationship as with nBM [Fig. 2(B)]. This identical effect of inhibitors acting either on succinate uptake (nBM) or on complex III (AA, MX) has an important significance. Indeed, it means that the variation of redox state of CoQ (more oxidized with increasing concentrations of nBM and more reduced with increasing concentrations of MX or AA) has no effect on the LA-induced H^+ leak (the putative UCP activity) since it remains constant at 10-μM LA in all titrations. This is in accordance with results obtained for kidney mitochondria (containing UCP2) where the basal proton conductance is not affected by the redox state of endogenous CoQ even when endogenous FFA are not chelated by BSA (Echtay and Brand, 2001)

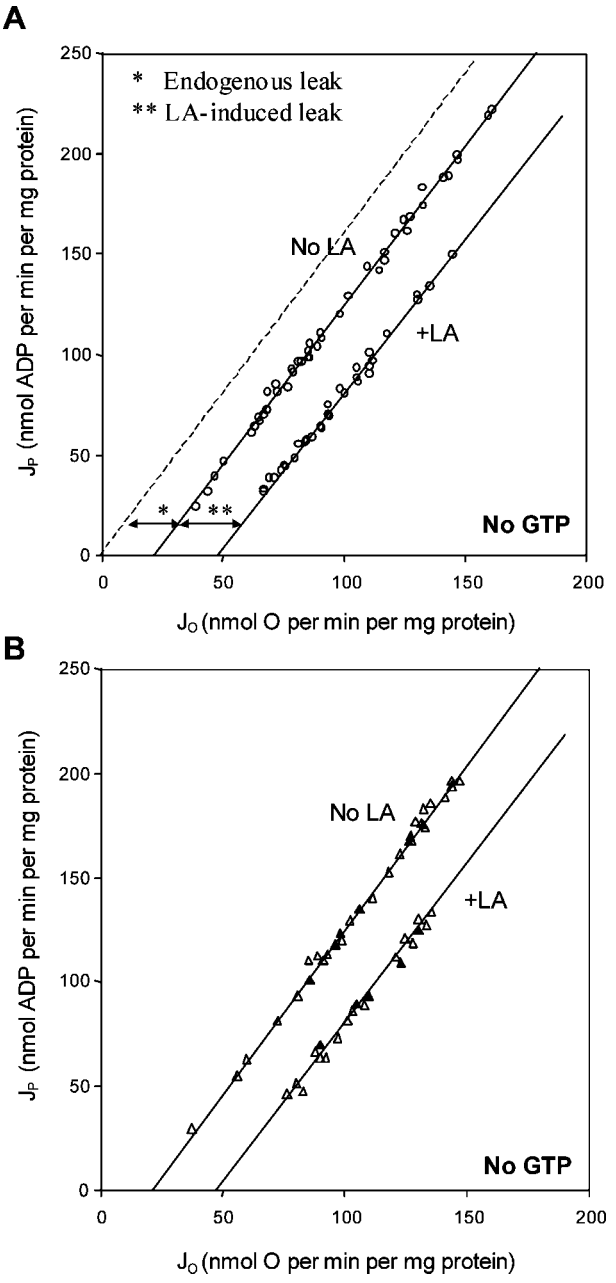

Fig. 2. Relationship between the rate of ADP phosphorylation (J_p) and the rate of oxygen uptake in state 3 respiration (J_o) in the absence of GTP. (A), titration of respiration with *n*-butyl malonate (nBM) from 0 to 1 mM, in the absence (no LA) or presence (+ LA) of 10-μM linoleic acid. Solid straight lines are least square regression lines. (B), titration of respiration with AA (Δ) from 0 to 65 nM or with MX ($\blacktriangle$) from 0 to 80 nM in the absence (no LA) or presence (+ LA) of 10-μM LA. Straight lines are those calculated in (A). In (A) and (B) the respiration sustained by the residual endogenous leak (*) is about 20-nmol O $\times$ min^{-1} $\times$ mg^{-1} protein, and the respiration sustained by the LA-induced leak (**) is around 30-nmol O $\times$ $min^{-1}\times$ mg^{-1} protein. Dotted line is a virtual titration without leak. Data deal with 11 mitochondrial preparations that also provide data for Fig. 3.

Effect of Linoleic Acid During Respiration Titration in the Presence of GTP

In order to check if the LA-induced H^+ leak observed during state 3 respiration can be attributed to muscle UCPs' activation, we have investigated whether this leak is sensitive to purine nucleotides or not. For these assays, we have chosen 2-mM GTP. Titrations of state 3 respiration with nBM show that 2-mM GTP has no effect in the absence of LA [Fig. 3(A)] on J_p *versus* J_o relationship. This means that 2-mM GTP has no protonophoric

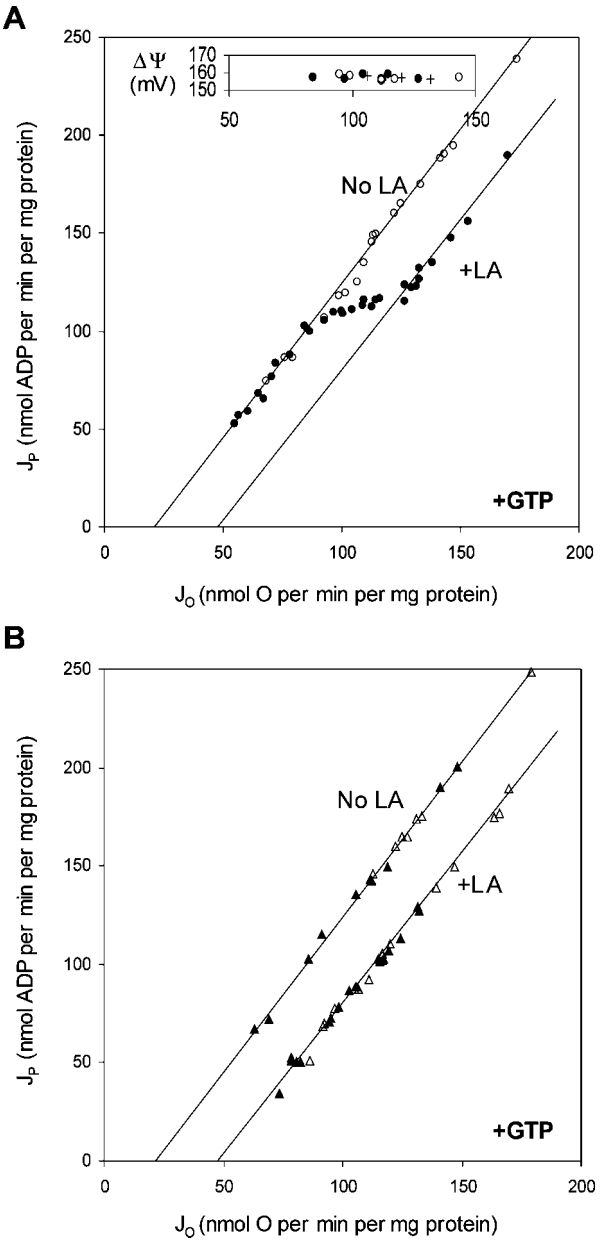

Fig. 3. Relationship between the rate of ADP phosphorylation (J_p) and rate of oxygen uptake in state 3 respiration (J_o) in the presence of 2-mM GTP. Straight lines are those calculated in Fig. 2(A). (A), titration of respiration with nBM in the absence (○) or presence (●) of 10-μM LA. Inset, membrane potential ($\Delta\Psi$) versus J_o in the presence of GTP and in the absence (○) or presence (●) of LA, and in the absence of GTP and presence of LA (+). The mean value of $\Delta\Psi$ was 158 ± 2 mV SD, $n = 20$). (B), titration of respiration with AA (△) or MX (▲) as in Fig. 2(B).

effect by itself and does not affect the intrinsic coupling ratio of the cytochrome pathway (H^+/O) and of the ATP synthase (ADP/ H^+). This finding is very important in order to allocate a possible GTP effect on the FFA-induced proton leak to UCP excluding parasitical effects of this nucleotide on the oxidative phosphorylation system. In the presence of 10-μM LA, the GTP-specific full inhibition of the LA-induced H^+ leak in state 3 respiration is observed at a low J_o obtained with high nBM concentrations, but not at a high J_o obtained without nBM or with low nBM concentrations [Fig. 3(A)]. The inhibitory effect of GTP is a strong indication that the LA-induced H^+ leak can be allocated to the activation of muscle UCPs by LA. The evolution of this inhibitory effect with state 3 respiration rate suggests that the GTP inhibitory efficiency is under the control of a factor that varies with state 3 respiratory rate, i.e., either the inhibitor concentration itself (nBM), membrane potential, or the CoQ redox state.

Control of the GTP Inhibitory Effect

Changes in the membrane potential with the decrease of J_o does not explain the progressive inhibition of the LA-induced H^+ leak in the presence of GTP as $\Delta\Psi$ in state 3 remains constant in the J_o range corresponding to the inhibitory transition of GTP [Fig. 3(A), inset]. Moreover, a possible direct effect of nBM on the LA-induced H^+ leak inhibition by GTP is excluded by the fact that state 4 respiration, enhanced by the presence of 10-μM LA, remains fully insensitive to GTP [Fig. 4(A)] even at the highest nBM concentration. Thus, the LA-induced H^+ leak inhibition by GTP during nBM titration is suspected to be related to CoQ redox state. Indeed, GTP has no effect on the LA-induced leak when state 3 respiration is decreased by complex III inhibitors, AA or MX [Fig. 3(B)], which are known to increase the reduced state of CoQ contrarily to nBM (Jarmuszkiewicz *et al.*, 2002b; Van den Bergen *et al.*, 1994). Figure 4(B) shows the change of CoQ reduction level *versus* state 3 respiratory rate during nBM titration in the presence of GTP (no LA), in the presence of LA (no GTP), or in the presence of both GTP and LA. In these three conditions, titration with nBM decreases the reduced form of CoQ and a single CoQ redox state *versus* J_o relationship is obtained within our titration range. This single relationship clearly demonstrates that the activity of the cytochrome pathway is not affected by LA and/or GTP as nBM acts upstream the cytochrome pathway.

In Fig. 5(A), it is shown that the inhibition by GTP of the LA-induced H^+ leak expressed in H^+ flux (J_{H+}) becomes effective within a narrow range of the CoQ redox state, as the transition between the fully inefficient and fully efficient inhibition by GTP occurs between 64% and 57% of CoQ reduction level. Consequently, the oxidative phosphorylation yield (ADP/O) in the presence of

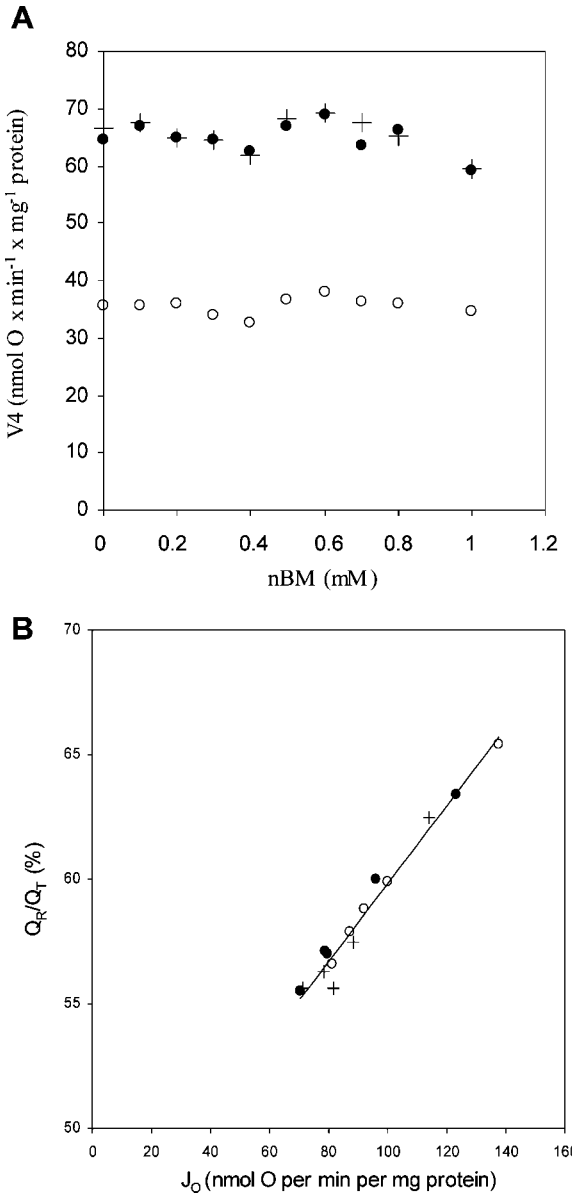

Fig. 4. Influence of nBM concentration on state 4 respiration (A) and variation of reduction level of CoQ with state 3 respiration (B). Measurements in the absence of LA and presence of 2-mM GTP (○), in the presence of 10-μM LA and 2-mM GTP (●), and in the absence of GTP and presence of 10-μM LA (+). (A), State 4 (V4) respiration was measured in the presence of carboxyatractyloside and oligomycin (1 and 2.5-μg mg^{-1} protein, respectively). (B), reduction level of CoQ (Q_R/Q_T) *versus* state 3 respiration (J_o). Data deal with a single representative mitochondrial preparation. The straight line is the least square regression line.

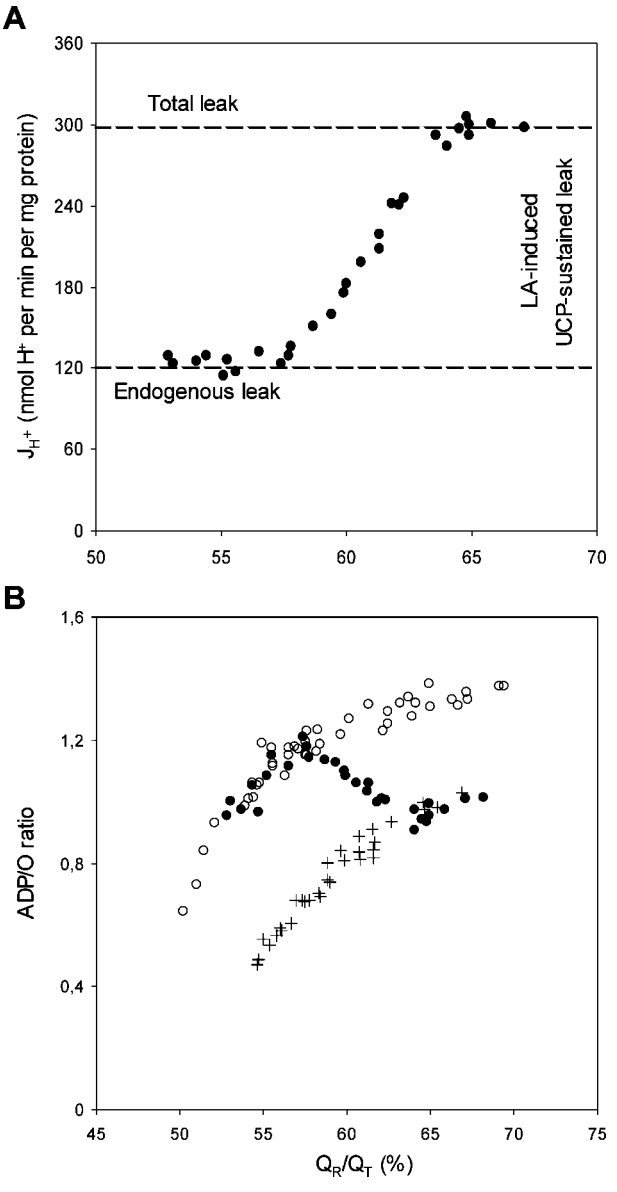

Fig. 5. Influence of CoQ reduction level on the proton leak (A) and on the oxidative phosphorylation yield (B). (A), proton leak rate (J_H^+) *versus* CoQ reduction level (Q_R/Q_T) in the presence of 10-μM LA and 2-mM GTP [from data of Figs. 3(A) and 4(B)]. J_H^+ was calculated from the H^+ leak-sustained respiratory rates and based on the stoichiometric $6H^+/O$ ratio of the cytochrome pathway, *i.e.*, $J_H^+ = 6 \times J_o \times [1-(ADP/O)/1.55]$, where 1.55 represents a slope of regression lines obtained in Fig. 2(A). Horizontal lines indicate the uninhibited total leak (around 300-nmol $H^+ \times min^{-1} \times mg^{-1}$ protein) observed for CoQ reduction level higher than 64 % and the endogenous residual leak (around 120-nmol $H^+ \times min^{-1} \times mg^{-1}$ protein) observed for CoQ reduction level below 57%. (B), ADP/O ratio *versus* Q_R/Q_T in the absence of LA and presence of 2-mM GTP (○), in the presence of 10-μM LA and 2-mM GTP (●), and in the absence of GTP and presence of 10-μM LA (+) [from data of Figs. 2(A), 3(A), and 4(B)].

both LA and GTP is modulated within the same narrow range of CoQ redox state [Fig. 5(B)], i.e., the oxidative phosphorylation yield is lowered by the LA-induced H^+ leak (most likely FFA-activated UCP-sustained) at high

CoQ reduction level and is maximized by GTP inhibition of the LA-induced H^+ leak at low CoQ reduction level.

DISCUSSION

For the first time, we provide a strong support that (i) in isolated muscle mitochondria respiring in state 3, the LA-induced GTP-sensitive UCP activity is able to uncouple the oxidative phosphorylation and that (ii) the inhibition of this FFA-induced UCP activity by GTP could be under control of the redox state of endogenous membranous CoQ.

It must be pointed out that in state 3 respiration the activation of muscle UCPs by 10-μM LA does not decrease the membrane potential [Fig. 1, Fig. 3(A) inset] in a detectable way but has nevertheless an effect on the ATP synthesis yield (ADP/O ratio) due to a high sensitivity of ATP synthase to minute decreases (steep dependence) of $\Delta\Psi$. Respiratory rate in state 3 (Fig. 1) is not increased by LA because the cytochrome pathway is insensitive to such minute decreases in $\Delta\Psi$. Furthermore, the 10-μM LA-induced leak (which is constant) is not sensitive to the minute $\Delta\Psi$ decreases occurring when respiratory rate is decreased by titration with nBM, MX, or AA within the reported inhibition range. Of course, the linear relationships shown in Figs. 2 and 3 must fail at higher inhibition levels because the protonmotive force and consequently the proton leaks will decrease and ultimately vanish together with the phosphorylation rate for nil or close to respirations. Nevertheless, a linear extrapolation till the abscissa (Figs. 2 and 3) provides valuable assessments of the proton leak-sustained respirations (plus or minus 10-μM LA) at the almost constant protonmotive force prevailing in the reported experimental conditions. These proton leak-sustained respirations are lower than state 4 respirations (plus or minus 10-μM LA) that occur at a higher protonmotive force. The fact that proton leaks remain not negligible in state 3 in the absence of LA cannot be predicted *a priori*. For instance, the residual endogenous proton leak of FFA-depleted tomato fruit mitochondria (Jarmuszkiewicz *et al.*, 2000a) and *Acanthamoeba castellanii* mitochondria (Jarmuszkiewicz *et al.*, 1998b) does not contribute significantly to state 3 respiration. In such a case, the ADP/O ratio remains constant during nBM titration, *i.e.*, the J_p versus J_o relationship is a straight line passing through the origin (the dotted line of "virtual titration" in Fig. 1).

As already reported in the Introduction, the contribution of the ADP/ATP, phosphate, and dicarboxylate carriers in the LA-induced uncoupling in state 3 respiration is likely negligible because of the presence of their specific substrates. In this work, we bring a new strong argument against participation of these carriers in the LA-induced uncoupling in state 3 respiration in isolated mitochondria as GTP is able to specifically inhibit the LA-induced proton leak. Indeed, if the ADP/ATP carrier that is known to be able to bind GTP (McKee *et al.*, 2000) was responsible for the GTP inhibited LA-induced H^+ leak through full inhibition of the carrier i.e. when the GTP inhibition is 100%, it would be impossible to measure ADP/O ratio because J_o would be equal to state 4 respiration. The same considerations also exclude the phosphate carrier. On the other hand, if the dicarboxylate carrier was responsible for the GTP effect through full inhibition of the carrier, both J_o and J_p would be null. Concerning the aspartate/glutamate carrier, so far no data show that it is inhibited by purine nucleotides. Thus, we can conclude that the LA-induced proton leak observed in isolated muscle mitochondria is most likely mediated by muscle UCPs.

It could be argued that in our experiments, the presence of 80-μM ATP (to activate the succinate dehydrogenase) and 177-μM ADP (needed for the ADP pulse) would have inhibited almost all activity of UCP. Indeed K_i values lower than 1 μM (Echtay *et al.*, 2001) or around 100 μM (Jaburek and Garlid, 2003) have been reported for ATP and/or ADP when UCP2 and UCP3 were reconstituted into liposomes. Dissociation constants of ATP and GTP bound to isolated UCP3 and/or UCP2 have been reported to be in the 1–6-μM range (Jekabsons *et al.*, 2002; Žácková *et al.*, 2003). Skeletal muscle mitochondria bound GTP with K_d equal to 0.14 μM in the presence of carboxyatractylate preventing binding to the ADP/ATP carrier (Žácková *et al.*, 2003). It is noteworthy that with regard to such high affinities for PN, UCP2 and UCP3 should be almost fully inhibited in the presence of normal cellular PN concentrations, therefore leading to a questionable inefficiency of UCP-mediated uncoupling *in vivo*. As pointed out by Jaburek and Garlid (2003) a physiologically relevant definition of UCP activation should be a reduction of the PN affinities by a biological compound that would interact with the nucleotide binding site of UCP. A physiologically relevant compound could be the reduced CoQ. Indeed when it decreases [when respiratory rates decrease, see Fig. 4(B)] the inhibition efficiency of GTP increases and the activity of muscle UCPs becomes GTP-sensitive when the CoQ reduction level is below 64% (in state 3 respiration) and fully inhibited when it is below 57% (Fig. 5). It is likely that the inhibition of FFA-activated muscle UCPs by ADP and ATP is also under the control of factor(s) decreasing their efficiency and allowing UCPs' activity to occur in our experimental conditions with moderate adenine nucleotide concentrations (80-μM ATP + 177-μM ADP). Alternatively, an unknown "UCP-like" protein could be put forward to

explain our observations. Although, even if not completely excluded, this perspective would only shift the problem. At the present stage of investigation, it is more meaningful to discuss our results as a probable activation/inhibition of muscle UCPs.

The molecular mechanism of the CoQ redox state effect on UCP inhibition by PN has still to be elucidated. Importantly, it is not at all related to a direct effect of CoQ as a UCP "obligatory" cofactor (Echtay *et al.*, 2000, 2001) that has been recently shown to be a result of solvent effects (Jaburek and Garlid, 2003). Furthermore, the activity of UCP1 has been shown to be similar when expressed in CoQ-containing and CoQ-deficient yeast mitochondria (Esteves *et al.*, 2004). In the present study, we show that in isolated muscle mitochondria, the CoQ redox state has no effect at the level of FFA-induced UCP activity in the absence of GTP (Fig. 2) and it only regulates the activity indirectly through the efficiency of inhibition by GTP (Fig. 3). This corresponds to the physiologically relevant definition of UCP activity regulation proposed by Jaburek and Garlid (2003).

According to our findings, muscle UCPs may be active in isolated mitochondria under state 4 respiration, but their activity could be insensitive to GTP due to the high reduction level of CoQ in this resting respiration. Thus, the lack of inhibition of the FFA-induced proton leak by PN observed in mitochondria of various organisms (Jarmuszkiewicz *et al.*, 1999, 2002b) does not necessarily mean that there is no UCP activity (as already mentioned by Jekabsons and Horwitz, 2001). This insensitivity to PN could be simply due to the high reduction level of endogenous CoQ in those mitochondria. In contrast, the UCP2 and UCP3 activities induced through superoxide production (Echtay *et al.*, 2002; Echtay and Brand, 2001) are sensitive to PN at high CoQ reduction level. Surprisingly, FFA are not required for UCP3 activation when superoxide is produced endogenously in the mitochondrial matrix (Talbot *et al.*, 2004). A working hypothesis can be proposed that *in situ* muscle UCPs may have two kinds of active forms that are differently regulated: the FFA-activated form (observed in the present study) and the superoxide-activated form. On the contrary, UCP1 *in situ* is sensitive to PN either activated by FFA or by superoxide (Echtay *et al.*, 2002).

To conclude, our results suggest that CoQ redox state could be the possible "unknown factor" (Žácková *et al.*, 2003) working as a metabolic sensor able to modulate the inhibition of UCPs by PN in muscle mitochondria and that reduced CoQ could decrease the affinity of FFA-activated UCP binding site for PN. What could be the physiological usefulness of such a sophisticated new dimension in the regulation of UCP? We may hypothesize that the UCP-sustained proton leak is inhibited by PN only when the oxidizable substrate availability is low and the ATP demand is high, in order to preserve ATP synthesis efficiency. On the contrary, at no limitation in the substrate supply or at the low ATP demand, the inhibition by PN cannot occur because of the high reduced state of CoQ and, as a consequence of UCP activity, is accompanied by a decrease in superoxide anion production and a nonobligatory nonshivering thermogenesis due to respiratory rate increase.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Belgian FRFC, Fonds Spéciaux ULg, accord communauté Wallonie-Bruxelles/Pologne, the Polish KBN, and by grants from Brazilian agencies: FAPESP, CNPq, PRONEX, FAEP/UNICAMP. R.N. and P.D. received Belgian FRIA fellowships. L.C.A. received master CNPq fellowship.

REFERENCES

Almeida, A. M., Jarmuszkiewicz, W., Khomsi, H., Arruda, P., Vercesi, A. E., and Sluse, F. E. (1999). *Plant Physiol.* **119**, 1323–1330.

Andreyev, A. Y., Bondareva, T. O., Dedukhova, V. I., Mokhova, E. N., Skulachev, V. P., Tsofina, L. M., Volkov, N. I., and Vygodina, T. V. (1989). *Eur. J. Biochem.* **182**, 585–592.

Argyropoulos, G., and Harper, M. E. (2002). *J. Appl. Physiol.* **92**, 2187–2198.

Boss, O., Samec, S., Paoloni-Giacobino, A., Rossier, C., Dulloo, A., Seydoux, J., Muzzin, P., and Giacobino, J.-P. (1997). *FEBS Lett.* **408**, 39–42.

Echtay, K. S., and Brand, M. D. (2001). *Biochem. Soc. Trans.* **29**, 763–768.

Echtay, K. S., Roussel, D., St.-Pierre, J., Jekabsons, M. B., Cadenas, S., Stuart, J. A., Harper, J. A., Roubuck, S. J., Morrison, A., Pickering, S., Clapham, J. C., and Brand, M. D. (2002). *Nature* **415**, 96–99.

Echtay, K. S., Winkler, E., Frischmuth, K., and Klingenberg, M. (2001). *Proc. Natl. Acad. Sci.* **98**, 1416–1421.

Echtay, K. S., Winkler, E., and Klingenberg, M. (2000). *Nature* **408**, 609–613.

Esteves, T. C., Echtay, K. S., Jonassen, T., Clarke, C. F., and Brand, M. D. (2004). *Biochem. J.* **379**, 309–315.

Fleury, C., Neverova, M., Collins, S., Raimbault, S., Champigny, O., Levi-Meyrueis, C., Boillaud, F., Seldin, M. F., Surwit, R. S., Ricquier, D., and Warden, C. H. (1997). *Nat. Genet.* **15**, 269–272.

Gornall, A. G., Bradawill, C. J., and David, M. M. (1949). *J. Biol. Chem.* **177**, 751–766.

Huang, S.-G., Lin, Q.-S., and Klingenberg, M. (1998). *J. Biol. Chem.* **273**, 859–864.

Jaburek, M., and Garlid, K. D. (2003). *J. Biol. Chem.* **278**, 25825–25831.

Jaburek, M., Varecha, M., Gimeno, R. E., Dembski, M., Ježek, P., Zhang, M., Burn, P., Tartaglia, L. A., and Garlid, K. D. (1999). *J. Biol. Chem.* **274**, 26003–26007.

Jarmuszkiewicz, W., Almeida, A. M., Sluse-Goffart, C. M., Sluse, F. E., and Vercesi, A. E. (1998a). *J. Biol. Chem.* **273**, 34882–34886.

Jarmuszkiewicz, W., Almeida, A. M., Vercesi, A. E., Sluse, F. E., and Sluse-Goffart, C. M. (2000a). *J. Biol. Chem.* **275**, 13315–13320.

Jarmuszkiewicz, W., Antos, N., Swida, A., Czarna, M., and Sluse, F. E. (2004). *FEBS Lett.* **569**, 178–184.

Jarmuszkiewicz, W., Behrendt, M., Navet, R., and Sluse, F. E. (2002a). *FEBS Lett.* **532**, 459–464.

Jarmuszkiewicz, W., Hryniewiecka, L., and Sluse, F. E. (2002b). *J. Bioenerg. Biomembr.* **34**, 221–226.

Jarmuszkiewicz, W., Milani, G., Fortes, F., Schreiber, A. Z., Sluse, F. E., and Vercesi, A. E. (2000b). *FEBS Lett.* **467**, 145–149.

Jarmuszkiewicz, W., Sluse-Goffart, C. M., Hryniewiecka, L., Michejda, J., and Sluse, F. E. (1998b). *J. Biol. Chem.* **273**, 10174–10180.

Jarmuszkiewicz, W., Sluse-Goffart, C. M., Hryniewiecka, L., and Sluse, F. E. (1999). *J. Biol. Chem.* **274**, 23198–23202.

Jarmuszkiewicz, W., Sluse-Goffart, C. M., Vercesi, A. E., and Sluse, F. E. (2001). *Biosci. Rep.* **21**, 213–221.

Jekabsons, M. B., Echtay, K. S., Brand, M. D. (2002). *Biochem. J.* **366,** 565–571.

Jekabsons, M. B., and Horwitz, B. A. (2001). *Biochim. Biophys. Acta* **1503**, 314–328.

Ježek, P. (2002). *Int. J. Biochem. Cell Biol.* **34**, 1190–1206.

Ježek, P., Costa, A. D., and Vercesi, A. E. (1997). *J. Biol. Chem.* **272**, 24272–24278.

Kamo, N., Muratsugu, M., Hongoh, R., and Kobatake, Y. (1979). *J. Membr. Biol.* **49**, 105–121.

Klingenberg, M., and Echtay, K. S. (2001). *Biochim. Biophys. Acta* **1504**, 128–143.

Laloi, M., Klein, M., Riesmeier, J. W., Müller-Röber, B., Fleury, C., Bouillaud, F., and Ricquier, D. (1997). *Nature* **389**, 135–136.

Mao, W. G., Yu, X. X., Zhong, A., Li, W. L., Brush, J., Sherwood, S. W., Adams, S. H., and Pan, G. (1999). *FEBS Lett.* **443**, 326–330.

McKee, E. E., Bentley, A. T., Smith, R. M., Jr., Kraas, J. R., and Ciaccio, C. E. (2000). *Am. J. Physiol. Cell Physiol.* **279**, 1870–1879.

Nicholls, D. G., and Rial, E. A. (1999). *J. Bioenerg. Biomembr.* **31**, 399–406.

Samartsev, V. N., Mokhova, E. N., and Skulachev, V. P. (2000). *FEBS Lett.* **412**, 179–182.

Sanchis, D., Fleury, C., Chomiki, N., Goubern, M., Huang, Q. L., Neverova, M., Gregoire, F., Easlick, J., Raimbault, S., Levi-Meyrueis, C., Miroux, B., Collins, S., Seldin, M., Richard, D., Warden, C., Bouillaud, F., and Ricquier, D. (1998). *J. Biol. Chem.* **273**, 34611–34615.

Skulachev, V. P. (1998). *Biochim. Biophys. Acta* **1363**, 100–124.

Sluse, F. E., and Jarmuszkiewicz, W. (2002). *FEBS Lett.* **510**, 117–120.

Talbot, D. A., Lambert, A. J., and Brand, M. D. (2004). *FEBS Lett.* **556**, 111–115.

Tonkonogi, M., and Salhin, K. (1997). *Acta Physiol. Scand.* **161**, 345–353.

Van den Bergen, C. W., Wagner, A. M., Krab, K., and Moore, A. L. (1994). *Eur. J. Biochem.* **226**, 1071–1078.

Vercesi, A. E., Martins, I. S., Silva, M. A. P., Leite, H. M. F., Cuccovia, I. M., and Chamovich, H. (1995). *Nature* **375**, 24.

Vidal-Puig, A., Solanes, G., Grujic, D., Flier, J. S., and Lowell, B. B. (1997). *Biochem. Biophys. Res. Commun.* **235**, 79–82.

Wieckowski, M. R., and Wojtczak, L. (1997). *Biochem. Biophys. Res. Commun.* **232**, 414–417.

Žácková, M., Kramer, R., and Ježek, P. (2000). *Int. J. Biochem. Cell Biol.* **32**, 499–508.

Žácková, M., Škobisová, E., Urbánková, E., and Ježek, P. (2003). *J. Biol. Chem.* **278**, 20761–20769.