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Variations in energization parameters and proton conductance induced by cold adaptation and essential fatty acid deficiency in mitochondria of brown adipose tissue in the rat

Marc Goubern [1], Jocelyne Yazbeck [1], Marie-France Chapey [1], Philippe Diolez [2] and François Moreau [2]

[1] *Laboratoire d'Adaptation Energétique à l'Environnement, E.P.H.E., and* [2] *Laboratoire de Biologie et de Biochimie Cellulaire Végétale, CNRS URA 8, Université P. et M. Curie, Paris France)*

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Male weanling Long-Evans rats were fed on a low-fat semipurified diet (control diet, 2% sunflower oil; essential fatty acid (EFA) deficient diet, 2% hydrogenated coconut oil) for 9 weeks. In order to modulate need for non-shivering thermogenesis, groups of rats on each diet were exposed at 28 °C (thermoneutrality) and at 5 °C (cold acclimation) for the last 5 weeks. In brown adipose tissue (BAT) mitochondria, several parameters of mitochondrial energization, protonmotive force (Δp) and its components ΔpH and membrane potential, $\Delta\psi$, were investigated. Simultaneous measurement of oxygen consumption and $\Delta\psi$ (the main component of Δp) was performed by varying α-glycerophosphate concentration and the force/flux relationship of the mitochondria was established by comparison of proton conductance, C_mH^+, over the whole range of protonmotive force, Δp. In the absence of GDP, at 28 °C, EFA deficiency induced a marked increase in C_mH^+. Cold acclimation led to comparable enhanced C_mH^+ in control and EFA-deficient mitochondria. In the presence of GDP which binds and inhibits the BAT 32 kDa uncoupling protein, C_mH^+ was the same in 28 °C and 5 °C control mitochondria, but EFA deficiency led to an enhanced GDP independent C_mH^+ at 28 °C and to a lesser extent at 5 °C. These results are discussed with reference to substantial changes in mitochondrial lipid composition induced by the deficiency.

Introduction

It is known that mitochondria from brown adipose tissue (BAT) play a crucial role in cold-induced or diet-induced thermogenesis in many mammalian species. In this tissue the respiration rate is controlled by a 32 kDa protein located in the inner mitochondrial membrane which acts as a proton translocator. As a consequence, proton extrusion linked to substrate oxidation is not used to generate a protonmotive force driving ATP synthesis, but is dissipated to produce heat [1].

Since the pioneer work by Wesson and Burr in 1931 [2] it has been established that essential fatty acid (EFA) deficiency enhances energy metabolism. Moreover, essential fatty acid deficient rats show diminished growth, while their food intake remains virtually unchanged. These effects are observed at thermal neutrality (28 °C) where thermogenic need is minimal, as well as in the cold (5 °C) where non-shivering thermogenesis is switched on [3–5]. Previous studies by this laboratory have been focused on the effects of essential fatty acid deficiency on BAT mitochondrial metabolism. Important changes in lipid composition have been shown in mitochondria isolated from essential fatty acid deficient rat [5]. A significant increase in GDP binding on the 32 kDa protein has been found to be a consequence of the deficiency at 28 °C as well as at 5 °C. However, additional parameters of brown adipose tissue activity such as measurement of mitochondrial swelling or respiration rate controlled by purine nucleotides, suggested the possibility of a modified effect of GDP coupling in brown adipose tissue mitochondria from essential fatty acid deficient rats. This point requires further investigation.

Abbreviations: BAT, brown adipose tissue; EFA, essential fatty acid; TPP, tetraphenylphosphonium

Correspondence: M. Goebern, Laboratoire de l'Adaptation Energétique à l'Environnement, 11 Place Marcelin Berthelst, 75231 Paris Cedex 05, France.

To analyse the effects of essential fatty acid deficiency on the bioenergetic properties of BAT mitochondria, several parameters of mitochondrial energization including the protonmotive force Δp and its components ΔpH and membrane potential $\Delta\psi$ were investigated. Further, by varying the rate of proton current and simultaneously measuring membrane potential (the main component of Δp) the flux/force relationships occuring in these mitochondria were established, these provided useful information concerning the proton conductance, C_mH^+, of these organelles.

This contrasts with classical work on cold-induced thermogenesis [6], such an approach having never been used in studies on pharmacologically or nutritionally-induced BAT thermogenesis.

The aim of this research was to determine the main parameters of energy transduction including the proton conductance in brown adipose tissue mitochondria of essential fatty acid deficient rats and to analyse the regulatory effect of GDP. In addition these effects were studied under conditions of both thermal neutrality and cold adaptation.

Material and Methods

Animals and diets

Male weanling Long-Evans rats (average weight 35 g) were used.

Half of the rats received a diet deprived of essential fatty acid (EFA deficient rats) (in % weight): casein 20, methionine 0.1, sucrose 68.4, mineral mixture 5, vitamin mixture 2, cellulose 2.5, hydrogenated coconut oil 2. For control rats, the fat source was sunflower oil. They had access to food and tap water ad libitum. The animals were maintained at 23°C for the first 4 weeks. Then, half of the rats on each diet were exposed for 5 weeks at 28°C (thermal neutrality), the other half at 5°C (cold acclimation). At death, animals were decapitated and exsanguinated. Interscapular brown adipose tissue was rapidly dissected out, weighed and used for experiments.

Methods

Interscapular brown adipose tissue was homogenized in ice-cold sucrose buffer (0.25 M sucrose, 5 mM Tes, 0.1 mM EDTA (pH 7.2)).

Mitochondria were isolated according to Cannon and Lindberg [7]. After the last washing, mitochondria were stored in the sucrose-based medium.

Oxygen consumption and membrane potential were determined simultaneously. Measurements were carried out in a glass vessel (final volume 1.5 ml) in a medium containing 100 mM sucrose, 20 mM K-Tes, 4 mM KH_2PO_4, 2 mM $MgCl_2$, 1 mM EGTA, 1% w/v fatty acid free serum albumin, 5 μM rotenone. 0.5 mg mitochondrial proteins were added per assay. Further additions are detailed in the tables and graphs. Oxygen uptake was measured polarographically with a Clark-type electrode (Hansatech, U.K.) and membrane potential with a laboratory-constructed tetraphenylphosphonium (TPP^+)-selective electrode with a remote Ag|AgCl-saturated reference electrode [8]. The calibration of the TPP^+ electrode was effected with several additions of TPP^+ before addition of mitochondria each addition doubling the concentration of TPP^+ in the medium up to 5 μM. Results were corrected to take into

TABLE I

O_2 consumption, matrix volume, membrane potential $\Delta\psi$, ΔpH gradient and protonmotive force Δp in respiring brown adipose tissue mitochondria of control and essential fatty acid (EFA) deficient rats at 28°C and 5°C

α-Glycerophosphate (2 mM) was used as substrate, O_2 consumption in nmol O_2/min per mg protein. Number of animals or pools in brackets. Significant effect of cold: P 0.05 *, P 0.01 **, P 0.001 ***. Significant effect of EFA deficiency: P 0.05 $^+$, P 0.01 $^{++}$, P 0.001 $^{+++}$. Values are mean ± S.E.

	Thermal neutrality (28°C)		Cold adaptation (5°C)	
	control (5)	EFA-deficient (5)	control (5)	EFA-deficient (5)
Without GDP				
O_2 consumption	23 ±2	27 ±3	37 ±2 **	25 ±2 $^{++}$
Matrix (µl/mg)	1.04±0.05	1.15±0.08	1.17±0.16	1.04±0.03
$\Delta\psi$ (mV)	143 ±4	118 ±2 $^{+++}$	110 ±6 **	100 ±4 **
59ΔpH (mV)	26 ±4	4 ±2 $^{++}$	1 ±0.3 ***	5 ±2
Δp (mV)	169 ±4	122 ±2 $^{+++}$	111 ±6 ***	105 ±4 **
With GDP (1.2 mM)				
O_2 consumption	22 ±3	15 ±2	16 ±2	14 ±2
Matrix (µl/mg)	1.08±0.06	1.42±0.13 $^+$	1.18±0.15	1.26±0.02
$\Delta\psi$ (mV)	176 ±5	152 ±6 $^+$	181 ±4	161 ±4 $^{++}$
59ΔpH (mV)	52 ±2	38 ±2 $^{++}$	43 ±3 *	45 ±2 *
Δp (mV)	228 ±5	190 ±6 $^{++}$	224 ±4	206 ±4 $^+$

account the activity coefficient of TPP^+ in the matrix according to Rottenberg [9], (about 50 mV in our experimental conditions).

The pH gradient was determined by a [^{14}C]sucrose, [^{3}H]acetate dual-isotope modification of the method of Nicholls [10]. 0.5 mg mitochondrial proteins was incubated for 2 min in the medium described above, containing 0.05 μCi of [^{14}C]sucrose and 0.5 μCi of [^{3}H]acetate.

Further additions are detailed in the text. After incubation, aliquots were added to small centrifuge tubes containing silicon oil prepared by mixing Dow Corning silicon fluid 200 and 550 to 1.032 specific gravity and are centrifuged in a Beckman table-top centrifuge. After freezing, the tubes were cut at the level of silicon oil and the bottom fraction (containing the mitochondrial pellet) and top fraction were counted separately. ^{14}C counts were used to determine the extra-matrix contamination of the pellet by incubation medium.

The volume of the matrix compartment of respiring mitochondria was determined to enable assessment of $\Delta\psi$ and ΔpH. A similar radio-isotope distribution was employed [11]. [^{14}C]Sucrose (0.05 μCi) was used to determine extramitochondrial fluid and ^3H_2O (0.5 μCi) to estimate the total water space. The difference between this gives the sucrose impermeable volume which was taken as the matrix volume. Values appear in Table I.

Protein was determined by Lowry's method [12].

To calculate the proton conductance of the mitochondrial inner membrane C_mH^+ (nmol protons per mg protein flowing through the membrane per minute per mV of protonmotive force Δp), proton current J_{H^+} was calculated from the respiratory rate, J_0, on the assumption that six protons were extruded by the respiratory chain per two electrons transferred to oxygen (α-glycerophosphate oxidation). C_mH^+ was calculated according to the following equation:

$$C_mH^+ = \frac{J_{H^+}}{\Delta p} = \frac{J_0(H^+/O)}{\Delta p}$$

where J_0 in nat O/min per mg protein and $\Delta p = \Delta\psi - 59\Delta$pH (in mV at 25°C).

To make comparisons easier, $\Delta\psi$, ΔpH and Δp are always expressed in electrical units (mV).

Data were expressed as mean ± S.E. The significance of the differences between different groups was analyzed using Student's t test.

Results

Basic parameters of the energy transduction process of brown adipose tissue mitochondria exhibiting an essential fatty acid deficiency are presented in Table I. The relative extents of 59ΔpH and $\Delta\psi$ were determined

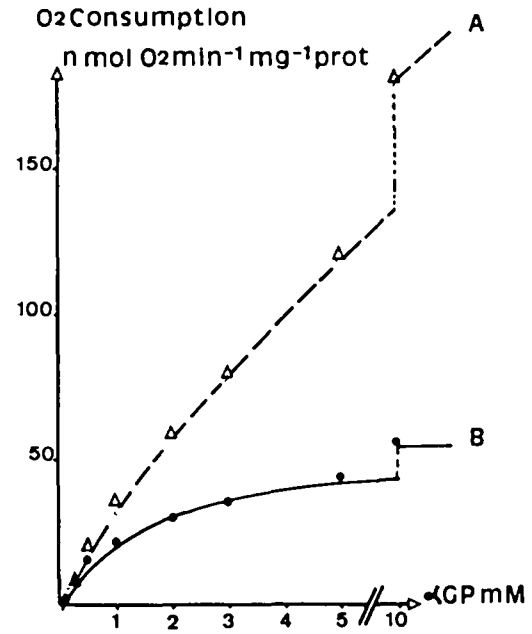

Fig. 1. Variations of α-glycerophosphate (αGP) oxidation rate with substrate concentration in brown adipose tissue mitochondria from the cold-adapted control rat in the presence of EGTA. (A) In the absence of GDP; (B) in the presence of 1.2 mM GDP.

with DL-α-glycerophosphate (αGP) as substrate (2 mM), both in the presence or in the absence of a purine nucleotide (GDP). In addition, matrix volumes are indicated. In the absence of GDP, for rats at thermal neutrality, 59ΔpH was very low in essential fatty acid deficient mitochondria (less than 4% of Δp) whereas this component represented about 15% of Δp in control mitochondria. For cold-adapted rats, no detectable 59ΔpH was observed in control or essential fatty acid deficient rats, thus $\Delta\psi$ represents the unique component of Δp. Compared to control rats at 28°C, Δp was lowered by cold adaptation (34%) and essential fatty acid deficiency (28%). At 5°C, essential fatty acid deficiency did not influence further modifications. In the presence of GDP, both 59ΔpH and $\Delta\psi$ were enhanced in all groups. The contribution of 59ΔpH to Δp represented about 20% in all four groups. However, 59ΔpH, $\Delta\psi$ and Δp were significantly lower in mitochondria from essential fatty acid deficient rats at 28°C (14%, 27% and 17%, respectively) than in 28°C controls.

In the absence of exogenous Ca^{2+} (endogenous Ca^{2+} being chelated with (EGTA), the mitochondrial α-glycerophosphate dehydrogenase exhibits a low affinity for its substrate [13]. Thus, it is possible to modulate respiration by varying αGP concentration from 0.1 to 10 mM as seen in Fig. 1. The effect of 1.2 mM GDP shows that mitochondrial membrane proton permeability is the limiting factor of respiration in these conditions. Fig. 2 shows that 1.2 mM GDP was the optimal concentration to completely block the 32 kDa uncou-

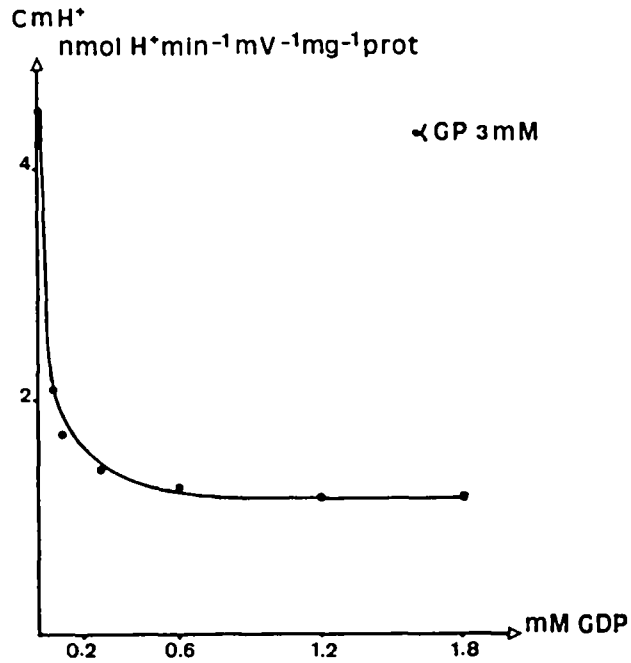

Fig. 2. Proton conductance, C_mH^+, of brown adipose tissue mitochondria from cold-adapted control rat as a function of GDP concentration. C_mH^+ was determined as described in Material and Methods. α-glycerophosphate (3 mM) was used as substrate.

pling protein (no further decrease of proton conductance being obtained for higher concentrations).

Fig. 3 shows representative experiments in which values of αGP oxidation rate were plotted against values of $\Delta\psi$. In the absence of GDP (Fig. 3A), as the respiratory rate increased (from 5 to 125 nmol/O_2 min per mg protein) the steady state of membrane potential increased to a greater extent (about 175 mV) in control rats at 28°C than in the three other groups (about 150 mV). Whatever the oxidation rate, mitochondria from essential fatty acid deficient rats at 28°C showed a relationship between membrane potential and respiratory rate similar to that in organelles isolated from cold-adapted rats, essential fatty acid deficient or not. In these last three groups, the lower values of $\Delta\psi$ obtained over the whole range of αGP oxidation rate indicated high degree of uncoupling of mitochondrial respiration. In the presence of optimal GDP concentration completely blocking 32 kDa protein (Fig. 3B), both higher values of membrane potential and lower oxidation rate were observed in all groups. As expected, the greatest effect of GDP was observed in mitochondria from 5°C rats, whereas the smallest occurred in mitochondria from 28°C control rats, leading to the same relationship. In contrast, GDP did not completely

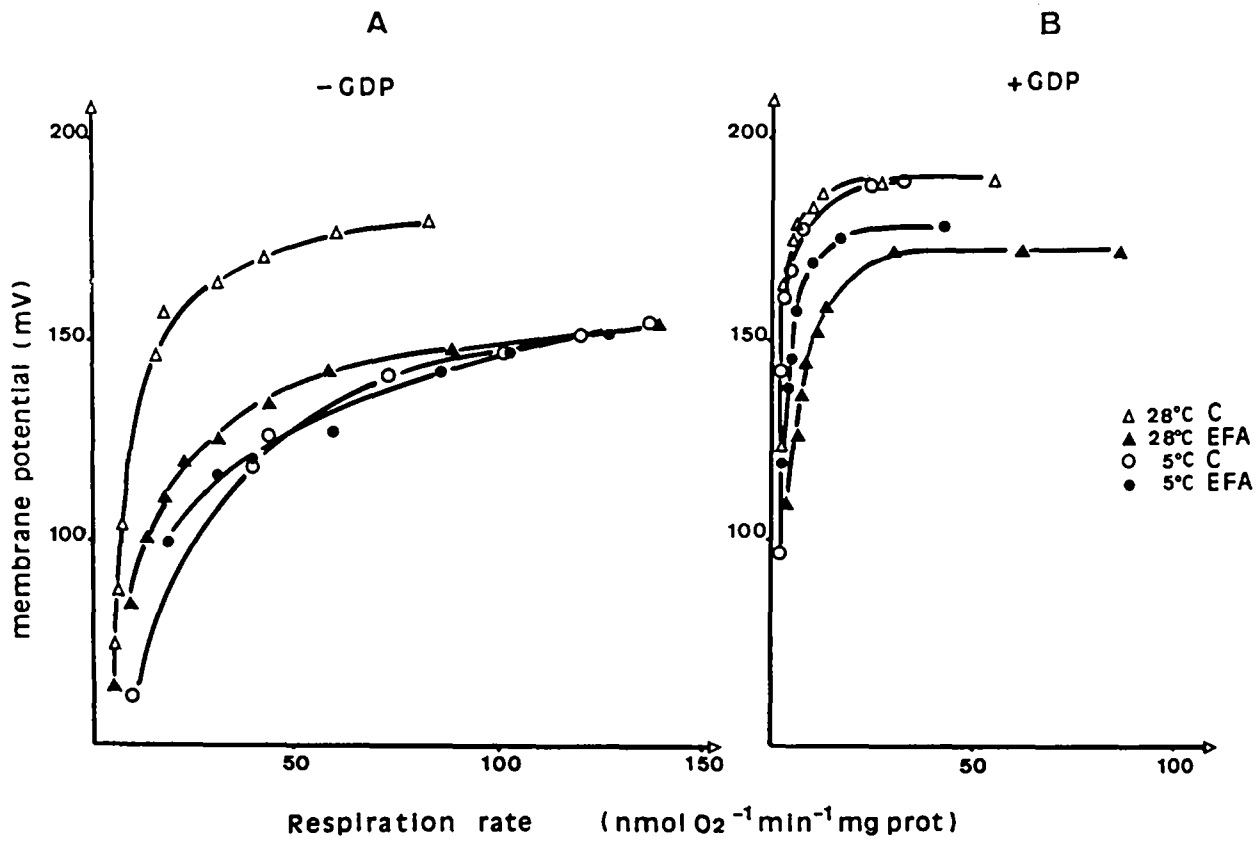

Fig. 3. Representative variations of respiration rate with membrane potential $\Delta\psi$ in brown adipose tissue mitochondria of control (C) and essential fatty acid (EFA) deficient rats at 28°C and 5°C. Respiration rate was modulated with additions of α-glycerophosphate (final concentration 0.1–10 mM). (A) In the absence of GDP; (B) in the presence of 1.2 mM GDP.

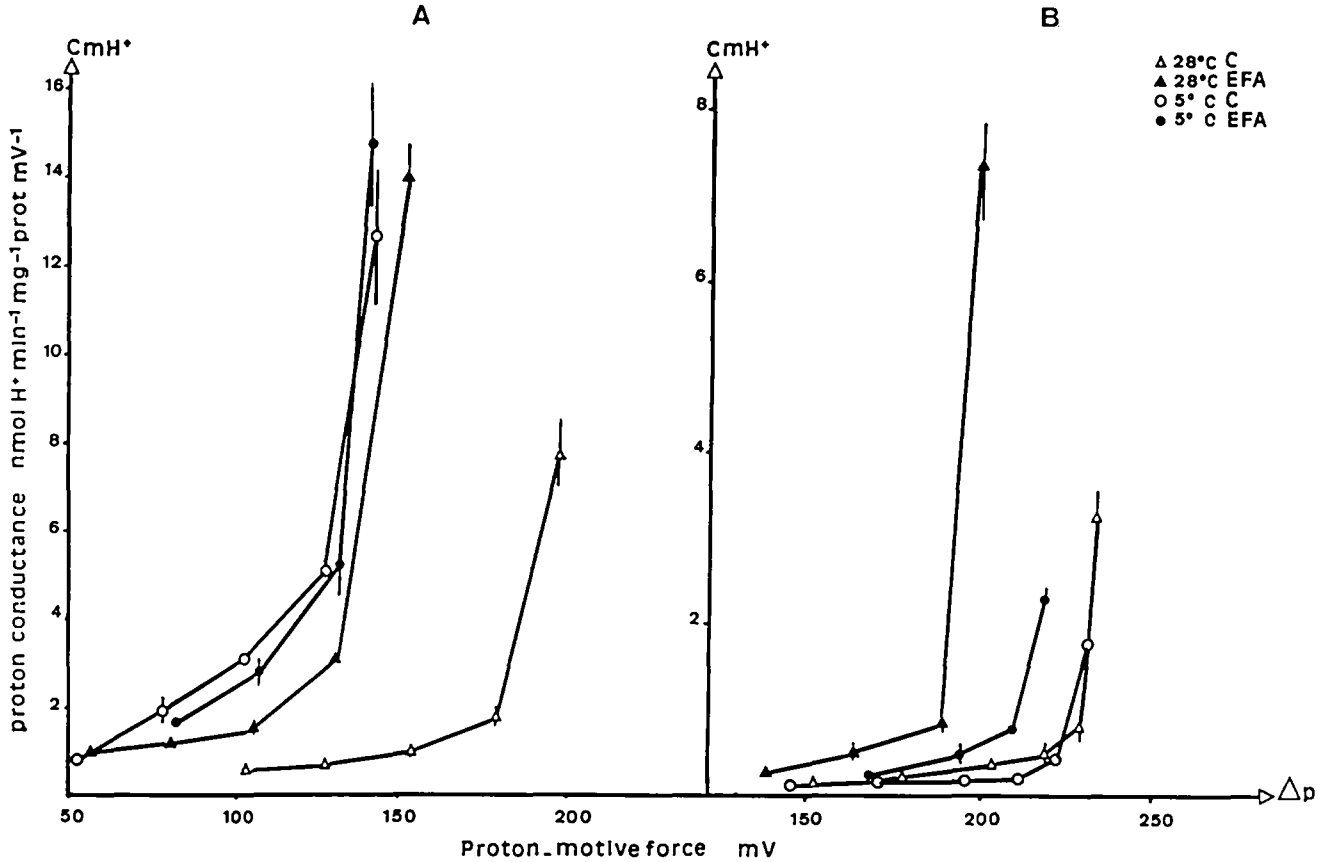

Fig. 4. The variation of proton conductance C_mH^+ with protonmotive force Δp in brown adipose tissue mitochondria of control (C) and essential fatty acid (EFA) deficient rats at 28°C and 5°C. (A) In the absence of GDP; (B) in the presence of 1.2 mM GDP. Five animals or pools per group, values are mean ± S.E.

destore $\Delta\psi$ in mitochondria of essential fatty acid deficient rats.

Using data shown in Table I and Fig. 3, the proton conductance was estimated assuming a constant H^+/O stoichiometry for αGP oxidation.

Since $59\Delta pH$, variations seen as a function of respiratory rate were very small under our experimental conditions (within 5 mV), values of $59\Delta pH$ from Table I were associated with values of $\Delta\psi$ measured as in Fig. 3 to calculate Δp and then C_mH^+. Omitting the very small $59\Delta pH$ variations due to respiration has no significant effect on comparisons between groups.

Fig. 4 shows the relationship between the calculated values of proton conductance, C_mH^+, and those of the protonmotive force, Δp. For all groups of mitochondria, C_mH^+ appeared to be dependent on Δp and was strongly enhanced when Δp reached its maximum value. As expected in the absence of GDP (Fig. 4A), C_mH^+ was higher in mitochondria from 5°C control rats than in 28°C controls over the whole range of Δp. A comparison between the flux/force relationships obtained in the different groups was practicable between 100 and 130 mV, approximately. Within these parameters, it is clear that essential fatty acid deficiency at thermal neutrality enhanced the proton conductance of brown adipose tissue mitochondria to a similar extent as in cold adaptation. In the presence of GDP (Fig. 4B), conductance curves of mitochondria from both 28°C and 5°C controls were superposable and C_mH^+ values were much lower than in the absence of GDP, even for high values of Δp. In contrast, when carried out at 28°C, essential fatty acid deficiency caused enhanced proton conductance over the whole range of Δp. Finally, except for high values of Δp, the proton conductance of mitochondria from essential fatty acid deficient rats was less affected at 5°C than at thermal neutrality.

Discussion

Few works have so far used any measurement of energization parameters to demonstrate the functional effect of the amount of brown adipose tissue uncoupling protein as modified by physiologically or pharmacologically induced changes [14–16]. In this paper, a more comprehensive approach is adopted. The establishment of force/flux relationships which led to the consideration of both GDP-sensitive and GDP-insensitive proton conductance over the whole range of mem-

brane potential clearly shows that proton conductance is enhanced in brown adipose tissue mitochondria of essential fatty acid deficient rats at thermal neutrality and to a lesser extent at 5°C.

Isolated brown adipose tissue mitochondria actively oxidize palmitoylcarnitine (in the presence of malate) or αGP [1]. In this work, αGP was used, although a direct role of this substrate in cold-induced thermogenesis has not been demonstrated. However, the oxidation of the better thermogenic substrate (palmitoylcarnitine) requires a low osmotic KCl medium which may affect the integrity of essential fatty acid deficient mitochondria. In contrast, αGP is oxidized by a dehydrogenase located on the outside of the inner mitochondrial membrane. Thus, there is no requirement for substrate permeation and no modification of matrix volume with subsequent changes in respiration rate [17]. Moreover, αGP is adequately oxidized in a sucrose-based medium with a low potassium concentration, which avoids possible interference with the TPP^+ electrode.

The increased fragility and sensitivity of essential fatty acid deficient mitochondria to isolation procedure has been described for the liver and heart, giving conflicting results as to their properties [18–20]. Isolation of these mitochondria in hypertonic medium which inhibits extensive swelling and structural damage apparently prevented the impairment of oxidative phosphorylation in organelles of these two organs [21,22]. In the case of brown adipose tissue, the use of a classical sucrose-based medium provides a highly condensed matrix. It is likely that such a treatment results in isolated mitochondria of satisfactory quality and that the modification of residual conductance evidenced in this work has functional significance. In electron microscopy, brown adipose tissue mitochondria isolated from the four groups had the condensed matrix and enlarged intracrystal spaces as seen in previous descriptions of warm- and cold-adapted rats [23]. No particular ultrastructural damage was seen in essential fatty acid deficient groups (data not shown).

In a medium containing inorganic phosphate, the contribution of $59\Delta pH$ to Δp is, as predicted, relatively low [24]. However, this parameter shows considerable variations depending on the 32 kDa protein pathway for proton reentry. Opening this pathway by cold adaptation abolishes $59\Delta pH$, whereas its closing by either warm adaptation or GDP addition to mitochondria raises $59\Delta pH$ to about 50 mV. Thus, at first sight, it seems difficult to neglect this component in proton conductance calculation. However, continuous and simultaneous measurements of ΔpH and $\Delta\psi$ were not possible over the whole range of respiration rates studied.

As expected, brown adipose tissue mitochondria from cold-adapted rats exhibit a high rate of substrate oxidation, no detectable ΔpH and a low value of membrane potential. However, in contrast to hamster or guinea-pig mitochondria [7,25], brown adipose tissue mitochondria from 5°C-adapted rats are not completely deenergized and still exhibit some uncoupler-sensitive respiration [4]. This enhanced proton conductance appearing during cold adaptation is entirely GDP-dependent.

An increase of GDP dependent proton conductance occurs in essential fatty acid deficient mitochondria, suggesting that the 32 kDa protein is also involved in the respiration of essential fatty acid deficient mitochondria in rats reared at thermal neutrality. These results are consistent with our previous observations indicating that mitochondria from the 28°C essential fatty acid deficient rat exhibit a high number of GDP binding sites (indicating an increase of 32 kDa protein) and show an increased GDP inhibition in both swelling (induced by valinomycin under non-respiring conditions) and NADH-linked substrate oxidation [3].

At variance, in the cold-adapted rat, essential fatty acid deficiency does not modify this GDP-dependent proton conductance, although an increase of GDP binding sites was shown to exist [5]. However, we have previously shown a marked 33% reduction in O_2 consumption in fully uncoupled mitochondria (by uncoupler FCCP) from essential fatty acid deficient rats, compared with controls (unpublished data). Comparable alterations have been reported for brown adipose tissue of riboflavin-deficient rats [26]. Thus, it is likely that the lower respiration rate (and thus proton current) resulting from these alterations reduces the calculated values of conductance in essential fatty acid deficient cold-adapted rats.

In the presence of GDP which binds and inhibits the 32 kDa uncoupling protein, an additional GDP independent proton conductance can be shown to exist in essential fatty acid deficient mitochondria in rats maintained at thermal neutrality and to a lower extent in cold-adapted rats. This can be tentatively ascribed to an increase in the basal residual conductance of these mitochondria. Such an effect may reflect the great changes occurring in the phospholipid bilayers of the inner mitochondrial membrane [5]. In fact, the large modifications induced by essential fatty acid deficiency in the lipidic environment of proton channels may be the reason for the enhanced basal conductance. It is possible that such nutritional modifications to the lipidic environment of the 32 kDa protein limit the blocking effect of GDP on proton conductance.

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