

# Interaction of adenine nucleotides with the adenine nucleotide translocase regulates the developmental changes in proton conductance of the inner mitochondrial membrane

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2-h-old neonatal liver mitochondria, when depleted of adenine nucleotides, showed an 'ohmic' current-voltage relationship and a higher passive proton permeability of the membrane, resembling fetal mitochondrial behaviors for the proton conductance. Incubation of fetal mitochondria with ATP, GDP or carboxyatractyloside promoted a significant reduction in the passive proton permeability of the membrane and the appearance of the characteristic biphasic behavior for the proton conductance. It is concluded that the postnatal increase in intramitochondrial adenine nucleotide concentration promotes, by the interaction of the nucleotides with the adenine nucleotide translocase, the reduction in the passive proton permeability of the mitochondrial membrane, allowing efficient energy conservation in the neonatal liver.

ATP/ADP antiporter; Adenine nucleotide; Proton conductance; Passive proton permeability; Liver development

## 1. INTRODUCTION

The first hours of life in the tissues of mammalian neonates are characterized by sudden changes in the metabolic pathways relevant for energy provision. Differentiation of mitochondria [1,2] allows the achievement of a functional energy conserving organelle [1] that supplies the required energy for the development of the metabolic pathways and cellular functions needed for successful adaptation to extrauterine life. Postnatal mitochondrial differentiation in rat liver, i.e. the acquisition of the ultrastructural, molecular and functional features of adult mitochondria, is accomplished by the synergistic action of two main processes: (i) a preferential postnatal increase in the rates of protein synthesis for mitochondrial proteins, involved in both the bioenergetic [1] and metabolic functions [3] of the organelle, and (ii) the postnatal increase in intramitochondrial adenine nucleotide concentrations [1,4,5]. The former process seems to be controlled at a translational level [6]. On the other hand, the mitochondrial enrichment in adenine nucleotides, a mechanism independent of protein synthesis [1], promoted the ultra-

structural changes that result in the rapid postnatal matrix volume contraction of the organelle [1]. In addition, it has been suggested that adenine nucleotides interact with the inner mitochondrial membranes to transform these into functional energy-conserving systems [7–9]. This is a reasonable suggestion since the passive proton permeability of the membrane experiences a profound reduction shortly after birth [10]. The aim of the present investigation was two-fold: (i) to demonstrate that the passive proton permeability of the inner mitochondrial membrane is regulated by adenine nucleotides at this stage of development and (ii) to identify the target protein for adenine nucleotide action on the bioenergetic behavior of these membranes.

## 2. MATERIALS AND METHODS

Term newborns ( $5.2 \pm 0.1$  g) were obtained by rapid hysterectomy from cervically dislocated timed-pregnant rats [1]. Liver mitochondria were isolated [1] from 0- and 2-postnatal-h-old newborns.

Protein concentration and mitochondrial respiration, membrane potential ( $\Delta\psi$ ), and ATP+ADP concentrations, were assayed as reported previously [1,10]. Proton currents ( $J_{H^+}$ ) were estimated from the respiratory rates assuming a  $H^+/O$  stoichiometry of 6 [11,12] and were determined simultaneously with electrode measurements of  $\Delta\psi$  [10]. For the determination of  $J_{H^+}-\Delta\psi$  relationships, mitochondrial respiratory rates were titrated against  $\Delta\psi$  by addition of the respiratory inhibitor malonate (0–25 mM) [10]. The effects of 5 min pre-incubation at 0°C of ATP (1  $\mu$ mol/mg protein) + oligomycin (2 nmol/mg protein), GDP (1  $\mu$ mol/mg protein), oligomycin (2 nmol/mg protein) and carboxyatractyloside (10 nmol/mg protein) in  $J_{H^+}-\Delta\psi$  relationships were studied in 0-h-old mitochondria.  $J_{H^+}-\Delta\psi$  relationships were also determined in  $K^+$ -pyrophosphate (0.01, 0.1 and 0.5 mM) adenine nucleotide-depleted [13] mitochondria from 2-h-old rats.

**Abbreviations:** BSA, bovine serum albumin; CAtr, carboxyatractylate;  $\Delta\psi$ , membrane potential; EGTA, [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid;  $J_{H^+}$ , proton respiratory rates;  $L_{H^+}$ , passive proton permeability; UCP, uncoupling protein.

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Estimation of the passive proton permeability ( $L_{H^+}$ ) of the membranes was carried out from the slope of the experimental data that fitted into a line at low values of  $\Delta\psi$  [10,14].

### 3. RESULTS

Malonate titration of respiratory rates against the membrane potential in static head of 2-h-old neonatal liver mitochondria exhibited the characteristic adult 'non-ohmic' behavior for the proton conductance [10] and close to adult values for the estimated passive proton permeability of the membrane [10] (see also insert to Fig. 1). In order to ascertain that the reported developmental changes in  $J_{H^+}$ - $\Delta\psi$  behavior and in the passive proton permeability [10] resulted from changes in the intramitochondrial ATP+ADP concentrations [1,4,5], 2-h-old neonatal mitochondria were incubated in the presence of various  $K^+$ -pyrophosphate concentrations and the  $J_{H^+}$ - $\Delta\psi$  behavior and passive proton permeabilities determined (Fig. 1). This treatment is known to promote an adenine nucleotide translocase-catalyzed exchange of extramitochondrial pyrophosphate for intramitochondrial adenine nucleotides, thus promoting an adenine nucleotide depletion dependent upon the concentration of pyrophosphate present [13]. In agreement with previous findings [13],  $K^+$ -pyrophosphate promoted a concentration-dependent ATP+ADP depletion from 2-h-old neonatal mitochondria (Table I). Under these conditions, when 70% of the intramitochondrial ATP+ADP has been depleted, the proton conductance of the membrane changes from the characteristic biphasic (Fig. 1A) to the 'anomalous' (Fig. 1C) linear behavior reported to occur in fetal mitochondria [10] (see also insert to Fig. 2). These changes resulted in a significant 3-fold increase in the passive proton permeability of the membrane in 2-h-old neonatal mi-

Table I

Effect of adenine nucleotide depletion on ATP+ADP concentration in the 2-h-old neonatal mitochondria

$K^+$ -pyrophosphate (mM)	ATP + ADP (% initial)
None	100
0.01	57 $\pm$ 3*
0.1	40 $\pm$ 1**
0.5	29 $\pm$ 4***

Liver mitochondria (1 mg/ml) from 2-h-old neonates were incubated for 5 min at 37°C in the absence or presence of various  $K^+$ -pyrophosphate concentrations. At the end of incubation mitochondria were isolated by centrifugation and resuspended in the same medium (250 mM sucrose, 1 mM Tris-HCl, 0.3 mM EGTA, 0.2% BSA, pH 7.4) devoid of  $K^+$ -pyrophosphate, for the determination of ATP+ADP concentration. ATP+ADP concentration in 2-h-old neonatal mitochondria incubated in the absence of  $K^+$ -pyrophosphate was  $3.6 \pm 0.6$  nmol/mg protein. The results shown are means  $\pm$  SEM. \* $P < 0.0005$  when compared to undepleted mitochondria. \*\* $P < 0.0025$  when compared to 0.01 mM  $K^+$ -pyrophosphate and \*\*\* $P < 0.025$  when compared to 0.1 mM  $K^+$ -pyrophosphate-depleted mitochondria by Student's *t*-test.

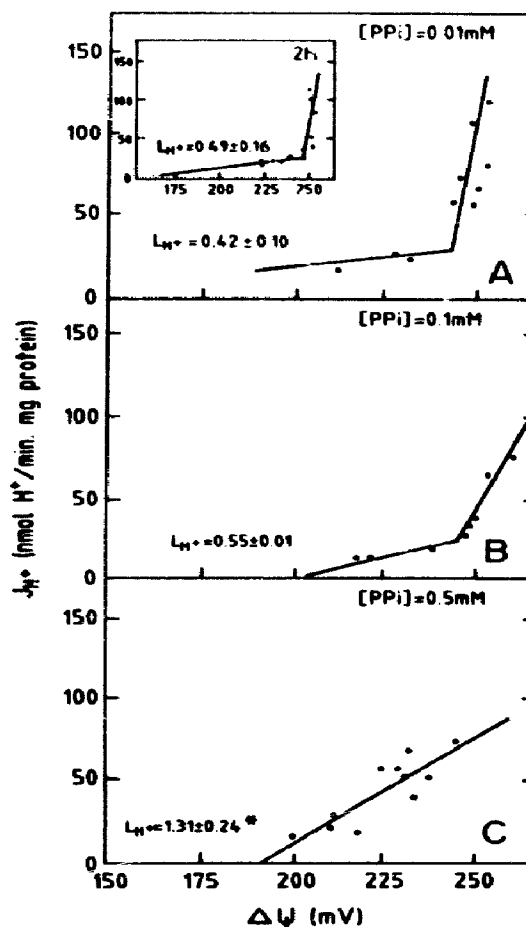


Fig. 1. Effect of adenine nucleotide depletion on the passive proton permeability and proton conductance of the inner mitochondrial membrane in 2-h-old neonatal rat liver mitochondria. 2-h-old neonatal rat liver mitochondria were incubated with various  $K^+$ -pyrophosphate (PPi) concentrations (A, 0.01 mM; B, 0.1 mM; C, 0.5 mM) to promote adenine nucleotide depletion (see legend to Table I). Adenine nucleotide-depleted mitochondria (1 mg/ml) were incubated at 30°C in a standard oxygen electrode buffer with 2.5  $\mu$ M rotenone and 8 mM sodium succinate for the simultaneous determination of state 4 respiratory rates and membrane potentials ( $\Delta\psi$ ). Mitochondrial respiratory rates were titrated by addition of the respiratory inhibitor malonate (0–25 mM) and plotted against  $\Delta\psi$ . Proton fluxes ( $J_{H^+}$ ) were calculated from respiratory rates assuming a constant  $H^+/O$  stoichiometry of 6. Passive proton permeabilities ( $L_{H^+}$ , nmol  $H^+$ /min·mg protein·mV) were calculated from the slope of the experimental data that fitted into a line at low values of membrane potential. The results shown are means  $\pm$  SEM. \* $P < 0.0005$  when compared to 2-h-old undepleted neonatal mitochondria. (Insert in A) The  $J_{H^+}$ - $\Delta\psi$  relationship and  $L_{H^+}$  value for 2-h-old neonatal adenine nucleotide-undepleted mitochondria.

tochondria (Fig. 1), reaching the values reported for this parameter in fetal mitochondria [10] (see also insert to Fig. 2). Further, the maximum proton currents assayed

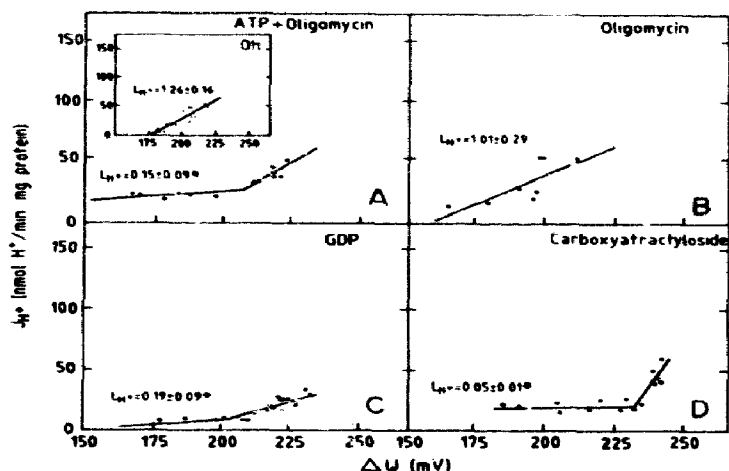


Fig. 2. Effect of the incubation of fetal rat liver mitochondria with ATP, oligomycin, GDP and carboxyatractylide on the passive proton permeability and proton conductance. 0-h-old rat liver mitochondria (1 mg/ml) were incubated with ATP + oligomycin (A), oligomycin (B), GDP (C) and carboxyatractylide (D), as indicated. Mitochondrial respiratory rates were titrated by the addition of malonate (0–25 mM) and plotted against  $\Delta\psi$ . For other details see legend to Fig. 1. Passive proton permeabilities ( $L_{H^+}$ , nmol  $H^+$ /min·mg protein·mV) were calculated. The results shown are means  $\pm$  SEM. \* $P < 0.0005$  when compared to non-incubated 0-h-old neonatal rat liver mitochondria. (Insert in A) The  $J_{H^+}$ – $\Delta\psi$  relationship and  $L_{H^+}$  value for 0-h-old non-incubated fetal liver mitochondria.

showed a significant drop in ATP+ADP-depleted mitochondria (Fig. 1C).

The close parallelism that exists between the developmental changes in intramitochondrial adenine nucleotides [1,4,5] and the rapid postnatal decrease in the passive proton permeability of the membrane [10], together with the results reported so far (Fig. 1, Table I), led us to further study the effect of purine nucleotides and of carboxyatractylide and oligomycin, on the  $J_{H^+}$ – $\Delta\psi$  behavior and passive proton permeability of the fetal mitochondria (Fig. 2). Fetal mitochondria exhibited a linear behavior for the proton conductance and a 6-fold higher value for the estimated passive proton permeability than adult mitochondrial membranes [10] (see also insert to Fig. 2). Incubation of fetal mitochondria with ATP+oligomycin promoted the appearance of the biphasic  $J_{H^+}$ – $\Delta\psi$  behavior in proton conductance and a drastic reduction in the estimated passive proton permeability (Fig. 2A). Static head maximum proton currents were much lower than in 2-h-old neonatal (insert to Fig. 1) or adult [10] mitochondria, in agreement with previous findings [1,10]. The former effects of ATP+oligomycin are due to the nucleotide alone, since oligomycin incubated mitochondria showed no changes for both the  $J_{H^+}$ – $\Delta\psi$  behavior and for the estimated  $L_{H^+}$  (Fig. 2B, compared to insert to

Fig. 2). Interestingly, GDP incubation promoted a reduction in the passive proton permeability of the membrane (Fig. 2C) similar to that obtained with ATP incubation (Fig. 2A); the biphasic behavior in  $J_{H^+}$ – $\Delta\psi$  was also manifested (Fig. 2C). Fetal mitochondria incubated with CATr showed a 25-fold reduction in the passive proton permeability of the membrane (Fig. 2D). The effect of CATr on the  $L_{H^+}$  of fetal mitochondria was even more pronounced than that of ATP or GDP incubated mitochondria (Fig. 2A and C, respectively), reaching a value for  $L_{H^+}$  that was even lower than that reported for adult mitochondria [10]. Further, CATr incubation promoted (i) the appearance of the biphasic behavior in  $J_{H^+}$ – $\Delta\psi$  and (ii) an increase in the membrane potential (Fig. 2D) of the fetal mitochondria, in agreement with recent findings [15–17].

#### 4. DISCUSSION

It has long been suggested that adenine nucleotides affect liver mitochondrial function in the perinatal period [1,5,7–10,18–21]. In fact, it has been claimed that the postnatal enrichment in intramitochondrial adenine nucleotides [1,4,5] acts as the trigger factor of the changes in the properties of the inner mitochondrial membrane, whereby the fetal mitochondria switch from a 'leaky' energy conserving state to a 'non-leaky' neonatal state [7–9]. We have recently shown that during the first hour postnatal, neonatal rat liver mitochondria suffer a profound reduction in the passive proton permeability of their membranes [10], changing from a state of higher energy dissipation of the proton electrochemical gradient (fetal mitochondria) to an almost mature state of energy conservation (1-h-old mitochondria). The results described herein, (a) that adenine nucleotide-depleted mitochondria from 2-h-old rats (Fig. 1) show both a  $L_{H^+}$  and an 'ohmic' proton conductance behavior similar to that of fetal mitochondria, and (b) that fetal mitochondria pre-incubated with ATP (Fig. 2) show both a  $L_{H^+}$  and a biphasic ('ohmic' and 'non-ohmic') proton conductance behavior, similar to that reported for neonatal (Fig. 1, insert) or adult mitochondria ([10] and references therein), strongly support the hypothesis that adenine nucleotides are responsible for regulating the developmental changes in  $L_{H^+}$  and proton conductance [10] of the inner mitochondrial membrane. In addition, the almost complete inhibition of the  $L_{H^+}$  and the appearance of the biphasic behavior for the proton conductance in fetal mitochondria pre-incubated with CATr, a specific inhibitor of the ATP/ADP antiporter, indicate that most, if not all of these changes, are triggered by conformational changes of the adenine nucleotide translocase upon the interaction of the antiporter with the nucleotides.

Mitochondrial changes in  $L_{H^+}$  are a mechanism by which the organisms could optimize heat production or

ATP synthesis according to environmental conditions [22–24]). That is, the higher the  $L_{H^+}$  the higher utilization of the proton electrochemical gradient as a source for heat production. Thus, the findings reported in this paper support Skulachev's proposal for the involvement of the ATP/ADP antiporter in the uncoupling of oxidative phosphorylation [22], a thermogenic mechanism that could operate in mitochondria of those tissues (skeletal muscle and liver) that lack the specific thermogenic protein (UCP) that is found in brown adipose tissue mitochondria [25,26]. This mechanism could play an important thermogenic role in some organisms (birds) [27] or under certain physiological situations (fetal development [10] and acute thermal responses [22–24]) where brown adipose tissue thermogenesis is absent or reduced. The UCP and the ATP/ADP antiporter show structural and, to some extent, functional homology [28] (for reviews see [22,29]). GDP is a regulatory molecule of UCP activity, and in contrast to CATr, has been shown to not inhibit the palmitate-induced stimulation of respiration in mitochondria from skeletal muscle and liver [15]. The surprising finding that GDP pre-incubation of fetal mitochondria promotes a decrease in  $L_{H^+}$  and introduces the characteristic biphasic proton conductance behavior (Fig. 2C), similar to that triggered by ATP (Fig. 2A), further reinforces the functional homology between UCP and the ATP/ADP antiporter as thermoregulatory elements of uncoupled respiration [15–17,22]. At least, under a situation where the adenine nucleotide content of mitochondria is below 30% of its normal level; that is, in the fetal liver [1,4,5] or under in vitro conditions of adenine nucleotide depletion (Fig. 1, Table I).

On the other hand, the sudden proton current increase observed in adult or neonatal mitochondria at high values of membrane potential is believed to be the result of the opening of voltage-gated ion channels in the inner mitochondrial membrane [30,31] to prevent its dielectric breakdown. The disappearance of the 'non-ohmic' behavior for the proton conductance in neonatal adenine-nucleotide-depleted mitochondria (Fig. 1C, Table I) and the appearance of the characteristic 'non-ohmic' current-voltage relationship in ATP or CATr pre-incubated fetal mitochondria (Fig. 2A,D), suggest, first, that adenine nucleotides are regulatory molecules of the activity of such channels, and secondly, that the ATP/ADP antiporter itself is able, under certain conditions (low mitochondrial levels of adenine nucleotides), to form such a channel; an observation in agreement with recent proposals for the activity of the ATP/ADP antiporter [32].

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