Molecular mechanism of uncoupling in brown adipose tissue mitochondria

The non-identity of proton and chloride conducting pathways

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Specific permeability properties of the inner membrane of brown adipose tissue mitochondria were analysed with the aid of simultaneous pH measurements outside mitochondria and of mitochondria swelling. It was shown that valinomycin-induced potassium diffusion potential drives a parallel passive uptake of chlorides and extrusion of proton. Electrogenic H^+-extrusion was independent on anion transport, no competition was found between the two processes and the former process exerted a lower sensitivity to the inhibitory effect of GDP. The existence of two distinct, independent pathways for translocation of protons and halide anions across the membrane is suggested.

Brown adipose tissue *Mitochondria* *Uncoupling protein* *Proton conductance* *Anion conductance* *pH measurement*

1. INTRODUCTION

Thermogenesis in brown adipose tissue (BAT) results from physiological uncoupling of oxidative phosphorylation [1,2]. The energy of respiration-generated electrochemical gradient of protons across the inner mitochondrial membrane is, in BAT, dissipated due to the specific, regulatable conductance for protons [3,4], independent of H^+-ATPase function [5–7]. This pathway is most probably represented by an abundant membrane protein with an M_r of 32000 (uncoupling protein) [8] that binds GDP and other purine nucleotides [9,10] and that is strictly specific for BAT mitochondria [11,12]. The uncoupling protein is also involved in the typical high permeability of BAT mitochondria for chloride and some other anions [13].

The interaction of purine nucleotides with the uncoupling protein decreases both H^+- and halide anion-conductance of isolated BAT mitochondria [3,13,14], while the removal of free fatty acids, which are always present in these mitochondria, specifically diminishes only H^+-conductance [4,13]. It is very likely that free fatty acids serve in vivo as an acute regulator of the rate of energy dissipation [4,15].

The induction of potassium diffusion potential in BAT mitochondria suspended in KCl medium results in intensive swelling due to the Cl^--uptake. In these experiments, chloride was assumed to be the only ion translocated [13,15], in spite of the fact that H^+-extrusion was observed under similar conditions in liposomes [16] or submitochondrial particles [17] containing H^+-conducting channel of H^+-ATPase. Accordingly, it was concluded that in BAT mitochondria passive Cl^--transport competes

Abbreviations: BAT, brown adipose tissue; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; BSA, bovine serum albumin

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with H^+-transport suggesting that both types of ions are translocated via a common channel [2,13,18]. However, a proposal was made recently that free fatty acids induce decoupling between the two processes [15].

In general, the H^+-conductance of BAT mitochondria has so far been evaluated indirectly; e.g., by following mitochondrial swelling [13], respiratory rate [3] and/or membrane potential [4]. Here, direct measurements of pH outside mitochondria (H^+-transport) were performed simultaneously with measurements of mitochondrial swelling (anion-transport) under the conditions of passive movements of ions driven by potassium diffusion potential. The results show that in this system BAT mitochondria accumulate chloride ions and in parallel they extrude protons with comparable rates. Both processes are related to the function of the uncoupling protein, but they are independent and uncompetitive, indicating the existence of two distinct ion-translocating pathways.

2. MATERIALS AND METHODS

Antimycin, CCCP, oligomycin, valinomycin, rotenone, GDP and BSA (fatty acid-free) were purchased from Sigma, all other chemicals were of analytical grade. Syrian hamsters (120–150 g) exposed for 3–4 weeks to 5°C were used for the preparation of BAT [19] and liver mitochondria [20].

Passive transport of anions (swelling, [13]) and protons (pH measurements) in isolated mitochondria were followed at 25°C in the same cuvette of a Perkin Elmer MPF-3 fluorometer equipped with an additional analogue output and mixing device. H^+-transport was measured with the aid of a combined pH electrode (Beckmann no.39505) connected to a mV-meter (Keithly 614 electrometer). The overall time response of the apparatus (90% change) was below 1 s. For calibration, 5 nmol HCl were added at the end of each trace. Swelling was followed as a decrease of light scattering at 540 nm (3 nm slits on both monochromators, two polarising filters used in parallel orientation). Both signals (pH, light scattering) were recorded simultaneously using a two-channel chart recorder. All experiments were performed in salt media of comparable osmotic properties (150 mM KCl or 110 mM K_2SO_4) containing 8 μM rotenone, 1 μM antimycin, 2.5 μM oligomycin. During the 15 s after the addition of mitochondria (final conc. 1 mg protein/ml) the pH of the medium was adjusted to 7.0 (by HCl, H_2SO_4 or KOH). The reaction was started 60 s after the addition of mitochondria by 0.5 μg valinomycin or nigericin/ml. The final volume was 2.2 ml.

The kinetic parameters of both swelling and pH measurements were calculated as shown in fig.1B: The initial rate (V_o; pH measurements – nmol $H^+ \cdot min^{-1} \cdot mg\ protein^{-1}$; swelling – % of light scattering change due to the addition of mitochondria/min, as in [13]) was obtained by subtraction of the rate before the addition of ionophore (a) from the rate after its addition (b); the extent value (c) refers to the maximum change of pH or light scattering due to the addition of ionophore (the change due to the basic drift, a, is subtracted); $T_{1/2}$ (d) refers to the time required to reach half of the extent value (c).

Protein was determined [21] using BSA as a standard.

3. RESULTS AND DISCUSSION

Fig.1 shows typical simultaneous measurements of passive proton and anion movements as induced

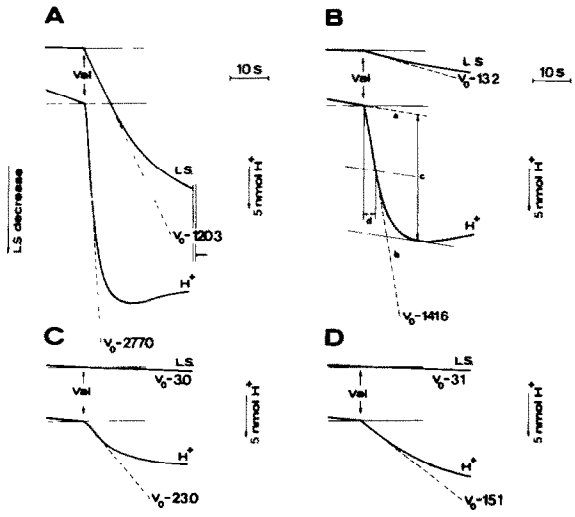

Fig.1. Parallel measurements of valinomycin-induced Cl^--uptake (changes of light scattering; L.S.) and H^+-extrusion (H^+) of BAT (A,B) and liver (C,D) mitochondria suspended in KCl (A,C) or K_2SO_4 (B,D,) media.

by potassium diffusion potential in BAT and liver mitochondria. The combination of direct pH measurement and parallel recording of light scattering revealed that in BAT mitochondria suspended in 150 mM KCl valinomycin caused extrusion of protons and uptake of chloride ions (fig.1A). The initial rates (V_o) of both processes were faster than those induced under identical conditions in liver mitochondria (fig.1C). Similarly, the extent of both processes was much higher in BAT mitochondria than in liver mitochondria (fig.1A,C). These results thus confirm the existence of a typically high Cl^--conductance of BAT inner mitochondrial membrane [13]. In addition, they demonstrate that in BAT mitochondria valinomycin-induced potassium diffusion potential is compensated for not only by Cl^--uptake, but also by H^+-extrusion. Of the two phenomena, only the former is fully consistent with the proposed model [13,15] of the electrogenic movements of ions across the membrane under these conditions.

The addition of valinomycin to the suspension of BAT mitochondria in the K_2SO_4 medium also resulted in a substantial H^+-extrusion (fig.1B). In contrast, both the V_o and the extent of valinomycin-induced H^+-movements were again very low in liver mitochondria (fig.1D). As far as the anion transport in the K_2SO_4 medium is concerned, there was practically no stimulation of swelling by valinomycin in both BAT (fig.1B) and liver (fig.1D) mitochondria indicating low permeability for sulfate ions in both types of mitochondria.

The fact that H^+-extrusion specific for BAT mitochondria is induced by valinomycin both in the presence (fig.1A) and absence (fig.1B) of anion transport indicated that the two processes should be independent. This proposal is further supported by the different time courses of Cl^-- and H^+-transport in KCl medium (fig.1A). Based on $T_{1/2}$ values of the two processes (H^+-extrusion, $T_{1/2} = 2.0 \pm 0.3$ s; Cl^- uptake, $T_{1/2} = 20.0 \pm 1.5$ s), the H^+-extrusion is completed during a 10-times shorter time period than the parallel Cl^--uptake.

Regarding the nature of valinomycin-induced H^+-extrusion in BAT mitochondria (H^+-ATPase and respiratory chain enzymes were inhibited in all experiments) it is to be stressed that the H^+-movements were intensive enough (table 1A,D) to represent the established H^+-conductance of BAT mitochondria. Thus the V_o values of H^+-extrusion in the KCl medium (table 1A, 305.0 ± 70.0 nmol $H^+ \cdot min^{-1} \cdot mg\ protein^{-1}$) were significantly higher than comparable rates of Cl^--uptake (70 nmol $\cdot min^{-1} \cdot mg\ protein^{-1}$) as calculated in [13] under similar experimental con-

Table 1

Initial rates of H^+-extrusion from BAT mitochondria in the KCl and K_2SO_4 media

Experimental conditions	V_o (nmol $H^+ \cdot min^{-1} \cdot mg\ protein^{-1}$)	No. of experiments
A 150 mM KCl; 0.5 μg valinomycin/ml	305.0 ± 70.0	8
B + 200 μM GDP	33.8 ± 5.2	4
C + 2 mg BSA/ml	86.9 ± 3.7	4
D 110 mM K_2SO_4; 0.5 μg valinomycin/ml	141.0 ± 12.0	10
E + 200 μM GDP	20.0 ± 11.0	4
F + 2 mg BSA/ml	35.5 ± 4.1	5
G + 0.5 μM CCCP	188.0 ± 42.0	4
H + 0.5 μM CCCP; 200 μM GDP	191.0 ± 28.0	4
I + 10 μM CCCP	200.0 ± 73.0	5
J + 10 μM CCCP; 200 μM GDP	172.0 ± 45.0	4
K + 10 μM CCCP; 2 mg BSA/ml	155.0 ± 21.0	2
L 110 mM K_2SO_4; 0.5 μg nigericin/ml	269.0 ± 48.0	8
M + 200 μM GDP	407.0 ± 105.0	5

A/B, A/C, A/D, D/E, D/F, I/K, L/D, $p < 0.001$; D/G, $p < 0.01$; D/I, L/M, $p < 0.05$

ditions. Furthermore, either in the presence of permeable (Cl^-) or impermeable (SO_4^{2-}) anion the V_o of H^+-extrusion was inhibited to about 25 and 10% by 2 mg BSA/ml (maximal effect) and 200 μM GDP, respectively (table 1B,C,E,F). The inhibition by GDP was fully abolished by the exogenous protonophor (CCCP), whether the uncoupler was added before or after valinomycin (table 1H,J; fig.2A,B). Similarly, BSA was ineffective in the presence of CCCP (table 1K). Therefore, the sensitivity of valinomycin-induced H^+-extrusion to BSA and GDP indicates clearly that the process measured here is related to the function of the specific H^+-conducting pathway involved in the physiological uncoupling of BAT mitochondria [3,4]. Importantly, CCCP further increases the basic control V_o values of H^+-extrusion (table 1D,G,I) which proves that in the present measurement of H^+-conductance, valinomycin-mediated K^+-translocation is not rate limiting.

The addition of nigericin instead of valinomycin to BAT mitochondria suspended in KCl (not shown) or in the K_2SO_4 medium (fig.2C; table 1L) also caused H^+-extrusion which, however, was clearly related to the K^+/H^+ exchange mediated by the ionophore proper. Correspondingly, the addition of GDP before nigericin had no inhibitory effect on H^+-extrusion (table 1M; Fig.2C). In fact, a rather slight stimulation of the V_o of H^+-extrusion was observed, most probably due to the inhibition of H^+-backflow via the uncoupling protein.

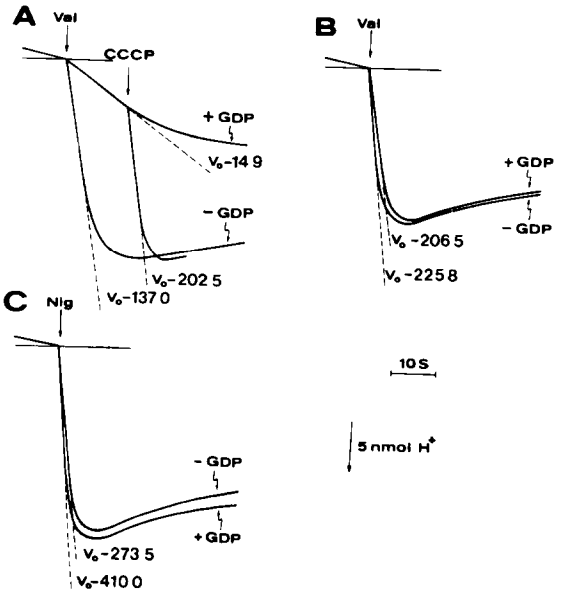

Fig.2. The effect of GDP (200 μM) on H^+-extrusion induced by valinomycin (A,B) or nigericin (C) in BAT mitochondria suspended in K_2SO_4. CCCP(0.5 μM) was added as indicated (A), or was included in the incubation medium (B).

On the basis of indirect measurements of electrogenic H^+- and Cl^--movements in non-respiring BAT mitochondria it has been suggested [2,18] that the two processes are independent. The results of this study are in accordance with such a proposal, however, they are not compatible with the suggested competition between Cl^-- and H^+-transport [13,15,22] when free fatty acids are removed from isolated BAT mitochondria. As is apparent from fig.1 and table 1A,D the V_o value of valinomycin-induced H^+-extrusion from BAT mitochondria were even lower in the K_2SO_4 medium than in the KCl medium, where the potassium diffusion potential is also compensated for by a simultaneous Cl^--uptake. This indicates that the two processes, electrogenic Cl^--uptake and H^+-extrusion are not competitive. As this difference between the two types of media was found both in the presence and absence of BSA (table 1A,C,D,F), it is also not necessary to assume any decoupling of the two processes caused by free fatty acids [15].

It is well established that purine nucleotides bind directly to a specific M_r 32000 protein of BAT mitochondria [9,10] and that the binding can be modulated by free fatty acids [15]. As these ligands influence both the H^+- and Cl^--translocation in BAT mitochondria, it should be apparent that the uncoupling protein is involved in both processes. However, the kinetics data presented here indicate that the two processes are not mutually related. Furthermore, the two processes are also differently sensitive to a common regulatory ligand, the purine nucleotide. As shown in fig.3, the Cl^--uptake is more sensitive than H^+-extrusion (apparent K_i = 2.2 and 6.0 μM GDP, respectively). Thus for example, at 5 μM GDP when Cl^--uptake is practically inhibited H^+-transport still retains at least 60% of its activity.

As stated above, the high H^+-conductance of BAT mitochondria has an apparent key role in the mechanism of BAT thermogenesis while the

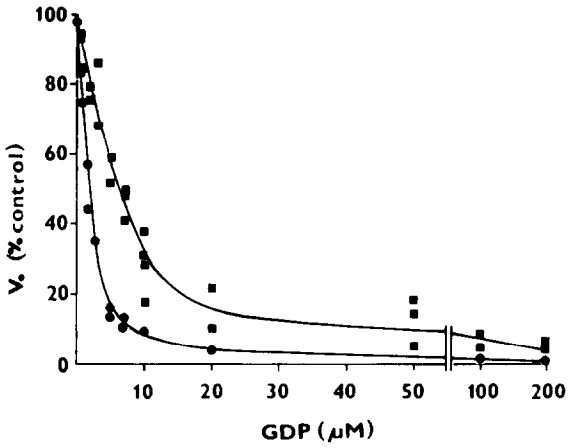

Fig.3. Valinomycin-induced swelling (●—●) of BAT mitochondria and H^+-extrusion (■—■) as a function of GDP concentration.

physiological importance of the high conductance for halide anions remains unclear. It is thus reasonable to conclude that H^+- and Cl^--conducting pathways of BAT mitochondria are formed by two independent transport entities which could be represented either by two-state transition of one channel or by two distinct types of channels. Interestingly, this assumption could also be helpful to understand why purine nucleotide inhibition of Cl^--transport, but not of H^+-transport, is abolished by acyl-CoA esters [4,23] and, inversely, why purine nucleotide inhibited H^+-transport but not Cl^--transport is reactivated by free fatty acids [4,23].

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