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Activation of respiration and loss of thermodynamic control in hyperthyroidism

Is it due to increased slipping in mitochondrial proton pumps?

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T_3 administration increases the extent of non-linearity in the flow–force relationship between pump proton conductance and protonmotive force. The effect is present also at the ATPase proton pump. These effects are not accompanied by changes in passive proton conductance. Incubation of mitocondria at 45°C also causes an increased non-linearity, accompanied by a partial increase of proton conductance. It appears that the increase of respiratory activity following T_3 administration is due to loss of thermodynamic control *within* or *at* the proton pumps, an effect which might be attributed to increased slipping.

Mitochondria; Hyperthyroidism; Temperature; Thermodynamics; Uncoupling; Slipping

1. INTRODUCTION

The mechanism by which thyroid hormones cause an increase of respiratory activity, and of basal metabolic rate in the organism, has remained an unsolved problem in spite of the fundamental progress in the field of energy coupling. While earlier research had considered thyroid hormones as *uncouplers*, the concept of thyroid hormones as regulators of the physiological *uncoupling* has been largely rejected [1] also in view of the evidence of an increased content of respiratory enzymes after T_3 administration.

Shears and Bronk [2] reported a correlation between increase of state 4 respiration and of Δp in mitochondria from T_3-treated rats. Since the relationship between respiration and Δp has both a linear and a non-linear region, it was postulated that the increase of Δp was due to the inhibition of the linear proton conductance pathway of the mitochondrial inner membrane and to the parallel increase of the non-linear proton conductance pathway. Brand et al. [3–5] found a decrease and an increase of the state 4 respiration and an opposite change in Δp in mitochondria from hypothyroid and hyperthyroid rats, respectively. They concluded that the mechanism of action of thyroid hormones is not concerned primarily with the amount of respiratory chain per mitochondrion but rather, with the slip/leak characteristics of the mitochondrial inner membrane. However, Hafner and Brand [6] have later negated the existence of slips as means of physiological energy regulation and hence their role in the hormonal effect.

The reason for the non-linearity of the respiration–Δp relationship at high Δp first discovered by Nicholls [7] has been a matter of great controversy in the last 10 years. Two interpretations have been confronting, namely of unspecific membrane proton leaks and of more specific slips in the pumps [8,9]. Since the change of the flow– force relationships in hyperthyroid rats occurs in the region of non-linearity, the understanding of the mechanism of the hormonal regulation requires the clarification also of that of the non-linearity.

We shall report below, data on the changes of flow–force relationships occurring in mitochondria from T_3-treated rats or incubated at high temperatures. We suggest that the increase of protein synthesis following T_3 administration is likely to be accompanied by an increase of the degree of slipping in the mitochondrial proton pumps.

Abbreviations: T_3, 3,3,5-triiodo-L-thyronine; Δp, proton-motive force; $\Delta\Psi$, transmembrane electrical potential gradient, or 'Nernst diffusion potential'; J_o, rate of respiration in state 4; J_{K+}, rate of K^+ efflux; J_{ATP}, rate of ATP hydrolysis; Ap_5A, P^1, P^5-di(adenosine-5')-pentaphosphate.

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2. EXPERIMENTAL

Hyperthyroidism was induced in male Wistar rats (approximately 300 g) by oral administration of 15 μg T_3/100 g body mass for 10 days [10]. Rats were deprived of food 18 h prior to the sacrifice. Liver

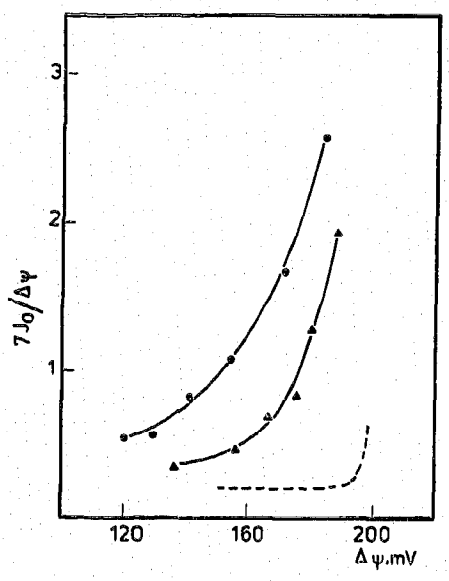

Fig. 1. Relationship between redox proton pump conductance and $\Delta\Psi$ in normal mitochondria at 45°C (●) and in mitochondria isolated from T_3-treated rats (▲). Mitochondria (1 mg/ml) were incubated in standard incubation medium. After 2 min, increasing concentrations of malonate (0–5 mM) were added and the rate of oxygen consumption and $\Delta\psi$ were measured. Dashed line represents the relationship as obtained from normal mitochondria incubated at 25°C.

mitochondria were prepared simultaneously from euthyroid and hyperthyroid rats according to standard procedures [11] in a medium containing 0.25 M sucrose, 10 mM Tris, 0.1 mM EGTA. The mitochondrial protein was assayed with the biuret method using serum albumin as standard. All the experiments were performed within 4 h of preparation. The standard medium used contained: 0.2 M sucrose, 30 mM Mops/Tris, 5 mM succinate, 5 mM P_i, 0.2 mM EGTA/Tris, 5 μM rotenone, 1 μg/mg oligomycin, pH 7.4. Other media used are indicated in the legends of figures. The respiratory rate of the redox chain and the transmembrane electrical potentials were determined as essentially described in Luvisetto et al. [12]. The calibration of the oxygen electrode as well as the determination of the oxygen concentration in the reaction media for each different temperature was carried out by oxidizing a known amount of spectrophotometrically standardized NADH in the presence of beef-heart submitochondrial particles [13]. The following average concentrations of oxygen were found: 474 and 346 nmol O/ml at 25 and 45°C respectively. The passive proton flow through the mitochondrial membrane was determined by measuring the initial rate of K^+ efflux upon addition of valinomycin to antimycin- inhibited mitochondria, in the presence of increasing amounts of K^+ in the external medium. The K^+ diffusion potentials were estimated by using the same procedure as described in Zoratti et al. [14]. Based on the null-point technique [14], the concentrations of intramitochondrial $[K^+]_i$ were determined as comprised in the range 100–120 mM both in normal rats or in hyperthyroid rats. The rate of ATP hydrolysis was measured by standard enzymatic methods as described in Luvisetto et al. [15]. To determine the P/O ratio, the rate of ATP synthesis was measured as essentially described in Luvisetto et al. [15]. All reagents were of maximal purity commercial grade. T_3, enzymes, inhibitors and valinomycin were purchased from Sigma.

3. RESULTS

Mitochondria isolated from T_3-treated rats or incubated at high temperature show both a marked increase of the state 4 respiratory rate with only little depression of the protonmotive force. Fig. 1 shows the effect of T_3 administration and of incubation of mitochondria at 45°C on the relationship between the redox proton pump conductance ($7J_o/\Delta\Psi$) and $\Delta\Psi$ as measured on the combined redox pumps at sites II and III. It is seen that in T_3-treated rats, and more markedly during the incubations at high temperature, there was a considerable enlargement of the extent of non-linearity, with values of $7J_o/\Delta\psi$ becoming several times higher with respect to those of normal mitochondria particularly at high $\Delta\Psi$. As will be discussed elsewhere the effect of T_3-treatment on the flow–force relationships of the various redox pumps is not equivalent.

Fig. 2 shows the effect of T_3 administration and of the incubation of mitochondria at 45°C on the passive proton conductance ($J_{K+}/\Delta\Psi$) as measured at increasing membrane potentials in valinomycin-treated, anaerobic mitochondria. It is seen that while T_3 administration caused little or negligible increase of passive proton conductance, incubation at high temperature did cause a marked increase of proton conductance both in the linear and in the non-linear region. The comparison between the results of Figs. 1 and 2 indicates that T_3-treatment and high temperature stimulate the state 4 respiratory rate by different mechanisms, without and with an involvement of the passive proton conductance in the former and in the latter case, respectively.

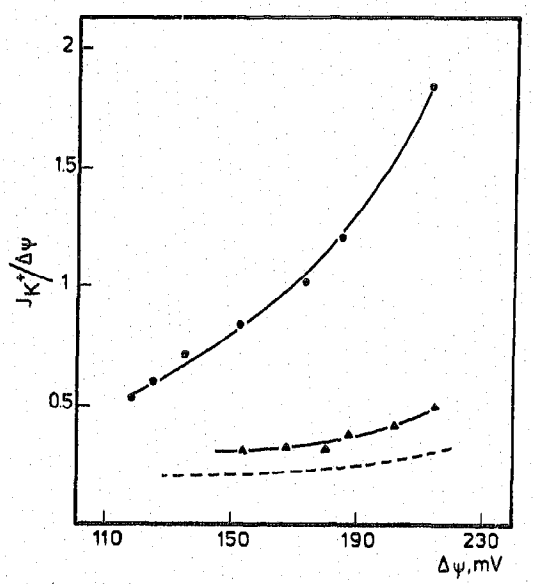

Fig. 2. Relationship between membrane proton conductance and $\Delta\Psi$ in normal mitochondria at 45°C (●) and in mitochondria isolated from T_3-treated rats (▲). Mitochondria (1 mg/ml) were incubated in the standard incubation medium in the presence of increasing concentrations of K^+ (30–500 μM) for 2 min. Antimycin (50 ng/mg) and, after 3 sec, valinomycin (150 ng/mg) were then added and the initial rate of K^+ efflux was measured. $\Delta\Psi$ represents the K^+ diffusion potential, $(RT/nF)\ln[K^+]_i/[K^+]_o$, calculated by considering $[K^+]_i$ equal to 100 mM in normal mitochondria at 25 and 45°C, and 120 mM in mitochondria isolated from T_3-treated mitochondria, respectively. Dashed line represents the relationship as obtained in normal mitochondria at 25°C.

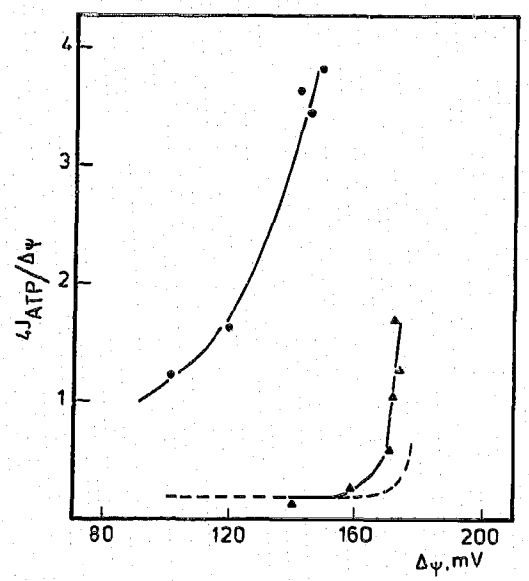

Fig. 3. Relationship between ATPase proton pump conductance and $\Delta\Psi$ in normal mitochondria at 45°C (●) and in mitochondria isolated from T_3-treated rats (▲). Mitochondria (1 mg/ml) were incubated in the standard incubation medium supplemented with 2 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 0.1 mM NADH, excess of pyruvate kinase and lactate dehydrogenase. Experimental procedure as in Fig. 1 with ATP (3 mM) instead of succinate (5 mM). Dashed line represents the relationship as obtained in normal mitochondria at 25°C.

Fig. 3 shows the effect of T_3 administration and of the incubation of mitochondria at 45°C on the relationship between the ATPase proton pump conductance ($4J_{ATP}/\Delta\Psi$) and $\Delta\Psi$. It is seen that while T_3 administration resulted in a modest, although constant and well appreciable, increased extent of non-linearity, the incubation at high temperature resulted in a very marked increase of non-linearity.

Finally in Table I are reported the P/O ratios measured in the same mitochondria assayed for the flow–force relationships reported in Figs. 1–3. It is seen that the P/O ratio was diminished less than 20% (and in many cases even less than that) after T_3 administration and about 30% during incubation at high temperature.

Table I

P/O ratios

Mitochondria	t (°C)	P/O
euthyroid rats	25	1.5 ± 0.1
euthyroid rats	45	1.0 ± 0.1
hyperthyroid rats	25	1.3 ± 0.1

Mitochondria (1 mg/ml) were incubated in the standard incubation medium supplemented with 40 μM Ap_5A and 1 mM EDTA instead of 0.2 mM EGTA. After 2 min of incubation ADP (1.5 mM) was added and the rate of respiration and of ATP synthesis was measured. P/O is the ratio between the rates of ATP synthesis and of respiration.

4. DISCUSSION

T_3 administration causes an early activation of respiration, preserved in isolated mitochondria (for review cf. [16]) and has been interpreted for a long time as due to an increased content of respiratory enzymes per mitochondrion. However, this interpretation is difficult to connect with the concepts, fundamental to biological energy transduction, of kinetic and thermodynamic control of state 3 and state 4 respirations, respectively. These concepts imply that, while activation of state 3 respiration can be linked to an increased content of respiratory enzymes, that of state 4 respiration is rather due to modifications of the factors controlling respiration (leaks/slips).

In the study of Hafner et al. [3], increase and decrease of protonmotive force in static head mitochondria from hypothyroid and hyperthyroid rats was accompanied by decrease or increase of the state 4 respiration, respectively. Moreover, the non-linear region of the flow–force relationship [3] was shifted toward higher or lower protonmotive force in hypothyroid or hyperthyroid rats, respectively.

The evidence favouring the concept that the non-linear flow–force (respiration–Δp or (proton conductance–Δp) relationship is due to slip in the pumps is as follows: (i) the passive proton conductance does not account for the state 4 respiration [15]; (ii) BSA diminishes the former but not the latter parameter [12]; (iii) each pump shows a specific flow–force relationship with a different region of non-linearity (particularly marked is the difference between redox and ATPase proton pumps); and (iv) a number of agents or conditions cause uncoupling without increasing the passive proton conductance. An explanation for the non-linearity, alternative to that of failures in the pump cycle (slip), is that of increased proton leaks taking place only during pump activity at the protein–lipid interfaces.

We have measured the flow–force relationships during respiration at sites II+III and during ATP hydrolysis, the relationship between rates of K^+ efflux and K^+ diffusion potentials, and the P/O ratios in mitochondria isolated from liver of euthyroid and hyperthyroid rats or incubated at high temperatures. This analysis provides the following new information: (i) both T_3 administration and incubation at high temperature are accompanied by only slight changes in the P/O ratios (particularly T_3 administration); (ii) T_3-administration and high temperature incubation have different capacity in inducing changes in the extent of non-linearity at the different pumps; and (iii) the modified extent of non-linearity is accompanied by an increase of passive proton conductance in the high temperature incubation but not in mitochondria from T_3-treated rats. That the marked increases of state 4 respiratory rate are accompanied by such a modest decline of the P/O ratio is due to the fact that the changes of flow–force relationship occur in a

highly non-linear region. Hence, the small depression of protonmotive force taking place during phosphorylation results in a large depression in the rate of proton cycling.

The comparison with the effect of the temperature increase suggests that the hormone induced loss of thermodynamic control concerns specific pump properties (composition and assembly of protein components of the pumps) and not the unspecific membrane conductance (changes occurring in the lipid components of the bilayer). In view of the number of RNAs appearing within minutes after hormonal injection (cf. [16]), it appears that T_3 administration causes rapid synthesis of specific pump components which leads to decreased thermodynamic control of some or all pumps. Whether the loss of respiratory control reflects a cycle taking place within the pump or at the pump protein–lipid interface remains open.

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