Hyperthyroidism increases the uncoupled ATPase activity and heat production by the sarcoplasmic reticulum Ca^{2+}-ATPase

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The sarcoplasmic reticulum Ca^{2+}-ATPase is able to modulate the distribution of energy released during ATP hydrolysis, so that a portion of energy is used for Ca^{2+} transport (coupled ATPase activity) and a portion is converted into heat (uncoupled ATPase activity). In this report it is shown that T_4 administration to rabbits promotes an increase in the rates of both the uncoupled ATPase activity and heat production in sarcoplasmic reticulum vesicles, and that the degree of activation varies depending on the muscle type used. In white muscles hyperthyroidism promotes a 0.8-fold increase of the uncoupled ATPase activity and in red muscle a 4-fold increase. The yield of vesicles from hyperthyroid muscles is 3–4-fold larger than that obtained from normal muscles; thus the rate of heat production by the Ca^{2+}-ATPase expressed in terms of g of muscle in hyperthyroidism is increased by a factor of 3.6 in white muscles and 12.0 in red muscles. The data presented suggest that the Ca^{2+}-ATPase uncoupled activity may represent one of the heat sources that contributes to the enhanced thermogenesis noted in hyperthyroidism.

Key words: ATP hydrolysis, Ca^{2+}-ATPase, Ca^{2+} transport, heat production, hyperthyroidism, thermogenesis.

INTRODUCTION

Interest in heat production and thermogenesis has increased during the past decade due to its implications in health and disease. Alterations of thermogenesis are noted in several disorders, such as control of body weight and endocrine dysfunction [1–4]. The thyroid hormone T_3 (3,5,3′-tri-iodo-L-thyronine) is involved in the thermal regulation of vertebrates, and in hyperthyroidism there is a decrease of body weight, and an increase of both the basal metabolism and the rate of heat production [5–10]. In small mammals and newborn humans, brown adipose tissue is the primary site of heat production. The mechanism of heat production in these cells involves the concerted action of T_3 and noradrenaline ('norepinephrine') and the expression of the mitochondrial uncoupling protein UCP1 [8–10]. T_3 is also involved in the thermal regulation of adult humans and other vertebrates devoid of brown adipose tissue. However, in these animals little is known about the cellular mechanisms by which T_3 regulates thermogenesis.

Skeletal muscle is the most abundant tissue of the human body, and a small change in the rate of heat production by this tissue represents a large contribution to the heat produced by the whole body. Of the total heat produced by resting muscles, 50 % (white muscles) to 20 % (red muscle) is derived from ATP hydrolysis by the sarcoplasmic reticulum Ca^{2+}-ATPase [1,2]. The amount of Ca^{2+}-ATPase found in the sarcoplasmic reticulum of white muscle is larger than that found in red muscle [11–15]. Three distinct genes encode the SERCA (sarco/endoplasmic reticulum Ca^{2+}-ATPase) isoforms, but the physiological significance of this isoform diversity is not clear. In normal animals, red muscles express both SERCA 1 and SERCA 2a, while white muscles express only the SERCA 1 gene. The SERCA 2b and SERCA 3 genes are expressed in non-muscular tissues such as blood platelets and lymphoid tissue [16–18]. The phenotype of white and red muscles and the transcription of SERCA 1 is regulated by T_3. In hyperthyroid animals red muscles are replaced by white muscles and SERCA 1 is overexpressed [19–22]. Besides regulating SERCA isoform expression, the injection of T_3 promotes an increase in the amount of Ca^{2+}-ATPase found in the sarcoplasmic reticulum of both white and red muscles [12,23,24]. We have observed [15,25–32] that the amount of heat released during the hydrolysis of each ATP molecule (calorimetric enthalpy, ΔH^{cal}) varies depending on the SERCA isoform used. For vesicles derived from blood platelets and red muscle the ΔH^{cal} of ATP hydrolysis varies between -11 and -14 kcal · mol^{-1} but for white muscle vesicles it varies between -20 and -24 kcal · mol^{-1}, i.e. twice the value measured with blood platelets and red muscle vesicles. This difference is related to the rate of uncoupled ATPase activity of SERCA 1 and SERCA 2 (Scheme 1, reaction 10). In SERCA 1 the rate of uncoupled ATPase activity is 4-fold faster than the rate of coupled ATPase activity. This is not observed in SERCA 2 [15,28]. When the ATPase activity is coupled with Ca^{2+} transport a part of the chemical energy released during ATP hydrolysis is used to pump Ca^{2+} (work) and a part is converted into heat. During the uncoupled ATPase activity there is no Ca^{2+} transport and more chemical energy is available to be converted into heat. Thus the total amount of energy released during ATP hydrolysis is always the same, but the heat produced varies depending on how much of this energy is used to pump Ca^{2+} [28,30–32].

The aim of this work was to evaluate whether or not T_3 plays a role in the amount of heat produced by muscle Ca^{2+}-ATPase during ATP hydrolysis, an effect that may contribute to the increased thermogenesis found in hyperthyroidism.

MATERIALS AND METHODS

Sarcoplasmic reticulum vesicles

During all the experiments performed, white male rabbits were treated in accordance with published regulations for animal

Abbreviations used: T_3, 3,5,3′-tri-iodo-L-thyronine; T_4, L-thyroxine; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase.

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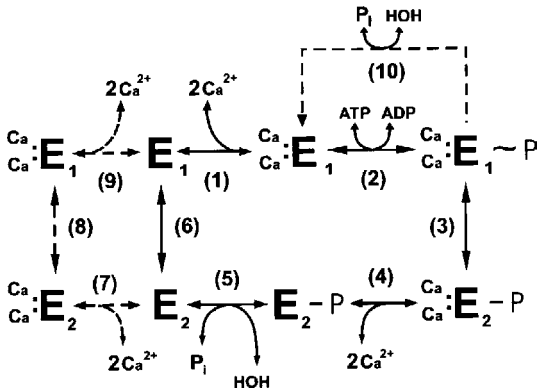

Scheme 1 The catalytic cycle of the Ca^{2+}-ATPase

The sequence includes two distinct enzyme conformations, E_1 and E_2. The Ca^{2+}-binding sites on the E_1 form face the external surface of the vesicle and have a high affinity for Ca^{2+} (K_a, 10^{-6} M at pH 7). In the E_2 form the Ca^{2+}-binding sites face the vesicle lumen and have a low affinity for Ca^{2+} (K_a, 10^{-3} M at pH 7). The enzyme form E_1 is phosphorylated by ATP but not by P_i and, conversely, the enzyme form E_2 is phosphorylated by P_i but not by ATP. When the Ca^{2+} concentration on the two sides of the membrane is inferior to 50 μM, reaction 4 is irreversible and this forces the sequence to flow forward from reaction 1 to 6. This is observed when leaky vesicles are used. With intact vesicles, the Ca^{2+} pumped by the ATPase is retained in the vesicle lumen. The high intravesicular Ca^{2+} concentration ($\approx$10 mM) permits the reversal of the catalytic cycle (reactions 5 to 1 backwards) during which a part of the accumulated Ca^{2+} leaves the vesicles in a process coupled with the synthesis of ATP from ADP and P_i. For vesicles derived from white muscle (SERCA 1), the rise of the intravesicular Ca^{2+} concentration leads to ramifications of the catalytic cycle, the uncoupled ATPase activity mediated by reaction 10 and the uncoupled Ca^{2+} efflux mediated by either reactions 7 to 9 or reactions 4, 3 and 10 [28,30–32,39–42,47–52].

laboratorial use. White and red muscles were dissected from rabbit hind limb. Vesicles derived from the longitudinal sarcoplasmic reticulum (light fraction) of the two types of muscle were prepared as described previously [15] and stored in liquid nitrogen. Prior to use, vesicles were diluted in a medium containing 50 mM Mops/Tris buffer, 100 mM KCl, 10 mM P_i and 10 μM $CaCl_2$.

Hyperthyroid rabbits

Eight rabbits having approximately the same body weight (mean $\pm$ S.E.M., 2.61 $\pm$ 0.08 kg) were kept in the same environment and fed *ad libitum* for 8 days. Hyperthyroidism was induced in four of these rabbits by daily subcutaneous injection of T_4 (L-thyroxine; 100–200 μg/kg of body weight), a dose used regularly to promote hyperthyroidism [12,20,33]. During the treatment period the body weights of the four control rabbits increased by 5%, while the weights of the T_4-treated rabbits decreased by 12.5%. This difference was statistically significant ($P < 0.01$). Total T_4 serum level were measured by specific radioimmunoassay using rabbit serum free from hormones to prepare the standard curve [34]. During treatment, the serum level of T_4 rose from 14 $\pm$ 3 to 204 $\pm$ 22 mg/l of serum.

Gel electrophoresis and Western blotting

Proteins were separated on a 7.5% polyacrylamide gel according to Laemmli [35]. Electrotransfer of protein from the gel to PVDF membranes was performed for 15 min at 250 mA/gel in 25 mM Tris, 192 mM glycine and 20% methanol using a Mini Trans-Blot cell from Bio-Rad. Membranes were blocked with 3% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 for 1 h at room temperature. Membranes were then washed and incubated for 1 h with anti-SERCA 1 or anti-SERCA-2 monoclonal antibodies at room temperature. The membranes were washed and blots were revealed using an ECL detection kit from Amersham Biosciences. Monoclonal antibodies for SERCA 1 (clone IIH11) and SERCA 2 (clone IID8) were obtained from Affinity BioReagents (São Paulo, Brazil).

Ca^{2+} uptake and $Ca^{2+}{}_{in} \leftrightarrow Ca^{2+}{}_{out}$ exchange

These were measured by the filtration method [28,36]. For ^{45}Ca uptake, trace amounts of ^{45}Ca were included in the assay medium. The reaction was arrested by filtering samples of the assay medium through Millipore filters. After filtration, the filters were washed five times with 5 ml of 3 mM $La(NO_3)_3$ and the radioactivity remaining on the filters was counted using a liquid scintillation counter. For $Ca^{2+}{}_{in} \leftrightarrow Ca^{2+}{}_{out}$ exchange, the assay medium was divided into two samples. A trace amount of $^{45}Ca^{2+}$ was added to only one of the samples and the reaction was started by the simultaneous addition of vesicles to both samples. The sample containing $^{45}Ca^{2+}$ was used to determine the incubation time required to fill the vesicles to the steady state level. The rate of $Ca^{2+}{}_{in} \leftrightarrow Ca^{2+}{}_{out}$ exchange was measured after the steady state was reached by adding a trace amount of $^{45}Ca^{2+}$ to the second sample containing vesicles loaded with non-radioactive Ca^{2+}. The exchange between radioactive Ca^{2+} from the medium and the non-radioactive Ca^{2+} contained inside the vesicles was measured by filtering samples of the assay medium through Millipore filters at different incubation intervals varying from 15 to 60 s after the addition of $^{45}Ca^{2+}$.

ATPase activity and ATP synthesis

ATPase activity was assayed by measuring the release of $^{32}P_i$ from [γ-^{32}P]ATP. The reaction was arrested with trichloroacetic acid (final concentration, 5%, w/v). The [γ-^{32}P]ATP not hydrolysed during the reaction was extracted with activated charcoal as described previously [37]. Two different ATPase activities can be distinguished in sarcoplasmic reticulum vesicles [38–42]. The Mg^{2+}-dependent activity requires only Mg^{2+} for its activation and is measured in the presence of 2 mM EGTA to remove contaminant Ca^{2+} from the medium. The Ca^{2+}-dependent ATPase activity, which is correlated with Ca^{2+} transport, is determined by subtracting the Mg^{2+}-dependent activity from the activity measured in the presence of both Mg^{2+} and Ca^{2+}.

ATP synthesis was measured using $^{32}P_i$ as described previously [42].

Heat of reaction

This was measured using an OMEGA Isothermal Titration Calorimeter from Microcal (Northampton, MA, U.S.A.). The calorimeter sample cell (1.5 ml) was filled with reaction medium, and the reference cell was filled with Milli-Q water. After equilibration at 35 °C, the reaction was started by injecting vesicles into the sample cell and the heat change was recorded for 20 min. The volume of vesicle suspension injected into the sample cell varied between 0.02 and 0.03 ml. The heat change measured during the initial 2 min after vesicle injection was discarded to avoid artifacts such as heat derived from the dilution of the vesicle suspension in the reaction medium and binding of ions to the Ca^{2+}-ATPase. The duration of these events is less than 1 min [26–28,30–32]. Calorimetric enthalpy (ΔH^{cal}) is calculated by dividing the amount of heat released by the amount of ATP hydrolysed. The units used are mol for substrate hydrolysed and kcal for heat released. Negative values indicate that the reaction is exothermic and positive values indicate that it is endothermic.

Table 1 Vesicles recovered, ATPase activity and heat released by vesicles from white and red muscles

Assay medium and experimental conditions were as described for Figure 2. Values are means ± S.E.M. from four experiments. The differences between muscles from control and hyperthyroid rabbits are statistically significant: *$P < 0.01$, †$P < 0.005$, ‡$P < 0.001$.

Muscle type	Vesicles (mg of protein/g of wet muscle)	Ca^{2+}-ATPase (μmol of P_i/g of wet muscle)	Heat released (mcal/g of wet muscle)
White			
Control	0.45 ± 0.08	0.67 ± 0.14	11.61 ± 0.24
Hyperthyroid	1.29 ± 0.15†	2.44 ± 0.10†	41.62 ± 2.10†
Red			
Control	0.21 ± 0.05	0.06 ± 0.02	0.81 ± 0.30
Hyperthyroid	0.83 ± 0.17*	0.49 ± 0.06†	9.70 ± 1.17‡

Experimental procedure

In different laboratories the rates of Ca^{2+} transport and ATP hydrolysis are usually measured at room temperature, a condition far from the physiological temperature of endothermic animals, which varies between 35 and 37 °C. The uncoupled ATPase activity of rabbit SERCA 1 is impaired when the temperature of the assay medium decreases below 30 °C [27,31]. Therefore, in this report all the experiments were performed at 35 °C. In a typical experiment the assay medium was divided into five samples, which were used for the simultaneous measurement of Ca^{2+} uptake, $Ca^{2+}{}_{in} \leftrightarrow Ca^{2+}{}_{out}$ exchange, substrate hydrolysis, ATP synthesis and heat release. The syringe of the calorimeter was filled with vesicles and the temperature of the syringe was allowed to equilibrate with that of the reaction cell of the calorimeter, a process that usually took between 8 and 12 min. During equilibration, the vesicles used for measurements of Ca^{2+} uptake, $Ca^{2+}{}_{in} \leftrightarrow Ca^{2+}{}_{out}$ exchange, ATP hydrolysis and ATP synthesis were kept at the same temperature, length of time and protein dilution as the vesicles kept in the calorimeter syringe. These different measurements were started simultaneously with vesicles to a final concentration varying between 5 and 10 μg of protein/ml. NaN_3 (5 mM), an inhibitor of mitochondrial ATP synthase, was added to the assay medium to avoid interference from possible contamination of the sarcoplasmic reticulum vesicles with this enzyme. The free Ca^{2+} concentration in the medium was calculated as described previously [43–45].

RESULTS

Gel electrophoresis and Western-blot analysis of muscle homogenates

The muscle homogenate used for the preparation of the sarcoplasmic reticulum vesicles was centrifuged at 10000 ***g*** for 20 min and the supernatant devoid of actomyosin and mitochondria was used for the electrophoresis. The gel electrophoresis revealed an enrichment of the 110-kDa band characteristic of SERCA in rabbit treated with T_4. This could be clearly seen in red muscle homogenates, but not so clearly in white muscle (Figure 1). In hyperthyroid rabbits the number of vesicles recovered after cell fractionation was greater than that recovered from the control rabbits, 2–3-fold in white muscles and 4–5-fold in red muscles (Table 1). Western-blot analysis of the gel confirms previous observations [19–24] that white muscles express only SERCA 1 in both control and hyperthyroid rabbits. Red muscles express both SERCA 1 and SERCA 2 and, after T_4 treatment, there was a down-regulation of SERCA 2 and an up-regulation of SERCA 1.

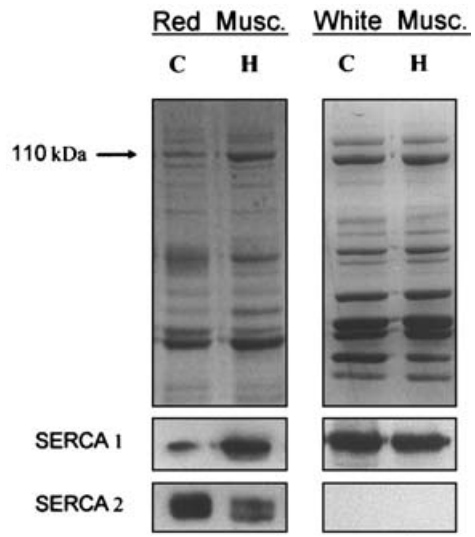

Figure 1 Gel electrophoresis and Western blot of red (left) and white (right) muscle homogenates

The amount of protein used for the gel electrophoresis was 10 μg of protein. C, control rabbits; H, hyperthyroid rabbits; SERCA 1 and SERCA 2 show Western blots where membranes were hybridized with anti-SERCA1 (upper panels) or anti-SERCA 2 (lower panels) monoclonal antibodies.

Ca^{2+} uptake, ATP hydrolysis and ATP synthesis

In agreement with previous reports [12,15] we found that vesicles derived from white muscles are able to accumulate more Ca^{2+} (compare Figures 2A and 2B) and to hydrolyse ATP at a faster rate than the vesicles derived from red muscles (compare Figures 3A and 4A). Nunes et al. [12] observed that vesicles derived from hyperthyroid red muscles are able to accumulate more Ca^{2+} and to hydrolyse ATP at a faster rate than the vesicles of control rabbits. This was confirmed in Figures 2(A) and 3(A) and in Tables 2 and 3. We now show that the effect of T_4 treatment varies depending on the muscle used. In contrast to red muscles, in white muscles (SERCA 1) hyperthyroidism promoted a 50% decrease in both the initial rate and the steady state level of Ca^{2+} uptake (Figure 2B and Table 2). Interestingly, the inhibition of Ca^{2+} uptake was not accompanied by a parallel inhibition of the ATPase activity; on the contrary, hyperthyroidism promoted a 44% increase in the rate of ATP hydrolysis (Figure 4A and Tables 3 and 4). This suggests that hyperthyroidism enhances the degree of SERCA 1 uncoupling, with more ATP being hydrolysed and less Ca^{2+} being accumulated by the vesicles.

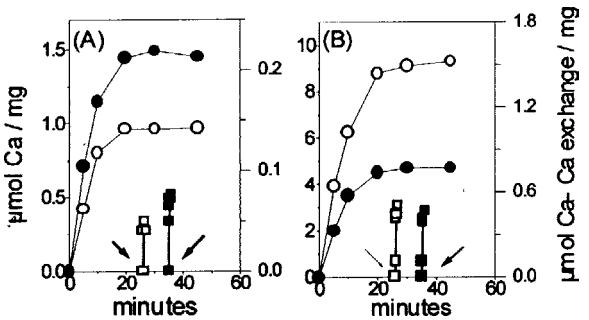

Figure 2 Ca^{2+} uptake and $Ca^{2+}{}_{in} \leftrightarrow Ca^{2+}{}_{out}$ exchange in vesicles from (A) red muscle and (B) white muscle

The assay medium composition was 50 mM Mops/Tris buffer (pH 7.0), 1 mM ATP, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.2 mM EGTA, 10 mM P_i, 100 mM KCl and 5 mM NaN_3. The reaction was performed at 35 °C and was started by the addition of vesicles (5 μg of protein/ml); open symbols, control rabbits; closed symbols, hyperthyroid rabbits. ●, ○, Ca^{2+} uptake; ■, □, $Ca^{2+}{}_{in} \leftrightarrow Ca^{2+}{}_{out}$ exchange. The rate of $Ca^{2+}{}_{in} \leftrightarrow Ca^{2+}{}_{out}$ exchange was measured as described in The Materials and methods section and the arrow indicates the addition of trace amounts of $^{45}Ca^{2+}$ to the tube containing vesicles loaded with non-radioactive Ca^{2+}. The calculated free Ca^{2+} concentration in the media was 5 μM [50]. The figure shows a representative experiment.

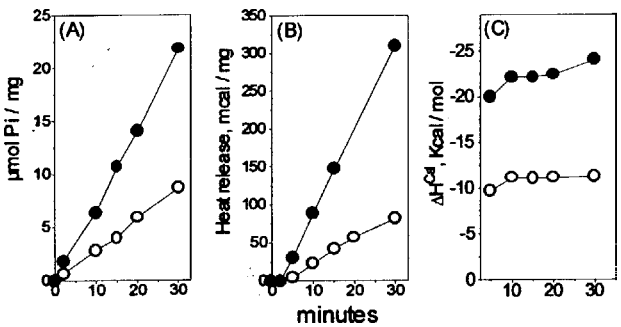

Figure 3 Red muscle: ATP hydrolysis (A), heat release (B) and ΔH^{cal} of ATP hydrolysis (C)

Assay medium composition and experimental conditions were as in Figure 2. ○, Control rabbits; ●, hyperthyroid rabbits. The figure shows a representative experiment.

Rate of heat production and ΔH^{cal} of ATP hydrolysis

The heat released during Ca^{2+} transport is derived from the hydrolysis of ATP [28,31,32]. The experiments of Figures 3 and 4 and Table 4 show that hyperthyroidism promotes an increase in the rates of ATP hydrolysis and heat production in both white and red muscle vesicles. In white muscle vesicles there was a 46% increase of the two activities. In vesicles derived from red muscles, however, the increment of the two activities was much higher than that observed in white muscles vesicles and the increment of the Ca^{2+}-dependent heat production was more pronounced than that of ATP hydrolysis, 407% and 232% respectively. The ΔH^{cal} value of ATP hydrolysis is calculated by dividing the amount of heat released by the amount of ATP hydrolysed. Therefore, in white muscle the enhancement of heat production was solely due to an increase in the ATP hydrolysis rate while in red muscle there was both an increase of ATP hydrolysis and a change of the ΔH^{cal} which went from -15.3 to -25.4 kcal/mol (Figure 3 and Table 4).

When extended to the whole muscle, the changes in ATPase activity and heat production promoted by the T_4 treatment were significantly magnified due to the increased expression of the

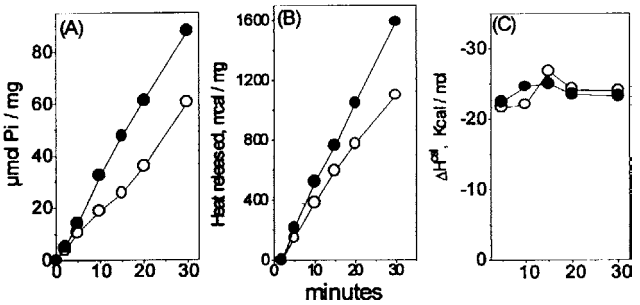

Figure 4 White muscle: ATP hydrolysis (A), heat release (B) and ΔH^{cal} of ATP hydrolysis (C)

Assay medium composition and experimental conditions were as in Figure 2. Values of ΔH^{cal} were calculated by dividing the amount of heat released by the amount of ATP hydrolysed. Negative values indicate that the reaction is exothermic. ○, Control rabbits; ●, hyperthyroid rabbits. The figure shows a representative experiment.

Table 2 Ca^{2+} uptake by vesicles from white and red muscles

Assay medium and experimental conditions were as described for Figures 2 and 3. Values are means ± S.E.M. from the number of experiments shown in parentheses. The differences between muscles from control and hyperthyroid rabbits were statistically significant: *$P < 0.001$, †$P < 0.01$, ‡$P < 0.05$.

Muscle	Initial rate (μmol of Ca^{2+}/mg per min)	Steady state (μmol of Ca^{2+}/mg)
White		
Control	0.75 ± 0.05 (7)	8.87 ± 0.03 (7)
Hyperthyroid	0.36 ± 0.02 (14)*	4.50 ± 0.02 (14)*
Red		
Control	0.09 ± 0.01 (10)	1.17 ± 0.10 (10)
Hyperthyroid	0.17 ± 0.03 (9)†	1.85 ± 0.30 (9)‡

Table 3 Steady state: $Ca^{2+}{}_{in} \leftrightarrow Ca^{2+}{}_{out}$ exchange, ATP hydrolysis and ATP synthesis in vesicles derived from white and red muscles

Assay medium and experimental conditions were as described for Figures 2 and 3. Values are means ± S.E.M. from *n* experiments, as shown. The differences between muscles from control and hyperthyroid rabbits were statistically significant: *$P < 0.05$.

Muscle	*n*	$Ca^{2+}{}_{in} \leftrightarrow Ca^{2+}{}_{out}$ exchange (μmol/mg per min)	ATP hydrolysis (μmol/mg per min)	ATP synthesis (μmol/mg of min)
White				
Control	4	0.54 ± 0.05	1.79 ± 0.21	0.09 ± 0.01
Hyperthyroid	6	0.46 ± 0.04	2.58 ± 0.30*	0.08 ± 0.01
Red				
Control	8	0.23 ± 0.04	0.30 ± 0.05	0.04 ± 0.01
Hyperthyroid	6	0.18 ± 0.03	0.62 ± 0.12*	0.04 ± 0.01

Ca^{2+}-ATPase. Thus when expressed in terms of g of muscle, in white muscles hyperthyroidism promoted a 3.6-fold increase in the rates of both ATP hydrolysis and heat production, while in red muscles the ATPase activity increased 8.4-fold and heat production increased 12.0-fold (Table 1). These values suggest that the Ca^{2+}-ATPase of skeletal muscle may be one of the sources contributing to the enhancement of non-shivering heat production noted in the hyperthyroid state. The aim of the following experiment was to ascertain which of the intermediary

Table 4 ATPase activity, heat released and ΔH^{cal} in the presence and absence of a Ca^{2+} gradient

Assay medium and experimental conditions were as described for Figures 2–4 and 6. Leaky vesicles refers to the addition of the ionophore A23187 to the medium (final concentration, 6 μM). Values are means ± S.E.M. from the number of experiments shown in parentheses. The differences between muscles from control and hyperthyroid rabbits were statistically significant: *$P < 0.05$, †$P < 0.001$.

Muscle	Vesicles	ATP hydrolysis (μmol/mg per min)	Heat released (mcal/mg per min)	ΔH^{cal} (kcal/mol)
White				
Control	Gradient	1.65 ± 0.16 (7)	37.95 ± 0.16 (7)	−23.00 ± 2.07 (7)
	Leaky	6.00 ± 0.52 (3)	66.27 ± 8.52 (3) 5	−11.22 ± 1.10 (3)
Hyperthyroid	Gradient	2.41 ± 0.31 (7)*	5.22 ± 3.17 (7)†	−22.55 ± 0.58 (7)
	Leaky	7.30 ± 0.40 (3)	70.04 ± 12.7 (3)	−9.63 ± 1.28 (3)
Red				
Control	Gradient	0.25 ± 0.04 (14)	3.56 ± 0.42 (14)	−15.28 ± 1.67 (14)
	Leaky	1.32 ± 0.30 (6)	16.90 ± 4.60 (6)	−12.69 ± 1.30 (6)
Hyperthyroid	Gradient	0.58 ± 0.09 (8)†	14.48 ± 3.16 (8)†	−25.43 ± 2.47 (8)†
	Leaky	1.50 ± 0.25 (8)	18.97 ± 3.9 (8)	−12.47 ± 0.89 (8)

Table 5 Coupled and uncoupled Ca^{2+} efflux and ATPase activity in vesicles from white and red muscles

Assay medium and experimental conditions were as described for Figure 2. For these calculations we used the stoichiometry of two Ca^{2+} ions pumped for each ATP molecule cleaved. The coupled Ca^{2+} efflux was calculated by multiplying the rate of ATP synthesis by 2, and the difference between the rate of $Ca^{2+}{}_{in} \leftrightarrow Ca^{2+}{}_{out}$ exchange and the rate of coupled Ca^{2+} efflux represents the uncoupled Ca^{2+} efflux (reactions 7–9 in Scheme 1). The rate of $Ca^{2+}{}_{in} \leftrightarrow Ca^{2+}{}_{out}$ exchange divided by 2 gives the rate of coupled ATP hydrolysis, i.e. the ATP cleaved to pump back the Ca^{2+} that leaves the vesicles during the $Ca^{2+}{}_{in} \leftrightarrow Ca^{2+}{}_{out}$ exchange (reactions 1–5 in Scheme 1). The difference between the total ATP hydrolysis and the coupled ATP hydrolysis gives the value of the uncoupled ATPase activity (reactions 2 and 10 in Scheme 1). For further details, see [28]. Values are means ± S.E.M. from *n* experiments, as shown. The differences between muscles from control and hyperthyroid rabbits were statistically significant: *$P < 0.02$, †$P < 0.001$.

		Ca^{2+} efflux (μmol/mg per min)		ATPase activity (μmol of P_i/mg per min)	
Muscle	*n*	Coupled	Uncoupled	Coupled	Uncoupled
White					
Control	4	0.16 ± 0.01	0.38 ± 0.02	0.27 ± 0.01	1.23 ± 0.20
Hyperthyroid	6	0.16 ± 0.02	0.30 ± 0.05	0.23 ± 0.01	2.18 ± 0.35*
Red					
Control	8	0.09 ± 0.02	0.11 ± 0.03	0.11 ± 0.02	0.14 ± 0.03
Hyperthyroid	6	0.10 ± 0.02	0.07 ± 0.04	0.09 ± 0.02	0.58 ± 0.10†

reactions of the Ca^{2+}-ATPase catalytic cycle is altered in hyperthyroidism.

ATP synthesis

The Ca^{2+} concentration in the lumen of intact vesicles reaches the millimolar range a few seconds after transport is initiated and this triggers the reversal of the catalytic cycle of the ATPase, during which a small fraction of the ATP cleaved is re-synthesized from ADP and P_i by the Ca^{2+}-ATPase [39–41,46]. At steady state, the rate of ATP synthesis was not altered by hyperthyroidism in either white or red muscle vesicles (Table 3).

$Ca^{2+}{}_{in} \leftrightarrow Ca^{2+}{}_{out}$, Ca^{2+} efflux and rates of coupled and uncoupled ATPase activity

These measurements were made after the Ca^{2+} uptake reached steady state (Figure 2 and Table 3). When the vesicles are still being filled, the rate of Ca^{2+} uptake measured represents a balance between the Ca^{2+} pumped inwardly by the ATPase and the Ca^{2+} that leaves the vesicles driven by the gradient formed across the membrane. During the initial minute of incubation these two rates are different and cannot be measured separately. Thus the stoichiometry between the fluxes of Ca^{2+} through the membrane and the rates of either ATP cleavage or ATP synthesis cannot be evaluated precisely. After the steady state is reached, the rate of efflux is the same as that of Ca^{2+} uptake and, by measuring the rate of $Ca^{2+}{}_{in} \leftrightarrow Ca^{2+}{}_{out}$ exchange, it is possible to determine the values of these two rates. The exchange represents the fraction of Ca^{2+} that leaves the vesicles and is pumped back inward by the ATPase [28,30,31]. Using the values of $Ca^{2+}{}_{in} \leftrightarrow Ca^{2+}{}_{out}$ exchange it is possible to estimate the rates of coupled and uncoupled Ca^{2+} efflux (Table 5). In different laboratories it has already been shown that the release of two Ca^{2+} ions from the vesicles drives the synthesis of one ATP molecule [39–41,47]. The coupled Ca^{2+} efflux (reactions 5–1 in Scheme 1) is therefore calculated by multiplying the rate of ATP synthesis (Table 3) by 2, and the difference between the rate of $Ca^{2+}{}_{in} \leftrightarrow Ca^{2+}{}_{out}$ exchange and the coupled Ca^{2+} efflux represents the uncoupled Ca^{2+} efflux (reactions 7–9 or reactions 4, 3 and 10 in Scheme 1). Following the same rationale it is possible to calculate the rates of ATP hydrolysis coupled and uncoupled to the translocation of Ca^{2+} (Table 4). Two Ca^{2+} ions are pumped inside the vesicles for each ATP molecule cleaved [39–41,47–49]. Thus the rate of $Ca^{2+}{}_{in} \leftrightarrow Ca^{2+}{}_{out}$ exchange shown on Table 3 divided by 2 give us the rate of coupled ATP hydrolysis, i.e. the ATP cleaved to pump back the Ca^{2+} that leaves the vesicles (reactions 1–5 in Scheme 1). The difference between the total Ca^{2+}-dependent ATP hydrolysis measured and the coupled ATP hydrolysis gives

the value of the uncoupled ATPase activity (reactions 2 and 10 in Scheme 1).

The rate of $Ca^{2+}{}_{in} \leftrightarrow Ca^{2+}{}_{out}$ exchange of the white muscle vesicles was twice as fast as that of the red muscle but, in spite of the hyperthyroidism effect in the Ca^{2+}-ATPase activity, there was no difference on the rate of $Ca^{2+}{}_{in} \leftrightarrow Ca^{2+}{}_{out}$ exchange between control and hyperthyroid rabbits (Table 3).

For the control rabbits, the rate of coupled ATPase of white muscle vesicles was 2.5 times faster than that of red muscle vesicles (Table 5) and the difference of uncoupled ATPase activity between vesicles derived from the two muscles was larger; white muscle vesicles being nine times faster than that of red muscle (Table 5). For the control white muscle vesicles the ratio between the rates of uncoupled (reaction 10 in Scheme 1) and coupled (reactions 1–5 in Scheme 1) ATPase activities was 4.5, i.e. for every 5.5 ATP molecules cleaved, the energy derived from only one molecule was used for the translocation of Ca^{2+} through the vesicles membrane (work) and most of the energy derived from the cleavage of the remaining 4.5 ATP molecules was dissipated as heat. For the red muscle vesicles the ratio between the rates of uncoupled and coupled ATPase activity was 1.3. The difference of uncoupled ATPase activity between white and red muscle vesicles accounts for the difference of heat output and ΔH^{cal} values detected in control rabbits during the ATP hydrolysis (Figures 3 and 4, and Table 4).

The rates of both coupled and uncoupled Ca^{2+} efflux as well as the rate of the coupled ATPase activity were not altered by hyperthyroidism (Figure 5 and Table 4). However, the rate of uncoupled ATPase activity, was enhanced in both types of muscle but this increment was particularly noticeable in red muscle vesicles. In vesicles derived from white muscles hyperthyroidism promoted a 77 % increase of the uncoupled ATPase activity rate while in red muscles the increase was of 414 %.

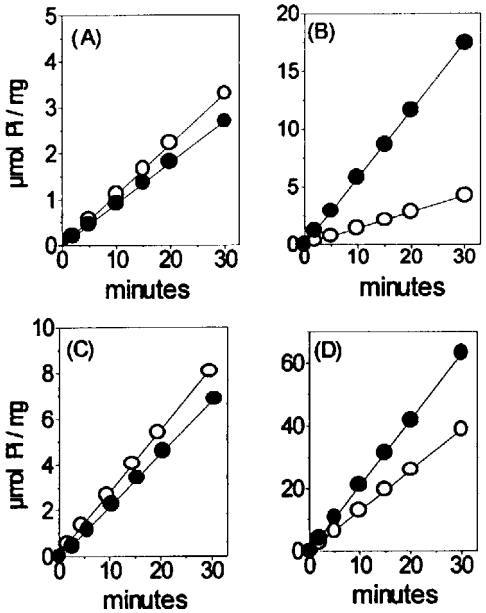

Figure 5 Rates of coupled and uncoupled ATPase activity

Vesicles derived from (**A** and **B**) red muscle and (**C** and **D**) white muscle. Open symbols, control rabbits; closed symbols, hyperthyroid rabbits. (**A** and **C**) Coupled ATPase activity and (**B** and **D**) uncoupled ATPase activity. The figure shows a representative experiment. The rates of coupled and uncoupled ATPase activity were calculated as described for Table 5.

Leaky vesicles

Activation of the uncoupled ATPase activity requires the presence of high Ca^{2+} concentrations in the vesicle lumen. In presence of the Ca^{2+} ionophore A23187 the vesicles are not able to accumulate Ca^{2+} and the uncoupled ATPase is abolished [48–52]. The experiment in Figure 6 shows that in the presence of A23187 the rates of ATP hydrolysis and heat production were the same in control and hyperthyroid rabbits and the ΔH^{cal} for ATP hydrolysis decreased to the range of -9.7 to -12.7 kcal/mol in both white and red muscle vesicles (Figure 6 and Table 4). This and the data of Figure 5 and Table 4 indicate that the increase in ATPase activity and heat production noted in hyperthyroid muscle are only noted when the vesicles are able to accumulate Ca^{2+} and are solely due to an increase in the uncoupled ATPase activity.

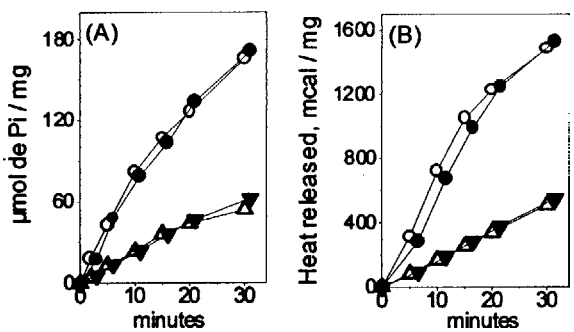

Figure 6 Rates of (A) ATP hydrolysis and (B) heat release in leaky vesicles

The assay medium and experimental conditions were as described for Figure 2 except that the ionophore A23187 was included in the assay medium to a final concentration of 6 μM. Open symbols refer to control rabbits and closed symbols to hyperthyroid rabbits. ○, ●, Vesicles derived from white muscle; △, ▼, vesicles derived from red muscles. The figure shows a representative experiment.

Lack of T_3 and T_4 effect *in vitro*

The findings described in Figures 2–6 were only observed when T_4 was injected into rabbits. In vesicles derived from normal rabbits the rates of Ca^{2+} transport, ATP hydrolysis and heat production of white and red muscles were not altered when either T_3 or T_4 were added to the assay medium in concentrations varying from 10^{-11} up to 10^{-2} M (results not shown).

DISCUSSION

Non-shivering thermogenic mechanisms are classified as either obligatory or facultative. Obligatory thermogenesis represent the energy dissipated as heat during the processes of energy interconversion that sustain life. Adaptative or facultative thermogenesis is the additional heat production that the body activates in response to cold or overfeeding [1–5]. T_3 plays a key role in both obligatory and adaptative thermogenesis. In obligatory thermogenesis thyroid hormones increase ATP turnover and expenditure [8] and during cold exposure the concentration of T_3 in brown adipocytes increases 3–4-fold. At least two different systems are known to be directly involved in the process of non-shivering thermogenesis; the UCP1 found in brown adipose tissue and the sarcoplasmic reticulum Ca^{2+}-ATPase.

The data presented suggest that activation of the SERCA-uncoupled ATPase activity may represent an important route

of heat production contributing to the increase in thermogenesis noted in hyperthyroidism. When extended to the living cell, the large uncoupled ATPase activity (reaction 10 in Scheme 1) could be considered an apparent futile cycle without a physiological purpose: ATP is cleaved without apparent work and then the ADP produced is phosphorylated by the mitochondria leading to an increase in oxygen consumption. However, during this cycle, a large amount of heat can be produced in muscles. Not only does each mol of ATP cleaved yield more heat (compare ΔH^{cal} values of Table 4), but in order to maintain a constant ATP concentration in the cell, the ADP derived from the uncoupled ATPase activity should lead to an increase in the mitochondrial respiration rate, and this would further enhance the rate of heat production.

The expression of the uncoupling protein isoform UCP3 found in muscle mitochondria is also regulated by T_3, but different from the isoform UCP1 found in brown adipose tissue, UCP3 seems not to be directly involved in the mechanism of heat production in muscle [53–61]. It has been assumed that the various UCP proteins could divert energy from ATP synthesis to thermogenesis by promoting the leakage of protons across the inner mitochondrial membrane. In order to compensate for this leak, the cell would then increase the rate of oxygen consumption to maintain the proton gradient at a level that is adequate for ATP synthesis. Recent reports [53–61], however, indicate that with the possible exception of UCP1, the physiological role of the various UCP isoforms is not related to heat production, but instead to the control of reactive oxygen species concentrations inside mitochondria, protecting them against oxidative damage.

The effect of T_3 on Ca^{2+} transport and uncoupled ATPase

The decrease of Ca^{2+} uptake and enhancement of the uncoupled ATPase activity noted in vesicles derived from white muscle (SERCA 1) suggest that hyperthyroidism promotes modifications in the rate constant of intermediary reactions of the catalytic cycle, namely an increase in the rate of $E_1 \sim P$ cleavage (reaction 10 in Scheme 1) and a decrease in the rate of conversion of the phosphoenzyme of 'high energy' into 'low energy' (reaction 3 in Scheme 1). At present we do not know how hyperthyroidism modifies the properties of the SERCA 1 of white muscle. Recently [52] it has been shown that sarcolipin, a small peptide found in the sarcoplasmic reticulum of skeletal muscle, uncouples the hydrolysis of ATP from Ca^{2+} transport. Perhaps the enhanced uncoupled ATPase activity promoted by T_3 is related to an overexpression of sarcolipin. Other possibilities are the modification of the membrane lipids that interact with the protein or a subtle modification of the Ca^{2+}-ATPase itself. In vesicles derived from red muscle there was both an increase of Ca^{2+} uptake and uncoupled ATPase activity. These modifications can be derived from a change in the ratio between the amount of SERCA 1 and SERCA 2a available in the muscle. However, at present we cannot exclude the possibility that hyperthyroidism could also promote a modification of the SERCA 2a isoform properties. In fact, in previous reports it was shown that the properties of SERCA 2 can be modified by the platelet-aggregating factor. In vesicles derived from human blood platelets platelet-aggregating factor promotes uncoupling of Ca^{2+} transport and an increase in the amount of heat produced during ATP hydrolysis [25].

The mechanism of energy conversion during the process of Ca^{2+} transport, the difference in values between the ΔH^{cal} measured with intact (gradient) and leaky (no gradient) vesicles and its correlation with the rates of coupled and uncoupled ATPase activity were discussed in detail in previous reports [15,28,30–32].

Hyperthyroidism and transport ATPases

In addition to the Ca^{2+}-ATPase, T_3 also regulates the concentration and the isoform expression of the Na^+/K^+ pumps [1,2,62], but as far as we know there are no direct measurements of heat production during ATP hydrolysis, nor it is known if this enzyme is able to cleave ATP without the translocation of ions across the membrane.

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