Energy Interconversion by the Sarcoplasmic Reticulum Ca^{2+}-ATPase: ATP Hydrolysis, Ca^{2+} Transport, ATP Synthesis and Heat Production*

LEOPOLDO DE MEIS**

Instituto de Ciências Biomédicas, Departamento de Bioquímica Médica,
Universidade Federal do Rio de Janeiro, Cidade Universitária – 21941-590 Rio de Janeiro, RJ, Brazil

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ABSTRACT

The sarcoplasmic reticulum of skeletal muscle retains a membrane bound Ca^{2+}-ATPase which is able to interconvert different forms of energy. A part of the chemical energy released during ATP hydrolysis is converted into heat and in the bibliography it is assumed that the amount of heat produced during the hydrolysis of an ATP molecule is always the same, as if the energy released during ATP cleavage were divided in two non-interchangeable parts: one would be converted into heat, and the other used for Ca^{2+} transport. Data obtained in our laboratory during the past three years indicate that the amount of heat released during the hydrolysis of ATP may vary between 7 and 32 Kcal/mol depending on whether or not a transmembrane Ca^{2+} gradient is formed across the sarcoplasmic reticulum membrane. Drugs such as heparin and dimethyl sulfoxide are able to modify the fraction of the chemical energy released during ATP hydrolysis which is used for Ca^{2+} transport and the fraction which is dissipated in the surrounding medium as heat.

key words: Ca^{2+}-ATPase, Ca^{2+} transport, energy interconversion, ATP hydrolysis, heat production, sarcoplasmic reticulum.

1. INTRODUCTION

The Ca^{2+}-ATPase found in the membrane of the sarcoplasmic reticulum of skeletal muscle is able to interconvert different forms of energy. This enzyme translocates Ca^{2+} from the cytoplasm to the lumen of the reticulum by using the chemical energy derived from ATP hydrolysis. After that Ca^{2+} is accumulated inside the reticulum, a Ca^{2+} gradient is formed across the membrane and this promotes the reversal of the catalytic cycle of the enzyme during which Ca^{2+} leaves the reticulum in a process coupled with the synthesis of ATP from ADP and Pi. During reversal of the catalytic cycle, the osmotic energy derived from the gradient is transformed back by the enzyme into chemical energy. In conditions similar to those found in the living cell at rest, a steady state is established during which the Ca^{2+} concentration is high inside the reticulum and low in the cytosol and the pump operates forward and backwards, cleaving and synthesizing ATP continuosly. In the bibliography, the simultaneous synthesis and hydrolysis of ATP measured in steady state conditions is referred to as the ATP $\leftrightarrow$ P_i exchange reaction (Hasselbach & Makinose 1961, Barlogie *et al.* 1971, Makinose & Hasselbach 1971, de Meis & Vianna 1979, Inesi 1985, de Meis 1993). Recently (Gould *et al.* 1987, Gould *et al.* 1978, Inesi &

*Invited paper

**Member of the Academia Brasileira de Ciências
E-mail: demeis@bioqmed.ufrj.br

de Meis 1989, Galina & de Meis 1991, De Meis *et al.* 1990, de Meis, 1991, de Meis & Inesi 1992, de Meis & Suzano 1994, Wolosker & de Meis 1995) it has been shown that during the ATP $\leftrightarrow$ P_i exchange only part of the Ca^{2+} efflux is coupled with the synthesis of ATP. The other part leaks through the Ca^{2+}-ATPase without promoting the synthesis of ATP, a process referred to as an uncoupled efflux. The rates of the coupled and the uncoupled Ca^{2+} effluxes can be modified by different drugs, including dimethyl sulfoxide and heparin.

Only a part of the chemical energy released during the hydrolysis of ATP is converted into other forms of energy such as osmotic energy. The other part is converted into heat, and this is used by the cell to maintain a constant and high body temperature. Nonshivering thermogenesis is a key component of temperature regulation in animals having little or no brown adipose tissue. During nonshivering thermogenesis most of the heat is derived from resting muscles but the mechanism of heat production is still unclear (Janský 1995, Chinet *et al.* 1992, Dumonteil *et al.* 1993, Dumonteil *et al.* 1995, Lowell & Splegelman 2000). It has been proposed that Ca^{2+} leaks from the sarcoplasmic reticulum and heat would then be derived from the hydrolysis of the extra amount of ATP needed to maintain a low myoplasmic Ca^{2+} concentration. In this formulation it is assumed that the amount of heat produced during the hydrolysis of an ATP molecule is always the same and is not modified by the formation of the gradient, as if the energy released by ATP hydrolysis were to e divided in two non-interchangeable parts: one would be converted into heat, and the other used for Ca^{2+} transport.

Data obtained in our laboratory during the past three years (de Meis *et al.* 1997, de Meis 1998a,b, Mitidieri & de Meis 1999) indicate that heat is produced during the uncoupled Ca^{2+} efflux. The coupled and the uncoupled Ca^{2+} efflux may represent two distinct routes of energy conversion, both mediated by the Ca^{2+} -ATPase in which the osmotic energy derived from the Ca^{2+} gradient is either used to synthesize ATP (coupled Ca^{2+} efflux) or is dissipated into the medium as heat (uncoupled Ca^{2+} efflux). Thus, it is possible to vary the amount of heat produced during ATP hydrolysis using drugs that change the rates of the coupled and uncoupled Ca^{2+} efflux.

2. HEAT PRODUCTION AND ATP SYNTHESIS BY THE Ca^{2+}-ATPase

A transmembrane Ca^{2+} gradient is formed when intact vesicles derived from the sarcoplasmic reticulum of rabbit white muscle are incubated in a medium containing ATP. This is not observed with leaky vesicles because the Ca^{2+} transported across the membrane readily diffuses back to the assay medium (Table I). Both in the presence and absence of a Ca^{2+} gradient the amount of heat produced during the hydrolysis of ATP was found to be proportional to the amount of ATP hydrolyzed (Fig. 1). However, in the presence of the gradient, the amount of heat released after the hydrolysis of each ATP molecule and the value of the ΔH^{cal} for ATP hydrolysis were found to be more negative than those measured with leaky vesicles (Fig. 1 and in Table I compare ΔH^{cal} values of leaky and intact vesicles). The calorimetric enthalpy (ΔH^{cal}) was calculated by dividing the amount of heat released by the amount of Ca^{2+} released by the vesicles. A negative value indicates that the reaction was exothermic and a positive value indicate that it was endothermic. This difference suggests that the vesicles were able to convert a part of the osmotic energy derived from the gradient into heat and the possibility was raised that the conversion could be mediated by the uncoupled leakage of Ca^{2+} through the ATPase. Thus, the coupled and the uncoupled Ca^{2+} effluxes could represent two distinct routes of energy conversion, both mediated by the Ca^{2+}-ATPase: one route in which the osmotic energy from the Ca^{2+} gradient is used to synthesize ATP and a second route in which the osmotic energy is converted into heat.

According to this reasoning it would be expected that drugs which change the rate of the uncoupled Ca^{2+} efflux should also change the amount

TABLE I
Ca^{2+} Uptake, ATP hydrolysis, ATP synthesis and heat production by rabbit sarcoplasmic reticulum vesicles.

Additions	n	Ca^{2+} Uptake, μmol/mg	ATP hydrolysis μmol/mg	ATP synthesis μmol/mg	Heat released mcal / mg	ΔH^{cal}, Kcal / mg
Leaky (no gradient)	5	None	28.4 ± 1.6	None	286.5 ± 27.6	-10.2 ± 1.3
Leaky + 20% DMSO	5	None	8.0 ± 1.1	None	91.8 ± 8.7	-11.4 ± 1.1
Intact (gradient)	5	3.4 ± 0.5	17.8 ± 3.0	0.5 ± 0.1	391.7 ± 36.1	-21.9 ± 1.5
Intact + 20% DMSO	6	4.9 ± 0.2	9.5 ± 1.9	0.8 ± 0.1	102.5 ± 11.3	-10.8 ± 1.4
Intact + 3μg/ml heparin	4	0.9 ± 0.2	9.8 ± 0.5	0.3 ± 0.1	297.1 ± 21.8	-30.2 ± 2.1

The reaction was performed at 35°C and the incubation time was 20 min. The assay medium composition was 50 mM MOPS-Tris buffer, pH 7.0, 0.1 mM $CaCl_2$, 1 mM ATP, 4 mM $MgCl_2$, and 10 mM P_i. The medium was divided into four samples. One was used for heat measurements. To the order three samples trace amounts of either ^{45}Ca, [$\gamma - ^{32}P$]ATP or $^{32}P_i$ were added for measurement of Ca^{2+} uptake, ATP hydrolysis and ATP synthesis, respectively. The four reactions were started simultaneously by the addition of vesicle protein (20 μg/ml). The values in the Table are the average $\pm$ SE. In the Table (n) is the number of experiments and DMSO is dimethyl sulfoxide. The calorimetric enthalpy (ΔH^{cal}) was calculated by dividing the amount of heat released by the amount of ATP cleaved by the vesicles. For experimental details see de Meis *et al.* 1997 and de Meis 1998b.

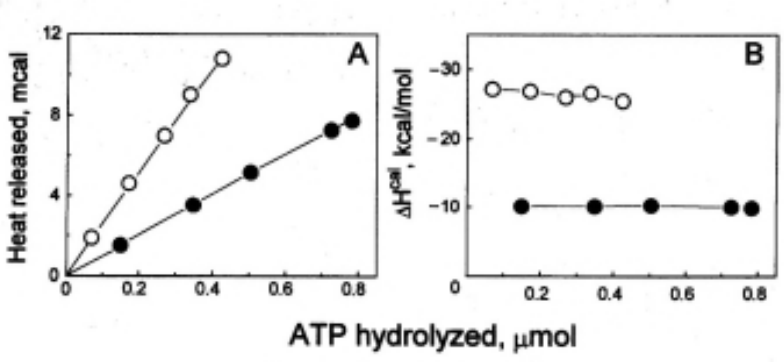

Fig. 1 – Heat release during ATP hydrolysis in presence and absence of a transmembrane Ca^{2+} gradient. The assay medium and experimental conditions were as in Table I using (❍) intact vesicles that accumulated Ca^{2+} and formed a transmembrane Ca^{2+}-gradient and (●) leaky vesicles which were not able to retain Ca^{2+} inside the vesicles. The figure shows a typical experiment where the amount of heat released (A) and the ΔH^{cal} values (B) were plotted as a function of at the amount of ATP cleaved at different incubation intervals. The calorimetric enthalpy (ΔH^{cal}) was calculated by dividing the amount of heat released by the amount of ATP cleaved by the vesicles. Note that when more negative the values of ΔH^{cal}, more heat was released during the hydrolysis of ATP. For experimental details see de Meis *et al.* 1997.

ATP synthesized during the ATP $\leftrightarrow$ P_i exhange reaction and the amount of heat produced during ATP hydrolysis i.e., the ΔH^{cal} of ATP hydrolysis. Dimethyl sulfoxide is able to couple the Ca^{2+}-ATPase (Inesi & de Meis 1989, de Meis *et al.* 1990, de Meis *et al.* 1980, de Meis 1989) leading to an

increased Ca^{2+} uptake, a decrease of the ATPase activity and an increase of ATP synthesis. We observed that in the presence of dimethyl sulfoxide the cleavage of ATP produces less heat and the ΔH^{cal} increases to the same value as that measured with leaky vesicles (Table I). Note that dimethyl sulfoxide did not change the ΔH^{cal} measured with leaky vesicles in the presence of 0.1 mM $CaCl_2$ (Table I). Heparin is an uncoupling drug that inhibits both the synthesis and the hydrolysis of ATP and increases the leakage of Ca^{2+} through the Ca^{2+}-ATPase (de Meis & Suzano 1994, Wolosker & de Meis 1995, Rocha *et al.* 1996). In the presence of 3 μg/ml heparin the vesicles still retained a small amount of Ca^{2+} and despite the significant decrease of the ATPase activity, the heat released during the different incubation intervals was similar to that measured with the control without heparin. Thus, the ΔH^{cal} measured with 3 μg/ml heparin was significantly more negative than that of the control (Table I). The degree of leakage increased when the heparin concentration was raised to 10 μg/ml and although the vesicles were still able to hydrolyse ATP, they were no longer able to accumulate Ca^{2+}. This promoted a decrease in the ΔH^{cal} to the same value as that measured with leaky vesicles (de Meis *et al.* 1997, de Meis 1998a,b).

3. CONTROL OF HEAT PRODUCTION IN ABSENCE OF A TRANSMEMBRANE Ca^{2+} GRADIENT

The Ca^{2+}-ATPase seems to be able to modulate the ΔH^{cal} of ATP hydrolysis even in the absence of a transmembrane Ca^{2+} gradient. In this case however, the amount of heat produced during ATP hydrolysis was always smaller than that measured with intact vesicles (without dimethyl sulfoxide in Table I). Leaky vesicles can catalyse both the hydrolysis and the synthesis of ATP when the Ca^{2+} concentration in the medium is raised to a level similar to that found inside the vesicles when a gradient is formed (about 2 mM) (de Meis & Vianna 1979, de Meis 1993, de Meis & Inesi 1992, de Meis *et al.* 1980, de Meis 1989, de Meis 1988, de Meis 1981, de Meis & Carvalho 1974, de Meis & Sorenson 1975). This promotes both a decrease of the rate of ATP hydrolysis and activation of the synthesis of ATP (Table II). Dimethyl sulfoxide is known to propitiate the reversal of the catalytic cycle (de Meis & Vianna 1979, de Meis *et al.* 1980, de Meis 1989, de Meis & Inesi 1985), decreasing the ratio between the velocities of ATP hydrolysis and of ATP synthesis. With the use of leaky vesicles a small increase (less negative) of the ΔH^{cal} was detected when the Ca^{2+} concentration in the medium was raised from 0.1 to 2.0 mM (Fig. 2 and Table II). The increase was more pronounced when dimethyl sulfoxide was added to the medium. These findings suggest that part of the chemical energy derived from the hydrolysis of ATP is retained by the enzyme to synthesize part of the ATP previously cleaved or it can be dissipated as heat and the selection between the two routes would be determined by the Ca^{2+} concentration in the medium and by the presence of P_i, one of the substrates needed for the synthesis of ATP.

4. EFFECT OF TEMPERATURE: Ca^{2+} TRANSPORT AND HEAT PRODUCTION BY ENDOTHERMIC (RABBIT) AND POIKILOTHERMIC (TROUT) ANIMALS

This was explored using vesicles derived from the sarcoplasmic reticulum vesicles of rabbit white muscle and trout muscle (de Meis 1998b). The activity of the two vesicles preparations increase with the temperature and after 40 min. incubation at 25°C the amounts of Ca^{2+} retained by the rabbit and trout vesicles are practically the same (Table III). The trout Ca^{2+}-ATPase is unstable at temperatures higher than 25°C and is inactivated after a few minutes incubation at 35°C (Chini *et al.* 1993). The rabbit ATPase however, is stable for more than one hour at 35°C. The physiological body temperature of the trout varies between 20° and 25°C while the rabbit is 37°C. Thus, in spite the fact that the two enzymes can pump similar amounts of Ca^{2+} at 25°C, at the physiological body temperature the rabbit sarcoplasmic reticulum is able to pump more Ca^{2+} (Table III) and at a faster rate than the reticulum of the trout (de Meis 1998b). After formation of the gradient both the rabbit and the trout Ca^{2+}-ATPases

TABLE II
Heat released and ATP synthesis in the absence of Ca^{2+} gradient.

Condition	ATP, μmol/mg.20 min^{-1} Hydrolysis	Synthesis	ΔH^{cal} (Kcal/mol P_i)
(a) 0.1 mM $CaCl_2$	5.34 ± 0.68	0	-12.25 ± 0.25
(b) 2.0 mM $CaCl_2$	2.31 ± 0.29	0.37 ± 0.03	-7.78 ± 0.23*
(c) 2.0 mM $CaCl_2$ without added P_i	2.43 ± 0.36	0	-11.93 ± 0.18

The reactions were performed at 25°C with rabbit leaky vesicles. The assay medium composition was 50 mM MOPS-Tris pH 7.0, 1 mM ATP, 0.1 mM ADP, 4 mM $MgCl_2$, 10 μM A23187, 20% (v/v) dimethyl sulfoxide and either with 2 mM P_i (a and b) or without added P_i (c). Other conditions were as described in the legend to Fig. 1. The values shown in the table represent the average ± S.E. of either seven (a and b) or three experiments (c). Note that negative values of ΔH^{cal} indicates that the reaction was exothermic and a positive value indicate that it was endothermic. The differences between the ΔH^{cal} values for ATP hydrolysis measured with 0.1 and 2.0 $CaCl_2$ (*) was significant (t test) with $p < 0.001$. For experimental details see de Meis 1998a.

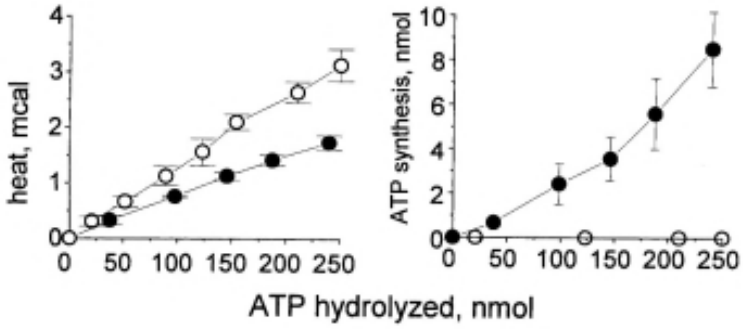

Fig. 2 – Heat release (A) and ATP resynthesis (B) during ATP hydrolysis. The assay medium composition was 50 mM MOPS/Tris buffer pH 7.0, 1 mM ATP, 0.1 mM ADP, 4 mM $MgCl_2$, 10 μM A23187, 20% dimethyl sulfoxide, 2 mM P and either (❍) 0.1 or (●) 2.0 mM $CaCl_2$. The reaction was started by the addition of rabbit leaky vesicles. The reaction was performed at 25°C. The values represent the average ± SE of four experiments. For experimental details see de Meis 1998a.

are able to synthesize a small amount of ATP and in all conditions tested, the rate of synthesis is 45 to 25 times smaller than the rate of ATP hydrolysis (Table III). Both, in the presence and in the absence of a Ca^{2+} gradient, the amount of heat released is proportional to the amount of ATP hydrolyzed. This can be visualized plotting either the heat released as a function of the amount of ATP hydrolyzed (Fig. 1) or calculating the ΔH^{cal} at each incubation interval (Table III and Fig. 3). The heat released for each ATP molecule hydrolyzed varies depending on the temperature of the assay and the source of the vesicles used. For the rabbit, the value of ΔH^{cal} measured at 35°C with intact vesicles is the double

TABLE III
Energy interconversion by the rabbit and trout Ca^{2+}-ATPase at different temperatures.

Animal and temperature	Ca^{2+} uptake μmol/mg	ATP hydrolysis μmol/mg.min^{-1}	ATP synthesis μmol/mg.min^{-1}	ΔH^{cal} Kcal/mol P_i
Rabbit				
35°C	3.25 ± 0.41 (6)	0.89 ± 0.15 (6)	0.03 ± 0.08 (6)	-20.78 ± 1.33 (41)
25°C	1.54 ± 0.10 (9)	0.42 ± 0.05 (9)	0.09 ± 0.01 (9)	-11.53 ± 0.54 (17)
Trout				
25°C	1.42 ± 0.14 (12)	0.67 ± 0.10 (12)	0.028 ± 0.02 (12)	-21.7 ± 1.15 (18)
15°C	0.94 ± 0.11 (5)	0.40 ± 0.06 (5)	0.010 ± 0.001 (5)	-11.1 ± 0.69 (9)

Values are means ± SE of the number of experiments shown in parenthesis. Assay medium composition and other experimental conditions were as described in Table I. Ca^{2+} uptake values are not initial velocities but steady-state level reached after 40 min incubation. For experimental details see de Meis 1998b.

of that measured with leaky vesicles. This difference was no longer detected when the temperature is decreased to 25°C as if in the rabbit, the mechanism that converts osmotic energy into heat production would be turned off when the temperature is decreased to a level far away from the physiologic body temperature (Table III and Fig. 3).

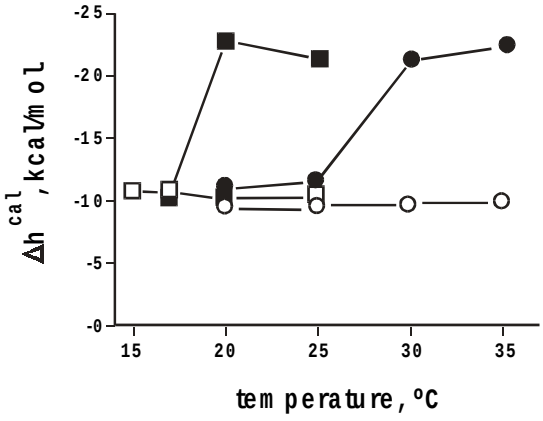

Fig. 3 – Effect of gradient and temperature on of ATP hydrolysis. The assay media and experimental conditions were as in Table III using trout (■, ❑) and rabbit (●, ❍) vesicles and either intact vesicles (solid symbols) or leaky vesicles (open symbols).

For the trout vesicles (poikilotherm), formation of a transmembrane Ca^{2+} gradient at 25°C leads to a change of the ΔH^{cal} for ATP hydrolysis to a value similar to that measured with the rabbit vesicles at 35°C. The difference of ΔH^{cal} values measured with trout vesicles in the presence and absence of a Ca^{2+} gradient is also abolished when the temperature of the medium was decreased but in this case, to a value below 17°C. The ΔH^{cal} measured with leaky vesicles did not vary with the temperature nor with the source of the vesicles used (Fig. 3). These data indicate that the amount of heat produced during ATP hydrolysis by the Ca^{2+}-ATPase increases when a gradient is formed across the sarcoplasmic reticulum membrane regardless of whether trout or rabbit a fish were used. The gradient dependent heat production however, seems to be arrested when the temperature of the medium is decreased more than 5°C below the physiological body temperature, i.e., below 30°C for the rabbit and below 20°C for the trout. The enhancement of heat production associated with the gradient could therefore play a physiological role in the maintenance of the body temperature but would not be a good emergency system to raise the body temperature after rapid cooling of the animal to an extreme point that leads to a large variation of the body temperature.

5. Ca^{2+} TRANSPORT AND HEAT PRODUCTION BY DIFFERENT Ca^{2+}-ATPase ISOFORMS

Three distinct genes encode the sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCA) isoforms, but

TABLE IV
Energy interconversion by the Ca^{2+}-ATPase of rabbit sarcoplasmic reticulum and human blood platelets endoplasmic reticulum.

Vesicles and Ca^{2+} concentration	Ca^{2+} uptake μmol/mg	ATP hydrolysis μmol/mg	Heat release, mcal	ΔH^{cal} Kcal/mol P_i
Skeletal muscle				
Ca^{2+}, zero	–	2.1 ± 0.1 (11)	20.6 ± 2 (11)	-10.20 ± 1.38 (11)
1 μM	1.85 ± 0.16 (5)	40.3 ± 2.5 (5)	$1,270.4 \pm 62.9$ (5)	-31.88 ± 1.22 (5)
10 μ	2.65 ± 0.43 (5)	46.1 ± 2.2 (5)	$1,054.1 \pm 147.4$ (5)	-22.67 ± 2.14 (5)
Blood platelets				
Ca^{2+}, zero	–	0.5 ± 0.1 (7)	4.2 ± 0.9 (7)	-12.30 ± 0.71 (7)
1 μM	0.14 ± 0.02 (3)	1.8 ± 0.3 (4)	15.9 ± 0.75 (4)	-9.91 ± 1.93 (4)
10 μM	0.22 ± 0.02 (9)	1.6 ± 0.2 (15)	17.1 ± 2.6 (15)	-10.99 ± 1.09 (15)

The incubation time at 35°C was 30 min. The assay medium composition was 50 mM MOPS/Tris buffer (pH 7.0), 4 mM $MgCl_2$, 100 mM KCl, 1 mM ATP, 5 mM NaN_3, 10 mM P_i, and 5 mM EGTA (zero Ca^{2+}) or 0.1 mM EGTA and either 0.063 or 0.112 $CaCl_2$. The calculated free Ca^{2+} concentration with these different mixtures of EGTA and $CaCl_2$ where 1 and 10 μM respectively. Values are the mean ± S.E. of the number of experiments shown in parentheses. For experimental details see Mitidieri & de Meis 1999.

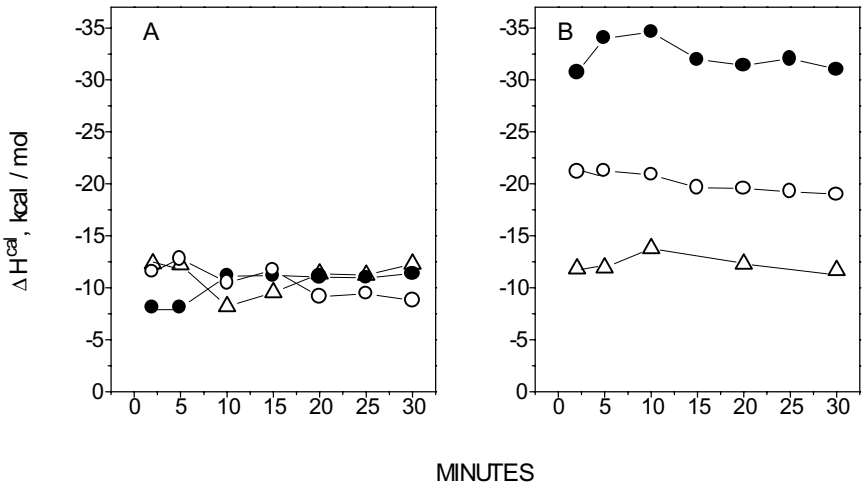

Fig. 4 – Effect of Ca^{2+} gradient on the values measured with platelet (A) and skeletal muscle (B) vesicles. The experimental conditions were as described in Table IV. The free Ca^{2+} concentrations were (Δ) zero, (●) 1 μM and (❍) 10 μM free Ca^{2+}.

the physiological meaning of isoforms diversity is not clear. The SERCA 1 gene is expressed exclusively in fast skeletal muscle whereas blood platelets and lymphoid tissues express SERCA 3 and SERCA 2b genes (MacLennan *et al.* 1985, Lytton & MacLennan 1988, Wuytack *et al.* 1994, Lytton *et al.* 1992). The catalytic cycle of the different SERCA can be reversed after that a Ca^{2+} gradient has been formed across the vesicles membrane and all of them are able to synthesize ATP from ADP and P_i during Ca^{2+} transport (Hasselbach & Makinose 1961, Barlogie *et al.* 1971, Makinose & Hasselbach

1971, de Meis & Vianna 1979, Inesi 1985, de Meis 1988, de Meis 1981, Hasselbach 1978). The vesicles derived from blood platelets endoplasmic reticulum are able to accumulate a smaller amount of Ca^{2+} than the vesicles derived from muscle (Table IV). During transport the two vesicle preparations catalyze simultaneously the hydrolysis and the synthesis of ATP from ADP and P_i. The rate of synthesis was several folds slower than the rate of hydrolysis. Using the same experimental conditions as those described in Tables IV and in presence of 1 μM free Ca^{2+}, the rates of ATP synthesis for platelets and muscle vesicles were 0.08 ± 0.01 (6) and 2.57 ± 0.22 (4) μmole of ATP/ mg protein.30 min^{-1} respectively. These values are the average $\pm$ S.E. of the number of experiments shown in parenthesis. The kinetics of Ca^{2+} transport and ATP synthesis have been analyzed in details in previous reports (Hasselbach & Makinose 1961, Barlogie *et al.* 1971, Makinose & Hasselbach 1971, de Meis & Vianna 1979, Inesi 1985, de Meis 1981, Hasselbach 1978). In this section we focused on the heat produced during transport. As for the muscle vesicles (SERCA 1), the Ca^{2+} transport by the vesicles derived from blood platelets endoplasmic reticulum (SERCA 3 and 2b) is exothermic and the amount of heat released during the different incubation intervals was proportional to the amount of ATP cleaved (Mitidieri & de Meis 1999). This could be visualized calculating the ΔH^{cal} using the values of heat release and P_i produced at different incubation intervals (Fig. 4). Two different ATPase activities can be distinguished in both platelets and muscle vesicles. The Mg^{2+}-dependent activity requires only Mg^{2+} for its activation and is measured in the presence of EGTA to remove contaminant Ca^{2+} from the assay medium. The ATPase activity which is correlated with Ca^{2+} transport requires both Ca^{2+} and Mg^{2+} for full activity (de Meis & Vianna 1979, Inesi 1985, Hasselbach 1978). In both vesicles preparations, the Mg^{2+}-dependent ATPase activity represents a small fraction of the total ATPase activity measured in presence of Mg^{2+} and Ca^{2+}. The amount of heat produced during the hydrolysis of ATP by the Mg^{2+}-dependent ATPase was the same regardless of whether muscle or platelets vesicles were used and the ΔH^{cal} value calculated in the two conditions (Table IV) was the same as that previously measured with soluble F1 mitochondrial ATPase (de Meis 1998a) and soluble myosin at pH 7.2 (Gajewski *et al.* 1986). For the vesicles derived from muscle (SERCA 1) the formation of a Ca^{2+} gradient increased the yield of heat production during ATP hydrolysis. This was not observed with the use of platelets vesicles (SERCA 2b and 3) where the yield of heat produced during ATP cleavage was the same in presence and absence of a transmembrane Ca^{2+} gradient (Fig. 4 and Table IV). For the muscle vesicles there was no difference in the ΔH^{cal} value of the Mg^{2+}-dependent ATPase and the Ca^{2+}-ATPase when the vesicles were rendered leaky (compare values of no gradient in Table I and zero Ca^{2+} in Table IV). With intact vesicles, the ΔH^{cal} value was more negative, i.e., more heat was produced during the hydrolysis of each ATP molecule when the free Ca^{2+} concentration in the medium was decreased from 10 to 1 μM (Fig. 4 and Table IV). During transport, the P_i available in the assay medium diffuses through the membrane of both muscle and platelets vesicles, to form Ca^{2+} phosphate crystals inside the vesicles. These crystals operate as a Ca^{2+} buffer that maintains the free Ca^{2+} concentration inside the two vesicles constant ($\sim$ 2 mM) at the level of the solubility product of calcium phosphate (de Meis 1981, de Meis *et al.* 1974). The energy derived from the gradient depends on the difference between the Ca^{2+} concentrations inside and outside the vesicles. The different values of ΔH^{cal} measured with the muscle vesicles with 1 and 10 μM Ca^{2+} suggest that when the free Ca^{2+} concentration in the medium is lower, the gradient formed across the vesicles membrane is steeper; thus more heat was produced and a more negative value of the ΔH^{cal} for ATP hydrolysis is observed. With vesicles derived from blood platelets, there is no extra heat production during Ca^{2+} transport regardless of the free Ca^{2+} concentration in the medium (Table IV). During transport, the free Ca^{2+} concentration

in the lumen of the platelets vesicles is the same as that of the muscle ($\sim$ 2 mM). Thus, the Ca^{2+} gradient formed across the membrane in the presence of 1 and 10 μM Ca^{2+} should be the same in the two vesicles preparations, but only in muscle vesicles the Ca^{2+} gradient increases the yield of heat production during ATP hydrolysis. These findings indicate that different from the muscle, the Ca^{2+}-ATPase of platelets is not able to convert the osmotic energy derived from the gradient into heat.

6. UNCOUPLED Ca^{2+} EFFLUX

Kinetics experiments indicate that the extra heat measured after the formation of a gradient in muscle vesicles is somehow related to the uncoupled Ca^{2+} efflux mediated by the Ca^{2+}-ATPase (de Meis *et al.* 1997, de Meis 1998b, Mitidieri & de Meis 1999). This can be measured arresting the pump by the addition of an excess EGTA to the medium (Fig. 5). In this condition, the free calcium available in the medium is chelated but ATP and other reagents remain at the same concentration as those used for measurements of ATP hydrolysis and heat production. The uncoupled efflux can also be measured diluting vesicles previously loaded with Ca^{2+} in a medium containing only buffer and EGTA (Fig. 6). For the muscle vesicles, the efflux promoted decreases when thapsigargin, a specific inhibitor of the Ca^{2+}-ATPase (Thastrup *et al.* 1987, Sagara *et al.* 1992), is added to the medium simultaneously with EGTA. The difference between the total efflux and the efflux measured in presence of thapsigargin represents the uncoupled efflux mediated by the Ca^{2+}-ATPase (de Meis & Inesi 1992, Wolosker & de Meis 1994) and in muscle vesicles it represents about 70% of the total Ca^{2+} efflux (Fig. 5 and Table V). The Ca^{2+} efflux of platelets vesicles is slower than that of muscle and is not impaired by thapsigargin, regardless of the method used to measure the efflux (Figs. 6 and Table V). These data suggest that Ca^{2+} leaks through the SERCA 1 of skeletal muscle but not through the SERCA 2B and 3 found in blood platelets. Therefore, the difference of heat production measured in muscle and platelet vesicles after formation of a transmembrane gradient (Table IV) could be due to the absence of uncoupled Ca^{2+} leakage through the Ca^{2+}-ATPase in platelets vesicles (thapsigargin sensitive efflux in Table V).

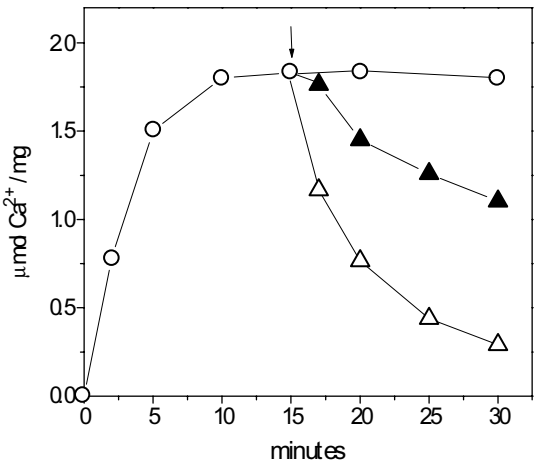

Fig. 5 – Ca^{2+} release from skeletal muscle vesicles. The assay medium composition was 50 mM MOPS/Tris pH 7.0, 2 mM $MgCl_2$, 1 mM ATP, 0.1 mM $CaCl_2$, 20 mM P_i. The reaction was started by the addition of vesicles at 35°C. (❍) control without additions. The arrow indicates the addition of either 5 mM EGTA (△) or 5 mM EGTA plus 1 M thapsigargin (▲).

7. PLATELET ACTIVATING FACTOR

Lipids derived from the breakdown of membrane phospholipids are able to increase the uncoupled efflux mediated by the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum (Cardoso & de Meis 1993). We therefore tested different lipids in platelets vesicles in search of a compound that could promote a thapsigargin sensitive Ca^{2+} efflux. The reasoning was that if we could promote the leakage of Ca^{2+} through the platelet Ca^{2+}-ATPase then, similar to the muscle vesicles, the platelet vesicles should become able to convert osmotic energy into heat. In the course of these experiments we found that DL-α-phosphatidylcholine, β-acetyl-γ-O-hexadecyl could promote such an efflux in platelets but not in muscle vesicles. This phospholipid belongs to a family of acetylated phospholipids

TABLE V
Ca^{2+} efflux from skeletal muscle and blood platelets vesicles.

Vesicles and PAF addition	n	Total efflux (A) nmol/mg.min^{-1}	5 μM TG (B) nmol/mg.min^{-1}	TG-sensitive (A-B)
Muscle				
Without PAF	7	203 ± 26	63 ± 19	140 ± 22
4 μM PAF	4	228 ± 38	97 ± 20	130 ± 38
Platelets				
Without PAF	6	40 ± 3	41 ± 6	0
4 μM PAF	4	$> 273 \pm 9$	61 ± 3	$> 212 \pm 10$

The assay medium composition and experimental conditions were as described in Fig. 7. In the table, TG refers to thapsigargin and n to the number of experiments. Values are average $\pm$ S.E.. For experimental details, see Mitidieri & de Meis 1999.

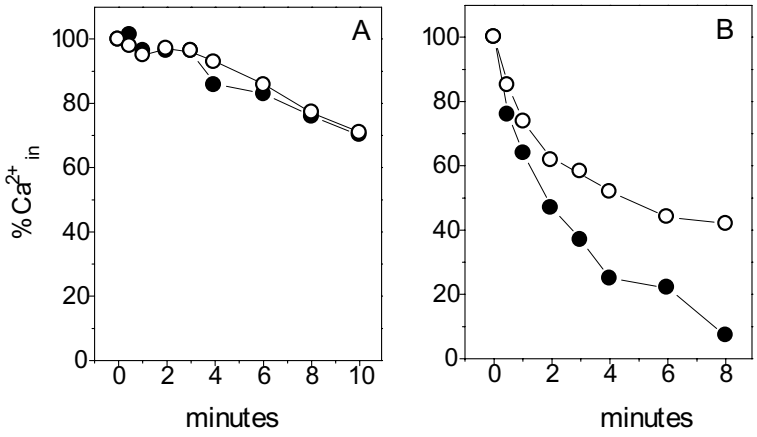

Fig. 6 – Ca^{2+} efflux from platelet (A) and skeletal muscle (B) vesicles. The vesicles were preloaded with ^{45}Ca and diluted to a final concentration of 30μg of protein/ml into a medium containing 50 mM MOPS/Tris pH 7.0 and 0.1 mM EGTA either in the absence (●) or presence (❍) of 1μM thapsigargin.

known as platelet activating factor (PAF) which are produced when cells involved in inflammatory process are activated. PAF was found to inhibit the Ca^{2+} uptake of both platelets and muscle vesicles (Tables VI). With the two vesicles, half maximal inhibition is obtained with 4 to 6 μM PAF. In contrast with the Ca^{2+} uptake, the ATPase activity of the two vesicles preparations is not inhibited by PAF (Mitidieri & de Meis 1999). The discrepancy between Ca^{2+} uptake and ATPase activity suggests that the decrease of Ca^{2+} accumulation is promoted by an increase of Ca^{2+} efflux and not by an inhibition of the ATPase. The amount of Ca^{2+} retained by the vesicles is determined by the differences between the rates of Ca^{2+} uptake and of Ca^{2+} efflux. The higher the efflux, the smaller the amount of Ca^{2+} retained by the vesicles. The addition of PAF during the course of Ca^{2+} uptake promotes the release of

TABLE VI
Effect of PAF on the Ca^{2+} uptake and ΔH^{cal} of ATP hydrolysis.

		Skeletal muscle vesicles		Blood platelets vesicles	
Ca^{2+}, μM	PAF, μM	Ca^{2+} uptake μmol/mg	ΔH^{cal}, Kcal / mol P_i	Ca^{2+} uptake μmol/mg	ΔH^{cal}, Kcal / mol P_i
1	0	1.83 ± 0.21 (4)	-32.99 ± 2.90 (4)	0.11 ± 0.03 (3)	-12.58 ± 1.29 (5)
	4	0.45 ± 0.19 (4)	-25.69 ± 1.71 (4)	0.03 ± 0.01 (3)	-20.04 ± 0.37 (3)
10	0	2.66 ± 0.44 (3)	-22.92 ± 2.24 (3)	0.20 ± 0.04 (3)	-10.70 ± 1.01 (3)
	6	0.68 ± 0.35 (5)	-16.91 ± 1.50 (5)	0.06 ± 0.01 (3)	-23.90 ± 1.06 (3)

The assay medium and experimental conditions were as in table IV. The values in the table are the average $\pm$ S.E. of the number of experiments shown in parentheses. The differences between the ΔH^{cal} values measured in the absence and in the presence of PAF with skeletal muscle were significant (t test) with $p < 0.05$ both with 1 and 10 μM Ca^{2+} and with blood platelets were significant $p < 0.005$ (1 μM Ca^{2+}) and $p < 0.001$ (10 μM Ca^{2+}). For experimental details see Mitidieri & de Meis 1999.

Ca^{2+} until a new steady state level of Ca^{2+} retention is achieved (Fig. 7). With both preparations, when higher the concentration of PAF added, the lower the new steady state level of Ca^{2+} filling. The release of Ca^{2+} promoted by PAF is not accompanied by a burst of ATP synthesis. On the contrary, PAF inhibits the synthesis of ATP driven by the coupled Ca^{2+} efflux (Mitidieri & de Meis 1999). This indicates that the Ca^{2+} release promoted by PAF was not promoted by an increase of the reversal of the pump. A major difference between the muscle and platelets vesicles was found when thapsigargin was added to the medium together with PAF. For the platelets vesicles, the rate of Ca^{2+} release measured after the addition of PAF was greatly decreased in presence of thapsigargin (Fig. 7 and Table V) indicating that most of the Ca^{2+} left the vesicles through the ATPase as an uncoupled Ca^{2+} efflux. This could be better seen after the initial minute of incubation. In fact, the rate of release in platelets vesicles was so fast that we could not measure the initial velocity of release with the method available in our laboratory. Thus, the values with PAF in Table V differ from the other values in that it does not reflect a true rate, but only the parcel of Ca^{2+} released during the first incubation minute. In muscle, the rate of Ca^{2+} efflux measured after the addition of PAF is slower than that measured with platelets vesicles (compare Figs. 7A and 7B) and the proportion between the Ca^{2+} effluxes sensitive and insensitive to thapsigargin measured with PAF is practically the same as that measured when the pump is arrested with EGTA (Table V). Having found a compound that induces the release of Ca^{2+} through the pump, we then measured the heat produced during ATP hydrolysis in the presence and absence of PAF (Table VI). The PAF concentrations selected were sufficient to enhance the rate of efflux without completely abolishing the retention of Ca^{2+} by the vesicles, i.e., without abolishing the formation of a Ca^{2+} gradient through the vesicles membrane. In such conditions PAF enhances the amount of heat produced during the hydrolysis of ATP by the blood platelets. In muscle vesicles however, PAF decreases the amount of heat produced during ATP hydrolysis. The ΔH^{cal} values measured with PAF and muscle vesicles were less negative than those measured in absence of PAF, but still more negative than the values measured in absence of Ca^{2+} gradient.

8. CONCLUSIONS

The Ca^{2+}-ATPase can regulate the interconversion of energy in such a way as to vary the fraction of the energy derived from ATP hydrolysis which is dissipated as heat. This can be observed both in the presence and in the absence of a transmembrane

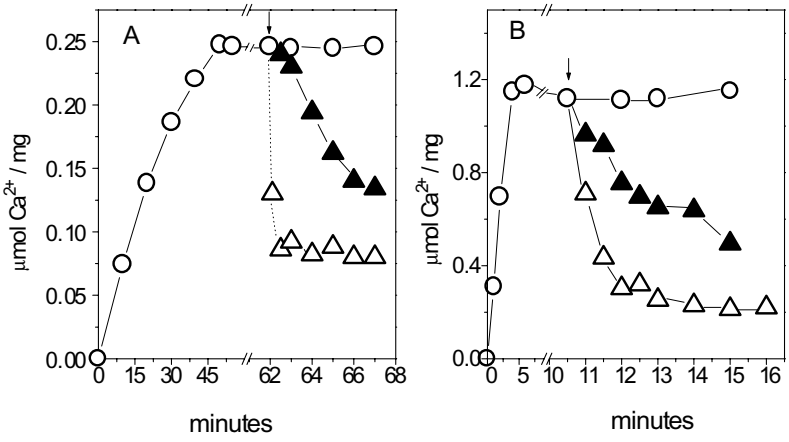

Fig. 7 – Ca^{2+} release after the addition of PAF. muscle (B) vesicles. The assay medium composition was 50 mM MOPS/Tris pH 7.0, 2 mM $MgCl_2$, 10 mM P_i, 40μM $CaCl_2$, 100 mM KCl and 3 mM ATP. The reaction was started by the addition of either platelets (A) or muscle (B) vesicles at 35°C. (❍) control without additions. The arrow indicates the addition of either 6μM PAF (△) or 6μM PAF plus 1 M thapsigargin (▲).

Ca^{2+} gradient and depending on the conditions used the ΔH^{cal} for ATP hydrolysis may vary from -7.8 (Table II) up to -31.9 kcal/mol P_i (Table IV).

The experiments described suggest the following sequences of energy conversion:

i) Ca^{2+} gradient - from the total chemical energy released during the ATP hydrolysis($\sim$ 30 kcal/mol P_i), about 1/3 is converted into heat ($\sim$ 10 kcal) provided that the Ca^{2+} concentration on both sides of the membrane is kept in the micromolar range (leaky vesicles in Tables I and II). The rest of the energy ($\sim$ 20 kcal/mol P_i) is probably used to translocate Ca^{2+} across the membrane (work). If the vesicles are leaky, the cycle is concluded in this step, because the Ca^{2+} transported diffuses back into the medium and the work performed during Ca^{2+} translocation is not converted into heat. However, if the membrane is intact, the energy used for the translocation of Ca^{2+} is converted into osmotic energy (Ca^{2+} gradient) and the Ca^{2+}-ATPase can then use this energy to either synthesize back a small part of the ATP previously cleaved or to produce heat. The balance between these two routes would be determined by the ratio between the coupled and uncoupled enzyme units. In one extreme (dimethyl sulfoxide in Table I), there would be a high degree of energy conservation, most of the energy derived from the hydrolysis of ATP being conserved by the vesicles as osmotic energy and practically all the Ca^{2+} that leaves the vesicles is used to synthesize back a part of the ATP previously cleaved. In the other extreme (3 μg/ml heparin), the SR operates as it was a "furnace", a small amount of Ca^{2+} is retained by the vesicles and most of the energy derived from ATP hydrolysis is dissipated into the medium as heat.

ii) No gradient - previous studies demonstrated that the soluble Ca^{2+}-ATPase is able to retain part of the energy derived from ATP hydrolysis even after that both ADP and P_i dissociate from the enzyme and the retained energy can be used for the synthesis of a new ATP molecule. This is promoted by the binding of Ca^{2+} to a low affinity site of the Ca^{2+}-ATPase located in a region of the protein facing the vesicles lumen (de Meis & Vianna 1979, de Meis *et al.* 1980, de Meis 1989, de Meis & Carvalho 1974, de Meis & Sorenson 1975, de Meis & Inesi 1985).

The data of Table II indicates that the synthesis of ATP in leaky vesicles is associated with an increase of the ΔH^{cal} value for ATP hydrolysis, suggesting that the binding of Ca^{2+} to the low affinity site of the enzyme may regulated the fraction of the energy that dissipates as heat and that which can be used for the synthesis of ATP.

iii) It seems that osmotic energy cannot be transformed spontaneously into heat and that a device is needed for this conversion. For the sarcoplasmic reticulum the device is probably the Ca^{2+}-ATPase itself, that in addition to interconvert chemical into osmotic energy, can also convert osmotic energy into heat. In leaky vesicles Ca^{2+} is translocated across the membrane during ATP hydrolysis and afterwards flows back to the medium through the permeabilized membrane. If the simple diffusion of Ca^{2+} through any kind of pore in the membrane would lead to heat production, then the same ΔH^{cal} value should have been found with intact and permeabilized vesicles (Table I).

iv) It is generally assumed that the energy released during the hydrolysis of ATP by the Ca^{2+}-ATPase can be divided in two non-interchangeable parts, one is converted into heat and the other is used to pump Ca^{2+} across the membrane. This was observed with the platelets vesicles before the addition of PAF (Table VI). The finding that the SERCA 1 can convert osmotic energy into heat revealed an alternative route that increases two to three folds the amount of heat produced during ATP hydrolysis therefore permitting the maintenance of the cell temperature with a smaller consumption of ATP. The data obtained with the blood platelets vesicles show that not all the SERCA isoforms are able to readily convert osmotic energy into heat. These vesicles however, can be converted by PAF into a system capable of increasing the heat production during ATP hydrolysis (Tables V and VI), suggesting that the mechanism capable of providing additional heat production can be turned on and off and this could represent a mechanism of thermoregulation specific of the cells expressing SERCA 2b and 3. Both in muscle and platelets vesicles there is a Ca^{2+} efflux which is not inhibited by thapsigargin. We do not know through which membrane structure this Ca^{2+} flows, but the data obtained with platelets before the addition of PAF indicate that during this efflux, osmotic energy is not converted into heat. In platelets, PAF promoted simultaneously the appearance of thapsigargin sensitive efflux and extra-heat production during ATP hydrolysis. These observations corroborate with the notion that the conversion of osmotic energy into heat can not be promoted by any kind of Ca^{2+} leakage and that a device is needed for this conversion.

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