CATALYTIC ACTIVITY AND HEAT PRODUCTION BY THE Ca^{2+}-ATPase FROM SEA CUCUMBER (*LUDWIGOTHUREA GRISEA*) LONGITUDINAL SMOOTH MUSCLE: MODULATION BY MONOVALENT CATIONS

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Summary

In muscle cells, excitation–contraction coupling involves the translocation of Ca^{2+} between intracellular compartments and the cytosol. Heat derived from the hydrolysis of ATP by the sarcoplasmic reticulum Ca^{2+}-ATPase of skeletal muscle plays an important role in the thermoregulation and energy balance of the cell. Although several Ca^{2+}-ATPase isoforms have been described in vertebrates, little is known about Ca^{2+} transport in invertebrates. In this report, a Ca^{2+}-ATPase is identified in the microsomal fraction obtained from sea cucumber (*Ludwigothurea grisea*) smooth muscle. The activity of this enzyme is enhanced three- to fivefold by K$^+$ and Na$^+$. During Ca^{2+} transport, the ATPase can synthesise ATP from ADP and inorganic phosphate (P$_i$) using the energy derived from the Ca^{2+} gradient formed across the microsomal membrane (ATP$\leftrightarrow$P$_i$ exchange). The apparent affinity of the enzyme for P$_i$ is increased by more than one order of magnitude by K$^+$. In the presence of K$^+$, the fraction of ATP synthesised during the exchange reaction by sea cucumber microsomes was found to be larger than that measured in microsomes derived from either rabbit or trout muscle. Like the isoforms found in skeletal muscle, the sea cucumber Ca^{2+}-ATPase can convert osmotic energy into heat. The amount of heat produced after the hydrolysis of each ATP molecule increases two- to threefold when a Ca^{2+} gradient is formed across the microsomal membrane.

Key words: heat production, sea cucumber, *Ludwigothurea grisea*, smooth muscle, Ca^{2+}-ATPase, energy conservation, K$^+$-dependence.

Introduction

The Ca^{2+}-ATPase of the sarcoplasmic reticulum of skeletal muscle that is found in both endothermic (rabbit) and ectothermic (trout) vertebrates is able to interconvert different forms of energy (Barlogie et al., 1971; Makinose, 1971; Makinose and Hasselbach, 1971; de Meis and Vianna, 1979; de Meis, 1998). During Ca^{2+} transport, the chemical energy derived from ATP hydrolysis is used by the Ca^{2+}-ATPase to pump Ca^{2+} into the vesicles, and during this process chemical energy is converted into osmotic energy. After accumulation of Ca^{2+} within the sarcoplasmic reticulum, the Ca^{2+} gradient formed across the membrane promotes the reversal of the catalytic cycle of the enzyme. During this reversal, some of the osmotic energy is either used to resynthesise some of the ATP previously cleaved (Barlogie et al., 1971; Makinose, 1971; Makinose and Hasselbach, 1971; de Meis and Vianna, 1979; de Meis, 1981) or dissipated into the surrounding medium as heat (Block, 1994; Janský, 1995; de Meis et al., 1997; de Meis, 1998; Mitidieri and de Meis, 1999). Ca^{2+} leaves the sarcoplasmic reticulum through the action of the Ca^{2+}-ATPase both during ATP synthesis from ADP and P$_i$ and during heat production (de Meis et al., 1997; de Meis, 1998; Mitidieri and de Meis, 1999).

In endothermic vertebrates, the heat produced by the Ca^{2+}-ATPase is used to maintain a constant and high body temperature (Block, 1994; Janský, 1995). During nonshivering thermogenesis, most of the heat is derived from resting muscle, but the mechanism of heat production remains unclear. It has been proposed that Ca^{2+} leaks from the sarcoplasmic reticulum, and heat would therefore be derived from the hydrolysis of the extra ATP needed to maintain a low myoplasmic Ca^{2+} concentration (Block, 1994; Janský, 1995). 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 Ca^{2+} gradient, as if the energy released by ATP hydrolysis were divided in two independent packets: one to be converted into heat and the other to be used for Ca^{2+} transport. Recently, microcalorimetric measurements of ATP hydrolysis have shown that in the presence of a Ca^{2+} gradient the heat produced during the hydrolysis of each ATP molecule is 2–3 times greater than that measured with vesicles in the absence of a Ca^{2+} gradient (de Meis et al., 1997; de Meis, 1998; Mitidieri and de Meis, 1999). These results indicate that the Ca^{2+}-

ATPase isoform found in vertebrate skeletal muscle is able to convert osmotic energy into heat. This interconversion is not promoted by the Ca^{2+}-ATPase isoforms found in human platelets, where the heat released during ATP hydrolysis in the absence of a Ca^{2+} gradient is the same as that measured in the presence of a Ca^{2+} gradient (Mitidieri and de Meis, 1999).

In this paper, we have examined the modulation of catalytic activity and heat production by the Ca^{2+}-ATPase of the longitudinal smooth muscle of the sea cucumber *Ludwigothurea grisea*. This is an abundant species of the coast of Rio de Janeiro (Brazil) and is of particular interest because, like other holothurians, it occupies an interesting position on the phylogenetic scale, being an invertebrate with many chordate-like features, including a conserved Ca^{2+} pump. Previous data from our laboratory have shown that Ca^{2+} transport mediated by the longitudinal muscle Ca^{2+}-ATPase of the sea cucumber is highly dependent on the K^+ concentration of the medium (Landeira-Fernandez and de Meis, 1997). In this work, we have studied the process of energy interconversion mediated by the sea cucumber Ca^{2+}-ATPase and compared its activity with that of the well-known Ca^{2+}-ATPase isoforms found in vertebrate skeletal muscle.

Materials and methods

Preparation of microsomes

Sea cucumbers, *Ludwigothurea grisea* (Selenka), were collected by snorkelling in the Urca region of Guanabara Bay, Rio de Janeiro, and immediately brought to the laboratory immersed in sea water. The longitudinal muscle was dissected from the body wall of 20 animals (mass approximately 50 g), cut into small pieces and homogenised in a Waring blender with 160 ml of an ice-cold buffer containing $10\,mmol\,l^{-1}$ Mops/Tris (pH 7.0), 10 % sucrose, $1\,mmol\,l^{-1}$ EDTA, $30\,mmol\,l^{-1}$ Na_2SO_4, $450\,mmol\,l^{-1}$ NaCl, $10\,mmol\,l^{-1}$ KCl and $1\,mmol\,l^{-1}$ phenylmethanesulphonyl fluoride. The microsomes were isolated as described by Eletr and Inesi (1972). Briefly, the homogenate was centrifuged at $14\,000\,g$ for 20 min at 4 °C. The supernatant was filtered through two layers of cheesecloth and centrifuged at $100\,000\,g$ for 40 min at 4 °C. The resulting pellet was suspended in 30 ml of ice-cold buffer containing $10\,mmol\,l^{-1}$ Mops/Tris (pH 7.0), $600\,mmol\,l^{-1}$ KCl and $1\,mmol\,l^{-1}$ EDTA, and incubated on ice for 60 min followed by centrifugation at $15\,000\,g$ for 20 min at 4 °C. The supernatant was further centrifuged at $100\,000\,g$ for 40 min at 4 °C. The microsomes that were collected as a pellet were suspended in 2 ml of ice-cold buffer containing $50\,mmol\,l^{-1}$ Mops/Tris (pH 7.0), $800\,mmol\,l^{-1}$ sucrose, $5\,mmol\,l^{-1}$ NaN_3 and $1\,mmol\,l^{-1}$ EDTA, and stored under liquid nitrogen until use. Microsomes from both rabbit and trout skeletal muscle were prepared using the same procedure. Protein concentration was determined by the method of Lowry et al. (1951), using bovine serum albumin as standard.

Uptake of Ca^{2+}

The uptake of Ca^{2+} was measured by the filtration method using ^{45}Ca and Millipore filters (0.45 μm) (Chiesi and Inesi, 1979). After filtration, the filters were washed five times with 5 ml of $3\,mmol\,l^{-1}$ $La(NO_3)_3$, and the radioactivity remaining on the filters was counted with a liquid scintillation counter. Unless specified otherwise, the standard assay medium contained $50\,mmol\,l^{-1}$ Mops/Tris (pH 7.0), $0.1\,mmol\,l^{-1}$ ATP, $20\,\mu mol\,l^{-1}$ $[^{45}Ca]Cl_2$, $1\,mmol\,l^{-1}$ $MgCl_2$ and $5\,mmol\,l^{-1}$ oxalate-Tris or $10\,mmol\,l^{-1}$ P_i-Tris as indicated in the figure legends. The reactions were performed at 25 °C and were started by the addition of microsomes to give a final protein concentration of $0.02\,mg\,ml^{-1}$.

ATPase activity

Hydrolysis of ATP was determined by measuring the release of $^{32}P_i$ from $[\gamma\text{-}^{32}P]ATP$ as previously described by Grubmeyer and Penesfky (1981). The Mg^{2+}-dependent activity was measured in the presence of $2\,mmol\,l^{-1}$ EGTA and subtracted from the total activity measured in the presence of both Mg^{2+} and Ca^{2+} to obtain the Ca^{2+}-dependent activity. Other conditions were as described under Ca^{2+} uptake. The ATPase activity of sea cucumber microsomes was very low and difficult to measure using colorimetric methods.

Synthesis of $[\gamma\text{-}^{32}P]ATP$

Synthesis of $[\gamma\text{-}^{32}P]ATP$ from ADP and $^{32}P_i$ was measured as described previously (de Meis, 1988). The $^{32}P_i$ was obtained from the Brazilian Institute of Atomic Energy. The specific activity used was $5000\,cts\,min^{-1}\,nmol^{-1}$ ATP.

Calorimetric measurements

Heat of reaction was measured using an OMEGA isothermal titration calorimeter from Microcal Inc. (Northampton, MA, USA) (de Meis et al., 1997; de Meis, 1998). The calorimeter cell was filled with reaction medium, and the reference cell was filled with Milli-Q water. After equilibration at the desired temperature, the reaction was started by injecting microsomes into the reaction cell. After 2 min, the heat change due to ATP hydrolysis was recorded for a period of 30 min, and the calorimetric enthalpy of hydrolysis (ΔH^{cal}) was calculated by dividing the amount of heat released (in kJ) by the number of moles of ATP hydrolysed. A negative value indicated that the reaction was exothermic and a positive value that it was endothermic.

Results

Ca^{2+} uptake and ATP hydrolysis: activation by monovalent cations

The Ca^{2+}-ATPase from sea cucumber is able to transport Ca^{2+} at the expense of ATP hydrolysis, but differs from the well-known vertebrate isoforms. Both Ca^{2+} uptake and ATP hydrolysis were activated several-fold by K^+ and Na^+, but not by Li^+ (Fig. 1). The degree of activation promoted by K^+ and Na^+ was found to vary depending on whether oxalate or P_i was used as the Ca^{2+}-precipitating agent (Table 1).

Oxalate and P_i are known to increase the Ca^{2+}-loading

Fig. 1. Activation of (A) Ca^{2+} uptake and (B) Ca^{2+}-dependent ATP hydrolysis by monovalent cations. The assay medium contained 50 mmol l^{-1} Mops/Tris (pH 7.0), 5 mmol l^{-1} oxalate, 1 mmol l^{-1} MgCl$_2$, 20 μmol l^{-1} CaCl$_2$ and 0.1 mmol l^{-1} ATP, and KCl (○), NaCl (△) or LiCl (□) was added as indicated. The medium was divided into two samples, and trace amounts of either ^{45}Ca or [γ-^{32}P]ATP were added to each sample for measurements of Ca^{2+} uptake (A) and ATP hydrolysis (B). In B, Ca^{2+}-dependent ATP hydrolysis was calculated by subtracting the values measured in the presence of 2 mmol l^{-1} EGTA (Mg^{2+}-dependent ATPase activity) from the values measured in the presence of CaCl$_2$ (total ATPase activity). Experiments were carried out at a temperature of 25 °C and a protein concentration of 0.02 mg ml^{-1}. Values are means of six (KCl), four (NaCl) or two (LiCl) experiments in A and three (KCl), two (NaCl) or three (LiCl) experiments in B performed using three different vesicles preparations. Vertical bars indicate ± S.E.M. and are not included for experiments with N values of 2.
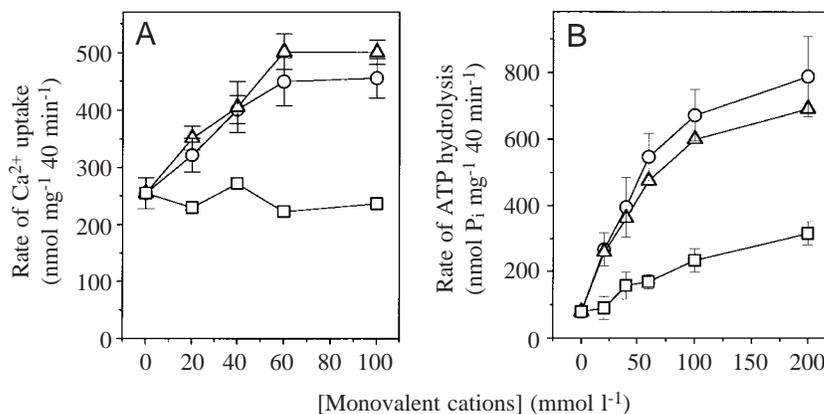
capacity of microsomes isolated from different tissues, including skeletal muscle, blood platelets and brain (de Meis et al., 1974; de Meis, 1981; Wolosker et al., 1997). During Ca^{2+} transport, they diffuse through the membrane and form crystals of calcium phosphate or calcium oxalate in the lumen of the vesicles (de Meis et al., 1974). In the presence of oxalate, the rate of Ca^{2+} uptake was faster ($P<0.001$) and the amount of Ca^{2+} retained by the vesicles was greater ($P<0.001$) than that measured in the presence of P$_i$ (Table 1). In the presence of oxalate, K$^+$ and Na$^+$ were able to activate both Ca^{2+} transport and ATP hydrolysis to the same extent (Fig. 1; Table 1). However, when P$_i$ was used, the activation of Ca^{2+} transport by K$^+$ was greater than the activation by Na$^+$ (Table 1). At present, we do not know the reason for this discrepancy.

The degree of activation promoted by monovalent cations was higher for ATP hydrolysis (approximately 4.5-fold) than for Ca^{2+} uptake (approximately two- to threefold). The concentration needed for half-maximal activation of both Ca^{2+} uptake and ATP hydrolysis was almost the same for K$^+$ and Na$^+$, being 30 mmol l^{-1} for Ca^{2+} uptake and 40 mmol l^{-1} for ATP hydrolysis.

Reversal of the catalytic cycle: ATP↔P$_i$ exchange

The Ca^{2+}-ATPase found in a variety of vertebrate tissues is able to catalyse simultaneously the hydrolysis and the synthesis of ATP during Ca^{2+} transport. This is referred to as the ATP↔P$_i$ exchange reaction (Makinose, 1971; Makinose and Hasselbach, 1971; de Meis, 1981). Our experiments showed that the sea cucumber ATPase was also able to synthesise a small amount of ATP during Ca^{2+} transport (Fig. 2A) but, unlike the Ca^{2+}-ATPase of vertebrates, the synthesis of ATP from ADP and P$_i$ was increased three- to fourfold by K$^+$ and Na$^+$. K$^+$ was found to increase the affinity of the Ca^{2+}-ATPase for P$_i$. In the absence of K$^+$, the apparent K_m for P$_i$ was greater than 15 mmol l^{-1} and could not be measured with the method used, but it decreased to 2 mmol l^{-1}

Table 1. *Activation promoted by K$^+$ and Na$^+$: differences between oxalate and inorganic phosphate*

Addition	Ca^{2+} uptake (nmol mg^{-1})		ATPase activity (nmol mg^{-1})	
	Oxalate	P$_i$	Oxalate	P$_i$
None	255±27 (10)	50±8 (8)	80±17 (3)	65±20 (7)
100 mmol l^{-1} KCl	456±34 (6)	187±20 (7)	673±77 (3)	300±40 (7)
100 mmol l^{-1} NaCl	501±21 (4)	113±13 (7)	600 (2)	360±18 (4)

The composition of the assay medium was the same as in Fig. 1, except that 5 mmol l^{-1} oxalate or 10 mmol l^{-1} inorganic phosphate (P$_i$) was included in the medium as indicated.

Values are means ± S.E.M. of the number of experiments shown in parentheses.

In the presence of P$_i$, the differences between the values of Ca^{2+} uptake measured with K$^+$ and Na$^+$ were statistically significant ($P<0.01$).

In each column, the differences between no addition and either 100 mmol l^{-1} KCl or 100 mmol l^{-1} NaCl were also statistically significant ($P<0.001$).

The reaction time was 40 min for measurements using oxalate and 60 min for measurements using P$_i$.

after the addition of 100 mmol l^{-1} KCl to the medium (Fig. 2B). Li$^+$ had almost no effect, indicating that the activation promoted by K$^+$ and Na$^+$ is not due to the increased ionic strength of the assay medium.

Energy conservation

A useful variable for evaluating the degree of energy conservation during Ca^{2+} transport is the relationship between the rates of ATP hydrolysis and ATP synthesis (ATP↔P$_i$ exchange). When the ratio is high, less ATP is recovered during
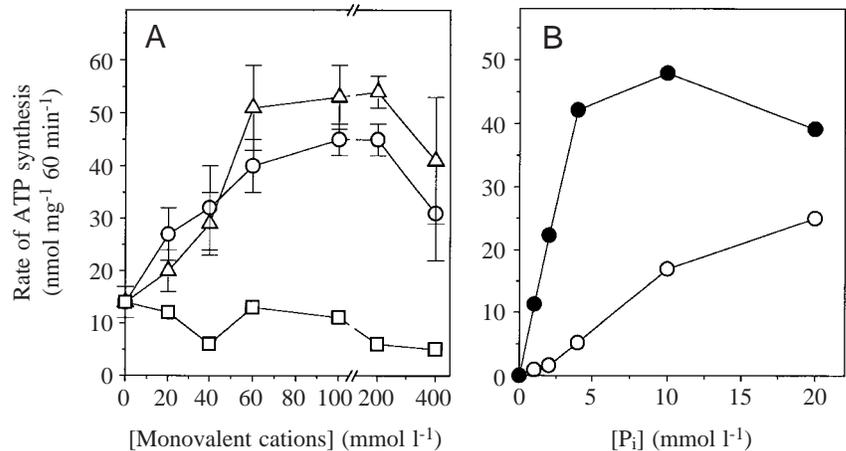
Fig. 2. ATP synthesis: (A) activation by monovalent cations and (B) P_i-dependence. The assay medium contained 50 mmol l^{-1} Mops/Tris (pH 7.0), 1 mmol l^{-1} MgCl$_2$, 50 µmol l^{-1} CaCl$_2$, 10 mmol l^{-1} $^{32}P_i$, 0.1 mmol l^{-1} ATP, 20 µmol l^{-1} ADP and KCl ($\bigcirc$), NaCl ($\triangle$) or LiCl ($\square$). (B) The reaction medium was the same as in A except that increasing concentrations of $^{32}P_i$ were used, either without KCl ($\bullet$) or with 100 mmol l^{-1} KCl ($\bigcirc$). In A, the values are means ± S.E.M. of four experiments and in B the average of two experiments.

Ca^{2+} transport and more energy is dissipated by the system (Barlogie et al., 1971; Makinose, 1971; Makinose and Hasselbach, 1971; de Meis and Vianna, 1979; de Meis, 1981). In previous reports, the ratio between the rates of ATP hydrolysis and ATP synthesis measured during the ATP↔P_i exchange reaction catalysed by the Ca^{2+}-ATPase of rabbit (35 °C) and trout (*Salmo gairdnerii*) (25 °C) skeletal muscle was found to vary between 24 and 32 (Table 2). The value for sea cucumber vesicles was 7.5 (Table 2). This low value indicates that, in this marine invertebrate, more ATP is synthesised and, consequently, more energy is conserved during Ca^{2+} transport than in the other systems shown in Table 2.

Heat production

It has recently been shown that, in addition to interconverting chemical and osmotic energy, the Ca^{2+}-ATPase of rabbit and trout skeletal muscle can also convert osmotic energy into heat. Calorimetric measurements revealed that the amount of heat released during ATP hydrolysis by these vesicle preparations depended on whether a Ca^{2+} gradient was formed across the vesicle membranes (de

Meis et al., 1997; de Meis, 1998; Mitidieri and de Meis, 1999). However, this was not observed with the Ca^{2+}-ATPase of human blood platelets, an enzyme that appears to be unable to convert osmotic energy spontaneously into heat (Table 3). Our results show that the sea cucumber ATPase is able to convert osmotic energy into heat in a manner similar to that observed with the skeletal muscle Ca^{2+}-ATPase isoforms. As shown in Fig. 3, addition of the Ca^{2+} ionophore A23187 to the medium promoted a small increase in the level of ATPase activity but a significant decrease in the rate of heat production. Both in the presence and in the absence of Ca^{2+} ionophore, the amount of heat produced was proportional to the amount of ATP cleaved. This can be visualised either by plotting the heat released as a function of the amount of ATP hydrolysed (Fig. 4A) or by calculating ΔH^{cal} using the values of heat released and P_i produced after different incubation times (Fig. 4B). Table 3 shows that the ΔH^{cal} for ATP hydrolysis measured with sea cucumber microsomes is very similar to those measured previously with vesicles derived from the sarcoplasmic reticulum of rabbit and trout skeletal muscle.

Table 2. *Ca^{2+} uptake and ratio between ATP hydrolysis and ATP synthesis catalysed by the Ca^{2+}-ATPase from different animals*

Microsome source and temperature	Ca^{2+} uptake (µmol mg^{-1})	ATP hydrolysis, *A* (µmol mg^{-1})	ATP synthesis, *B* (µmol mg^{-1})	Ratio *A/B*
Rabbit skeletal muscle, 35 °C	3.25±0.41 (6)	35.6±6.0 (6)	1.27±0.30 (6)	31.8
Trout skeletal muscle, 25 °C	1.42±0.14 (12)	26.8±4.0 (12)	1.12±0.08 (12)	23.9
Sea cucumber smooth muscle, 25 °C	0.19±0.02 (15)	0.30±0.04 (4)	0.040±0.003 (4)	7.5

The composition of the assay medium for sea cucumber was 50 mmol l^{-1} Mops/Tris buffer (pH 7.0), 1 mmol l^{-1} MgCl$_2$, 50 µmol l^{-1} Ca^{2+}, 100 mmol l^{-1} KCl, 10 mmol l^{-1} inorganic phosphate (P_i) and 0.1 mmol l^{-1} ATP. The medium was divided into three samples containing trace amounts of ^{45}Ca, [γ-^{32}P]ATP or [γ-^{32}P]P_i for measurements of Ca^{2+} uptake, ATP hydrolysis and ATP synthesis. The reaction was started by the addition of vesicles to give a final protein concentration of 0.02 mg ml^{-1}, and the reaction time was 60 min.

Values are means ± S.E.M. of the number of experiments shown in parentheses.

Data from vesicle preparations of skeletal muscle from rabbit and trout are from de Meis (1998) and, in this case, the reaction time was 40 min.

Table 3. *Effect of a Ca^{2+} gradient on the calorimetric enthalpy of hydrolysis of ATP hydrolysis*

|	ΔH^{cal} (kJ mol^{-1})			
Condition	Rabbit skeletal muscle, 35 °C[1]	Trout skeletal muscle, 25 °C[2]	Human platelets, 35 °C[3]	Sea cucumber smooth muscle, 25 °C
Intact vesicles (gradient)	−94.76±8.94 (5)	−90.71±4.81 (18)	−45.94±4.56 (15)	−121.26±1.09 (9)
Leaky vesicles (no gradient)	−50.91±5.39 (16)	−42.26±4.47 (9)	−51.41±2.97 (7)	−52.92±8.57 (9)

Values are means ± S.E.M. of the number of experiments shown in parentheses.

ΔH^{cal}, calorimetric enthalpy of hydrolysis.

The assay medium composition and other experimental conditions were as described in Fig. 4.

[1]Data taken from de Meis et al. (1997) and de Meis (1998); [2]data from de Meis (1998); [3]data from Mitidieri and de Meis (1999).

Fig. 3. ATPase activity (A) and heat released during ATP hydrolysis (B). The assay medium contained 50 mmol l^{-1} Mops/Tris (pH 7.0), 1 mmol l^{-1} MgCl$_2$, 100 mmol l^{-1} KCl, 0.1 mmol l^{-1} ATP, 5 mmol l^{-1} NaN$_3$, 5 mmol l^{-1} oxalate and 20 µmol l^{-1} CaCl$_2$, either without Ca^{2+} ionophore (Ca^{2+} gradient) (○) or with 2 µmol l^{-1} A23187 (no Ca^{2+} gradient) (●). The medium was divided into three samples. One of these was used for measuring heat production (B), while trace amounts of either ^{45}Ca or [γ-^{32}P]ATP were added to the remaining samples for measurements of Ca^{2+} uptake (not shown) or ATPase activity (A). The three reactions were started simultaneously by the addition of vesicles to give a final protein concentration of 0.02 mg ml^{-1}. The amount of Ca^{2+} accumulated by the vesicles after a 30 min incubation in the absence of the ionophore varied between 400 and 500 nmol mg^{-1}. There was no Ca^{2+} uptake in the presence of 2 µmol l^{-1} A23187. The assay temperature was 30 °C. The values are means ± S.E.M. of 6–8 experiments, performed with three different vesicles preparations.
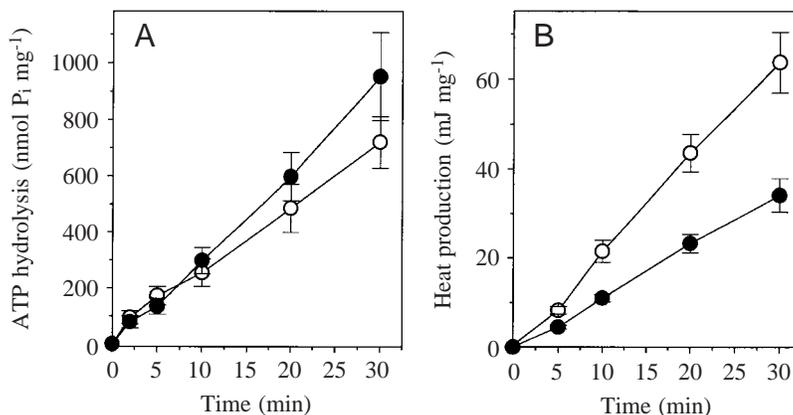
Discussion

Activation by monovalent cations and energy conservation

Our results show that the Ca^{2+}-ATPase from the longitudinal muscle of the sea cucumber, like the Ca^{2+}-ATPase isoforms of vertebrates, can interconvert different forms of energy. However, unlike the vertebrate ATPases, this enzyme only achieves its maximal activity in the presence of K$^+$. In previous reports, we measured the effects of K$^+$ on Ca^{2+}-ATPase isoforms found in rabbit and trout skeletal muscle. In these preparations, K$^+$ either has no effect or it promotes a small (6–10 %) activation of the rate of Ca^{2+} transport (de Meis and Suzano, 1994; Engelender et al., 1995; Rocha et al., 1996; de

Fig. 4. Heat released during ATP hydrolysis in the presence (○) and absence (●) of a Ca^{2+} gradient. (A) The values of heat released measured in Fig. 3 plotted as a function of the amount of ATP hydrolysed. (B) The calorimetric enthalpy of hydrolysis (ΔH^{cal}), calculated using data from Fig. 3 by dividing the amount of heat released by the amount of ATP hydrolysed, plotted against time. Values are means ± S.E.M. (N=6–8). The negative values of ΔH^{cal} indicate that the reaction was exothermic. The lines in A are linear regressions; the P values were 1.91×10^{-12} in the presence of a Ca^{2+} gradient and 2.898×10^{-9} in the absence of a Ca^{2+} gradient.
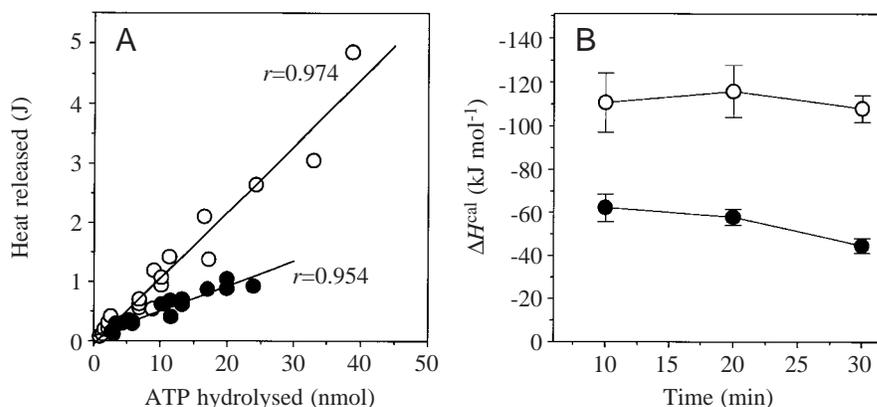
Meis, 1998). In the case of the sea cucumber, K^+ activates all the reactions catalysed by Ca^{2+}-ATPase, i.e. Ca^{2+} transport, Ca^{2+}-dependent ATP hydrolysis (Fig. 1; Table 2) and the ATP$\leftrightarrow$P$_i$ exchange reaction (Fig. 2). In the case of ATP synthesis measured during the exchange reaction, K^+-promoted activation seems to be caused by an increase in the apparent affinity of the enzyme for P$_i$ (Fig. 2B). Another feature of this enzyme is the high degree of energy conservation, as measured by the ratio between the rates of ATP hydrolysis and ATP synthesis. This value for the sea cucumber Ca^{2+}-ATPase was 3–5 times smaller than that for vesicles derived from skeletal muscle of either an ectothermic (trout) or an endothermic (rabbit) vertebrate (Table 2).

Heat production

Heat generation in animal tissues plays a key role in the regulation of several physiological processes, including body temperature, metabolic rate, body mass, energy balance and cold acclimation. Heat production without contractile activity (nonshivering thermogenesis) is found in a variety different mammals, birds and fishes, but the physiological mechanism for this heat generation is controversial and has yet to be elucidated. Brown adipose tissue plays a key role in cold adaptation and nonshivering heat production in small mammals and in young large mammals (Nicholls and Locke, 1984). In this tissue, a futile mitochondrial electron transport cycle is promoted by the uncoupling protein, which is expressed during cold exposure (Nicholls and Locke, 1984; Jezek et al., 1998). This protein dissipates the H^+ gradient formed across the inner mitochondrial membrane, preventing both the passage of H^+ through the F_1F_o-ATP synthase and the synthesis of ATP from ADP and P$_i$. As a result, oxidative metabolism is activated, leading to an increase in the rate of heat production (Nicholls and Locke, 1984).

In animals lacking brown adipose tissue, an important source of heat during nonshivering thermogenesis is derived from the hydrolysis of ATP by the sarcoplasmic reticulum Ca^{2+}-ATPase of skeletal muscles. Calorimetric measurements on rat soleus muscle (Chinet et al., 1992) indicate that 25–45 % of heat produced in resting muscle is related to the circulation of Ca^{2+} between the sarcoplasm and sarcoplasmic reticulum. In cold-acclimated ducklings, 70 % of the total heat production is derived from muscle. In these birds, the development of muscular nonshivering thermogenesis is associated with a 30–50 % increase in the activity of both the sarcoplasmic reticulum Ca^{2+}-ATPase and the ryanodine-sensitive Ca^{2+}-release channel (Dumonteil et al., 1993, 1995).

Some fish from the family Scombroidae (billfish, swordfish) have the capacity to dive into deep waters and, as a result, may experience changes in body temperature of as much as a 19 °C within a period of 2 h. Tolerance of such large temperature changes is unique to fishes. To buffer the effects of such thermal excursions, the swordfish reduces the temperature changes experienced by the brain and retina by warming these tissues with the heater organ derived from extraocular muscle (Carey, 1982; Block, 1986, 1987).

Little is known about the mechanism of heat production by ectothermic animals with a low metabolic rate that live in poorly oxygenated environments, as is the case for the sea cucumber studied in this work. This echinoderm buries itself in sand at the bottom of the sea and, when it moves, it does so at a very slow pace (Hill et al., 1982). The finding that the Ca^{2+}-ATPase of this marine invertebrate can convert osmotic energy into heat indicates a mechanism that can increase by two- to threefold the amount of heat produced during ATP hydrolysis (Figs 3, 4). This result also suggests that, in addition to its role in the contraction–relaxation cycle, the microsomal Ca^{2+}-ATPase could play a role in the production of heat in the sea cucumber body in a manner similar to that described for the Ca^{2+}-ATPase found in vertebrate (rabbit and trout) skeletal muscle.

Unlike the situation in vertebrates, the sea cucumber ATPase can produce heat with little consumption of ATP. As shown in Tables 2 and 3, the sea cucumber ATPase can both increase the rate of heat production and recover a large fraction of the ATP cleaved. This suggests that Ca^{2+} transport in the sea cucumber smooth muscle proceeds with a high degree of energy conservation, a condition probably related to the low content of mitochondria found in this tissue (Hill et al., 1978).

The ability to produce heat is usually observed in animals that have a naturally high metabolic rate or possess mechanisms to increase it, as observed in mammals and some fishes. In these animals, the generation of heat is always accompanied by an increase in the consumption of ATP (Block, 1994). The finding that sea cucumbers are able to generate heat using the Ca^{2+}-ATPase and a low consumption of ATP could represent a novel alternative pathway present in invertebrates capable of generating heat with a low energy cost.

In smooth muscle of *Beroe ovata* (a cnetnophore), Ca^{2+}-ATPase activity and Ca^{2+} storage were found, using cytochemical analyses, both in the endoplasmic reticulum and in the plasma membrane (Cario et al., 1996). The amino acid sequence of the Ca^{2+}-ATPase present in the invertebrate *Artemia* (a crustacean) reveals a high homology with that of rabbit sarcoplasmic reticulum (71 %) and little similarity to either Na^+/K^+-ATPase (24 %) or the plasma membrane Ca^{2+}-ATPase (25 %) (Palmero and Sastre, 1989). The Ca^{2+}-ATPase from the protozoan *Trypanosoma brucei* shows kinetic properties quite similar to the mammalian sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) isoforms so far described including those sensitive to vanadate, an inhibitor of P-type ATPases, and to thapsigargin, a specific SERCA inhibitor (Nolan et al., 1994). These data suggest that the mechanism of Ca^{2+} transport catalysed by the Ca^{2+}-ATPase is conserved throughout evolution. Heat production by the different Ca^{2+} pumps has, however, not yet been evaluated.

The main differences between the SERCA isoforms seems to be related to both their tissue-specific expression and their regulation of the catalytic cycle. At present, the causes of the differences between vertebrates and invertebrates have not been identified and further studies are necessary to investigate these.

References

Barlogie, B., Hasselbach, W. and Makinose, M. (1971). Activation of calcium efflux by ADP and inorganic phosphate. *FEBS Lett.* **12**, 267–268.

Block, B. A. (1986). Structure of brain and eye heater tissue in marlins, sailfish and spearfish. *J. Morph.* **190**, 169–189.

Block, B. A. (1987). The billfish brain and eye heater: A new look at non-shivering heat production. *News Physiol. Sci.* **2**, 208–213.

Block, B. A. (1994). Thermogenesis in muscle. *Annu. Rev. Physiol.* **56**, 535–577.

Carey, F. G. (1982). A brain heater in the swordfish. *Science* **216**, 1327–1329.

Cario, C., Malavar, L. and Hernandez-Nicase, M. L. (1996). Two distinct distribution patterns of sarcoplasmic reticulum in two functionally different giant smooth muscle cell of *Beroe ovata*. *Cell Tissue Res.* **282**, 435–443.

Chiesi, M. and Inesi, G. (1979). The use of quench reagents for resolution of single transport cycles in sarcoplasmic reticulum. *J. Biol. Chem.* **254**, 10370–10377.

Chinet, A. E., Decrouy, A. and Even, P. C. (1992). Ca^{2+} dependent heat production under basal and near-basal conditions in the mouse soleus muscle. *J. Physiol., Lond.* **455**, 663–678.

de Meis, L. (1981). *The Sarcoplasmic Reticulum: Transport and Energy Transduction*, vol. 2 (ed. E. Bittar), pp 1–163. New York: John Wiley & Sons.

de Meis, L. (1988). Approaches to study mechanisms of ATP synthesis in sarcoplasmic reticulum. *Meth. Enzymol.* **157**, 190–206.

de Meis, L. (1998). Control of heat production by the Ca^{2+}-ATPase of rabbit and trout sarcoplasmic reticulum. *Am. J. Physiol.* **43**, C1738–C1744.

de Meis, L., Bianconi, M. L. and Suzano, V. A. (1997). Control of energy fluxes by the sarcoplasmic reticulum Ca^{2+}-ATPase: ATP hydrolysis, ATP synthesis and heat production. *FEBS Lett.* **406**, 201–204.

de Meis, L., Hasselbach, W. and Machado, R. D. (1974). Characterisation of calcium oxalate and calcium phosphate deposits in sarcoplasmic reticulum vesicles. *J. Cell Biol.* **62**, 505–509.

de Meis, L. and Suzano, V. A. (1994). Uncoupling of muscle and blood platelets Ca^{2+}-transport ATPases by heparin. *J. Biol. Chem.* **269**, 14525–14529.

de Meis, L. and Vianna, A. L. (1979). Energy interconversion by the Ca^{2+}-transport ATPase of sarcoplasmic reticulum. *Annu. Rev. Biochem.* **48**, 275–292.

Dumonteil, E., Barré, H. and Meissner, G. (1993). Sarcoplasmic reticulum Ca^{2+}-ATPase and ryanodine receptor in cold-acclimated ducklings and thermogenesis. *Am. J. Physiol.* **265**, C507–C513.

Dumonteil, E., Barré, H. and Meissner, G. (1995). Expression of sarcoplasmic reticulum Ca^{2+} transport proteins in cold-acclimated ducklings. *Am. J. Physiol.* **269**, C955–C960.

Eletr, S. and Inesi, G. (1972). Phospholipid orientation in sarcoplasmic reticulum, membranes: spin label ESR and proton NMR studies. *Biochim. Biophys. Acta* **282**, 174–179.

Engelender, S., Wolosker, H. and de Meis, L. (1995). The Ca^{2+}-ATPase isoforms of platelets are located in distinct functional Ca^{2+} pools and are uncoupled by a mechanism different from that of skeletal muscle Ca^{2+}-ATPase. *J. Biol. Chem.* **270**, 21050–21055.

Grubmeyer, C. and Penesfky, H. S. (1981). The presence of two hydrolytic sites on beef heart mitochondrial adenosine triphosphatase. *J. Biol. Chem.* **256**, 3718–3727.

Hill, B., Sanger, J. W. and Chen, C. (1982). Close apposition of muscle cells in the longitudinal bands of the body wall of a holothurian, *Isostichopus badionotus*. *Cell Tissue Res.* **227**, 465–473.

Hill, R. B., Sanger, J. W., Yantorno, R. E. and Deutsch, C. (1978). Contraction in a muscle with negligible sarcoplasmic reticulum: the longitudinal retractor of the sea cucumber *Isostichopus badionotus* (Selenka), *Holothuria aspidochirota*. *J. Exp. Zool.* **206**, 137–150.

Janský, L. (1995). Humoral thermogenesis and its role in maintaining energy balance. *Physiol. Rev.* **75**, 237–259.

Jezek, P., Engstova, H., Zackova, M., Vercesi, A. E., Costa, A. D., Arruda, P. and Garlid, K. D. (1998). Fatty acid cycling mechanism and mitochondrial uncoupling proteins. *Biochim. Biophys. Acta* **1365**, 319–327.

Landeira-Fernandez, A. M. and de Meis, L. (1997). Ca^{2+}-transport in vesicles derived from Holothuroidea muscle. *FASEB J.* **11**, 587.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.

Makinose, M. (1971). Calcium efflux dependent formation of ATP from ADP and orthophosphate by the membranes of sarcoplasmic reticulum vesicles. *FEBS Lett.* **12**, 269–270.

Makinose, M. and Hasselbach, W. (1971). ATP synthesis by the reversion of the sarcoplasmic reticulum pump. *FEBS Lett*. **12**, 271–272.

Mitidieri, F. and de Meis, L. (1999). Ca^{2+} release and heat production by the endoplasmic reticulum Ca^{2+}-ATPase of blood platelets: Effect of the platelet activating factor. *J. Biol. Chem.* **274**, 28344–28350.

Nicholls, D. G. and Locke, R. M. (1984). Thermogenic mechanisms in brown fat. *Physiol. Rev.* **64**, 1–64.

Nolan, D. P., Reverald, P. and Pays, E. (1994). Overexpression and characterization of a gene for a Ca^{2+}-ATPase of the endoplasmic reticulum in *Trypanosoma brucei*. *J. Biol. Chem.* **269**, 26045–26051.

Palmero, I. and Sastre, L. (1989). Complementary DNA cloning of a protein highly homologous to mammalian sarcoplasmic reticulum Ca^{2+}-ATPase from the crustacean *Artemia*. *J. Mol. Biol.* **210**, 737–748.

Rocha, J. B. T., Wolosker, H., Souza, D. S. and de Meis, L. (1996). Alteration of Ca^{2+} fluxes in brain microsomes by K$^+$ and Na$^+$: Modulation by sulfated polysaccharides and trifluoperazine. *J. Neurochem.* **66**, 772–778.

Wolosker, H., Rocha, J. B. T., Engelender, S., Panizzutti, R. and de Miranda, J. (1997). Sarco/endoplasmic reticulum Ca^{2+}-ATPase isoforms: Diverse responses to acidosis. *Biochem. J.* **321**, 545–550.