Brown adipose tissue Ca\(^{2+}\)-ATPase: Uncoupled ATP hydrolysis and thermogenic activity

Leopoldo de Meis

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

Running title: Heat production by BAT Ca\(^{2+}\)-ATPase

Address correspondence to Leopoldo de Meis, Tel. and Fax: int + 55 21 2270-1635, E-mail demeis@bioqmed.ufjr.br
Abstract

In this report a sarco/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) was identified in rats brown adipose tissue. Electrophoretic analysis of brown fat microsomal protein yields a 110 kD band that is reactive to SERCA 1 antibody, but is not reactive to SERCA 2 antibodies. Nevertheless, the kinetics properties of the brown fat SERCA differ from the skeletal muscle SERCA 1 inasmuch they manifest a different Ca\textsuperscript{2+} affinity and a much higher degree of ATPase/Ca\textsuperscript{2+} uncoupling. A SERCA enzyme is not found in white fat. Fatty acids promoted Ca\textsuperscript{2+} leakage from brown fat vesicles. The heat released during ATP hydrolysis was $-24.7$ Kcal/mol when a Ca\textsuperscript{2+} gradient was formed across the vesicles membrane and $-14.4$ Kcal/mol in the absence of a gradient.

The data reported suggest that in addition to store Ca\textsuperscript{2+} inside the endoplasmic reticulum, the Ca\textsuperscript{2+}-ATPase may represent a source of heat production contributing to the thermogenic function of brown adipose tissue.
Introduction

Brown adipose tissue (BAT) is capable of rapidly converting fat stores to heat and has been used as a model system for the understanding of nonshivering heat production and mechanisms of energy wasting to control obesity. The thermogenic activity of BAT is mediated by α₁ and β₃-adrenergic receptors. Activation of α₁-adrenoreceptors is coupled to inositol 1,4,5-triphosphate (IP₃) production and release of Ca²⁺ from intracellular stores. β₃-adrenergic receptors promote an increase of cAMP, release of free fatty acids, and uncoupling of mitochondria through activation of uncoupling protein 1 (UCP1) (1-10). In addition to these effects, β₃-adrenergic receptors also increase the effect of Ca²⁺ release promoted by α₁-adrenoreceptors through a mechanism not yet known (9). Recent experiments (9,10) performed with cultured brown adipocytes indicates that the endoplasmic reticulum is the main intracellular Ca²⁺ store but, as far as we know, a sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA) isoform has not yet been characterized in BAT.

The amount of heat released during ATP hydrolysis varies depending on the SERCA isoform used (11-14). The total amount of energy released during ATP hydrolysis is always the same, but the heat produced varies depending on the rates of coupled and uncoupled ATPase activity (12, 13, 15-18). When coupled to Ca²⁺ transport a part of the chemical energy derived from ATP hydrolysis is used to pump Ca²⁺ across the membrane and a part is converted into heat (reactions 1 to 6 in Fig. 1). During the uncoupled ATPase activity (reaction 10 in Fig. 1), none of the energy derived from ATP hydrolysis is used to pump Ca²⁺ and more energy is
left available to be converted into heat (12, 13, 17, 18). In vesicles derived from either red muscle (SERCA 2A) or human blood platelets (SERCA 2B and 3) practically all the hydrolysis of ATP is coupled to Ca$^{2+}$ transport and the heat released during hydrolysis varies between 10 and 12 kcal/mol ATP cleaved (11, 13, 14). However, in vesicles derived from white muscle (SERCA 1) between 70 and 80% of the ATP cleaved is not coupled to Ca$^{2+}$ transport (reaction 10 in Fig. 1) and the amount of heat released raises to the range of 20 to 30 kcal/mol ATP cleaved (12, 17, 18).

In this report we identified a SERCA 1 in vesicles derived from BAT endoplasmic reticulum which has several kinetics properties that are different from those of white muscle SERCA 1.

METHODS

Vesicles derived from rat BAT, white adipose tissue and from rabbit skeletal muscle. Interscapular BAT was dissected from 20 rats and frozen at −72°C overnight. After thawing, BAT was homogenized in a mixture containing 10 mM MOPS/Tris buffer pH 7.0, 1 mM EDTA and 10g % sucrose. The homogenate was centrifuged at 10,000 g during 20 min, the pellet and the thick fat upper layer were discarded. Bovine serum albumin was added to the supernatant to a final concentration of 2.5 g % and the mixture was centrifuged at 65,000 g during 40 min. The pellet was resuspended in 8 ml of a solution containing 50 mM MOPS/Tris buffer pH 7.0, 100 mM KCl and 2.5 g % bovine serum albumin and centrifuged
at 10,000 g during 20 min - the pellet was discarded and the supernatant was centrifuged at 65,000 g during 40 min. The pellet was resuspended in a small volume solution containing 50 mM MOPS/Tris buffer pH 7.0, 0.8 M sucrose and 5 mM NaN$_3$ and frozen at -72$^\circ$C until used. A large excess of albumin was needed for the isolation of active BAT vesicles. When albumin was not used the vesicles obtained at the end of the fractionation process had a low Ca$^{2+}$-dependent ATPase activity and were not able to accumulate Ca$^{2+}$. The rates of Ca$^{2+}$ uptake and of Ca$^{2+}$-dependent ATPase activity of vesicles isolated as described above were not altered when more albumin was added to the assay medium in concentrations varying from 0.1 up to 2.5 g%, thus indicating that the amount of albumin used during the isolation procedure was sufficient for the protection of the vesicles. The activity of the vesicles was stable during the initial 3 to 4 days storage at -72$^\circ$C but decreased progressively during the subsequent days and after 10 days storage at -72$^\circ$C were no longer able to either accumulate Ca$^{2+}$ or to hydrolyze ATP. In 6 different groups of rats, the amount of microsomes protein obtained from each gram of BAT use was 0.86 $\pm$ 0.17 mg (mean $\pm$ S.E.). Vesicles derived from rat epididymis white adipose tissue were prepared as described above for BAT. In 3 different groups of rats, the amount of microsomes protein obtained from each gram of white adipose tissue was 0.12 $\pm$ 0.21 mg (mean $\pm$ S.E.). Vesicles derived from rabbit white and red muscles were prepared as previously described (14)

**Gel electrophoresis, Western blot and autoradiography.** Samples were separated in a 7.5% polyacrylamide gel according to Laemmli (19). Electrotransfer
of protein from the gel to nitrocellulose membrane was performed for 15 minutes at 250 mA per 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 hour at room temperature. Membranes were then washed and incubated for one hour with monoclonal antibodies anti-SERCA 1 or anti SERCA-2 at room temperature. The membranes were washed and blots were revealed using ECL detection kit from Amersham-pharmacia Biotec, UK (14). Monoclonal antibodies for SERCA 1 (clone IIH11) and SERCA 2 (clone IID8) were obtained from Affinity BioReagents, Inc. Brazil.

**Ca^{2+} uptake and Ca^{2+}_{in} \leftrightarrow Ca^{2+}_{out} exchange.** These were measured by the filtration method (12, 17, 18, 20). 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 5ml of 3mM 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. 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 the two media. The sample containing the radioactive Ca$^{2+}$ was used to determine the incubation time when the vesicles were filled and the steady state $^{45}$Ca$^{2+}$ uptake was reached. The rate of Ca$^{2+}_{in} \leftrightarrow$Ca$^{2+}_{out}$ exchange was measured after steady state was reached by adding 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 5, 10, 15, 20 and 25 sec after the addition of $^{45}$Ca$^{2+}$.

**ATPase activity.** This was assayed by measuring the release of $^{32}$P$_i$ from [$\gamma$-$^{32}$P]ATP. The reaction was arrested with TCA, final concentration 5% (w/v). The [$\gamma$-$^{32}$P]ATP not hydrolyzed during the reaction was extracted with activated charcoal as previously described (21). Similar to sarcoplasmic reticulum vesicles (22, 23), two different ATPase activities could be distinguished in vesicles derived from BAT. The Mg$^{2+}$-dependent activity requires only Mg$^{2+}$ for its activation and is measured in the presence of 10 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.** This was measured using $^{32}$Pi as previously described (23).

**Heat of reaction.** This was measured using an OMEGA Isothermal Titration Calorimeter from Microcal, Inc. (Northampton, MA). 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 in the sample cell varied between 0.02 and 0.03 ml. The heat change measured during the initial 2 min after vesicles injection was discarded in order to avoid artifacts such as heat derived from the dilution of the vesicles suspension in the reaction medium and binding of ions to the Ca$^{2+}$-
ATPase. The duration of these events is less than one minute (11, 12, 24). Calorimetric enthalpy ($\Delta H^{\text{cal}}$) is calculated dividing the amount of heat released by the amount of ATP hydrolyzed. The units used are mols for substrate hydrolyzed and kcal for heat released. Negative values indicate that the reaction is exothermic and positive values indicate that it is endothermic. The enthalpy of buffer protonation ($\Delta H^p$) was measured at 35°C by measuring the heat released following the addition of known amounts of HCl to the assay medium and the value found was -3.8 kcal / mol. The concentration of the different magnesium complexes and ionic species of ATP, ADP, and P$_i$ were calculated as previously described (25-27) and from these values the fraction of ATP cleaved that generates free protons at pH 7.0, was estimated to be less than 30%. Thus, the heat derived from buffer protonation during ATP cleaved was about 1 kcal/mol ATP cleaved.

**Experimental procedure.** All experiments were performed at 35°C. The vesicles were diluted in a media containing 50 mM MOPS/Tris buffer, 100 mM KCl, 10 mM P$_i$ and 10 µM CaCl$_2$. In a typical experiment the assay media was divided in five samples, which were used for the simultaneous measurement of Ca$^{2+}$ uptake, Ca$^{2+}_{\text{in}}$$\leftrightarrow$Ca$^{2+}_{\text{out}}$ exchange, substrate hydrolysis, ATP synthesis and heat release. The syringe of the calorimeter was filled with vesicles and the temperature difference between the syringe and the reaction cell of the calorimeter was allowed to equilibrate, a process that usually took between 8 and 12 min. During equilibration, the vesicles used for measurements of Ca$^{2+}$ uptake, Ca$^{2+}_{\text{in}}$$\leftrightarrow$Ca$^{2+}_{\text{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 of 0.01 mg/ml. NaN$_3$ (5 mM), an inhibitor of ATP synthase, was added to the assay medium in order 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 previously described (17, 25-27).

RESULTS

**Gel electrophoresis and Western blot analysis.** Vesicles derived from BAT rats endoplasmic reticulum revealed a 110 KD band characteristic of SERCA that reacted with monoclonal antibodies anti-SERCA 1 of white skeletal muscle but did not react with antibodies anti-SERCA 2 (Fig. 2). A 110 KD band that reacted with SERCA antibodies was not found in vesicles derived from rat white adipose tissue (data not shown).

**Ca$^{2+}$ uptake and Ca$^{2+}$ dependent ATP hydrolysis.** BAT vesicles were able to accumulate Ca$^{2+}$ using the energy derived from ATP hydrolysis. The rates of Ca$^{2+}$ transport and ATP hydrolysis varied among the different vesicles preparations tested. Representative experiment are shown in Figs. 3 to 10 and the average values of various vesicles preparations tested are shown in Tables 1 and 2. Similar to the vesicles isolated from muscle (22, 23, 28), during Ca$^{2+}$ transport BAT vesicles were able to synthesized a small amount of ATP from ADP and P$_i$.
using the energy derived from the Ca\(^{2+}\) gradient formed across the vesicles membrane (Table 1). There was no Ca\(^{2+}\) uptake nor ATP synthesis in the presence of Ca\(^{2+}\) ionophore A-23187 (Fig. 3). Vesicles derived from white adipose tissue were not able to accumulate Ca\(^{2+}\) and did not display a Ca\(^{2+}\) dependent ATPase activity (data not shown). Two different ATPase activities could be distinguished in BAT vesicles, a Mg\(^{2+}\)-dependent activity which required only Mg\(^{2+}\) for its activation and a Ca\(^{2+}\)-dependent activity which required both Mg\(^{2+}\) and Ca\(^{2+}\) for its full activation (Fig. 3B). In the subsequent experiments the Ca\(^{2+}\)-dependent ATPase activity was determined by subtracting the Mg\(^{2+}\)-dependent activity from the activity measured in the presence of both Mg\(^{2+}\) and Ca\(^{2+}\). Both Ca\(^{2+}\) uptake and the Ca\(^{2+}\)-dependent ATPase activity of BAT vesicles were inhibited by thapsigargin, a specific inhibitor of the different SERCA isoforms, and the thapsigargin concentration needed for half maximal inhibition of the two activities was 1 nM (Fig. 4). The Mg\(^{2+}\)-dependent ATPase activity was not inhibited by thapsigargin.

**Ca\(^{2+}\) affinity.** Both the initial rate and the steady-state level of Ca\(^{2+}\) uptake were found to vary depending on the free Ca\(^{2+}\) concentration in the medium. The basal cytosolic Ca\(^{2+}\) concentration of brown adipocytes varies between 0.05 and 0.10 μM and raises to 0.7 μM after adrenergic stimulation (9). A very small amount of Ca\(^{2+}\) was accumulate by the BAT vesicles when the free Ca\(^{2+}\) concentration in the medium varied between 0.07 to 0.11 μM but, in spite of the low transport rate, in presence of these low Ca\(^{2+}\) concentrations there was a significant Ca\(^{2+}\)-dependent ATPase activity (Fig. 5). The discrepancy between the rates of Ca\(^{2+}\) uptake and ATP hydrolysis decreased as the Ca\(^{2+}\) concentrations in the medium was raised to
higher values. The Ca\(^{2+}\) concentration needed for half maximal activation (apparent Km) of Ca\(^{2+}\) transport and Ca\(^{2+}\)-dependent ATPase activity were different (Fig. 5 C), 0.45 ± 0.04 \(\mu\)M and 0.15 ± 0.03\(\mu\)M respectively. These values constitute the mean ± S.E. of six experiments and the difference between them was statistically significant (t-test, p<0.001). The data for Ca\(^{2+}\) dependence of enzyme activity were best fit by a model requiring two highly cooperative Ca\(^{2+}\)-binding sites for the activity with Hill coefficient of 1.74 ± 0.19 for Ca\(^{2+}\) uptake and 1.85 ± 0.07 for the Ca\(^{2+}\)-dependent ATPase activity. In vesicles derived from rabbit white muscle (SERCA 1), the raise of the Ca\(^{2+}\) concentration in the vesicles lumen promotes an inhibition of the ATPase activity. In the bibliography (22, 23, 29) this inhibition is referred to as “back inhibition” and is abolished when the Ca\(^{2+}\) accumulation in the vesicles lumen is prevented by the addition of the Ca\(^{2+}\) ionophore A-23187 to the medium. The back inhibition was also observed in vesicles derived from BAT but the degree of inhibition was found to vary depending on the free Ca\(^{2+}\) concentration in the assay medium (Fig. 6). In presence of 0.15 \(\mu\)M free Ca\(^{2+}\), the rates of ATP hydrolysis in intact and leaky vesicles were practically the same, but in presence of saturating Ca\(^{2+}\) concentrations the ATPase activity of leaky vesicles was two to three folds faster than that of intact vesicles. Similar to the difference between Ca\(^{2+}\) transport and ATPase activity noted in Fig. 5, the difference of ATPase activity between intact and leaky vesicles was related to a disparity between the apparent Km for Ca\(^{2+}\). In four experiments the Km value measured for the Ca\(^{2+}\)-dependent ATPase activity of leaky vesicles was 0.41 ± 0.03 \(\mu\)M, a value which was practically the same as that measured for Ca\(^{2+}\)
transport and significantly higher (t-test, p<0.001) than the Km for ATP hydrolysis measured with intact vesicles (Fig. 5).

**Uncoupled ATPase activity.** This can be assessed by either measuring the ratio between the initial rates of Ca\(^{2+}\) uptake and ATP hydrolysis (Table 1) or by measuring the rates of Ca\(^{2+}\)\(_{\text{in}}\leftrightarrow\text{Ca}^{2+}\)\(_{\text{out}}\) exchange and ATP hydrolysis at steady state. In muscle vesicles the hydrolysis of one ATP molecule leads to the translocation of two Ca\(^{2+}\) ions across the membrane. This was determined in transient kinetics experiments where the transport of Ca\(^{2+}\) was measured during the first catalytic cycle of the ATPase (30-32). After a few seconds of reaction the Ca\(^{2+}\) concentration inside the vesicles rises to the millimolar range and this leads to Ca\(^{2+}\) leakage (reactions 7 to 9 in Fig. 1) and uncoupled ATPase activity (reaction 10). In vesicles derived from rabbit white muscle the leakage and uncoupled hydrolysis promote a decrease of the Ca\(^{2+}\)/ATP ratio to values varying between 0.3 and 0.7 (12-18, 30-35). The data of Fig. 5 and Table 1 show that in BAT vesicles the Ca\(^{2+}\)/ATP ratio measured after one min reaction varies depending on the Ca\(^{2+}\) concentration in the medium and that at all Ca\(^{2+}\) concentrations tested it is far smaller than that measured in muscle vesicles - in presence of Ca\(^{2+}\) concentrations similar to that found in resting adipocytes the Ca\(^{2+}\)/ATP ratio measured in BAT vesicles is 30 to 80 times smaller and in presence of 1.7\(\mu\)M Ca\(^{2+}\) it is 3 to 7 times smaller than the Ca\(^{2+}\)/ATP ratio reported for white muscle vesicles. During the first minute of incubation the vesicles are still being filled and the rate of Ca\(^{2+}\) uptake measured represents a balance between the Ca\(^{2+}\) pumped inside the vesicles by the ATPase and the rate of Ca\(^{2+}\) that leaves the vesicles driven by the gradient
formed across the membrane. Thus, the stoichiometry between the rates of ATP cleavage or ATP synthesis cannot be evaluated with precision. After the steady state is reached, the rate of efflux is the same as that of \( \text{Ca}^{2+} \) uptake, and by measuring the rate of \( \text{Ca}^{2+\text{in}} \leftrightarrow \text{Ca}^{2+\text{out}} \) exchange it is possible to determine the value of the two rates. The exchange represents the fraction of \( \text{Ca}^{2+} \) that leaves the vesicles and is pumped back inside the vesicles by the ATPase \((12, 13, 17, 18)\). In four experiments the rates of \( \text{Ca}^{2+} \) leakage and \( \text{Ca}^{2+}\)-dependent ATP hydrolysis measured with BAT vesicles at steady state in media containing 1.7 \( \mu \text{M} \) free \( \text{Ca}^{2+} \) were 9 \( \pm \) 2 and 370 \( \pm \) 30 nmol / mg. min\(^{-1}\) respectively. These values show that very little \( \text{Ca}^{2+} \) leaked from BAT vesicles after they were filled, and during steady state the energy derived from only one out of 365 ATP cleaved is used to pump back the \( \text{Ca}^{2+} \) that leaked from the vesicles. In white muscle vesicles at steady state one out of 3 to 6 ATP cleaved is used for \( \text{Ca}^{2+} \) transport \((12, 17, 18)\). These values show that BAT vesicles cleave far more ATP through the uncoupled route that the vesicles derived from white muscle.

**Effect of fatty acids.** The \( \text{Ca}^{2+} \) accumulation by BAT vesicles was impaired by the addition of low fatty acids concentrations to the medium \((\text{Fig. 7})\). The fatty acids tested were arachidic \((\text{C}_{20:0})\), stearic \((\text{C}_{18:0})\), linoleic \((\text{C}_{18:2})\) and linolenic \((\text{C}_{18:3})\) acid. The inhibitory activity of these four fatty acids was the same and in eighteen experiments, the concentration needed for half maximal inhibition of \( \text{Ca}^{2+} \) uptake was 2.9 \( \pm \) 0.2 \( \mu \text{M} \) regardless of the acyl chain length and degree of saturation of the fatty acid used. The decrease of uptake was accompanied by a small, but significant increase of the \( \text{Ca}^{2+} \) dependent ATPase activity. The different fatty acids
tested increase the membrane permeability leading to a rapid leakage of Ca\textsuperscript{2+} from the vesicles (Fig. 8). In previous reports (36) it was found that arachidic (C\textsubscript{20:0}) and stearic (C\textsubscript{18:0}) acid inhibited the Ca\textsuperscript{2+} uptake and increased the rate of Ca\textsuperscript{2+} efflux of white muscle vesicles but, the concentration of fatty acids needed for a 50% inhibition was higher than 20 μM, i.e., more than one order of magnitude higher than that needed to inhibit the BAT vesicles. Another difference between the two types of vesicles is that in muscle vesicles, but not in BAT vesicles, the inhibitory activity varies depending on degree of saturation of the fatty acid used (36).

**Heat production during ATP hydrolysis.** Heat was produced during ATP hydrolysis (Figs 9 and 10). The amount of heat produced was proportional to the amount of ATP cleaved (Fig. 9C). Thus, when higher the free Ca\textsuperscript{2+} concentration in the medium, more ATP was cleaved and more heat was produced by the BAT vesicles (Fig. 9A and 9B). The amount of heat released during the hydrolysis of each ATP molecule (ΔH\textsubscript{cal} values in Table 2) varied depending on whether or not a gradient was formed across the vesicles membrane. In the presence of a Ca\textsuperscript{2+} gradient between 23 and 26 Kcal were released per mol of ATP cleaved and in the absence of gradient the heat released decreases to the range of 12 to 14 kcal per mol of ATP (Fig. 10 and Table 2). Thus the ΔH\textsubscript{cal} values of ATP hydrolysis measured with BAT vesicles in presence and absence of a gradient were practically the same as those previously measured with vesicles derived from white muscle and different from those measured with vesicles derived from blood platelets and red muscle (SERCA 2 and 3) where the ΔH\textsubscript{cal} varies between 10 and 12 k cal.mol\textsuperscript{-1} both in the presence and absence of Ca\textsuperscript{2+} gradient (11-14, 24).
Discussion

**Ca$$^{2+}$$ affinity.** Previous reports (9, 10) demonstrated that adrenergic stimulation promotes an increase of adipocytes cytosolic Ca$$^{2+}$$ concentration from a basal level varying between 0.05 and 0.10 µM up to the range of 0.2 to 0.7 µM. When extended to the living cell, the values of Fig. 5 suggest that in resting cells, the Ca$$^{2+}$$-dependent ATPase activity of the brown adipocytes varies between 30 and 40% of its maximal activity and is fully active after adrenergic stimulation. The discrepancy between the Ca$$^{2+}$$-dependencies for Ca$$^{2+}$$ uptake and ATP hydrolysis noted in Fig. 5 seems to be a specific feature of the BAT SERCA 1 like isoform not found in the various SERCA isoform so far studied (37, 38). In intact BAT vesicles, activation of both Ca$$^{2+}$$ uptake and ATP hydrolysis is due to Ca$$^{2+}$$ binding to the high affinity site of the enzyme and the small difference between the apparent Ca$$^{2+}$$ dependence for the two activities is probably related to elimination of some steps of the cycle due to slippage of the pump through reaction 10 in Fig. 1.

**Effect of fatty acids.** In brown adipocytes the activation of β$$\_3$$-adrenergic receptors leads to an increase of the lipolysis rate with release of free fatty acids, activation of mitochondrial UCP1 by fatty acids, uncoupling of mitochondrial respiration and energy dissipation as heat (1-8). Perhaps, the fatty acid released during the β$$\_3$$-mediated response, in addition to activate UCP1, may also promote the release of Ca$$^{2+}$$ from the endoplasmic reticulum as shown in Fig. 8. This would explain why the amount of Ca$$^{2+}$$ released from the endoplasmic reticulum when both α$$\_1$$- and
β₃-adrenergic receptors are activated is larger than that measured when only the α₁-receptor is activated (9).

**Thermogenesis** - Activation of BAT thermogenic activity is associated with an increase of the mitochondrial respiration rate (1-9). This has been attributed to the leakage of protons across the inner mitochondrial membrane promoted by activation of UCP 1. In order to compensate for this leak, the cell would then increase the rate of oxygen consumption to maintain the proton gradient at a competent level for ATP synthesis (1-8). Heat is produced during the uncoupled ATPase activity of BAT vesicles (Figs. 9, 10 and Table 2) and, in order to maintain the cytosolic ATP concentration, in the living cell the ADP produced by the uncoupled ATPase activity should also lead to an increase of the mitochondrial oxygen consumption. Perhaps, in addition to the leakage of proton promoted by UCP1, the uncoupled ATPase activity of BAT SERCA may represent one of the routes of heat production that contributes for the thermogenic function of BAT cells.
References


**Footnotes**

(*) This work was supported by grants from PRONEX - Financiadora de Estudos e Projetos (FINEP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and by Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ). The author is grateful to Mr. Valdecir A. Suzano and Antônio Carlos Miranda for technical assistance.

(^1) The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; BAT, brown adipose tissue.
Table 1

Ca$^{2+}$ transport, ATP hydrolysis and ATP synthesis by BAT vesicles

<table>
<thead>
<tr>
<th>[Ca$^{2+}$] $\mu$M</th>
<th>Ca$^{2+}$ uptake Initial rate nmol/mg. min (A)</th>
<th>Ca$^{2+}$ uptake Steady state nmol/mg</th>
<th>Ca$^{2+}$ -ATPase nmol/mg. min (B)</th>
<th>ATP synthesis nmol/mg. min</th>
<th>Ca$^{2+}$/ATP A/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.8 ± 0.6 (6)</td>
<td>3.0 ± 1.5 (6)</td>
<td>89.2±12.2 (6)</td>
<td>-</td>
<td>0.009</td>
</tr>
<tr>
<td>1.7</td>
<td>31 ± 4 (20)</td>
<td>257 ± 25 (27)</td>
<td>325 ± 39 (20)</td>
<td>16 ± 2 (3)</td>
<td>0.095</td>
</tr>
</tbody>
</table>

Assay medium and experimental conditions were as described in Figs. 3 to 5.
Table 2

ATP hydrolysis and heat release in presence and absence of a Ca\(^{2+}\) gradient.

<table>
<thead>
<tr>
<th>Vesicles and activity</th>
<th>ATPase (\mu\text{mol/mg.min}^-1)</th>
<th>Heat released (\text{mcal/mg.min}^-1)</th>
<th>(\Delta H^{\text{cal}}) Kcal / mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact vesicles (gradient)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg(^{2+})-dependent activity</td>
<td>0.31 ±0.06</td>
<td>-3.53 ± 0.45</td>
<td>-13.19 ± 1.43(^a)</td>
</tr>
<tr>
<td>Ca(^{2+})-dependent activity</td>
<td>0.37 ± 0.07</td>
<td>-9.13 ± 1.84</td>
<td>-24.67 ± 2.17(^b)</td>
</tr>
<tr>
<td>Leaky vesicles (A-23187)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca(^{2+})-dependent activity</td>
<td>0.68 ± 0.25</td>
<td>-9.81 ± 3.55 (4)</td>
<td>-14.35 ± 0.72(^c)</td>
</tr>
</tbody>
</table>

The values in the table are mean ± SE of 7 experiments. (\(^b\to a\)) and (\(^b\to c\)), \(p<0.001\). The Ca\(^{2+}\)-dependent ATPase activity was measured in the presence of 1.6 \(\mu\text{M}\) free Ca\(^{2+}\). Assay medium and experimental conditions were as described in Figs. 8 and 9.
Legends to figures

Figure 1- The catalytic cycle of the Ca\(^{2+}\)-ATPase. The catalytic cycle of the Ca\(^{2+}\)-ATPase. The sequence includes two distinct enzymes conformations, E\(_1\) and E\(_2\). The Ca\(^{2+}\) binding sites in the E\(_1\) form face the external surface of the vesicle and have a high affinity for Ca\(^{2+}\) (\(K_a = 2 \times 10^{-7}\) 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 sites of the membrane is inferior to 50 \(\mu\)M, 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 (~10 mM) permits the reversal of the catalytic cycle (reactions 5 to 1 backwards) during which a part of the Ca\(^{2+}\) accumulated 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 raise of the intravesicular Ca\(^{2+}\) concentration leads to ramifications of the catalytic cycle, the uncoupled Ca\(^{2+}\) efflux mediated by reactions 7 to 9 and the uncoupled ATPase activity mediated by reaction 10. The amount of heat released during ATP hydrolysis varies depending on the phosphoenzyme cleaved and on whether or not the Ca\(^{2+}\) is translocated through the membrane. 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\(^{2+}\). When coupled to Ca\(^{2+}\) transport (reactions 1 to 6) a
part of the chemical energy derived from ATP hydrolysis is used to translocate Ca\(^{2+}\) across the membrane and a part is converted into heat. In this case the hydrolysis of ATP is completed after the cleavage of the low energy phosphoenzyme E\(_2\)-P and 10 to 12 kcal are released during the hydrolysis of each ATP molecule. During the uncoupled ATPase activity the high energy phosphoenzyme is cleaved (reaction 10), there is no Ca\(^{2+}\) translocation and all the energy derived from ATP hydrolysis is converted into heat. In this case the amount of heat released during the hydrolysis of one ATP mol raises to the range of 20 to 30 kcal (12, 13, 15, 22, 23, 32, 35, 39- 41).

**Fig. 2 - SDS-PAGE and western blot BAT vesicles.** Upper panel, BAT vesicles commassie blue stained gel electrophoresis; (B) western blot analysis of vesicles derived from BAT and from the sarcoplasmic reticulum of rabbit white (WM) and red muscle (RM). The membranes were probed with anti-SERCA1 monoclonal antibody (upper panel) or anti SERCA 2 monoclonal antibody (lower panel). The amount of vesicles protein used for the electrophoresis of BAT, WM and RM were 10, 0.1 and 3 µg respectively. Vesicles derived from rabbit white and red muscles were prepared as previously described (14).

**Fig. 3- Ca\(^{2+}\) uptake (A), ATPase activity (B) and ATP synthesis (C).** The assay medium composition was 50 mM MOPS-Tris (pH 7.0), 1 mM ATP, 2 mM MgCl\(_2\), 100 mM KCl, 10 mM P\(_i\), 5 mM NaN\(_3\), 0.10 mM EGTA and 0.09 mM CaCl\(_2\). The free Ca\(^{2+}\) concentration calculated was 1.6 µM. For the Mg\(^{2+}\)-dependent ATPase in
(B) the assay medium was the same but without CaCl_2 and the EGTA concentration was raised to 10 mM.

(A) Ca^{2+} uptake (●) control and (O) with 4 μM A-23187; (B) ATPase activity: (▲) total and (Δ) Mg^{2+}-dependent activity; (C) ATP synthesis, (■) control and (□) with 4 μM A-23187.

**Fig. 4 - Inhibition by thapsigargin.** The assay medium composition was as described in Fig. 3. (A) Ca^{2+} uptake; (B) ATPase activity, (▲) total, (Δ) Ca^{2+}-dependent and (□) Mg^{2+}-dependent activity; (C) The values of Ca^{2+} uptake (O) and Ca^{2+}-dependent ATPase activity (Δ) were expressed as % of the activity measured in absence of thapsigargin.

**Fig. 5 - Ca^{2+} dependence for Ca^{2+} uptake and Ca^{2+}-dependent ATPase activity.** The assay medium composition was 1 mM ATP, 2 mM MgCl_2, 100 mM KCl, 10 mM P_i, 5 mM NaN_3, 0.10 mM CaCl_2 and different EGTA concentrations to achieve the different free Ca^{2+} concentrations shown in the figure. The Mg^{2+}-dependent ATPase activity was measured as in fig. 2 and subtracted from the total ATPase activity measured in presence of both Mg^{2+} and Ca^{2+}. (A) Ca^{2+} uptake and (B) Ca^{2+}-dependent ATPase activity in the presence of either (O) 0.08, (●) 0.23 or (Δ) 1.71 μM free Ca^{2+}. (C) The values of Ca^{2+} uptake (●) and Ca^{2+}-dependent ATPase activity (O) were expressed as % of the activity measured in presence of 1.7 μM free Ca^{2+}. 
Fig. 6 - Ca$^{2+}$- dependent ATPase activity in presence (▲, ●) and absence (△, ○) of a Ca$^{2+}$ gradient. The assay medium composition was as described in Fig. 3 with (△, ○) and (▲, ●) without 4 µM A-23187.

Fig. 7 - Effect of fatty acids. The assay medium composition was as described in Fig. 3. (A): Ca$^{2+}$ uptake (○, ●) and Ca$^{2+}$- dependent ATPase activity (Δ, ▲) in presence of various concentration of either (○, Δ) stearic C$_{18:0}$ or (●, ▲) linolenic C$_{18:3}$ acid. (B) Ca$^{2+}$ efflux promoted by linolenic acid: (○) control without fatty acid and (●) in presence of 2 µM linolenic C$_{18:3}$ acid. Arrows indicate the addition of 2 µM linolenic C$_{18:3}$ acid to control media.

Fig. 8 - Heat released during ATP hydrolysis. The assay medium composition was as described in Fig. 5 in the presence of either (Δ) 0.11, (●) 0.23 or (○) 1.71 µM free Ca$^{2+}$. (A) Ca$^{2+}$- dependent ATPase activity, (B) Ca$^{2+}$- dependent heat release. In (C) data of (A) and (B) were replotted.

Fig. 9- ATP hydrolysis and heat released in the presence (●) and absence (○) of a Ca$^{2+}$ gradient. The assay medium composition was as described in Fig. 3 with (○) and (●) without 4 µM A-23187. The free Ca$^{2+}$ concentration in the medium was 1.6 µM.
Fig. 1
Fig. 3

(A) Ca²⁺ uptake, μmol/mg

(B) ATPase, μmol Pi/mg

(C) ATP synthesis, μmol/mg
Fig. 4

(A) μmol Ca²⁺/mg·10 min⁻¹ vs [TG], nM

(B) μmol Pi/mg·10 min⁻¹ vs [TG], nM

(C) % activity vs [TG], nM
FIG 5

(A) mol P i / mg minutes

(B) mol / mg minutes

(C) % Activity

[Ca^{2+}], μM
Fig. 7

(A) 

\[ \frac{\mu\text{mol Ca}^{2+}}{\mu\text{mol Pi}} \] per mg 20 min\(^{-1} \]

versus [Fatty acid], \( \mu\text{M} \)

(B) 

\[ \frac{\mu\text{mol Ca}^{2+}}{\mu\text{mol Pi}} \] per mg 20 min\(^{-1} \]

versus minutes
Fig. 8

(A) ATPase, μmol Pi/mg

(B) Heat released, kcal/mg

(C) Heat released, kcal/mg
FIG. 9

(A) μmol Pi / mg minutes
(B) mcal / mg minutes
(C) mcal / mg μmol Pi / mg
Brown adipose tissue Ca2+-ATPase: Uncoupled ATP hydrolysis and thermogenic activity
Leopoldo de Meis

J. Biol. Chem. published online August 11, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M308280200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts