Identification of a Ca\textsuperscript{2+} -ATPase in Brown Adipose Tissue Mitochondria

REGULATION OF THERMOGENESIS BY ATP AND Ca\textsuperscript{2+}*

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In brown adipose tissue (BAT) adrenaline promotes a rise of the cytosolic Ca\textsuperscript{2+} concentration from 0.05 up to 0.70 \(\mu M\). It is not known how the rise of Ca\textsuperscript{2+} concentration activates BAT thermogenesis. In this report we compared the effects of Ca\textsuperscript{2+} in BAT and liver mitochondria. Using electron microscopy and immunolabeling we identified a sarcoplasmic endoplasmic reticulum (ER) Ca\textsuperscript{2+} -ATPase bound to the inner membrane of BAT mitochondria. A Ca\textsuperscript{2+} -dependent ATPase activity was detected in BAT mitochondria when the respiratory substrates malate and pyruvate were included in the medium. ATP and Ca\textsuperscript{2+} enhanced the amount of heat produced by BAT mitochondria during respiration. The Ca\textsuperscript{2+} concentration needed for half-maximal activation of the ATPase activity and rate of heat production were the same and varied between 0.1 and 0.2 \(\mu M\). Heat production was partially inhibited by the proton ionophore carbonyl cyanide \(p\)-trifluoromethoxyphenylhydrazone and abolished by thapsigargin, a specific ER Ca\textsuperscript{2+} -ATPase inhibitor, and by both rotenone and KCN, two substances that inhibit the electron transfer trough the mitochondrial cytochrome chain. In liver mitochondria, Ca\textsuperscript{2+} did not stimulate the ATPase activity nor increase the rate of heat production. Thapsigargin had no effect on liver mitochondria. In conclusion, this is the first report of a Ca\textsuperscript{2+} -ATPase in mitochondria that is BAT-specific and can generate heat in the presence of Ca\textsuperscript{2+} concentrations similar to those noted in the cell during adrenergic stimulation.

BAT\textsuperscript{3} 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 (1–9). The signal that activates the rate of heat production in BAT cells is the rise of the cytosolic Ca\textsuperscript{2+} concentration from a basal level of 0.05 \(\mu M\) up to the range of 0.2–0.7 \(\mu M\). This is promoted by \(\alpha_1\) - and \(\beta_3\)-adrenergic receptors located in the cell membrane. Activation of \(\alpha_1\)-adrenoceptors leads to the release of Ca\textsuperscript{2+} from intracellular stores into the cytosol, whereas \(\beta_3\)-adrenergic receptors promote the release of free fatty acids and increase the effect of Ca\textsuperscript{2+} release induced by \(\alpha_1\)-adrenoceptors (10, 11). At present we do not know how the rise of the cytosolic Ca\textsuperscript{2+} concentration activates the rate of heat production in BAT cells.

In a previous report (12) we identified a sarco/ER Ca\textsuperscript{2+} ATPase (SERCA 1) in vesicles derived from BAT ER. In this report we show that the rate of heat produced by BAT mitochondria is enhanced when the Ca\textsuperscript{2+} concentration in the medium is raised to a level similar to that observed in BAT cells during adrenergic stimulation. This effect is not observed in liver mitochondria, a tissue that is not specialized in heat production.

EXPERIMENTAL PROCEDURES

Isolation of Mitochondria from Rat BAT and Liver—Adult male Wistar rats were killed by decapitation. Mitochondria were isolated as previously described (13). Briefly, interscapular BAT and liver were removed and homogenized in a mixture containing 0.32 \(M\) sucrose, 1 \(mM\) EDTA, 10 \(mM\) MOPS/Tris buffer, pH 7.4, and 0.2 \(mg\) ml of fatty acid-free bovine serum albumin. The homogenate was centrifuged at 1,330 \(g\) for 30 min. The supernatant was carefully removed and centrifuged at 21,200 \(\times g\) for 10 min. The pellet was re-suspended in 15\% Percoll. A discontinuous density gradient was prepared manually by layering 3-ml fractions of the re-suspended pellet on two preformed layers consisting of 3.5 ml of 23\% Percoll above 3.5 ml of 40\% Percoll. Tubes were centrifuged for 5 min at 37,700 \(g\). The material equilibrating near the interface between 23 and 40\% Percoll layer was removed and gently diluted with the isolation buffer described above. The pellet was re-suspended in the isolation buffer. After centrifugation at 6,900 \(\times g\) for 10 min, the supernatant was decanted and the pellet re-suspended in the same buffer using a fine Teflon pestle. Protein was determined by the Folin-Lowry method using serum albumin as standard (14).

Vesicles Derived from BAT Endoplasmic Reticulum—These were prepared as previously described (12).

Transmission Electron Microscopy and Immunolabeling—BAT was chopped to 1-mm\textsuperscript{3} pieces and fixed in 0.7\% glutaraldehyde (v/v), 0.1\% picric acid, 1\% sucrose, 2\% paraformaldehyde, and 5 \(mM\) Ca\textsubscript{Cl\textsubscript{2}} in 0.1 \(M\) cacodylate buffer (pH 7.2), dehydrated in ethanol and embedded in Unicryl (Ted Pella, Redding, CA). Ultrathin sections were quenched in 50 \(mM\) NH\textsubscript{4}Cl for 30 min and incubated in the presence of monoclonal anti-Serca-1 antibodies (clone IIH11) from Affinity BioReagents, Inc., Brazil. After several washes in phosphate-buffered saline/1\% albumin, sections were incubated in the presence of 10 \(nm\) of gold-labeled goat anti-mouse IgG (BB International, UK), washed, and observed in a JEO\textsubscript{L} 1210 electron microscope. This method allows an adequate diffusion of the antibody but decreases the preservation of the material; therefore, it decreases the quality of the image. Isolated mitochondria were centrifuged at 150 \(\times g\) for 15 min. The pellet was chopped into 1-mm\textsuperscript{3} pieces and treated as described above.

Gel Electrophoresis and Western Blot—Samples were separated in a 7.5\% polyacrylamide gel according to Laemmli (15). Electrotransfer of...
protein from the gel to polyvinylidene difluoride membrane was performed for 15 min 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% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 for 1 h at room temperature. Membranes were then washed and incubated for 1 h with monoclonal antibody anti-SERCA 1 at room temperature. The membranes were washed, and blots were revealed using an ECL detection kit from Amersham Biosciences (16). Monoclonal antibody for SERCA 1 (clone VE121G9) was obtained from Affinity BioReagents, Inc.

Determination of \( \Delta \Psi \)—Mitochondrial membrane potential was measured by using the fluorescence signal of the cationic dye safranine O, which is accumulated and quenched inside energized mitochondria (17). Fluorescence was detected with an excitation wavelength of 495 nm (slit 5 nm) and an emission wavelength of 586 nm. Other details were as described previously (13).

ATPase Activity—This was assayed as previously described (18) using \([\gamma-^3P]ATP\). Measurements were performed at 35 °C, and the reaction was arrested with trichloroacetic acid, final concentration 5% (w/v). In all experiments the amount of ATP cleaved never exceeded 20% of the total amount of ATP added in the assay medium.

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 mitochondria into the sample cell, and the heat change was recorded for 30 min. The volume of mitochondria suspension injected in the sample cell varied between 0.03 and 0.05 ml. The heat change measured during the initial 5 min after mitochondria injection was discarded to avoid artifacts such as heat derived from the dilution of the mitochondria suspension in the reaction medium and binding of ions to mitochondria. The duration of these events is <1 min (12, 19). Negative heat values indicate that the reaction is exothermic, and positive values indicate that it is endothermic. The enthalpy of buffer protonation (\(\Delta F^0\)) 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 \(-3.8 \text{ kcal/mol}\). The concentration of the different magnesium complexes and ionic species of ATP, ADP, P, and Ca\(^{2+}\) were calculated as previously described (20–22), and from these values the fraction of ATP cleaved that generates free protons at pH 7.0, was estimated to be <30%. Thus, the heat derived from buffer protonation during ATP cleaved was \(-1 \text{ mcal/}\mu\text{mol of ATP cleaved.}\n
Ca\(^{2+}\) Uptake—This was measured by the filtration method (23). For \(\text{Ca}^{45}\) uptake, trace amounts of \(\text{Ca}^{45}\) were included in the assay medium. The reaction was arrested by filtering samples of the assay medium through Millipore filters. After filtration, the filters were washed five times with 5 ml of 3 mM La(NO\(_3\))\(_3\), and the radioactivity remaining on the filters was counted using a liquid scintillation counter.

Oxygen Uptake Measurements—Oxygen consumption was measured at 35 °C in a Strathkelvin oxymeter as previously described (13).

Determination of Mitochondrial Hydrogen Peroxide Generation—Mitochondrial H\(_2\)O\(_2\) production was assessed by the scopoletin oxidation method (13, 24).

Experimental Conditions—These were similar to those found in the cell, i.e., pH 7.0–7.4, 2 mM P, 4 mM MgCl\(_2\), and 100 mM KCl and different EGTA and CaCl\(_2\) concentrations. The free Ca\(^{2+}\) concentration in the medium was calculated as described previously (20–22). In most experiments ATP was included in the medium. In addition to being utilized by mitochondrial ATPases, ATP impair the coupling protein 1 (UCP1) found in BAT mitochondria. In all experiments we compared the effects obtained with BAT and liver mitochondria. The aim was to evaluate whether or not the effects observed were specific for BAT mitochondria. All experiments were performed at 35 °C.

RESULTS

Transmission Electron Microscopy and Immunolabeling—In a previous work (12) we found that vesicles derived from BAT ER retain a membrane-bound Ca\(^{2+}\)-ATPase that in Western blot reacted with antibodies for SERCA 1. This work began with an attempt to visualize in electron microscopy the BAT SERCA 1 attached to the membrane of the ER (25). When ultrathin sections were analyzed, to our surprise, we found that, in addition to the ER, the anti-SERCA 1 antibodies also reacted with BAT mitochondrial cristae. This could be visualized using 10 nm gold-labeled anti-mouse IgG. Labeling could be clearly identified in sections of whole tissue (Fig. 1) and in isolated mitochondria, where controls with (Fig. 2, a and d) and without anti-SERCA 1 antibody (Fig. 2, a and d) were compared. The presence of SERCA in BAT mitochondria was confirmed in different preparations examined.

Western blot analysis of BAT mitochondria preparations revealed a faint band with the same mobility than SERCA 1 (Fig. 3). This band was detected in most, but not all BAT mitochondria preparations tested. In

FIGURE 1. BAT electron microscopy and immunocytochemistry. a, ultrathin section of whole tissue demonstrating a nucleus (N), several mitochondria (M), lipid deposits (LD), and ER (arrows) peripheral to the nucleus. b, immunolabeling using anti-SERCA 1 antibody showing specific labeling in mitochondria. No labeling was observed in LD. Bars: 1.0 μm in a and 0.2 μm in b.

FIGURE 2. Isolated mitochondria. Immunolabeling using anti-SERCA 1 antibody (c and d) and the corresponding controls (a and b) with gold-labeled anti-mouse IgG but without anti-SERCA 1. Intense labeling was observed on the mitochondria cristae (c and arrowheads in d). No labeling was detected in the control without anti-SERCA 1. Bars: 500 nm in a and c and 50 nm in b and d.
Heat Production by BAT Ca\textsuperscript{2+}-ATPase

**FIGURE 3.** Gel electrophoresis and Western blot. Upper panel, the amount of protein used in SDS-PAGE gel electrophoresis was 20 \( \mu \text{g} \) for endoplasmic reticulum (ER) and 60 \( \mu \text{g} \) for BAT and liver mitochondria. The gel was stained with Coomassie Brilliant Blue. The prominent band found at 66 kDa of all BAT samples corresponds to albumin that was always used in large excess during ER and mitochondria preparations. Lower panel, immunodetection was obtained with SERCA 1-specific monoclonal antibody. In control experiment, the band was not visible when other antibody were used as for instance anti-SERCA 2.

**Heat Production**—A small amount of heat was produced when either BAT or liver mitochondria were incubated with ATP in the absence of respiratory substrate (Fig. 6 and Table 1). The heat produced by BAT mitochondria increased when pyruvate and malate were included in the assay medium (Table 1). A surprising new finding was that Ca\textsuperscript{2+} enhanced the rate of heat produced by BAT mitochondria with pyruvate and malate. The activation of heat production by Ca\textsuperscript{2+} was further enhanced when ATP was included in the media containing pyruvate and malate (Table 1 and Fig. 6). The increment promoted by ATP and Ca\textsuperscript{2+} exceeded the sum of the heat produced by the single addition of either ATP or respiratory substrates, indicating that, in the presence of pyruvate and malate, ATP magnifies the thermal effect of Ca\textsuperscript{2+}. The amount of heat produced by liver mitochondria was smaller than that measured with BAT mitochondria in all the conditions tested in Table 1, i.e. only ATP, only respiratory substrates and a mixture of both ATP and respiratory substrates. In media containing ATP and no respiratory substrates, Ca\textsuperscript{2+} promoted a small increase in the rate of heat production. However, different from BAT, when respiratory substrates were used, Ca\textsuperscript{2+} decreased the rate of heat production in liver mitochondria, and ATP did not modify the effect of Ca\textsuperscript{2+} (Table 1).

**Ca\textsuperscript{2+} Dependence**—In three experiments the Ca\textsuperscript{2+} concentration needed for half-maximal activation of both Ca\textsuperscript{2+}-dependent ATPase activity and Ca\textsuperscript{2+}-dependent heat production by BAT mitochondria was found to vary between 0.1 and 0.2 \( \mu \text{M} \). This was measured using media containing ATP, pyruvate, and malate. When extrapolated to living cells, the data of Fig. 7 indicate that the concentration of Ca\textsuperscript{2+} needed to enhance heat production of isolated BAT mitochondria is in the same range as the cytosolic Ca\textsuperscript{2+} concentration measured in BAT cells during adrenergic stimulation (10, 11).

**Oxygen Consumption**—In the absence of ATP, BAT mitochondria consumed oxygen at a fast rate when pyruvate and malate were added to the medium, indicating a lack of respiratory control. The addition of either ADP (Fig. 8A) or Ca\textsuperscript{2+} (Fig. 8B) did not alter the rate of oxygen consumption. In the presence of ATP, however (Fig. 8C), the rate of oxygen consumption elicited by the respiratory substrates decreased, indicating that ATP restored the respiratory control of BAT mitochondria. Different from BAT, in liver mitochondria ATP accelerated and Ca\textsuperscript{2+} decreased the rate of oxygen consumption (state 4), and its restoration by ATP, has already been described, and it is attributed to the binding of ATP to UCP1 (6, 28–33). The effect of ATP in Fig. 8C contrasts with the experiment of Fig. 4D where ATP was not able to restore the \( \Delta \Psi \) measured with safranine. The mitochondria protonmotive force (\( \Delta \Psi \)), as defined by Mitchell (34, 35), involves two different parameters, an electrochemical and a pH difference between the two sides of the membrane: \( \Delta \Psi = \Delta \Psi + 2.2(RT/F) \Delta \text{pH} \). Therefore, in the experimental conditions shown in Fig. 8C, BAT mitochondria were able to form a \( \Delta \text{pH} \) but not a \( \Delta \Psi \).

In Fig. 8C, the rate of oxygen consumption decreased with ATP, but the subsequent addition of Ca\textsuperscript{2+} sharply accelerated the rate of both oxygen utilization and heat released (Tables 1 and 2). A different pattern was observed with liver mitochondria (Fig. 8D and Table 2). The addition of pyruvate and malate elicited the consumption of oxygen (state 2), a small amount of ADP (50 \( \mu \text{M} \)) increased the rate of oxygen utilization (state 3), and at prolonged incubation intervals the rate decreased to a value similar to that observed before the addition of ADP (state 4). Different from BAT, in liver mitochondria ATP accelerated and Ca\textsuperscript{2+} decreased the rate of oxygen consumption (Fig. 8, compare C, E, and F).

The heat generated during respiration depends, at least, on two different parameters, (i) the rate of oxygen consumption and (ii) the amount of heat produced during the utilization of each oxygen molecule (ratio of kilocalories/moles of O\textsubscript{2} in Table 2). In BAT mitochondria, the
The effect of ATP varied depending on the addition of Ca\(^{2+}\). In the absence of Ca\(^{2+}\), ATP slowed down the rate of O\(_2\) consumption induced by pyruvate and malate but increased the heat/O\(_2\) ratio. This indicates that most of the energy derived from each oxygen molecule consumed was converted into heat. When Ca\(^{2+}\) was included, ATP accelerated the rate of O\(_2\) utilization, but from the parcel of energy released, only a fraction was converted in heat and the rest was converted in another form of energy not detected by the methods used.

**Effect of FCCP**—The aim of these experiments was to verify if the effect of Ca\(^{2+}\) and ATP on heat formation were related to a formation of energy other than heat.

**TABLE 1**

| Additions     | Tissue | ATPase activity | Heat production |
|---------------|--------|-----------------|-----------------|
|               |        | \(\text{Mg}^{2+}\) | \(\text{Mg}^{2+} + \text{Ca}^{2+}\) | \(\text{Ca}^{2+}\)-dependent | \(\text{Mg}^{2+}\) | \(\text{Mg}^{2+} + \text{Ca}^{2+}\) | \(\text{Ca}^{2+}\)-dependent |
| ATP           | BAT    | 0.79 ± 0.11 (10)| 0.83 ± 0.10 (10)| 0.11 ± 0.04 (10) | -18 (2) | -19 (2) | -1 (2) |
| Pyruvate + malate |      | 2.83 ± 0.47 (9) | 1.11 ± 0.42 (9) | 0.30 ± 0.04 (24) | -95 ± 12 (4) | -161 ± 12 (4) | -71 ± 16 (4) |
| ATP + pyruvate + malate | 1.14 ± 0.08 (24) | 1.43 ± 0.11 (24) | 0.32 ± 0.14 (7) | -119 ± 20 (16) | -295 ± 38 (16) | -176 ± 32 (16) |
| ATP          | Liver  | 1.69 ± 0.37 (9) | 2.83 ± 0.47 (9) | 1.11 ± 0.42 (9) | 47 (2) | 74 (2) | 27 (2) |
| Pyruvate + malate |      | 1.43 ± 0.11 (24) | 0.32 ± 0.14 (7) | -119 ± 20 (16) | 295 ± 38 (16) | 176 ± 32 (16) |
| ATP + pyruvate + malate | 1.36 ± 0.20 (7) | 1.54 ± 0.18 (7) | 0.22 ± 0.06 (7) | -42 ± 6 (6) | 21 ± 2 (6) |
Heat Production by BAT $Ca^{2+}$-ATPase

**FIGURE 5.** BAT ATPase activity with and without of respiratory substrates. A, the assay medium composition was 50 mM MOPS/Tris buffer, pH 7.0, 4 mM MgCl$_2$, 100 mM KCl, 10 mM P$_i$, 1 mM ATP, and one of the following: , 10 mM EGTA; , 0.1 mM EGTA and 0.1 mM CaCl$_2$ (free Ca$^{2+}$ = 3.9 μM); , 6 mM pyruvate, 6 mM malate, and 10 mM EGTA; or , 6 mM pyruvate, 6 mM malate, 0.1 mM EGTA, and 0.1 mM CaCl$_2$ (free Ca$^{2+}$ = 3.9 μM). Reaction was started by the addition of BAT mitochondria to a final concentration of 50 μg/ml and arrested after different incubation intervals at 35 °C by the addition of trichloroacetic acid, final concentration of 5%. B, $Ca^{2+}$-dependent ATPase calculated subtracting the ATPase values measured in A with an excess of EGTA from those measured in the presence of 3.9 μM free Ca$^{2+}$ (A) without respiratory substrate and (C) with 6 mM pyruvate, 6 mM malate.

**FIGURE 6.** Heat production by BAT mitochondria with and without of respiratory substrates. Assay medium, experimental conditions, and symbols are the same as in Fig. 5 (A and B).

**FIGURE 7.** $Ca^{2+}$ dependence for ATP hydrolysis and heat production. The assay medium composition was 50 mM MOPS/Tris buffer, pH 7.4, 4 mM MgCl$_2$, 100 mM KCl, 2 mM P$_i$, 1 mM ATP, 1 mM pyruvate and 1 mM malate, 50 μg/ml BAT mitochondria, 0.1 mM CaCl$_2$, and the following EGTA concentrations: 10 μM, 0.140, 0.130, 0.120, and 0.115 mM to yield the free Ca$^{2+}$ concentrations of 0.0004, 0.012, 0.016, 0.024, 0.032, and 0.061 μM. A, ATPase activity; B, heat released.

ΔpH in BAT mitochondria. The proton ionophore FCCP promoted a 50% decrease of the heat produced, but the activation by Ca$^{2+}$ was not modified and the total amount of heat released was still larger than that of liver mitochondria (Fig. 9). The degree of inhibition did not vary when the FCCP concentration was raised from 1 up to 5 μM. This suggests that the gradient was not an absolute requirement for heat production as in the case of ATP synthesis from ADP and P$_i$, but it enhanced the effects of ATP and Ca$^{2+}$ in liver mitochondria FCCP did not inhibit, but on the contrary, promoted a small increase of heat production (Fig. 9C).

**Inhibition of Heat Production by Drugs—Thapsigargin** is a specific inhibitor of the various SERCA isoforms. The BAT ATPase activity measured in the presence of Mg$^{2+}$ was not modified by thapsigargin. However, both the Ca$^{2+}$-dependent ATPase activity and the heat produced by BAT mitochondria in the presence of ATP, pyruvate, and malate were inhibited by thapsigargin (Figs. 10 and 11A and Table 3). The concentration of thapsigargin needed for half-maximal inhibition of BAT mitochondria was found to vary between 1 and 2 μM. This is two to three orders of magnitude higher than the concentration needed to inhibit the SERCA isoforms found in vesicles derived from the ER of BAT and skeletal muscle (12). Contrasting with BAT, the small amount of heat produced by liver mitochondria was not impaired by thapsigargin (Fig. 11B and Table 3).

Rotenone and KCN inhibit the electron flow through the mitochondrial cytochrome chain. Both drugs strongly inhibited the heat produced by BAT mitochondria (Fig. 12), indicating that the energy-transducing system responsible for heat production depends on the electrons transfer through the mitochondrial cytochromes chain. In addition to heat production, rotenone promoted a 60% inhibition of the mitochondria ATPase activity (average of two experiments, data not shown).

**TABLE 2**

Oxygen consumption and heat production by BAT and liver mitochondria

For oxygen consumption, the composition for the Mg$^{2+}$ medium was 50 mM MOPS/Tris buffer, pH 7.4, 4 mM MgCl$_2$, 100 mM KCl, 2 mM P$_i$, 0.1 mM EGTA, and 0.25 mg/ml of either BAT mitochondria (A–C) or liver mitochondria (D–F). Arrows indicate the addition of 1 mM pyruvate and 1 mM malate (P+M); ADP, 50 μM; Ca$^{2+}$, 0.1 mM CaCl$_2$ (free Ca$^{2+}$ = 3.9 μM); or ATP, 1 mM.

| Additions | Heat released | O$_2$ consumed | Ratio |
|-----------|---------------|----------------|-------|
| BAT mitochondria | Mg$^{2+}$ | -95 ± 12 (4) | 1.76 ± 0.17 (7) | -54 |
| Mg$^{2+}$ + Ca$^{2+}$ | -161 ± 12 (4) | 1.79 ± 0.19 (5) | -90 |
| ATP + Mg$^{2+}$ | -119 ± 20 (16) | 0.39 ± 0.05 (8) | -305 |
| ATP + Mg$^{2+}$ + Ca$^{2+}$ | -295 ± 38 (16) | 2.22 ± 0.34 (8) | -133 |
| Liver mitochondria | EGTA + Mg$^{2+}$ | -44 | 0.27 ± 0.03 (4) | -163 |
| Mg$^{2+}$ + Ca$^{2+}$ | -27 | 0.10 ± 0.02 (4) | -270 |
| ATP + Mg$^{2+}$ | -42 ± 6 (6) | 0.51 ± 0.03 (6) | -82 |
| ATP + Mg$^{2+}$ + Ca$^{2+}$ | -21 ± 2 (6) | 0.20 ± 0.02 (5) | -105 |
The assay medium composition was 50 mM MOPS/Tris buffer, pH 7.4, 4 mM MgCl₂, 100 mM KCl, 2 mM Pi, 1 mM ATP, 1 mM pyruvate, 1 mM malate, 0.1 mM EGTA, 0.1 mM CaCl₂ (free Ca²⁺ = 3.9 μM), or 10 mM EGTA. A, without FCCP and B, with 2 μM FCCP. In C the assay medium was the same as in A and B with excess EGTA, no CaCl₂, and 50 μg/ml liver mitochondria. Δ, without FCCP and Δ, with 2 μM FCCP.

**FIGURE 12.** Inhibition of BAT heat production by rotenone and KCN. The assay medium composition was 50 mM MOPS/Tris buffer, pH 7.0, 4 mM MgCl₂, 100 mM KCl, 2 mM Pi, 1 mM pyruvate, 1 mM malate, 0.1 mM EGTA, 0.1 mM CaCl₂ (free Ca²⁺ = 3.9 μM), or 1 mM ATP without inhibitor (○), with 10 μM rotenone (●), and with 1 mM KCN (△).

**TABLE 3** Inhibition of heat release by thapsigargin

| Mitochondria | Mg²⁺ | Mg²⁺ + Ca²⁺ | Ca²⁺-dependent |
|--------------|------|-------------|----------------|
| BAT          | −154 ± 63 (4) | −312 ± 50 (7) | −174 ± 52 (4) |
| BAT + 5 μM thapsigargin | −11 ± 11 (4) | −16 ± 7 (6) | −15 ± 13 (4) |
| Liver        | −35 ± 3 (3) | −14 ± 2 (3) |                |
| Liver + 5 μM thapsigargin | −39 ± 3 (3) | −10 ± 4 (3) |                |

DISCUSSION

Several reports (34, 35) have described a close association between the ER and mitochondria and that Ca²⁺ can be transferred from the ER to the mitochondria matrix. However, as far as we know, there are no reports describing a Ca²⁺-ATPase located within the mitochondria. The data presented indicate that the Ca²⁺ released in the cytosol of adipocytes during adrenergic stimulation interact directly with the mitochondria activating the rate of heat production. The main findings that correlate the Ca²⁺-ATPase activity, Mg²⁺ dependent, with excess EGTA, no CaCl₂, and 50 μg/ml liver mitochondria. Δ, without FCCP and Δ, with 2 μM FCCP.

Reactive Oxygen Species and Endoplasmic Reticulum Ca²⁺-ATPase Contamination—BAT mitochondria did not generate a measurable formation of reactive oxygen species in media containing ATP, pyruvate, malate, and Ca²⁺ (data not shown). This was tested in three different BAT mitochondrial preparations. The Ca²⁺-ATPase activity measured in the BAT mitochondrial fraction was not due to a contamination with vesicles derived from the ER because: (i) in a previous report (12) it was shown that the thapsigargin concentration needed for half-maximal inhibition of the ER Ca²⁺-ATPase was 1 mM, whereas for BAT mitochondria, half-maximal inhibition was only attained with 1–2 μM thapsigargin (Fig. 10A), i.e. a three orders of magnitude higher concentration than that needed for the ER; (ii) the Ca²⁺-ATPase activity, Ca²⁺-dependent, was inhibited by FCCP, rotenone, and KCN as observed for BAT mitochondria (data not shown).

**FIGURE 11.** Effect of thapsigargin on the rate of heat production by BAT (A) and liver mitochondria (B). In A the assay medium composition was 50 mM MOPS/Tris buffer, pH 7.4, 4 mM MgCl₂, 100 mM KCl, 2 mM Pi, 1 mM ATP, 1 mM pyruvate, 1 mM malate, 0.1 mM EGTA, 0.1 mM CaCl₂ (free Ca²⁺ = 3.9 μM). The reaction was started by the addition of 50 μg/ml without thapsigargin (○) and with 5 μM thapsigargin (●).

**FIGURE 9.** Effect of FCCP in BAT (A and B) and in liver (C) mitochondria. In A and B the assay medium composition was 50 mM MOPS/Tris buffer, pH 7.4, 4 mM MgCl₂, 100 mM KCl, 2 mM Pi, 1 mM ATP, 1 mM pyruvate, 1 mM malate, 50 μg/ml BAT mitochondria, and either 0.1 mM EGTA and 0.1 mM CaCl₂ (free Ca²⁺ = 3.9 μM) or 10 mM EGTA (○). A, without FCCP and B, with 2 μM FCCP. In C the assay medium was the same as in A and B with excess EGTA, no CaCl₂, and 50 μg/ml liver mitochondria. Δ, without FCCP and Δ, with 2 μM FCCP.
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centrations lower than 10 μM, thapsigargin had no effect on both types of mitochondria (38).

During the past decade (1, 2, 9), heat production by BAT has been correlated with thermogenin or uncoupling protein 1 (UCP1). This protein is inserted in the mitochondria membrane, and when activated, it would act as an H$^+$ pore. During proton leakage osmotic energy would be converted into heat and the decrease of the gradient promoted by the leakage would accelerate the various reactions involved in O$_2$ consumption with heat production, because a large amount of heat could be derived from the continuous synthesis and hydrolysis of ATP in the mitochondria matrix.

The experiments described in this report suggest that in rat BAT mitochondria the presence of an H$^+$ gradient is not an absolute requirement for heat production, because a large amount of heat could be detected even after the addition of the proton ionophore FCCP (Fig. 9). Finally, on the basis of the data presented, we hypothesize that BAT mitochondria would be able to synthesize ATP during the electron flux through the cytochromes without the need to form a ΔΨ or ΔpH, and the ATP synthesized would then be cleaved by the Ca$^{2+}$-ATPase before leaving the mitochondria. Thus, heat would be derived from the continuous synthesis and hydrolysis of ATP in the mitochondria matrix.

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