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ATP Synthesis and Heat Production during Ca^{2+} Efflux by Sarcoplasmic Reticulum Ca^{2+}-ATPase

Leopoldo de Meis[1]
Departamento de Bioquímica Médica, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Cidade Universitária, Rio de Janeiro 21941-590, Brazil

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Vesicles derived from the sarcoplasmic reticulum of rabbit white skeletal muscle were loaded with Ca^{2+} and used to measure the rates of Ca^{2+} efflux, heat production, and ATP synthesis from ADP and P_i. It was found that the Ca^{2+}-ATPase can function in three different forms: (i) it absorbs heat from the medium (5 Kcal/mol Ca^{2+}) when the efflux was coupled with ATP synthesis; (ii) it converts the energy derived from the gradient into heat (30 Kcal/mol Ca^{2+}) when Mg^{2+} is removed from the medium and the synthesis of ATP is impaired; and (iii) the ATPase becomes uncoupled when the different ligands of the enzyme are removed from the medium. As a result, there is no ATP synthesis and no heat production or absorption during Ca^{2+} efflux. The Ca^{2+} efflux, ATP synthesis, and heat production were inhibited by thapsigargin, a specific inhibitor of the Ca^{2+}-ATPase.

Key Words: **energy interconversion; Ca^{2+}-ATPase; gradient; ATP synthesis; heat production.**

The Ca^{2+}-ATPase found in the sarcoplasmic reticulum of skeletal muscle is a membrane bound protein which is able to interconvert different forms of energy (1–4). This enzyme translocates Ca^{2+} from the cytoplasm to the lumen of the reticulum by using the chemical energy derived from ATP hydrolysis. The catalytic cycle of the ATPase can be reversed after a Ca^{2+} gradient is formed across the vesicles membrane. During the reversal, Ca^{2+} leaves the reticulum through the ATPase at a fast rate and ATP is synthesized from ADP and Pi using the energy derived from the Ca^{2+} gradient. Data obtained in different laboratories show that only a fraction of the Ca^{2+} that leaves the vesicles is coupled with the synthesis of ATP (5–8). Little is known on the conversion of chemical and osmotic energy into heat during the process of Ca^{2+}-transport. Recently it was shown that the amount of heat produced after the hydrolysis of each ATP molecule increases two to three fold when a Ca^{2+} gradient is formed across the vesicles membrane (9–11). These experiments were performed in steady state conditions, during which Ca^{2+} is translocated both inward and outward the vesicles and the Ca^{2+}-ATPase catalyze simultaneously the hydrolysis and the synthesis of ATP. Therefore, we were not able to identify which of these events could account for the difference of heat produced with and without gradient. In this report the amounts of heat released during the unidirectional efflux of Ca^{2+} was measured. The aim was to verify if in addition to convert osmotic energy into chemical energy, the Ca^{2+}-ATPase can also convert the energy derived from the Ca^{2+} gradient into heat.

METHODS

Sarcoplasmic reticulum vesicles. These were derived from the longitudinal sarcoplasmic reticulum of rabbit hindleg white skeletal muscle and were prepared as previously described (12). The vesicles were stored in liquid nitrogen until use. The efflux of Ca^{2+} measured with these vesicles was not altered by ryanodine, indicating that they did not contain a significant amounts of ryanodine-sensitive Ca^{2+} channels. The vesicles also did not exhibit the phenomenon of Ca^{2+}-induced Ca^{2+} release found in the heavy fraction of the sarcoplasmic reticulum. Vesicles were preloaded with either $^{40}Ca^{2+}$ or $^{45}Ca^{2+}$ in a medium containing 50 mM Mops/Tris (pH 7.0), 100 mM KCl, 20 mM P_i, 0.1 mM $CaCl_2$, 4 mM $MgCl_2$, 1 mM ATP and 0.05 mg of vesicles protein/ml. After 30 min incubation at 35°C, the vesicles were centrifuged at 40,000*g* for 40 min, the supernatant was discarded and the pellet was kept in ice and resuspended before starting the experiment in a small volume of the loading mixture to reach the final vesicles concentration of 1.0 to 1.5 mg protein/ml.

Ca^{2+} efflux. This was measured by the filtration method using vesicles previously loaded with ^{45}Ca and Millipore filters (13). 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.

ATPase activity and ATP synthesis. The methods for measuring the ATPase activity using [γ-^{32}P]ATP and ATP synthesis from ADP and $^{32}P_i$ are described elsewhere (14).

Heats of reaction. These were measured using an OMEGA Isothermal Titration Calorimeter from Microcal Inc. (Northampton, MA) (9–11). The calorimeter cell (1.5 ml) was filled with reaction

[1] To whom correspondence should be addressed. Fax: +55 21 270-8647. E-mail: demeis@bioqmed.ufjr.br.

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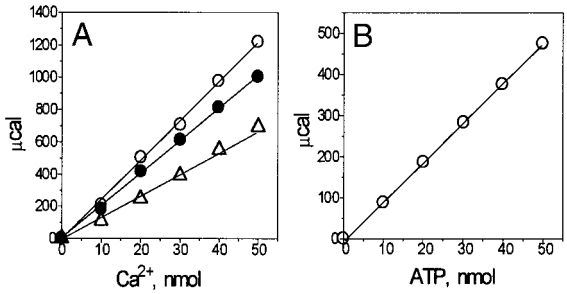

FIG. 1. Heat released during binding of Ca^{2+} to EGTA (A) and during formation of glucose 6-P (B). The assay medium composition was 50 mM Mops/Tris buffer (pH 7.0), 5 mM EGTA, and either (●) 5 mM EDTA; (○) 0.1 mM ADP, 10 mM P_i, 4 mM $MgCl_2$, 100 mM KCl, 5 mM NaN_3, 20 mM glucose, and 10 units/ml hexokinase; or (△) 0.1 mM ADP, 10 mM P_i, 5 mM EDTA, 100 mM KCl, 5 mM NaN_3, 20 mM glucose, and 10 units/ml hexokinase. In B, the assay medium was the same as in A (○). Heat release was measured after the addition of a different amount of either $CaCl_2$ in A, or ATP in B, to 1.5 ml assay medium. In the graph, the final concentrations of Ca^{2+} and ATP in the calorimeter cell varied between 6.67 μM (10 nmol addition) up to 33.33 μM (50 nmol addition).

medium, and the reference cell was filled with Milli-Q water. After equilibration at 35°C, the reaction was started by injecting vesicles previously loaded with Ca^{2+} into the reaction cell to a final concentration of 10 μg/ml and the heat change during Ca^{2+} efflux was recorded for 20 to 25 min. The volume of vesicles suspension injected in the cell varied between 0.02 and 0.03 ml. The heat change measured during the initial 2 min after the vesicles injection was discarded in order to avoid artifacts such as the heat derived from the dilution of the medium containing the loaded vesicles into the efflux medium and the binding of ions to the Ca^{2+}-ATPase. The duration of these events is less than one minute. The calorimetric enthalpy (ΔH^{cal}) was calculated by dividing the amount of heat released by the amount of Ca^{2+} released by the vesicles. The units used were moles for Ca^{2+} released and kcal for the heat released. A negative value indicates that the reaction is exothermic and a positive value indicate that it is endothermic.

Experimental procedure. All experiments were performed at 35°C. In a typical experiment the efflux media was divided in three samples. Trace amounts of ^{32}Pi was added to the sample used for measuring the synthesis of ATP. The other two were used for measurements of Ca^{2+} efflux and heat release. The syringe of the calorimeter was filled with the vesicles preloaded with Ca^{2+} 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. After equilibration, the reaction was started by injecting the vesicles into the reaction cell. During equilibration, the loaded vesicles used for measurements of Ca^{2+} efflux and ATP synthesis were kept at the same temperature, and for the same length of time and protein dilution as the loaded vesicles kept in the calorimeter syringe. The three reactions of Ca^{2+} efflux, ATP synthesis and heat production were started simultaneously using vesicles loaded with either ^{45}Ca (Ca^{2+} efflux) or ^{40}Ca (heat release and ATP synthesis). The heat released during the efflux experiments was corrected by the heat derived from both the binding of Ca^{2+} to EGTA and the heat derived from the formation of glucose 6-phosphate from ATP and glucose (Fig. 1). During efflux, the Ca^{2+} that leaves the vesicles binds to the EGTA present in the media and the protons released during the formation of the Ca. EGTA bind to the Mops/Tris buffer used. The amount of heat produced during these two reactions was determined injecting known amounts of $CaCl_2$ to the different efflux media used (Fig. 1A). In experiments where the efflux of Ca^{2+} was coupled to the synthesis of ATP from ADP and Pi, an excess of glucose and hexokinase was added to the assay media in order to convert the ATP synthesized into glucose 6-P and ADP. This was needed in order to avoid the accumulation of ATP in the media (14). The heat released during the formation of glucose 6-P was determined injecting known amounts of ATP to the efflux media (Fig. 1B).

NaN_3, an inhibitor of ATP synthase, and P^1, P^5-di(adenosine 5′) pentaphosphate, a specific inhibitor of adenylate kinase, were added to the assay medium in order to avoid interference derived from possible contamination of the sarcoplasmic reticulum vesicles with these enzymes.

RESULTS

Passive Ca^{2+} efflux. Two different forms of Ca^{2+} efflux can be identified when vesicles previously loaded with calcium are incubated in a medium containing EGTA, a Ca^{2+} chelating substance. One of them is not mediated by the Ca^{2+}-ATPase nor by any known specific membrane structure and is not inhibited by thapsigargin, a specific inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase (3–8, 11, 15–23). In earlier reports (3, 15, 18, 19) this efflux was referred to as passive Ca^{2+} efflux. The second form of Ca^{2+}-efflux is inhibited by thapsigargin. As shown in previous reports (5–8, 11, 15–21), this efflux represents the fraction of the total Ca^{2+} efflux which is mediated by the Ca^{2+}-ATPase. In the subsequent experiments difference between the

TABLE 1

Energy Transduction during the Thapsigargin Sensitive Ca^{2+} Efflux

Additions to efflux media	*n*	TG sensit. Ca^{2+} efflux μmol/mg	ATP synthesis μmol/mg	Heat, mcal/mg	Ca^{2+} efflux ΔH^{cal}, Kcal/Ca^{2+} mol
ADP, P_i, K^+, Mg^{2+}	11	0.77 ± 0.09	0.62 ± 0.06	+3.86 ± 1.01	+5.01 ± 1.05
ADP, P_i, K^+, EDTA	14	0.11 ± 0.03	none	−3.30 ± 0.48	−30.00 ± 1.62
EDTA*, K^+	5	0.15 ± 0.04	none	−4.56 ± 0.58	−30.40 ± 1.72
EDTA*	13	0.63 ± 0.03	none	+1.16 ± 0.54	+1.84 ± 0.44

Note. The values of Ca^{2+} effluxes are the difference between the rates measured with and without 1 μM thapsigargin (TG). Other additions to the assay medium were 50 mM Mops/Tris buffer pH 7.0, 5 mM EGTA, 5 mM sodium azide, with or without (*) 20 mM glucose and 11 units hexokinase/ml. When added to the medium the concentrations of ADP, Pi, KCl, $MgCl_2$, and EDTA were 0.1 mM, 10 mM, 100 mM, 4 mM, and 5 mM, respectively. The reaction time at 35°C was 9 min. Other conditions were as in Figs. 2 and 3. The values are mean ± SE. In the table, *n* refers to the number of experiments.

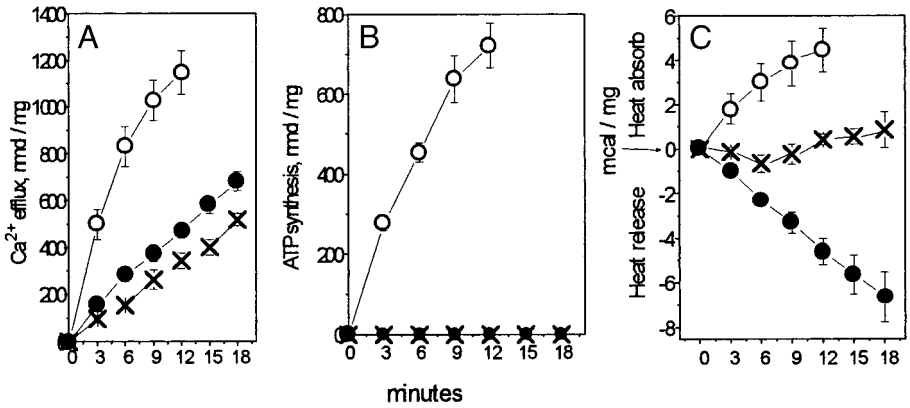

FIG. 2. Ca^{2+} efflux (A), ATP synthesis (B), and heat release (C). The assay medium composition was 50 mM Mops/Tris buffer (pH 7.0), 5 mM EGTA, and (○) 0.1 mM ADP, 10 mM P_i, 4 mM $MgCl_2$, 100 mM KCl, 5 mM NaN_3, 10 μM P^1, P^5-di(adenosine 5′) pentaphosphate, 20 mM glucose, and 10 units/ml hexokinase. (●) same as in (○) but without $MgCl_2$ and with 5 mM EDTA; (×) same as in (●) but with 1 μM thapsigargin. The reaction was started by the addition of Ca^{2+} loaded vesicles, total of 20 μg/ml. The values shown in the figure are mean ± SE of six experiments.

Ca^{2+} effluxes measured with and without the inhibitor will be referred to as thapsigargin sensitive Ca^{2+} efflux. The composition of the four different efflux media used in this report are shown in Table 1. After the addition of thapsigargin, the rate of the passive Ca^{2+} efflux measured with the four media was the same and in 16 experiments, after 9 min incubation at 35°C the amount of Ca^{2+} released by the vesicles was 0.26 ± 0.05 μmol/mg protein (average ± SE). The experiments of Figs. 2 and 3 show that there was no ATP synthesis and no heat production or release during the passive Ca^{2+} efflux.

Conversion of osmotic energy into either chemical energy or heat during the thapsigargin sensitive Ca^{2+} efflux. The sarcoplasmic reticulum Ca^{2+}-ATPase can use the energy derived from a Ca^{2+} gradient to synthesize ATP from ADP and P_i (1–4, 14, 15, 19). This was demonstrated by incubating vesicles previously loaded with Ca^{2+} in a medium containing ADP, P_i, Mg^{2+}, and EGTA. In these conditions, Ca^{2+} leaves the vesicles in a process coupled to the synthesis of ATP from ADP and Pi. This experiment was reproduced in Figs. 2A and 2B but in addition to the Ca^{2+} efflux and ATP synthesis we also determined whether or not this was associated with the release or the absorption of heat from the medium, a measurement that as far as we know, has not been previously done. The experiments of Fig. 2C and Table 1 show that the synthesis of ATP promoted by the Ca^{2+} efflux is an endothermic reaction. The heat absorbed from the assay medium was proportional to the amounts of ATP synthesized (compare Figs. 2B and 2C). Both the synthesis of ATP and the heat absorption were abolished after the addition of thapsigargin to the medium (Fig. 2). The heat absorbed from the medium was proportional to the amount of thapsigargin sensitive Ca^{2+} efflux (Fig. 4) and 5 Kcal were absorbed for each mol of Ca^{2+} released from the vesicles (see ΔH^{cal} values in Table 1). The rate of thapsigargin sensitive Ca^{2+} efflux decreased and ATP was no longer synthesized when Mg^{2+} was not added and EDTA was included in the medium in order to chelate the small amount of Mg^{2+} introduced in the medium together with the Ca^{2+} loaded vesicles (Fig. 2 and Table 1). In this condition, the Ca^{2+} efflux was exothermic (Fig. 2C), the amount of heat released was proportional to the amount of Ca^{2+} released through the thapsigargin sensitive route (Fig. 4) and 30 Kcal were produced for each mole of Ca^{2+} released by the vesicles (Table 1).

Uncoupling of the Ca^{2+}-ATPase. In previous reports (5–8, 11, 20–23) it was shown that Ca^{2+} leaks through the Ca^{2+}-ATPase at a rate similar to that measured during the reversal of the Ca^{2+} pump when the concentrations of the different enzyme ligands in the medium, namely K^+, ADP, P_i, Mg^{2+} and ATP were decreased to a very low level. This leakage of Ca^{2+} was arrested by thapsigargin or decreased to a slow rate

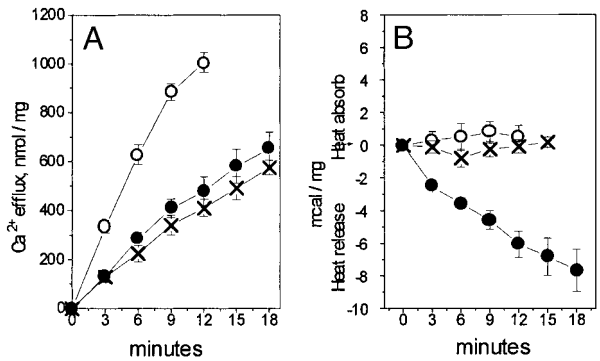

FIG. 3. Uncoupling of the Ca^{2+} ATPase: Ca^{2+} efflux (A) and heat release (B). The assay medium composition was 50 mM Mops/Tris buffer (pH 7.0), 5 mM EGTA, and 5 mM EDTA without (○, ×) and with 100 mM KCl (●). In (×), 1 μM thapsigargin was added to the efflux medium. The values are mean ± SE of five experiments.

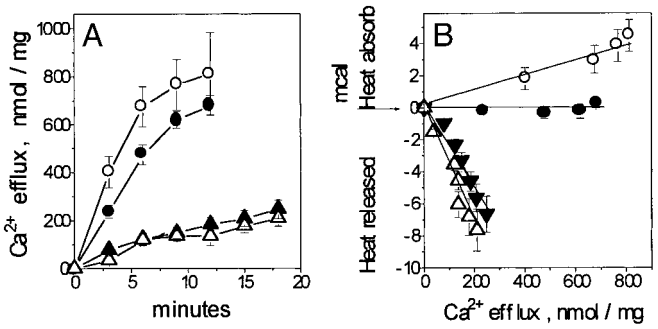

FIG. 4. Heat production during thapsigargin sensitive Ca^{2+} efflux. The experimental values are from Figs. 2 and 3. (A) Thapsigargin sensitive Ca^{2+} efflux and (B) heat release as a function of the amount of Ca^{2+} released during the thapsigargin sensitive Ca^{2+} efflux. The assay medium composition was 50 mM Mops/Tris buffer (pH 7.0), 5 mM EGTA, and either (○) 0.1 mM ADP, 10 mM P_i, 4 mM $MgCl_2$, 100 mM KCl, 5 mM NaN_3, 10 μM P^1, P^5-di(adenosine 5′) pentaphosphate, 20 mM glucose, and 10 units/ml hexokinase; (▲) same as in (○) but without $MgCl_2$ and with 5 mM EDTA; (●) 5 mM EDTA; or (△) 5 mM EDTA and 100 mM KCl.

after the addition to the medium of either 100 mM K^+, 50 μM Ca^{2+}, or by a mixture containing millimolar concentrations of either P_i and Mg^{2+} or P_i and ADP (5–8, 11, 20–23). We now show that the Ca^{2+}-ATPase is not able to convert osmotic energy into heat during the fast thapsigargin sensitive Ca^{2+} efflux measured in the absence of the enzyme ligands. In the experiments of Figs. 3 and 4 there was no release nor absorption of heat during the Ca^{2+} efflux measured in presence of buffer, EGTA and EDTA. However, an amount of heat equivalent to 30 kcal was produced for each mol of Ca^{2+} released by the vesicles after that the rate of Ca^{2+} efflux was decreased by the addition of either KCl (Fig. 3) or a mixture of ADP, P_i, and KCl to the medium (Figs. 2 to 4 and Table 1). The rate of the thapsigargin sensitive Ca^{2+} efflux and heat release measured in presence of K^+ and in presence of a mixture containing K^+, P_i, and ADP were practically the same (Table 1). Potassium is one of the ligands of the Ca^{2+}-ATPase. The binding of K^+ to the ATPase modify the kinetic parameters of at least four different intermediary reaction of the enzyme catalytic cycle; it decreases the apparent Ca^{2+} affinity of the high affinity Ca^{2+} binding site of the ATPase (3, 24), impair the conversion of the ADP-sensitive phosphoenzyme into ADP-insensitive phosphoenzyme (25, 26), accelerates the rate of hydrolysis of the ADP-insensitive phosphoenzyme (27) and increases the rate conversion of conversion of the enzyme form E2 into E1 (28).

Controls. In the experiments of Ca^{2+} efflux, the vesicles were preloaded actively in a medium containing ATP. The true substrate of the Ca^{2+}-ATPase is the complex Mg.ATP and the enzyme does not use free ATP as substrate (3, 4, 15). In control experiments, trace amount of (γ-^{32}P)ATP were added to the efflux media containing EDTA, EGTA, and KCl. We did not detect any (γ-^{32}P) ATP hydrolysis, indicating that the small amount of ATP introduced in the efflux media as contaminant of the loaded vesicles does not contribute to the heat produced during Ca^{2+} efflux. With the method used, we could detect the hydrolysis of up to 0.1 nanomol of ATP, an amount far too small to account for the heat production detected in Figs. 2 to 4 in presence of EDTA, EGTA, ADP, P_i, and KCl.

DISCUSSION

The data presented in this report indicate that the Ca^{2+}-ATPase can function in three different states. In the first state, the ATPase uses the energy derived from the gradient to synthesize ATP, absorbing heat from the medium. A second state is observed in the absence of Mg^{2+} and in presence of either K^+ or a mixture containing ADP, P_i, and K^+. In this state the Ca^{2+}-ATPase converts the energy derived from the gradient into heat. The third state was observed in the absence of the enzyme ligands (Fig. 4) where the enzyme is uncoupled and cannot convert the energy derived from the gradient into either chemical energy or into heat. The experiments of Fig. 2A and Table 1 show that heat was absorbed from the environment when the Ca^{2+} efflux was coupled with the synthesis of ATP from ADP and P_i. This finding suggests that in addition to the energy derived from the gradient, the Ca^{2+}-ATPase is able to convert some of the thermal energy available in the medium into the chemical energy needed for the synthesis of ATP. This possibility is supported by early reports (29, 30) showing that the Ca^{2+}-ATPase is able to catalyze the synthesis of ATP from ADP and P_i after a rapid temperature transition. In these reports synthesis was measured in the absence of a transmembrane Ca^{2+} gradient and led to the conclusion that the Ca^{2+}-ATPase can convert thermal energy into chemical energy.

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