Uncoupled ATPase Activity and Heat Production by the Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase

REGULATION BY ADP*

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Sarcoplasmic reticulum vesicles of rabbit skeletal muscle accumulate Ca\(^{2+}\) at the expense of ATP hydrolysis. The heat released during the hydrolysis of each ATP molecule varies depending on whether or not a Ca\(^{2+}\) gradient is formed across the vesicle membrane. After Ca\(^{2+}\) accumulation, a part of the Ca\(^{2+}\)-ATPase activity is not coupled with Ca\(^{2+}\) transport (Yu, X., and Inesi, G. (1995) J. Biol. Chem. 270, 4361–4367). I now show that both the heat produced during substrate hydrolysis and the uncoupled ATPase activity vary depending on the ADP/ATP ratio in the medium. With a low ratio, the Ca\(^{2+}\) transport is exothermic, and formation of the gradient increases the amount of heat produced during the hydrolysis of each ATP molecule cleaved. With a high ADP/ATP ratio, the Ca\(^{2+}\) transport is endothermic, and formation of a gradient increased the amount of heat absorbed from the medium. Heat is absorbed from the medium when the Ca\(^{2+}\) efflux is coupled with the synthesis of ATP (5.7 kcal/mol of ATP). When there is no ATP synthesis, the Ca\(^{2+}\) efflux is exothermic (14–16 kcal/Ca\(^{2+}\) mol). It is concluded that in the presence of a low ADP concentration the uncoupled ATPase activity is the dominant route of heat production. With a high ADP/ATP ratio, the uncoupled ATPase activity is abolished, and the Ca\(^{2+}\) transport is endothermic. The possible correlation of these findings with thermogenesis and anoxia is discussed.

This work deals with two interconnected subjects: (i) the mechanism of energy interconversion by enzymes and (ii) heat generation, a process that plays a key role in the metabolic activity and energy balance of the cell. The biological preparation used was vesicles derived from the sarcoplasmic reticulum of rabbit white skeletal muscle. These vesicles retain a membrane-bound Ca\(^{2+}\)-ATPase, which is able to interconvert different forms of energy. During Ca\(^{2+}\) transport, the chemical energy derived from ATP hydrolysis is used by the ATPase to pump Ca\(^{2+}\) across the vesicle membrane, leading to the formation of a transmembrane Ca\(^{2+}\) gradient (see reactions 1–6 forward in Figs. 1 and 2). In this process, chemical energy derived from ATP hydrolysis is converted into osmotic energy. After Ca\(^{2+}\) accumulation, the catalytic cycle of the enzyme can be reversed, and the accumulated Ca\(^{2+}\) leaves the vesicles through the Ca\(^{2+}\)-ATPase synthesizing ATP from ADP and P\(_i\) (read reactions 6 to 1 backward in Figs. 1 and 2). During synthesis, osmotic energy is converted back into chemical energy (1–6). In the steady state, the Ca\(^{2+}\) concentrations inside the vesicles and in the assay medium remain constant, but the ATPase operates simultaneously forward (ATP hydrolysis and Ca\(^{2+}\) uptake) and backwards (Ca\(^{2+}\) efflux and ATP synthesis), and chemical and osmotic energy are continuously interconverted by the ATPase.

The catalytic cycle of the ATPase varies depending on the Ca\(^{2+}\) concentration in the vesicle lumen. When the free Ca\(^{2+}\) concentration inside the vesicles is kept in the micromolar range, the reaction cycle flows as shown in Fig. 1 (2–5). The main feature of this cycle is that the hydrolysis of each ATP molecule is coupled with the translocation of two Ca\(^{2+}\) ions across the membrane (4–7). This was best measured in pre-steady state experiments in which the luminal Ca\(^{2+}\) has yet to rise (8–10). The enzyme cycles through two more sets of intermediary reaction when intact vesicles are used, and the Ca\(^{2+}\) concentration inside the vesicles rises to the millimolar range (see Fig. 2). These are ramifications of the catalytic cycle and are denoted as dashed lines in Fig. 2. In one of them, a part of the Ca\(^{2+}\) accumulated by the vesicles leaks through the enzyme without catalyzing the synthesis of ATP. This is referred to as uncoupled Ca\(^{2+}\) efflux and is represented by reactions 7–9 in Fig. 2 (11–14). In 1995, Yu and Inesi (10) and later Fortea et al. (15) observed that the progressive rise in the luminal Ca\(^{2+}\) concentrations promotes another ramification of the catalytic cycle sequence leading to ATP hydrolysis without Ca\(^{2+}\) translocation. According to these authors, the uncoupled ATP hydrolysis is derived from the cleavage of the phosphoenzyme form 2Ca\(^{2+}\)E\(_i\)–P (reaction 10 in Fig. 2).

In recent reports (16–20), it was shown that chemical and osmotic energy are not the only two forms of energy interconverted by the ATPase. During the steady state, a fraction of both chemical and osmotic energy is converted by the ATPase into heat. The total amount of energy released during ATP hydrolysis is always the same, but the fraction of the total energy that is converted into either chemical or osmotic energy or heat seems to be modulated by the ATPase. The main experimental finding that led to this conclusion was that the amount of heat released during the hydrolysis of each ATP molecule varies depending on whether or not a transmembrane gradient is formed across the vesicle membrane. In the absence of a Ca\(^{2+}\) gradient (leaky vesicles; see Fig. 1) between 10 and 12 kcal are released for each mol of ATP cleaved, and in the presence of a Ca\(^{2+}\) gradient (intact vesicles; see Fig. 2) the amount of heat released increases to the range of 20–24 kcal/mol of ATP cleaved. At present, it is not clear why the amount

Received for publication, April 13, 2001, and in revised form, May 2, 2001
Published, JBC Papers in Press, May 7, 2001, DOI 10.1074/jbc.M103318200

* 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 costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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of heat produced during the hydrolysis of each ATP molecule increases after Ca\(^{2+}\) accumulation. One of the catalytic routes involved in heat production seems to be the uncoupled Ca\(^{2+}\) efflux (20). In this case, the energy derived from ATP hydrolysis is first converted into osmotic energy (reactions 1–4 in Fig. 2), and then during the uncoupled Ca\(^{2+}\) efflux (reactions 7–9), osmotic energy is converted into heat. This work now raises the possibility that the uncoupled ATP hydrolysis discovered by Yu and Inesi (10) may represent a second route of heat production. If the hydrolysis of ATP is completed before Ca\(^{2+}\) translocation through the membrane (reaction 10 in Fig. 2), then there is no conversion of chemical into osmotic energy, and during catalysis more chemical energy should be left available to be converted into heat. In order to test this hypothesis, I measured the rates of uncoupled Ca\(^{2+}\) efflux and uncoupled ATP hydrolysis in the presence of different ADP concentrations. It is known (3, 4, 21) that reaction 2 in Fig. 2 is highly reversible (K\(_{\text{eq}}\) \approx 1). Therefore, during catalysis, the fraction of enzyme that accumulates in the form 2Ca\(_{\text{E}}\),P depends on the ratio between the ADP and ATP concentrations available in the medium. While ATP phosphorylates the enzyme form 2Ca\(_{\text{E}}\)1 (reaction 2 forward), ADP drives the reversal of the reaction converting 2Ca\(_{\text{E}}\),P back to 2Ca\(_{\text{E}}\)1. The rise in the intravesicular Ca\(^{2+}\) concentration promotes inhibition of the ATPase activity and an increase in the steady state level of the enzyme form 2Ca\(_{\text{E}}\),P. This is referred to in the bibliography as back inhibition (1, 3–6), and it is the increase of 2Ca\(_{\text{E}}\),P level noted during the back inhibition that promotes the uncoupled ATP hydrolysis through reactions 2 and 10 in Fig. 2 (10, 15). Because reaction 2 is highly reversible, it should be expected that an increase of the ADP concentration in the medium should prevent the accumulation of 2Ca\(_{\text{E}}\),P, and if in fact the uncoupled ATP hydrolysis proceeds through reaction 10 (10, 15) and if this cleavage produces more heat than the coupled ATP hydrolysis (reactions 3–5 in Figs. 1 and 2) as I hypothesize, then both the uncoupled ATP hydrolysis and the amount of heat produced during the cleavage of each ATP molecule should decrease in the presence of a high ADP concentrations. This working hypothesis was tested in this report using different ATP-regenerating systems.

**MATERIALS AND METHODS**

**Sarcoplastic Reticulum Vesicles**—These were derived from the longitudinal sarcoplasmic reticulum of rabbit hind limb white skeletal muscle and were prepared as previously described (22). 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 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 (13, 22).

**Ca\(^{2+}\)-Loaded Vesicles**—Vesicles were preloaded with either 45Ca\(^{2+}\) or 44Ca\(^{2+}\) using different assay media as described in the figure legends for Figs. 3 to 10. After 30–40 min of 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 vesicle concentration of 1.0–1.5 mg of protein/ml. The vesicles loaded with 44Ca\(^{2+}\) were used for measurement of Ca\(^{2+}\) efflux, and the vesicles loaded with 45Ca\(^{2+}\) were used for calorimetric measurements and for measurement of ATP synthesis from ADP and 32P.

**Ca\(^{2+}\): Uptake, Ca\(^{2+}\): Eﬄux, and Ca\(^{2+}\)\textsubscript{out} Exchange**—This was measured by the filtration method (23). For 44Ca uptake, trace amounts of 44Ca were included in the assay medium. For 44Ca efflux, vesicles previously loaded with 44Ca were used. The reaction was arrested by filtering samples of the assay medium in 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. For the Ca\(^{2+}\)\textsubscript{in} \textraightarowright Ca\(^{2+}\)\textsubscript{out} exchange, the assay medium was divided in two samples. Trace amounts of 45Ca\(^{2+}\) were 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 where the vesicles were filled and the steady state of 45Ca\(^{2+}\) uptake was reached. The rate of Ca\(^{2+}\)\textsubscript{in} \textraightarowright Ca\(^{2+}\)\textsubscript{out} exchange was measured after that steady state was reached by adding a trace amount of 44Ca\(^{2+}\) to the second sample containing vesicles loaded with nonradioactive Ca\(^{2+}\). The exchange was thus measured in the radioactive Ca\(^{2+}\) pool with the nonradioactive Ca\(^{2+}\) contained inside the vesicles was measured by filtering samples of the assay medium in Millipore filters 10, 20, 30, 40, 60, and 120 s after the addition of 45Ca\(^{2+}\).

**ATPase Activity and Cleavage of PEP**—Glc-6-P, and Fru-1,6-P—These were assayed using either a colorimetric method or by measuring the increase of 32P, from either ATP, Glc-6-P, or Fru-1,6-P (24–26). The 32P, produced was extracted from the medium with ammonium molybdate and a mixture of isobutyl alcohol and benzene. When the colorimetric method was used, P, was not included in the assay medium. In the various experimental conditions used, the same results were obtained with either the colorimetric method or with the use of radioactive substrate, regardless of the ATP concentrations and ATP-regenerating system used. The values of ATPase activity shown in the figures and tables are the Ca\(^{2+}\)-dependent activity responsible for ATP transport. The Mg\(^{2+}\)-dependent activity was measured in the presence of 2 mM EGTA. The Ca\(^{2+}\)-dependent activity was determined by subtracting the Mg\(^{2+}\)-dependent activity from the activity measured in the presence of both Mg\(^{2+}\) and Ca\(^{2+}\). In the different experimental conditions used, the Mg\(^{2+}\)-dependent activity represented 2–10% of the total activity measured.

**ATP Synthesis**—This was measured using 32P, as previously described (24).

**Heat of Reaction**—This was measured using an OMEGA Isothermal Titration Calorimeter from Microcal Inc. (Northampton, MA) (16–20). The calorimeter 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 reaction cell, and the heat change during either Ca\(^{2+}\) uptake or Ca\(^{2+}\) efflux was recorded for 20–30 min. The volume of vesicle suspension injected in the cell varied between 0.02 and 0.03 ml. The heat change measured during the initial 2 min after vesicle injection was discarded in order to avoid any heat that was derived from the 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 vesicles used for measurements of Ca\(^{2+}\) uptake, Ca\(^{2+}\)\textsubscript{in} \textraightarowright Ca\(^{2+}\)\textsubscript{out} exchange, ATP hydrolysis, and ATP synthase were kept at the same temperature, length of time, and protein dilution as the vesicles kept in the calorimeter syringe. The different reactions were started simultaneously using either empty vesicles or Ca\(^{2+}\)-loaded vesicles. For the experiments where the unidirectional Ca\(^{2+}\) efflux was measured, the heat released during the efflux was corrected for the heat derived from both the binding of Ca\(^{2+}\) to EGTA and the heat derived from the formation of Glc-6-P from ATP and glucose as previously described (20).

**NAD\(^+**—An inhibitor of ATP synthase, and P** P** P** P** diadenosine-5'- pentaphosphate, a specific inhibitor of adenylate kinase, were added to the assay medium in order to avoid interference from possible contamination of the sarcoplasmic reticulum vesicles with these enzymes.

The free Ca\(^{2+}\) concentration in the medium was calculated using the association constants of Schwartzzenbach et al. (27) in a computer program described by Fabiato and Fabiato (28) and modified by Sorensen et al. (29).
Heat Production by the Ca\textsuperscript{2+}-ATPase

TABLE I

| Additions | ATP hydrolysis (a) | ATP synth. (b) | Net ATP hydrolysis (a-b) | Ca\textsuperscript{2+} \rightleftharpoons Ca\textsuperscript{2+} extr | Ca\textsuperscript{2+}/ATP ratio |
|-----------|-------------------|----------------|--------------------------|-----------------------------|------------------|
| 50 μM ADP + 2 mM PEP | 826 ± 109 | None | 826 ± 109 | 173 ± 37 | 0.26 ± 0.06 |
| ATP, 1 mM | 494 ± 60 | 50 ± 3 | 459 ± 22 | 285 ± 20 | 0.65 ± 0.08 |
| 50 μM ADP + 5 mM Fructose-1,6-P | 184 ± 9 | 87 ± 10 | 109 ± 10 | 273 ± 37 | 2.33 ± 0.22 |
| 50 μM ADP + 5 mM Glucose-6-P | 226 ± 39 | 36 ± 7 | 110 ± 17 | 237 ± 25 | 2.31 ± 0.43 |

*The difference between the rates of Ca\textsuperscript{2+} \rightleftharpoons Ca\textsuperscript{2+} extr exchange measured in the presence of 2 mM PEP and 1 mM ATP was statistically significant (p < 0.05).

TABLE II

| Additions | Coupled | Uncoupled | Net Ca\textsuperscript{2+}/ATPase |
|-----------|---------|-----------|-------------------------------|
| 50 μM ADP + 2 mM PEP | 0 | 173 ± 37 | 89 ± 18 | 737 ± 98* |
| ATP, 1 mM | 101 ± 5 | 184 ± 22 | 142 ± 10 | 312 ± 56* |
| 50 μM ADP + 5 mM Fructose-1,6-P | 178 ± 25 | 92 ± 48 | 129 ± 25 | 1 ± 1 |
| 50 μM ADP + 5 mM Glucose-6-P | 97 ± 22 | 147 ± 38 | 122 ± 8 | 22 ± 15 |

*The difference between the uncoupled ATPase activity measured with PEP and with 1 mM ATP was statistically significant (p < 0.01).

ATP-regenerating System—A large excess of pyruvate kinase, hexokinase, or phosphofructokinase was used in order to assure that ATP was regenerated at a faster rate than it was cleaved by the Ca\textsuperscript{2+}-ATPase. In control experiments, the rates of substrate hydrolysis and Ca\textsuperscript{2+} uptake were measured in the presence of different concentrations of the ATP-regenerating enzymes, and the concentration of enzyme used in all experiments described was 5–10 times higher than that needed for maximal activity (25, 26).

RESULTS

Ca\textsuperscript{2+} Transport in the Presence of 1 mM ATP and PEP

Most of the measurements performed in this work were made after the Ca\textsuperscript{2+} uptake reached the steady state. When the vesicles are still being filled, the rate of Ca\textsuperscript{2+} uptake measured represents a balance between the Ca\textsuperscript{2+} pumped inside the vesicles by the ATPase and the rate of Ca\textsuperscript{2+} that leaves the vesicles driven by the gradient formed across the membrane. During the initial minutes of incubation, these two rates are different and cannot be measured separately. Thus, the stoichiometry between the fluxes of Ca\textsuperscript{2+} through the membrane and the rates of either 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 Ca\textsuperscript{2+} uptake, and by measuring the rate of Ca\textsuperscript{2+} \rightleftharpoons Ca\textsuperscript{2+} extr exchange it is possible to determine the value of the two rates. The exchange represents the fraction of Ca\textsuperscript{2+} that leaves the vesicles and is pumped back inside the vesicles by the ATPase.

The initial velocities of Ca\textsuperscript{2+} uptake measured with 1 mM ATP and 50 μM ATP plus PEP as the ATP-regenerating system were the same (see Fig. 3A). However, when the steady state was reached, the amount of Ca\textsuperscript{2+} retained by the vesicles with PEP was larger than that accumulated with 1 mM ATP. In six experiments, the steady state levels of Ca\textsuperscript{2+} uptake were 3.90 ± 0.25 μmol/mg with PEP and 2.73 ± 0.07 μmol/mg with 1 mM ATP. These values are the mean ± S.E. The rate of Ca\textsuperscript{2+} \rightleftharpoons Ca\textsuperscript{2+} extr exchange measured in the presence of PEP was slower than that measured with 1 mM ATP and no ATP-regenerating system (Table I). The time course of ATP hydrolysis was found to vary depending on the condition used. With the use of PEP, the rate of hydrolysis did not vary with the incubation interval, being practically the same before and after the steady state of Ca\textsuperscript{2+} uptake was reached. With 1 mM ATP, however, a significant decrease in the ATPase activity was detected after the vesicles were filled with Ca\textsuperscript{2+} (see Fig. 3B). This difference was probably related to the accumulation of ADP in the medium during the course of the reaction that drives reaction 2 in Fig. 2 backwards (1–6, 13, 21). The apparent K_m of the enzyme form 2Ca:2E for ATP is in the range of 1–3 μM, and K_m of the form 2Ca:2E−P for ADP is in the range of 10–30 μM (2, 3). In the incubation interval of 10–40 min (see Fig. 3), 15–40% of the ADP added was cleaved (i.e. the ADP concentration rose from 0.15 to 0.40 mM), being therefore sufficient to promote the reversal of reaction 2 shown in Fig. 2. In the presence of PEP, there is practically no accumulation of ADP in the medium.

The Ca\textsuperscript{2+} concentration in the lumen of intact vesicles reaches the millimolar range in a few seconds after the transport is initiated (1–6, 8, 9). This triggers the reversal of the catalytic cycle of the ATPase (30–32), during which Ca\textsuperscript{2+}...
Leaves the vesicles through the ATPase, and ATP is synthesized from ADP and P_i (see Fig. 3B). With the use of PEP, there was no measurable synthesis of ATP due to the absence of ADP, one of the substrates needed for the synthesis. With 1 mM ATP, the amount of ADP accumulated during steady state was sufficient to maximally activate the synthesis of ATP from ADP and P_i (30, 31), and about 10% of the ATP cleaved during steady state was synthesized back due to the reversal of the Ca^{2+} pump (Table I).

Knowing the rates of Ca^{2+}_{in} \rightleftharpoons Ca^{2+}_{out} ATP hydrolysis and ATP synthesis it was possible to estimate the steady state values of the following.

The Net ATP Hydrolysis—This represents the true amount of ATP cleaved to maintain the Ca^{2+} gradient formed across the vesicle membrane. This was calculated by subtracting the rate of ATP synthesis from the rate of ATP hydrolysis (Table I).

The Ratio between the Rates of ATP Hydrolysis and ATP Synthesis—This ratio gives a measure of the degree of energy conservation of the system (3, 30–32). The more ATP is synthesized, the smaller is the ratio between the rates of hydrolysis and synthesis and the more energy is conserved by the system; i.e. the steady state can be conserved for a longer period of time because the net decline of the ATP concentration in the medium proceeds at a slower rate. With the use of PEP as an ATP-regenerating system, there is no energy conservation, because none of the substrate cleaved is synthesized back by the system. With 1 mM ATP, in six experiments the ratio between the rates of ATP hydrolysis and ATP synthesis was 11 ± 2.

The Ratio between the Rates of Ca^{2+} Uptake and ATP Hydrolysis—This was calculated by dividing the rate of Ca^{2+}_{in} \rightleftharpoons Ca^{2+}_{out} exchange by the rate of net ATP hydrolysis (Table I).
Both with ATP and PEP, the values of the \( \text{Ca}^{2+}/\text{ATP} \) ratio were smaller than 2, but with 1 mM ATP the coupling ratio was higher than that measured with PEP, suggesting that the presence of ADP in the medium promotes a better coupling of the system.

The Rates of \( \text{Ca}^{2+} \) Efflux Coupled with the Synthesis of ATP

In different laboratories, it has already been shown that the release of two \( \text{Ca}^{2+} \) ions from the vesicles drives the synthesis of one ATP molecule (1–6). The coupled \( \text{Ca}^{2+} \) efflux was therefore calculated by multiplying the rate of ATP synthesis by 2, and the difference between the rate of \( \text{Ca}^{2+} \text{in} \leftrightarrow \text{Ca}^{2+} \text{out} \) exchange and the coupled \( \text{Ca}^{2+} \) efflux represents the uncoupled \( \text{Ca}^{2+} \) efflux (reactions 7–9 in Fig. 2). With the use of PEP, there was no ATP synthesis. Therefore, all of the efflux measured was uncoupled. With 1 mM ATP, about one-third of the \( \text{Ca}^{2+} \) that leaves the vesicle during the steady state is coupled with the synthesis of ATP (Table II).

The Rates of ATP Hydrolysis Coupled and Uncoupled with the Translocation of \( \text{Ca}^{2+} \)

For these calculations, I used the values of net ATP hydrolysis and the stoichiometry of two \( \text{Ca}^{2+} \) ions pumped for each ATP molecule cleaved. Thus, the rate of \( \text{Ca}^{2+} \text{in} \leftrightarrow \text{Ca}^{2+} \text{out} \) exchange in Table I divided by 2 gives the rate of coupled ATP hydrolysis, i.e. the ATP cleaved to pump back the \( \text{Ca}^{2+} \) that leaves the vesicles during the \( \text{Ca}^{2+} \text{in} \leftrightarrow \text{Ca}^{2+} \text{out} \) exchange (reactions 1–5 in Fig. 2). The difference between the net ATP hydrolysis and the coupled ATP hydrolysis gives the value of the uncoupled ATPase activity (reactions 2 and 10 in Fig. 2). With the use of PEP, about 90% of the ATP cleaved was not coupled with the transport of \( \text{Ca}^{2+} \), while with 1 mM ATP about 69% of the net ATPase activity was uncoupled (Table II). These data confirm the findings of Yu and Inesi (10) and Fortea et al. (15) that after \( \text{Ca}^{2+} \) accumulation, a significant fraction of the ATP cleaved by the \( \text{Ca}^{2+} \)-ATPase is not coupled with the translocation of \( \text{Ca}^{2+} \) through the membrane and in addition shows that the rate of the uncoupled ATPase activity decreases when ADP accumulates in the medium.

\( \text{Ca}^{2+} \) Transport in the Presence of High ADP Concentration

In order to measure the \( \text{Ca}^{2+} \) transport in the presence of a high ADP/ATP ratio, I used sugar phosphates as the ATP-regenerating system. In previous reports (25, 26), it was shown that the \( \text{Ca}^{2+} \)-ATPase can use both Glc-6-P and hexokinase or Fru-1,6-P and phosphofructokinase as ATP-regenerating systems. The affinity of the \( \text{Ca}^{2+} \)-ATPase for ATP is sufficiently high \( (K_a = 10^{-6} \text{M}) \) to permit the formation of the enzyme-substrate complex even in the presence of the very low concentration of ATP formed from ADP and either Glc-6-P or Fru-1,6-P. During \( \text{Ca}^{2+} \) uptake, the ADP formed from ATP is phosphorylated by the sugar phosphate in order to maintain the equilibrium concentration of ATP. Thus, in steady state conditions, the \( \text{Ca}^{2+} \) transport proceeds as if it was supported by the cleavage of the sugar phosphate as shown for Glc-6-P by the addition of Reactions 1 and 2.

\[
\begin{align*}
\text{Glc-6-P + ADP} & \leftrightarrow \text{glucose + ATP} \\
\text{ATP + HOH} & \leftrightarrow \text{ADP + P} \\
\text{Glc-6-P + HOH} & \leftrightarrow \text{glucose + P},
\end{align*}
\]

Reactions 1–3

The difference between the use of sugar phosphate or PEP as ATP-regenerating systems is the amount of ADP available in the medium during the reaction. While with PEP there is practically no ADP available, with the sugar phosphate most of the nucleotide in the medium is in the form of ADP (Table III). The initial rates of \( \text{Ca}^{2+} \) transport and steady state level of \( \text{Ca}^{2+} \) accumulation obtained with the use of either Glc-6-P or Fru-1,6-P were 2–4-fold smaller than those measured with either 1 mM ATP or 10 \( \mu \text{M} \) ATP and PEP (compare Figs. 3A and 4A). Comparing the two sugar phosphates, the vesicles were able to accumulate more \( \text{Ca}^{2+} \) and at a faster rate with Fru-1,6-P (see Fig. 4A), and when the steady state was reached, the amount of \( \text{Ca}^{2+} \) retained by the vesicles in six experiments with Fru-1,6-P and Glc-6-P was 1.37 \( \pm 0.03 \) and 1.00 \( \pm 0.03 \) \( \mu \text{mol/mg}, \) respectively. The rates of \( \text{Ca}^{2+} \text{in} \leftrightarrow \text{Ca}^{2+} \text{out} \) exchange measured at steady state with Glc-6-P and Fru-1,6-P were practically the same as those measured with 1 mM ATP (see Fig. 4A and Table I), but the rates of \( \text{Ca}^{2+} \)–dependent ATP hydrolysis were slower than those observed with 1 mM ATP (Table I). In the presence of sugar phosphate, a significant amount of ATP was synthesized during the steady state. The reactions involved in the regeneration of ATP shown above flow both forward and backwards, and the radioactive phosphate of the \( \gamma\text{-ATP} \) synthesized during reversal of the \( \text{Ca}^{2+} \) pump is continuously transferred to either glucose or Fru-6-P added to the assay medium, forming \( ^{32}\text{P}\text{Glc-6-P} \) and \( ^{32}\text{P}\text{Fru-1,6-P}, \) which are diluted in the large pool of nonradioactive sugar phosphate available in the medium. In contrast to the rates of hydrolysis, the rates of ATP synthesis measured with the sugar phosphate were similar to those measured with 1 mM ATP (Table I). As a result, the ratios between the rates of substrate hydrolysis and synthesis were smaller than those measured with ATP. This new finding shows that a high ADP concentration favors the energy conservation of the \( \text{Ca}^{2+} \) transport system. In fact, while with 1 mM ATP the ratio between the rates of ATP cleavage and ATP synthesis was 11.0, with the use of Glc-6-P and Fru-1,6-P in six experiments the values found were 5.6 \( \pm 0.7 \) and 2.4 \( \pm 0.2 \), respectively. When the rates of \( \text{Ca}^{2+} \) efflux and ATP synthesis were compared, it was found that the rates of coupled and uncoupled \( \text{Ca}^{2+} \) efflux measured with 1 mM ATP and Glc-6-P were practically the same, but with Fru-1,6-P there was a significant predominance of the coupled

| Additions to assay medium | Vesicles | Net ATPase | Back inhibition | Heat release | Heat absorption | \( \Delta H^{\text{cal}} \) |
|--------------------------|----------|------------|----------------|-------------|----------------|-----------------|
| 10 \( \mu \text{M} \) ATP + 2 mM PEP | Intact 12 | 931 \( \pm \) 47 | 18.8 | 13,624 \( \pm \) 560 | -15.46 \( \pm \) 1.26 |
|                          | Leaky 14 | 1,147 \( \pm \) 42 | 7,955 \( \pm \) 770 | -7.07 \( \pm \) 0.69 |
| 1 mM ATP                 | Intact 13 | 570 \( \pm \) 58 | 54.8 | 12,639 \( \pm \) 416 | -22.85 \( \pm \) 1.25 |
|                          | Leaky 16 | 1,260 \( \pm \) 103 | 15,519 \( \pm \) 927 | -12.18 \( \pm \) 1.29 |
| 100 \( \mu \text{M} \) ADP + 5 mM Fru-1,6-P | Intact 11 | 86 \( \pm \) 5 | 56.3 | 579 \( \pm \) 76 | +7.03 \( \pm \) 0.71 |
|                          | Leaky 9  | 197 \( \pm \) 7 | 255 \( \pm \) 12 | +1.02 \( \pm \) 0.04 |
| 100 \( \mu \text{M} \) ADP + 5 mM Glc-6-P + hexokinase | Intact 7 | 108 \( \pm \) 9 | 75.7 | 1,094 \( \pm \) 203 | +10.18 \( \pm \) 1.13 |
|                          | Leaky 9  | 445 \( \pm \) 63 | 374 \( \pm \) 113 | +0.54 \( \pm \) 0.03 |
efflux over the uncoupled Ca\textsuperscript{2+} efflux (Table II). However, the most interesting finding was that the uncoupled ATPase activity was practically abolished in the presence of both Glc-6-P and Fru-1,6-P (Table II), and as a result, the ratio between net ATP cleavage and Ca\textsuperscript{2+} uptake in \( \text{Ca}^{2+} \text{ \leftrightarrow \text{Ca}^{2+}} \) exchange was 2 (Table I). These findings show that the ramification of the catalytic cycle proposed by Yu and Inesi (10) is abolished when the ADP concentration is higher than that of ATP, thus providing an experimental condition that permits verifying whether or not the amount of heat produced during the hydrolysis of the enzyme form \( 2\text{Ca}^{2+}\text{E}_1^{-}\text{P} \) (reaction 10 in Fig. 2) is larger than that measured during the hydrolysis of \( \text{E}_2\text{P} \) (reactions 5 and 6 in Figs. 1 and 2).

Control Experiments

The following experiments demonstrate that the Ca\textsuperscript{2+} accumulation, \( P_1 \) production, and ATP synthesis measured in Fig. 4 and Tables I and II were in fact derived from the hydrolysis of ATP generated by ADP and either Glc-6-P or Fru-1,6-P: (i) there was no Ca\textsuperscript{2+} uptake nor \( P_1 \) production if either the sugar phosphate (Glc-6-P or Fru-1,6-P) or the enzymes needed for regenerating ATP (hexokinase or phosphofructokinase) were omitted from the medium; (ii) there was no Ca\textsuperscript{2+} uptake nor \( P_1 \) production when the concentrations of glucose and Fru-6-P in the medium were raised from 50 \( \mu \text{m} \) to 5 \( \text{m} \) (this promotes a decrease of the calculated ATP concentration in the medium from 3.1 to 0.03 \( \mu \text{m} \) in the case of Glc-6-P and from 5.0 to 0.05 \( \mu \text{m} \) in the case of Fru-1,6-P (25, 26)); (iii) thapsigargin, a highly specific inhibitor of the sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase, inhibited the Ca\textsuperscript{2+} transport supported by 1 \( \mu \text{m} \) ATP and by the three different ATP-regenerating systems used in Figs. 3 and 4; and (iv) there was no ATP synthesis if the vesicles were rendered leaky by the addition of the Ca\textsuperscript{2+}-ionophore A23187 regardless of whether 1 \( \mu \text{m} \) ATP or a sugar phosphate was used as an ATP-regenerating system.

Back Inhibition and Heat Production in the Presence and Absence of a Ca\textsuperscript{2+} Gradient

In these experiments, the rate of heat release and the rate of substrate hydrolysis were measured simultaneously in leaky vesicles (no gradient) and intact vesicles (gradient). For intact vesicles, the values of hydrolysis were corrected for the ATP or sugar phosphate synthesized back at the different incubation intervals (net hydrolysis). In agreement with previous reports (1–6, 30–32), there was no synthesis of ATP in the absence of a Ca\textsuperscript{2+} gradient. The rate of hydrolysis of the leaky vesicles was always higher than that measured with intact vesicles (gradient). This difference is related to the back inhibition promoted by the rise of the intravesicular Ca\textsuperscript{2+} concentration (1, 3–6, 13). I now show that the back inhibition is modulated by ADP (Table IV). With the use of PEP as the ATP-regenerating system (no ADP available) the rise in the intravesicular Ca\textsuperscript{2+} concentration promoted a 18.8% inhibition of ATPase activity. In the presence of ADP and depending on the substrate used, the back inhibition increased to the range of 54.8–75.7%. This indicates that in the absence of ADP, the decrease of the coupled ATPase activity (reactions 1–5 in Fig. 2) is compensated by the fast rate of uncoupled ATPase activity (reaction 10). As the ADP concentration in the medium rises, the uncoupled ATPase activity is arrested, and the back inhibition of the coupled ATPase becomes more visible.

The transport of Ca\textsuperscript{2+} was exothermic when measured in the presence of either 1 \( \mu \text{m} \) ATP or 10 \( \mu \text{m} \) ATP and 2 \( \mu \text{m} \) PEP (Table IV and Fig. 5). The amount of heat released in the presence and absence of a Ca\textsuperscript{2+} gradient was proportional to the amount of ATP hydrolyzed, both during the initial incubation...
tion intervals, where the vesicles accumulate $\text{Ca}^{2+}$, and after prolonged incubation intervals, where the vesicles were filled and the steady state was reached. This could be visualized by plotting the heat release as a function of the amount of ATP hydrolyzed (Fig. 5C). In earlier reports (16–20), it was found that the heat released for each ATP molecule hydrolyzed by intact vesicles was larger than that measured with leaky vesicles. This was confirmed in Table IV. I now show that the difference between intact and leaky vesicles is also observed when the ATP cleaved is continuously regenerated by PEP. The amount of heat produced during the hydrolysis of each PEP molecule cleaved in the presence of a gradient was 2 times larger than that measured in the absence of a gradient (Fig. 5 and $\Delta F^{\text{cal}}$ values in Table IV). A different result was obtained when Fru-1,6-P and Glc-6-P were used as the ATP-regenerating system. The $\text{Ca}^{2+}$ transport supported by the sugar phosphate was endothermic, and the amount of heat absorbed from the environment with intact vesicles was larger than that measured with leaky vesicles (Fig. 6 and Table IV). In a control experiment, the hydrolysis of Glc-6-P and Fru-1,6-P catalyzed by leaky vesicles and hexokinase was compared with the hydrolysis catalyzed by alkaline phosphatase and no hexokinase (Fig. 7). In the first condition, the sugar phosphate had to transfer its phosphate first to ADP, forming ATP, and then the ATP formed was cleaved by the ATPase. With the use of alkaline phosphatase, the sugar phosphate was directly cleaved by the enzyme. In seven experiments, the $\Delta F^{\text{cal}}$ values obtained for the cleavage of Fru-1,6-P and Glc-6-P by alkaline phosphatase were $+0.78 \pm 0.14$ and $+0.57 \pm 0.04$, respectively. These values are the same as those measured with leaky vesicles in Table IV, indicating that the difference in heat absorption measured in the presence and absence of a $\text{Ca}^{2+}$ gradient in Table IV and Fig. 6 was related to $\text{Ca}^{2+}$ accumulation by the vesicles and not to the transfer of phosphate from the sugar phosphate to ADP.

Reversal of the $\text{Ca}^{2+}$ Pump and Energy Interconversion during Unidirectional $\text{Ca}^{2+}$ Efflux

In an early report, Rossi et al. (33) observed that the stoichiometry of $\text{Ca}^{2+}$ transport may vary depending on the substrate used to energize the $\text{Ca}^{2+}$ pump. These authors found a coupling ratio of 1 with the use of either $p$-nitrophenolphosphate, methylumbelliferophosphate, or furylacryloylphosphate. The aim of the following experiments was to verify whether or not the molar ratio between $\text{Ca}^{2+}$ efflux and ATP synthesis measured in the presence of sugar phosphates was in fact 2, as assumed for the calculations of the coupled and uncoupled activities in Table II. The experiment of Fig. 8 was performed using Fru-1,6-P as the ATP-regenerating system. After the vesicles were loaded with $\text{Ca}^{2+}$, an aliquot of the assay medium was mixed with an excess of EGTA and an excess of Fru-6-P in order to drastically decrease the concentrations of both $\text{Ca}^{2+}$ and ATP in the medium. Following this maneuver, the pumping was arrested, and the $\text{Ca}^{2+}$ accumulated by the vesicles leaked to the medium at a fast rate. The $\text{Ca}^{2+}$ efflux was coupled with the synthesis of ATP, and both the rate of $\text{Ca}^{2+}$ efflux and ATP synthesis were impaired when thapsigargin was included in the efflux media. In eight different experiments, using either Glc-6-P or Fru-1,6-P as the ATP-regenerating system, the ratio between the rates of efflux and synthesis was $2.16 \pm 0.25$ (Fig. 8). In a previous report (20), the heat produced during the unidirectional $\text{Ca}^{2+}$ movement from the vesicle lumen to the medium was measured by diluting vesicles previously loaded with $\text{Ca}^{2+}$ in efflux media containing different concentrations of ADP, $P_i$, or $K^+$. These experiments revealed that the $\text{Ca}^{2+}$-ATPase can function in three different forms: (i) it absorbs heat from the medium when the efflux is coupled with ATP synthesis ($\Delta H^{\text{cal}} +5.01$ kcal/mol of $\text{Ca}^{2+}$ released); (ii) it converts the energy derived from the gradient into heat when Mg$_2^+$ is removed from the medium and the synthesis of ATP is impaired, and (iii) the ATPase is no longer able to interconvert energy when the different ligands of the enzyme are removed from the medium, and as a result, there is no ATP synthesis and no heat production or absorption. I now repeated these experiments, loading the vesicles with Glc-6-P as shown in Fig. 4 and, after centrifugation, diluting the loaded vesicles in efflux media containing either a mixture of glucose and hexokinase or 1 mM ATP (Table V). In agreement with the previous report, there was heat absorption from the medium when ADP, $P_i$, and Mg$_2^+$ were included in the medium, thus favoring the synthesis of ATP (Fig. 9). The rate of $\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$-loaded vesicles. In this condition, the $\text{Ca}^{2+}$ efflux was exothermic, and the amount of heat released was proportional to the amount of $\text{Ca}^{2+}$ released (Fig. 10). The rate of $\text{Ca}^{2+}$ efflux decreased after the addition of thapsigargin to the medium, and in this condition there was no measurable heat release or heat absorption during the efflux (Figs. 9 and 10 and Table V). The $\Delta H^{\text{cal}}$ values calculated using the uncoupled efflux measured in the absence of thapsigargin varied between $-13.6$ and $-16.3$ kcal/mol of $\text{Ca}^{2+}$ released. These values decreased to $-20.3$ and $-24.6$ kcal/mol of $\text{Ca}^{2+}$ when the difference of efflux measured in the presence and absence of thapsigargin was used to calculate the $\Delta F^{\text{cal}}$ value (20). Knowing the $\Delta F^{\text{cal}}$ values for the coupled and uncoupled $\text{Ca}^{2+}$ efflux, it was possible to estimate the relative contribution of the efflux and of the substrate hydrolysis to the heat produced during steady state. In the presence of either PEP or 1 mM ATP, most of the heat produced was derived from the hydrolysis of ATP (Table VI); the $\text{Ca}^{2+}$ efflux contributed with only 18.9 and 17.0%, respectively, of the total heat released during steady state.
Heat Production by the Ca\(^{2+}\)-ATPase

The vesicles were loaded with Ca\(^{2+}\) in media containing Glc-6-P and hexokinase as described in Fig. 4. Other additions to the assay medium and experimental conditions were as in Figs. 9 and 10. Values are means ± S.E. of the number of experiments (n) shown in the table. HK, hexokinase; TG, thapsigargin.

Additions to efflux media | n | Ca\(^{2+}\) efflux | ATP synthesis | Heat release | Heat absorption | ΔH\(^{ad}\)
---|---|---|---|---|---|
4 mM Mg\(^{2+}\) + 20 mM glucose + HK | 8 | 193 ± 19 | 106 ± 11 | 1,002 ± 130 | +5.65 ± 0.48 |
5 mM EDTA + 20 mM glucose + HK | 15 | 47 ± 5 | 0 | 630 ± 62 | −13.58 ± 1.83 |
5 mM EDTA + 1 mM ATP | 11 | 46 ± 4 | 0 | 737 ± 86 | −16.27 ± 1.72 |
4 mM Mg\(^{2+}\) + 20 mM glucose + HK + TG | 6 | 16 ± 5 | 0 | 23 ± 20 | 0 |
5 mM EDTA + 20 mM glucose + HK + TG | 9 | 12 ± 2 | 0 | 0 | 0 |

**DISCUSSION**

**Thermogenesis**—The data presented indicate that in the presence of a low ADP concentration, a very large fraction of the ATP cleaved by the Ca\(^{2+}\)-ATPase is not used to pump Ca\(^{2+}\) across the reticulum membrane. When extended to the living cell, the cleavage of ATP through reaction 10 could be considered an apparently futile cycle without a physiological purpose. ATP is cleaved without apparent work, and then the ADP produced is phosphorylated by the mitochondria, leading to an increase in oxygen consumption. The data now described suggest that the uncoupled ATPase activity may represent an important route of heat production that contributes to the thermogenic control of the cell. Not only does the yield of heat produced during the hydrolysis of ATP double (Table IV), but the ADP produced leads to an increase in the mitochondrial respiration with more heat production. There are several experimental reports linking the sarcoplasmic reticulum to thermogenesis. What follows is a brief description of the evidence.

At least two different systems are known to be involved in the process of nonshivering thermogenesis, and these are the uncoupling proteins (UCP) and the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase of skeletal muscle (34–38). Different UCP isoforms have already been identified. These include UCP1, specific to brown adipose tissue; UCP2, found in most tissues; and UCP3, which is highly expressed in skeletal muscle. Of the three isoforms, only UCP1 is clearly involved in heat production. The physiological role of UCP2 and UCP3 is still controversial (35–38). The different UCP isoforms promote the dissipation of the proton electrochemical gradient formed across the inner mitochondrial membrane during respiration. In order to restore the gradient and to prevent the decrease of the cytosolic ATP concentration, the proton leakage promoted by the UCP leads to an increase in the mitochondrial respiration rate, increase of fatty acid oxidation, and heat production. Similar to the UCP, a high uncoupled ATPase activity can also lead to an increase of the mitochondrial respiration rate to maintain the cytosolic ATP concentration (34, 35). In fact, Table II shows that in presence of PEP, the uncoupled ATPase activity can be up to 1 order of magnitude higher than the coupled activity needed to maintain Ca\(^{2+}\) inside the reticulum. Skeletal muscle is by far the most abundant tissue of the human body and accounts for over 50% of the total oxygen consumption in a resting human being and up to 90% during very active muscular work. Calorimetric measurements of rat soleus muscle indicate that 25–45% of heat produced in resting muscle is related to Ca\(^{2+}\) recirculation between sarcoplasm and sarcoplasmic reticulum (39). Conditions that promote a change in the rate of heat production in animals are usually associated with changes in expression of both the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase and UCP proteins. Thus, during cold adaptation, UCP1 and UCP2 but not UCP3 are overexpressed (37, 38, 40–42). Similarly, in cold-acclimated ducklings, there is a 30–50% increase of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase, and in these animals, 70% of the total heat production is derived from...
membrane. The overall reactions catalyzed by the Ca$^{2+}$-ATPase and UCPI are decreased in hypothyroid rats. In these animals, the injection of the thyroid hormone 3,5,3’-triiodo-l-thyronine increases the expression of both Ca$^{2+}$-ATPase and UCPI (40, 45–48). A curious system that highlights the importance of the sarcoplasmic reticulum Ca$^{2+}$-ATPase in heat production is the tissue of billfishes. In specialized heater tissues (34). During the daily fluctuations in temperature, the swordfish reduces the temperature changes experienced by the brain and retina by warming these tissues with the heater organ. The heater tissue is composed of modified muscle cells in which the contractile filaments are virtually absent and the cell volume is packed with mitochondria and a highly developed sarcoplasmic reticulum.

Energy Interconversion—For enzymes that are able to interconvert energy, it is generally assumed that the chemical energy released during ATP hydrolysis is divided in two noninterchangeable parts, one used by the enzyme to perform work and the other converted into heat. This does not seem to be the case of the Ca$^{2+}$-ATPase. The data presented in this and previous reports (16–20, 49) indicate that the enzyme is able to handle the energy released during ATP hydrolysis in such a way as to modulate the fraction used to pump Ca$^{2+}$ across the membrane, the fraction that is dissipated in the surrounding medium as heat, and the fraction that is used to synthesize back part of the ATP cleaved. In this view, the total amount of energy released during ATP hydrolysis is always the same, but the enzyme could be able to regulate the interconversion of the different forms of energy. The fraction converted into heat during the hydrolysis of each ATP molecule varies depending on the conditions used; it is maximal with intact vesicles and in the absence of ADP and decreases as the ADP/ATP ratio in the medium increases up to a point where the Ca$^{2+}$ transport is converted from an exothermic to an endothermic process (Table IV).

The Catalytic Cycle—During catalysis, ATP can be cleaved through different sets of intermediary reactions, depending on whether or not a Ca$^{2+}$ gradient is formed across the vesicle membrane. The overall reactions catalyzed by the Ca$^{2+}$-ATPase in the presence and absence of a transmembrane Ca$^{2+}$ gradient are different. This can be concluded by comparing the sum of all the reactions of the catalytic cycle as it flows in absence of a gradient (Fig. 1, reactions 1–6) or in the presence of a gradient (Fig. 2, reactions 2 and 10 or reactions 1–5 and 7–9): no gradient, 2Ca$^{2+}$out + ATP ⇄ ADP + P$_i$ + 2Ca$^{2+}$in; gradient, ATP ⇄ ADP + P$_i$.

With leaky vesicles, a part of the energy derived from ATP hydrolysis is used to translocate Ca$^{2+}$ across the membrane (work), and a part is dissipated as heat. The low Ca$^{2+}$ concentration (10 μM) available on the two sides of the membrane is not sufficient to permit a significant binding of Ca$^{2+}$ to the enzyme forms E$_{2-P}$ and E$_{2}$, and as a result reactions 3 and 4 are irreversible, the catalytic cycle flows continuously forward without branching, and the cleavage of each ATP molecule is accompanied by the translocation of two Ca$^{2+}$ ions across the membrane.

With intact vesicles, the energy used for Ca$^{2+}$ translocation is converted into osmotic energy, and after the vesicles are filled with Ca$^{2+}$, the net hydrolysis of ATP is not associated with a net translocation of Ca$^{2+}$ across the membrane. This is clearly the case of the uncoupled ATPase activity (reactions 2 and 10), which, according to the data of Table II, is the major route of ATP hydrolysis at steady state in the presence of either PEP or 1 mM ATP. After the vesicles are filled, a part of the Ca$^{2+}$ that binds to the enzyme form E$_1$ is pumped across the membrane (reactions 3 and 4 forward), but the high Ca$^{2+}$ concentration available inside the vesicles will promote the binding of Ca$^{2+}$ to the enzyme forms E$_{2-P}$ and E$_{2}$. The Ca$^{2+}$ binding to E$_{2-P}$ promotes the reversal of reactions 4 and 3, leading to an increase in the steady state level of E$_{2-P}$, which is then hydrolyzed through reaction 10 in Fig. 2 (10, 15). Alternatively, the Ca$^{2+}$ that binds to the enzyme form E$_2$ leaves the vesicles through the uncoupled efflux, and the energy derived from ATP hydrolysis used to translocate Ca$^{2+}$ is first converted into osmotic energy (reactions 1–5 forward), and then, as shown in Fig. 10 and Table IV, osmotic energy is converted by the enzyme into heat (reactions 7–9). In both cases, the net ATP hydrolysis is not coupled with a net Ca$^{2+}$ translocation, and ultimately all of the energy derived from the ATP cleaved is converted into heat.

What may appear to be difficult to understand is the notion that in leaky vesicles part of the chemical energy released during ATP hydrolysis is converted into work. This was analyzed in detail in early reports when the reversal of the catalytic cycle was discovered and the intermediary reactions of the Ca$^{2+}$-ATPase catalytic cycle were identified (2–4, 50). In short, binding energy and chemical energy are interconverted during catalysis. The association constant ($K_a$) for the binding of Ca$^{2+}$ to the enzyme form E$_1$ is 10$^6$ M, and the binding energy ($\Delta G$) derived from reaction 1 in Fig. 1 is −16.9 kcal. After phosphorolytic reaction by ATP, the enzyme undergoes a conformational change that leads to a decrease in the enzyme affinity for Ca$^{2+}$ and dissociation of the cation on the inner side of the membrane. $K_a$ of reaction 4 is 10$^5$ M, and if the Ca$^{2+}$ concentration is kept in the micromolar range on the two sides of the membrane, then two calcium ions dissociate from the phosphoenzyme and the $\Delta G$ of reaction 4 forward is +8.5 kcal. The difference in the $\Delta G$ of binding and dissociation is −8.4 kcal. This value represents the free energy needed for the translocation of Ca$^{2+}$ ions through the membrane, and during catalysis this is provided by the fraction of the energy derived from ATP hydrolysis that is converted into work. This sequence is altered after formation of the gradient, and the cleavage of ATP is no longer coupled with sequential binding and dissociation of Ca$^{2+}$ from the enzyme, as can be deduced from the addition of

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**Table VI**

| Additions                  | Heat derived from Ca$^{2+}$ efflux | Heat measured | Heat derived from ATPase |
|----------------------------|-----------------------------------|---------------|--------------------------|
|                           | Coupled (a)                       | Uncoupled (b) |                           |
| 50 μM ADP + 2 mM PEP      | 0                                 | −2.58         | −2.58                    | 13.82 | −13.04 |
| ATP, 1 mM                 | +0.57                             | −2.75         | −2.88                    | 12.84 | −10.66 |
| 50 μM ADP + 5 mM Fru-1,6-P| +1.01                             | −1.37         | −0.36                    | +0.58 | +0.94  |
| 50 μM ADP + 5 mM Glc-6-P  | +0.55                             | −2.19         | −1.64                    | +1.09 | +2.73  |
the different intermediate reactions of the catalytic cycle.

**ADP Regulation and Anoxia**—A failure of mitochondrial respiration such as noted in anoxia and ischemia promotes an increase in the cytosolic ADP/ATP ratio. ADP is needed for several cellular functions, and during anoxia the cell has to adapt to a lower consumption of ATP in order to survive. A decrease in the heat production rate is one of the earliest events noted in anoxia (51–53). Not only will this decrease the consumption of ATP, but a drop in cell temperature promotes a simultaneous decrease in the metabolic activity and ATP demand of the cell. The experiments of Tables II and IV show that an increase in the ADP/ATP ratio promotes a decrease of both uncoupled ATPase activity and yield of heat generated during ATP hydrolysis. In the presence of a high ADP/ATP ratio, such as that attained when sugar phosphates are used as the ATP-regenerating system, the Ca\(^{2+}\) transport is no longer exothermic but, on the contrary, absorbs heat from the medium. Thus, when extrapolated to the living cell, the thermogenic activity of the Ca\(^{2+}\)-ATPase can convert thermal energy into chemical energy. At present, we do not know the mechanism of this conversion.

Heat Production by the Ca\(^{2+}\)-ATPase

Heat Absorption—This was observed in two experimental situations, when the Ca\(^{2+}\) uptake was supported by Glc-6-P and Fru-1,6-P (Fig. 6 and Table IV) and when ATP was synthesized during active Ca\(^{2+}\) efflux (Fig. 9 and Table V). This finding suggests that Ca\(^{2+}\)-ATPase can convert thermal energy into either osmotic or chemical energy; i.e. it is able to use the thermal energy available in the medium to help the pumping of Ca\(^{2+}\) into the vesicles (Figs. 4 and 6 and Table IV) and to synthesize ATP from ADP and P\(_i\) (Fig. 9 and Table V). This possibility is supported by early reports (54, 55) 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. At present, we do not know the mechanism of this conversion. Possible parameters involved are the different \(\Delta G^0\) and \(\Delta G\) values of the phosphate compounds used. The \(\Delta G^0\) values reported in the bibliography for ATP hydrolysis vary between \(-7.0\) and \(-8.0\) kcal/mol, and values for PEP, Fru-1,6-P, and Glc-6-P are \(-14.0\), \(-2.8\), and \(-2.5\) kcal/mol, respectively (25, 56, 57). The \(\Delta G\) values calculated taking into account the concentrations of reactants and products available in the medium during steady state vary between \(-10.1\) and \(-11.9\) for the experiments performed with 1 mM ATP, between \(-18.0\) and \(-18.6\) for PEP, between \(-7.1\) and \(-7.4\) for Fru-1,6-P, and between \(-6.8\) and \(-7.0\) for Glc-6-P.

Finally, the possibility that Fru-1,6-P and Glc-6-P may be used in an ATP-regenerating system as salvage routes during anoxia has been discussed in earlier reports (25, 26).
Uncoupled ATPase Activity and Heat Production by the Sarcoplasmic Reticulum Ca

2+-ATPase: REGULATION BY ADP

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J. Biol. Chem. 2001, 276:25078-25087.
doi: 10.1074/jbc.M103318200 originally published online May 7, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103318200

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