Correlation between Uncoupled ATP Hydrolysis and Heat Production by the Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase

COUPLING EFFECT OF FLUORIDE*

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The sarcoplasmic reticulum Ca\(^{2+}\)-ATPase transports Ca\(^{2+}\) using the chemical energy derived from ATP hydrolysis. Part of the chemical energy is used to translocate Ca\(^{2+}\) through the membrane (work) and part is dissipated as heat. The amount of heat produced during catalysis increases after formation of the Ca\(^{2+}\) gradient across the vesicle membrane. In the absence of gradient (leaky vesicles) the amount of heat produced/mol of ATP cleaved is half of that measured in the presence of the gradient. After formation of the gradient, part of the ATPase activity is not coupled to Ca\(^{2+}\) transport. We now show that NaF can impair the uncoupled ATPase activity with discrete effect on the ATPase activity coupled to Ca\(^{2+}\) transport. For the control vesicles not treated with NaF, after formation of the gradient only 20% of the ATP cleaved is coupled to Ca\(^{2+}\) transport, and the caloric yield of the total ATPase activity (coupled plus uncoupled) is 22.8 kcal released/mol of ATP cleaved. In contrast, the vesicles treated with NaF consume only the ATP needed to maintain the gradient, and the caloric yield of ATP hydrolysis is 3.1 kcal/mol of ATP. The slow ATPase activity measured in vesicles treated with NaF has the same Ca\(^{2+}\) dependence as the control vesicles. This demonstrates unambiguously that the uncoupled activity is an actual pathway of the Ca\(^{2+}\)-ATPase and not a contaminant phosphatase. We conclude that when ATP hydrolysis occurs without coupled biological work most of the chemical energy is dissipated as heat. Thus, uncoupled ATPase activity appears to be the mechanistic feature underlying the ability of the Ca\(^{2+}\)-ATPase to modulated heat production.

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The balance between the hydrolysis of the phosphoenzymes and the amount of heat released during Ca$^{2+}$ transport is lower than 50%. This relation indicates that uncoupled activity and reversal of the Ca$^{2+}$ transport become tightly coupled to the hydrolysis of ATP with little heat dissipation.

**Materials and Methods**

**Sarcoplasmic Reticulum Vesicles**—These were derived from the longitudinal sarcoplasmic reticulum (light fraction) of rabbit hind limb white skeletal muscle. They were prepared as previously described (32).

**Heat Production by Uncoupled ATPase Activity**

Heat production by uncoupled ATPase activity measured with intact vesicles is given by the equation:

\[ Q = -n \cdot Q_{ATP} \]

where \( Q \) is the amount of heat produced, \( n \) is the number of ATP molecules hydrolyzed, and \( Q_{ATP} \) is the amount of heat released per ATP hydrolyzed.

**Experimental Procedure**—All experiments were performed using an OMEGA Isothermal Titration Calorimeter from Microcal, Inc. (Northampton, MA) (18, 20–22). The calorimeter sample cell (1.5 ml) was filled with reaction medium and the heat change was recorded for 20 min. The volume of vesicle suspension in the reaction medium was measured by filtering samples of the assay medium through Millipore filters 5, 10, 15, 20, and 25 s after the addition of 45Ca$^{2+}$.

**ATPase Activity and ATP Synthesis**—This was assayed by measuring the release of 32P$^-$ from [γ-32P]ATP. The reaction was arrested with trichloroacetic acid, filtered, and the final concentration of [γ-32P]ATP was determined using a liquid scintillation counter. For Ca$^{2+}$ uptake, the assay medium was divided into two samples. Trace amount of 45Ca$^{2+}$ was added to one of the samples, and the reaction was started by the simultaneous addition of vesicles to both media. The sample containing the radioactive Ca$^{2+}$ was used to determine the incubation time when the vesicles are filled and the steady state 45Ca$^{2+}$ uptake is reached. The rate of Ca$^{2+}$ uptake to Ca$^{2+}$ released by the reaction was measured using an OMEGA Isothermal Titration Calorimeter from Microcal, Inc. (18, 20–22).

**Experimental Procedure**—All experiments were performed at 35 °C. The Mg$^{2+}$-dependent activity requires only Mg$^{2+}$ for its activation and is measured in the presence of 2 mM EGTA to remove contaminant Ca$^{2+}$ from the medium. The Ca$^{2+}$-dependent ATPase activity, which is correlated with Ca$^{2+}$ transport, is determined by subtracting the Mg$^{2+}$-dependent activity from the activity measured in the presence of both Mg$^{2+}$ and Ca$^{2+}$. ATP synthesis was measured using 32P$^-$ as previously described (37).

**Heat of Reaction**—This was measured using an OMEGA Isothermal Titration Calorimeter from Microcal, Inc. (18, 20–22). The calorimeter sample cell (1.5 ml) was filled with reaction medium and the heat change was recorded for 20 min. The volume of vesicle suspension in the reaction medium was measured by filtering samples of the assay medium through Millipore filters 5, 10, 15, 20, and 25 s after the addition of 45Ca$^{2+}$.

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interference from possible contamination of the sarcoplasmic reticulum vesicles with this enzyme. The free Ca²⁺ concentration in the medium was calculated using the association constants of Schwartzenbach et al. (39) in a computer program described by Fabiato and Fabiato (40) and modified by Sorensen et al. (41).

RESULTS

Effects of Fluoride on the Rates of Ca²⁺ Uptake and ATP Hydrolysis—In agreement with previous reports (27, 28, 30, 31), we observed that fluoride inhibits the sarcoplasmic reticulum Ca²⁺-ATPase (Figs. 3A and 4). Now, we show that contrasting to the inhibition of the ATPase activity, both the initial rate of Ca²⁺ uptake and the maximal amount of Ca²⁺ accumulated by the vesicles are enhanced by fluoride (Figs. 3B, 4, and Table I). The maximal effect is obtained in presence of 20 mM NaF (Fig. 4). The inhibition of the ATPase activity increases as the vesicles are filled with Ca²⁺, and maximal inhibition is observed after the steady state Ca²⁺ uptake is reached (Table I). These combined effects of fluoride on the ATPase activity and Ca²⁺ uptake indicate that in the presence of fluoride the vesicles are able to accumulate more Ca²⁺ with less ATP hydrolysis (Table I). In fact, the ratio between the initial rates of Ca²⁺ uptake and ATP hydrolysis is 0.26 ± 0.06 (5) in the absence of NaF and increases to 1.63 ± 0.13 (7) in the presence of 20 mM NaF. These values are mean ± S.E. of the number of experiments shown in parenthesis. Notice in Fig. 3A that the inhibition of the ATPase activity becomes more pronounced after 5 min incubation. Nevertheless, the vesicles are able to pump Ca²⁺ even after prolonged incubation intervals with NaF. This was shown by diluting the vesicles in a medium containing 20 mM NaF and adding small amounts of Ca²⁺ at different incubation intervals (Fig. 5). It was found that the Ca²⁺ added is rapidly taken up by the vesicles even after 20 min of incubation (Fig. 3A).

The uncoupled ATPase activity described by Yu and Inesi (13) is better detected after the vesicles are filled with calcium (15). A possible explanation to the effects of NaF noted in Figs. 3–5 and Table I is that fluoride inhibits the uncoupled Ca²⁺-ATPase activity to an extent larger than the ATPase activity actually responsible for Ca²⁺ uptake (reactions 1–6 in Fig. 2). This could be confirmed in experiments in which the vesicles are preincubated with NaF, and then the concentration of fluoride in the assay medium is decreased to a very low level.

Preincubation with Fluoride—Earlier studies indicate that fluoride binds tightly to the enzyme form E₂ but not to the form 2Ca⁺E₁ (27, 31, 42). The enzyme-fluoride complex formed in the absence of Ca²⁺ is very stable and can be isolated free of other components by 48 h of dialysis at 4 °C (27). This protective effect of Ca²⁺ is confirmed in Fig. 6 where vesicles were preincubated without ATP and with 20 mM NaF either in presence or absence of Ca²⁺. After different intervals, samples of the mixture were diluted 75-fold in the assay media containing ATP used to measure the enzyme activities. The presence of Ca²⁺ during the preincubation period prevented the inhibition of the Ca²⁺-ATPase by fluoride. When Ca²⁺ is substituted by EGTA, both the rate of Ca²⁺ uptake and the maximal amount of Ca²⁺ accumulated by the vesicles is diminished by fluoride (Fig. 7A). However, the ATPase activity is inhibited to a far larger extent than Ca²⁺ uptake. Both control vesicles and vesicles preincubated with NaF are able to resynthesize part of the ATP cleaved (Fig. 7). The values of net ATP hydrolysis shown on Tables II and III represent the true amount of ATP cleaved during Ca²⁺ transport and are calculated by subtracting the rate of ATP synthesis from the rate of ATP hydrolysis. For control vesicles, the ratio between the initial rate of Ca²⁺ uptake and net ATP hydrolysis varies between 0.68 (Table II) and 0.33 (Table III), values similar to those described in the bibliography (43). We now show that after fluoride treatment, this ratio raises to values close to 2, the optimal stoichiometric value for Ca²⁺ transport. Taken together, these data indicate that after 20 min of preincubation with EGTA and fluoride the vesicles accumulate Ca²⁺ at a slower rate than the control vesicles, but the transport is optimized with low energy dissipation, i.e. less ATP cleavage is needed to fill the vesicles with Ca²⁺ (Table II) and, after steady state, to maintain the gradient (Table III).

The difference between the initial rates and maximal amount of Ca²⁺ uptake noted in Figs. 3B and 7A are probably related to the amount of NaF available in the assay medium. In Fig. 3B the NaF concentration varies from 5 to 20 mM, while in Fig. 7, after dilution of the preincubation mixtures, the NaF concentration is 0.27 mM for both control and NaF-preincubated vesicles. Fluoride diffuses through the membrane, and, similar to phosphate and oxalate (44), complexes with calcium acting as a calcium-precipitating agent thus decrease the luminal free Ca²⁺ concentration and increase the loading capacity of the vesicles. In fact, in the absence of either Pi or oxalate, 20 mM fluoride greatly increased the loading capacity of the vesicles (data not shown). Thus, under the conditions of Fig. 7, we measured only the effect derived from the binding of fluoride to the enzyme. However, in Fig. 3 fluoride has two effects: it binds to the enzyme and acts as a Ca²⁺-precipitating agent increasing the loading capacity of the vesicles.

Ca²⁺ Dependence—Effects of Thapsigargin and Ca²⁺ Ionophore—The slow ATPase activity detected in intact vesicles preincubated with NaF and EGTA has the same Ca²⁺ dependence as the control vesicles. The K value for Ca²⁺ is 0.34 μM, both for control and vesicles preincubated with NaF (Fig. 8). The Ca²⁺-dependent ATPase activity of intact vesicles preincubated with NaF was inhibited by thapsigargin, a specific inhibitor of the sarcoplasmic or endoplasmic reticulum Ca²⁺-ATPase ATPases (data not shown). The effect of fluoride varies depending on the membrane permeability for Ca²⁺. There is no
Heat Production by Uncoupled ATPase Activity

TABLE I

Effect of fluoride on the rates of ATP hydrolysis and Ca\textsuperscript{2+} uptake

The assay medium composition and experimental conditions were as in Figs. 3 and 4. Initial velocity, refers to the initial 3-min incubation. Steady state refers to the rate of ATP cleavage during the interval of 15 to 20 min of incubation, i.e., after that the vesicles were filled with Ca\textsuperscript{2+} and there was no net uptake. The values are mean ± S.E. of the number of experiments shown in parenthesis.

| NaF (mM) | Ca\textsuperscript{2+}-ATPase | Ca\textsuperscript{2+} uptake |
|----------|-----------------------------|-----------------------------|
|          | Initial velocity            | Steady state                | Initial velocity | Steady state    |
|          | μmol P/μg min\textsuperscript{-1} |                        | μmol/mg         |                   |
| 0        | 1.77 ± 0.13 (5)             | 1.20 ± 0.12\textsuperscript{(4)} | 0.53 ± 0.04\textsuperscript{(3)} | 4.02 ± 0.37\textsuperscript{(3)} |
| 20       | 1.43 ± 0.24 (5)             | 0.14 ± 0.06\textsuperscript{(5)} | 2.53 ± 0.40\textsuperscript{(3)} | 7.69 ± 1.00\textsuperscript{(5)} |

\textsuperscript{a}The difference of the value was statistically significant with p < 0.001.
\textsuperscript{b}The difference of the value was statistically significant with p < 0.005.
\textsuperscript{c}The difference of the value was statistically significant with p < 0.01.

FIG. 5. Stepwise Ca\textsuperscript{2+} addition. The reaction mixture composition was 50 mM MOPS-Tris buffer (pH 7.0), 4 mM MgCl\textsubscript{2}, 20 mM NaF, 10 mM P\textsubscript{i}, 100 mM KCl, 5 mM Na\textsubscript{2}HPO\textsubscript{4}, 10 mM ATP, and 0.01 mg of vesicles protein/ml. Arrows indicate the addition of 45CaCl\textsubscript{2} to a final concentration of 5 μM. The first addition of 45CaCl\textsubscript{2} was carried out immediately after dilution of the vesicles.

FIG. 6. Preincubation with 20 mM NaF. Vesicles were preincubated in the presence of 20 mM NaF and with either (○) a mixture of 0.20 mM EGTA and 0.22 mM CaCl\textsubscript{2}, (16 μM free Ca\textsuperscript{2+}), or (●) a mixture of 0.20 mM EGTA and 0.22 mM 45CaCl\textsubscript{2}, 10 mM P\textsubscript{i}, 100 mM KCl, 5 mM NaN\textsubscript{3}, and 1 mM ATP. The final protein concentration in the assay mixture was 0.01 mg/ml. Calcium uptake (A) and ATPase activity (B) were assayed at 35 °C for 20 min.

measurable Ca\textsuperscript{2+}-dependent ATPase activity when vesicles preincubated with 20 mM NaF and EGTA are diluted in an assay medium containing the Ca\textsuperscript{2+} ionophore A23187. In seven different measurements the total ATPase activity measured in the presence of Ca\textsuperscript{2+} plus Mg\textsuperscript{2+} and the Mg\textsuperscript{2+}-dependent activity measured in the presence of Mg\textsuperscript{2+} and 2 mM EGTA were 1.32 ± 0.31 and 1.10 ± 0.21 μmol P/μg/min 15 min\textsuperscript{-1}, respectively. This difference is not statistically significant (p > 0.1).

ATP Synthesis—The Ca\textsuperscript{2+} concentration in the lumen of intact vesicles reaches the millimolar range a few seconds after the transport is initiated (3, 4, 7–9), and this triggers the reversal of the catalytic cycle of the ATPase (3, 6, 45–47) during which ATP is synthesized from ADP and P\textsubscript{i}. Fluoride inhibited the synthesis of ATP, but this inhibition was smaller than that of ATP hydrolysis (compare Fig. 7, B and C). During the initial incubation intervals, the rate of ATP synthesis by control vesicles is about 2-folds faster than that of vesicles pretreated with fluoride (Fig. 7C and Tables II and III). This difference decreases when the vesicles are filled with Ca\textsuperscript{2+} and the steady state is reached. The ratio between the rates of ATP hydrolysis and ATP synthesis (Tables II and III) is one of the parameters used to measure the degree of energy conservation of this system (7, 46, 47). The smaller this ratio, the more energy is being conserved by the system, i.e., the steady state can last longer because the net decline of the ATP concentration in the medium proceeds at a slower rate. The vesicles preincubated with fluoride are able to conserve more energy than the control vesicles because they are able to synthesize back a larger fraction of the ATP cleaved than the control vesicles both during the initial incubation intervals (Table II) and after the steady state is reached (Table III).
Calcium uptake, Ca\textsuperscript{2+} in \rightleftharpoons Ca\textsuperscript{2+} out exchange, ATPase activity, and ATP synthesis by control and NaF-preincubated vesicles. Calcium uptake (A, left ordinate), Ca\textsuperscript{2+} in \rightleftharpoons Ca\textsuperscript{2+} out exchange (A, right ordinate), ATPase activity (B), and ATP synthesis (C) were measured as described in “Materials and Methods.” Vesicles were preincubated during 20 min in media containing 50 mM MOPS-Tris buffer (pH 7.0), 4 mM MgCl\textsubscript{2}, 10 mM Pi, 100 mM KCl, 5 mM NaN\textsubscript{3}, and 2 mM EGTA and either without NaF (○, △) or with 20 mM NaF (●, ▽). The reaction medium composition was as in Fig. 3. In A (△, ●), Ca\textsuperscript{2+} in \rightleftharpoons Ca\textsuperscript{2+} out exchange. Values in the figure are mean ± S.E. of five experiments.

**Table II**

| Preincubation | Ca\textsuperscript{2+}-ATPase (a) | ATP synthesis (b) | Net ATP hydrolysis (c = (a − b)) | Ca\textsuperscript{2+} uptake (d) | ATP Hydrolysis/Synthesis (a/b) | Ca\textsuperscript{2+}/ATP (d/c) |
|---------------|-------------------------------|------------------|---------------------------------|-------------------------------|-------------------------------|-------------------------------|
| EGTA          | 1.40 ± 0.10\textsuperscript{a} | 0.16 ± 0.04      | 1.23 ± 0.12                     | 0.82 ± 0.03\textsuperscript{b} | 9.60 ± 1.40\textsuperscript{p} | 0.68 ± 0.07\textsuperscript{d} |
| EGTA + NaF    | 0.14 ± 0.01\textsuperscript{a} | 0.07 ± 0.01      | 0.07 ± 0.01                     | 0.14 ± 0.01\textsuperscript{b} | 2.23 ± 0.48\textsuperscript{p} | 1.89 ± 0.41\textsuperscript{d} |
| a The difference between the value was statistically significant with \( p < 0.001 \).  
| b The difference between the value was statistically significant with \( p < 0.001 \).  
| c The difference between the value was statistically significant with \( p < 0.001 \).  
| d The difference between the value was statistically significant with \( p < 0.02 \).  

**Table III**

Vesicles preincubated with 20 mM fluoride and rates of Ca\textsuperscript{2+} in \rightleftharpoons Ca\textsuperscript{2+} out exchange, ATPase activity and ATP synthesis at steady state

Preincubation and activities were measured as described for Figs. 6 and 7. The rates were measured 15–20 min after starting the reaction, i.e., after that the vesicles were filled with Ca\textsuperscript{2+} and there was no net uptake. Values are mean ± S.E. of five experiments.

| Preincubation | Ca\textsuperscript{2+}-ATPase (a) | ATP synthesis (b) | Net ATP hydrolysis (c = (a − b)) | Ca\textsuperscript{2+} in \rightleftharpoons Ca\textsuperscript{2+} out exchange (d) | ATP Hydrolysis/Synthesis (b/c) | Ca\textsuperscript{2+}/ATP (b/\( \Delta H \)) |
|---------------|-------------------------------|------------------|---------------------------------|---------------------------------|-------------------------------|-------------------------------|
| EGTA          | 1.17 ± 0.27\textsuperscript{a} | 0.12 ± 0.04      | 1.05 ± 0.24                     | 0.36 ± 0.06                     | 11.05 ± 1.80\textsuperscript{b} | 0.33 ± 0.01\textsuperscript{c} |
| EGTA + NaF    | 0.29 ± 0.03\textsuperscript{a} | 0.11 ± 0.03      | 0.18 ± 0.05                     | 0.39 ± 0.03\textsuperscript{b}  | 3.58 ± 0.96\textsuperscript{b} | 1.84 ± 0.08\textsuperscript{d} |
| a The difference between the value was statistically significant with \( p < 0.01 \).  
| b The difference between the value was statistically significant with \( p < 0.01 \).  
| c The difference between the value was statistically significant with \( p < 0.01 \).  

The data on Table IV show that pretreatment of the vesicles with NaF promotes a small increase of the uncoupled Ca\textsuperscript{2+} efflux and strongly inhibits the uncoupled ATPase activity. The coupled ATPase activity is the same for control and pretreated vesicles; however, control vesicles hydrolyzed five times more ATP than that needed to pump back the Ca\textsuperscript{2+} that leaks from the vesicles (uncoupled ATPase) while the NaF-treated vesicles hydrolyzed only the ATP needed to maintain Ca\textsuperscript{2+} inside the vesicles. This result suggests that at steady state, fluoride inhibits preferentially the uncoupled ATPase activity. This explains why for fluoride pretreated vesicles the ratio between Ca\textsuperscript{2+} pumped by the vesicles and the rate of ATP hydrolysis is close to the optimal value 2 (Tables II and III).

Heat Production in Presence and Absence of Ca\textsuperscript{2+} Gradient—The Mg\textsuperscript{2+}-dependent ATPase activity measured in the presence of Mg\textsuperscript{2+} and excess EGTA is not modified by 20 mM NaF. Both the rates of hydrolysis and heat released by vesicles preincubated in the presence or absence of NaF are the same (Fig. 9 and Table V). In these experiments the values of heat released/mol of ATP hydrolyzed (\( \Delta H^{\text{calc}} \)) found are the same as those previously measured for the Mg\textsuperscript{2+}-dependent ATPase activities of vesicles derived from either skeletal muscle or blood platelets (20, 21). In the presence of Mg\textsuperscript{2+} and Ca\textsuperscript{2+}, the addition of NaF promoted a drastic decrease of both the rates of ATP hydrolysis and heat release. The decrease in heat production, however, is more pronounced than that in the ATPase activity, thus, for the Ca\textsuperscript{2+}-dependent ATPase activity, the amount of heat released for each mole of ATP cleaved (\( \Delta H^{\text{calc}} \)) by vesicles pretreated with NaF is six to seven times smaller than that measured with control vesicles. This is observed either when NaF is added directly to the assay (Fig. 10) or when the vesicles are preincubated with NaF (Fig. 11 and Table V).

In previous reports (15, 18–22) it has been shown that the collapse of the Ca\textsuperscript{2+} gradient leads to an increase of the Ca\textsuperscript{2+}-dependent ATPase activity without concomitant increase in heat release, and as a result, the yield of heat produced during the hydrolysis of each ATP molecule in the presence of a gradient is 2-fold larger than that measured in the presence of the Ca\textsuperscript{2+} ionophore A23187 (leaky vesicles). This is confirmed in Figs. 10 and 11 and on Table V. As shown above, there is no measurable Ca\textsuperscript{2+}-dependent ATPase activity when vesicles preincubated with EGTA and NaF are diluted in media containing A23187, i.e., the amount of heat released and \( \Delta H^{\text{calc}} \) are those determined by the Mg\textsuperscript{2+}-dependent ATPase. Therefore, with NaF it is not possible to compare the values of \( \Delta H^{\text{calc}} \) with and without gradient.
Energy Balance—In previous work (15, 22), the heat produced during the unidirectional Ca\(^{2+}\)/H\(^{+}\) movement from the vesicle lumen to the medium by diluting vesicles previously loaded with Ca\(^{2+}\)/H\(^{+}\) into efflux media containing different concentrations of ADP, Pi, Mg\(^{2+}\)/H\(^{+}\), or K\(^{+}\)/H\(^{+}\) has been measured. These experiments revealed that the Ca\(^{2+}\)/H\(^{+}\)-ATPase can work in at least two different forms: i) it absorbs heat from the medium when the efflux is coupled to ATP synthesis (\(\Delta H^\text{cal} = 5.7\) kcal/mol Ca\(^{2+}\) released) and 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. In such a condition, the Ca\(^{2+}\) efflux is exothermic (\(\Delta H^\text{cal} = 14.9\) kcal/mol Ca\(^{2+}\) released). Knowing the \(\Delta H^\text{cal}\) values for coupled and uncoupled Ca\(^{2+}\) efflux, it is possible to estimate the relative contributions of the efflux and that of substrate hydrolysis to the heat produced during steady state either in the presence or absence of fluoride (Table VI). Under both conditions, the amount of heat produced by the Ca\(^{2+}\) efflux is small. In the absence of fluoride (control) all the heat produced is derived from the hydrolysis of ATP, and in this condition most of the ATP is cleaved through the un-

![Figure 8](image1)

**Fig. 8.** Ca\(^{2+}\) dependence of the ATPase activity of control (A) and vesicles preincubated with 20 mM NaF (B). Preincubation was as described in Fig. 7. For the total ATPase activity (○, ●), the assay medium composition was 50 mM MOPS-Tris (pH 7.0), 4 mM MgCl\(_2\), 0.20 mM EGTA, 10 mM P\(_6\), 100 mM KCl, 5 mM Na\(_2\)HPO\(_4\), 1 mM ATP, and different CaCl\(_2\) concentrations to achieve the different free Ca\(^{2+}\) concentrations shown in the figure. The assay media for the Mg\(^{2+}\)-dependent activity (△, ▽) was the same as that used for the total ATPase but without CaCl\(_2\), and the EGTA concentration was raised to 2 mM. The Ca\(^{2+}\)-dependent ATPase activity (○, ●) was calculated subtracting the Mg\(^{2+}\)-dependent from the total activity ATPase activity. Values are mean ± S.E. of three experiments.

![Figure 9](image2)

**Fig. 9.** Mg\(^{2+}\)-dependent ATPase activity (A), heat production (B), and \(\Delta H^\text{cal}\) (C). Preincubation without and with 20 mM NaF and assay media were as described in Fig. 8 for the Mg\(^{2+}\)-dependent activity. (○) Control and (●) are vesicles preincubated with 20 mM NaF. The figure shows a typical experiment.

![Figure 10](image3)

**Fig. 10.** Total ATPase activity (A), heat production (B), and \(\Delta H^\text{cal}\) (C) in absence (○) and presence (●) of 20 mM NaF. Assay medium was as described in Fig. 3. The figure shows a typical experiment. Inset in B, heat production in presence of 20 mM NaF plotted in an enhanced scale. The figure shows a typical experiment.
coupled route (Table IV). Therefore, the hydrolysis of ATP through reactions 2 and 10 in Fig. 2 is probably the catalytic route that mostly contributes to the heat released during ATP hydrolysis in the control experiments. The low heat production noted after fluoride treatment is probably derived from the uncoupled Ca\(^{2+}\) efflux.

**DISCUSSION**

**Correlation with the Bibliography**—Most of the earlier experiments with NaF were performed using leaky vesicles, a condition in which the inhibitory activity of fluoride is enhanced and the recovery of the Ca\(^{2+}\)-ATPase activity after removal of fluoride from the medium is unfavorable (27, 28, 30). Murphy and Coll (28) first described that the effect of fluoride is prevented by the binding of Ca\(^{2+}\) to the high affinity binding sites on the enzyme form \(E_1\). This is confirmed in Fig. 6, where vesicles solubilized with the detergent C12E9 (polyoxyethylene9-lauryl ether) Murphy and Coll (27) noted that the activity of fluoride-pretreated vesicles is more rapidly recovered after the excess fluoride is removed from the medium by 48 h of dialysis in the presence of millimolar Ca\(^{2+}\) concentration. Under these conditions, the reversal of fluoride inhibition by high Ca\(^{2+}\) concentration is very slow, with a \(t_{1/2}\) of 16 h at 37 °C. We observed that partial protection of the coupled ATPase activity is obtained provided that the integrity of the vesicle membrane is preserved. This protection is specific for the coupled ATPase, the uncoupled ATPase being completely inhibited by fluoride regardless of the membrane integrity. Notice in Fig. 7 that after dilution of vesicles preincubated with NaF, there is a lag phase before the vesicles are able to accumulate Ca\(^{2+}\). This was consistently noted in all experiments performed. At present we do not know the cause. One possibility is that during the initial incubation interval the vesicles accumulate Ca\(^{2+}\) at a very slow rate and the progressive rise in the internal Ca\(^{2+}\) concentration propitiates the activation of the coupled ATPase activity thus enhancing the rate of Ca\(^{2+}\) uptake.

**Coupling Ratio between Ca\(^{2+}\) Uptake and ATP Hydrolysis**—In the bibliography, this stoichiometry has been proved to be difficult to measure. A value of 2 was deduced from experiments measuring the binding of Ca\(^{2+}\) to the enzyme in the absence of ATP and from transient kinetics measurements in which the Ca\(^{2+}\) accumulated by the vesicles is determined in the initial milliseconds of reaction, i.e. before the Ca\(^{2+}\) concentration in the vesicles lumen reaches the millimolar range (9, 43, 49). Another experimental approach used to determine the stoichiometry has been to measure the reversal of the Ca\(^{2+}\) pump, in which the amount of ATP synthesized is correlated with the heat derived from the ATPase.
with the rate of Ca$^{2+}$ efflux (4, 6, 15). The Ca$^{2+}$ concentration inside the vesicles raises to the millimolar range after one or two catalytic cycles of the enzyme are completed (43). The Ca$^{2+}$-ATP ratio measured after a few seconds of incubation is smaller than 2, and this has been attributed to Ca$^{2+}$ leakage through the membrane and back fluxes of Ca$^{2+}$ through the ATPase protein itself. The recent discovery of the uncoupled ATPase activity indicates that the low Ca$^{2+}$-ATP values usually measured are not due to Ca$^{2+}$ leakage but rather to a dissociation between the catalytic and transport functions of the ATPase. Recently (15) it has been shown that the uncoupled ATPase is impaired when the ADP concentration in the medium is higher than that of ATP. In this condition, a Ca$^{2+}$-ATP ratio of approximately 2 is measured after the steady state is reached. In this report it is shown that the uncoupled ATPase activity can be selectively impared by fluoride with discrete effects on the coupled ATPase. As a result, the amount of ATP cleaved is practically only that necessary to pump Ca$^{2+}$, and a Ca$^{2+}$-ATP ratio near 2 can be measured both during the initial incubation intervals and after the steady state is reached and the vesicles are filled with Ca$^{2+}$. The identical Ca$^{2+}$ dependence of the activities inhibited more easily and less easily by fluoride demonstrate unambiguously that the uncoupled activity is an actual pathway of the native Ca$^{2+}$-ATPase, rather than due to partial denaturation or a contaminating phosphatase.

**Energy Interconversion**—The data presented in this and previous reports (15, 18, 20, 21) indicate that the enzyme is able to determine the fate of 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 to 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 would be able to regulate the interconversion of these different forms of energy. In this work it is shown that the amount of heat released during ATP hydrolysis by the Ca$^{2+}$-ATPase varies depending on whether or not the vesicles are preincubated with fluoride (Table V). With intact vesicles and in the absence of fluoride, 20–22 kcal are released during the hydrolysis of each ATP molecule, and after fluoride pretreatment, only 3 kcal/mol of ATP cleaved are released. The different values found with and without fluoride indicate that when no work is being performed (uncoupled ATPase), most of the energy is dissipated as heat. With fluoride practically all the ATPase activity is coupled to Ca$^{2+}$ transport, and therefore most of the energy is converted into work and little is dissipated as heat (coupled ATPase). Furthermore, the relationship between the rates of ATP hydrolysis and ATP synthesis is found to decrease about 3-fold after NaF treatment (Table III), indicating that the abolishment of the uncoupled ATPase activity by fluoride leads to an increase of the degree of energy conservation by the system. Finally, the possible physiological implications of the thermogenic activity of the Ca$^{2+}$-ATPase has been discussed in a recent report (15).

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