Insight into the Uncoupling Mechanism of Sarcoplasmic Reticulum ATPase Using the Phosphorylating Substrate UTP*

(Received for publication, November 1, 1999, and in revised form, January 31, 2000)

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Ca²⁺ transport and UTP hydrolysis catalyzed by sarcoplasmic reticulum Ca²⁺-ATPase from skeletal muscle was studied. A passive Ca²⁺ load inside microsomal vesicles clearly decreased the net uptake rate and the final accumulation of Ca²⁺ but not the UTP hydrolysis rate, causing energy uncoupling. In the absence of passive leak, the Ca²⁺/Pₐ coupling ratio was 0.7–0.8. UTP hydrolysis did not maintain a rapid component of Ca²⁺ exchange between the cytoplasmic and luminal compartments as occurs with ATP. The uncoupling process in the presence of UTP is associated with: (i) the absence of a steady state accumulation of ADP-insensitive phosphoenzyme; (ii) the cytoplasmic dissociation of Ca²⁺ bound to the ADP-sensitive phosphoenzyme; and (iii) the absence of enzyme inhibition by cyclopiazonic acid. All these characteristics confirm the lack of enzyme conformations with low Ca²⁺ affinity and point to the existence of an uncoupling mechanism mediated by a phosphorylated form of the enzyme. Suboptimal coupling values can be explained in molecular terms by the proposed functional model.

The ion motive ATPases are molecular devices involved in energy coupling processes (1). A decrease in energy conservation efficiency gives rise to the phenomenon of uncoupling. A self-evident source of uncoupling is the loss of ionic gradient through ionophoric activity (2–4), although a more subtle origin may be the catalytic mechanism itself (intramolecular uncoupling). Uncoupling has been observed in different energy-transducing systems and has been reported to depend on the experimental conditions tested. For instance, the sarcoplasmic reticulum (SR)¹ Ca²⁺-ATPase presents Ca²⁺ transport/ATP hydrolysis coupling ratios of 2, when measured under pre-steady state conditions (5). However, steady state measurements have given values ranging from 1.3 to 1.8 (6–8). The use of GTP (6), ITP (6), or other non-nucleotide substrates such as acetylphosphate (9), p-nitrophenylphosphate (10), methylumbelliferophosphate (10), or dinitrophenylphosphate (10) provided values near to 1. Likewise, some reports have given coupling ratios of around 2 for UTP and other nonphysiological substrates (11, 12).

Ca²⁺-ATPase intramolecular uncoupling in the presence of ATP has been related through indirect evidence to the cytoplasmic dissociation of Ca²⁺ from the phosphorylated intermediate (7, 8). However, a rapid efflux of luminal Ca²⁺ involving nonphosphorylated species of the enzyme has also been suggested as a possible uncoupling mechanism (13, 14). The reaction cycle in the presence of ATP is difficult to analyze because the substrate has complex kinetic effects that may be complicated by the existence of rapid Ca²⁺ exchange between the cytoplasmic and luminal compartments. We therefore selected UTP because it behaves in a more straightforward manner when it acts as phosphorylating agent and does not support rapid Ca²⁺ exchange. In this way, the study of the uncoupling mechanism was facilitated by the use of UTP.

Our experimental system consisted of sealed vesicles that were isolated from skeletal SR membrane. The preparation was enriched in Ca²⁺-ATPase protein, retained the native orientation of the membrane (15), and showed low passive permeability (16). In this study we explored different experimental conditions in the presence of UTP as an ATP surrogate. Data analysis of UTP hydrolysis, Ca²⁺ movement, and phosphoenzyme conformations provided clear illustrations of how the uncoupling mechanism works during enzyme cycling.

EXPERIMENTAL PROCEDURES

Isolation and Quantitation of Microsomes—Fast-twitch skeletal muscle from adult female New Zealand rabbit was used as starting material. Sealed right-side vesicles mainly derived from SR longitudinal tubules were prepared by the method of Eletr and Inesi (17). The final pellet was resuspended at 10–15 mg protein/ml, aliquoted, and stored at −80 °C until use. This preparation shows no significant level of passive leakage because of the absence of Ca²⁺ release channel (16). The SR protein was estimated by the colorimetric procedure of Lowry et al. (18) using bovine serum albumin as standard.

Free Ca²⁺ in the Media—Concentrations of free Ca²⁺ were adjusted by adding appropriated CaCl₂/EGTA mixtures according to the computer program developed by Fabiato (19). Calculations were based on the Ca²⁺-EGTA absolute stability constant (20) and the EGTA protonation equilibria (21). Relevant Ca²⁺ ligands and pH in the medium were also considered.

Passive Ca²⁺ Loading—This was achieved by equilibrating SR vesicles at 5 mg protein/ml in a medium containing 20 mM Mops, pH 7.0, 80 mM KCl, and 10, 20, or 40 mM CaCl₂. Radioactive ⁴⁵Ca²⁺ at −5,000 cpm/nmol was also included when indicated. Incubation lasted 4 h at 30 °C.

UTP Hydrolytic Activity—The release of inorganic phosphate in the min time scale was measured under stirring at 25 °C, according to Lin and Morales (22). The enzymatic reaction was started by diluting aliquots 100-fold of passively Ca²⁺-loaded vesicles (0.085 ml) in a medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl₂, 1 mM UTP, and sufficient CaCl₂ and/or EGTA to yield a final free Ca²⁺ in the external medium of 50 μM. This was calculated taking into account the external Ca²⁺ withdrawn with each aliquot of loaded vesicles. After dilution, the membrane protein concentration was 0.05 mg/ml. The same protocol was applied in experiments performed with Ca²⁺-un-
0.1 mM EGTA for vesicles loaded with 10 mM Ca\(^{2+}\). The hydrolysis was started at 25 °C by diluting aliquots 100-fold of Ca\(^{2+}\)-loaded or unloaded vesicles (0.085 ml). The dilution medium was 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl\(_2\), 1 mM UTP, and 0.151 mM EGTA for vesicles loaded with 20 mM Ca\(^{2+}\); 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl\(_2\), 1 mM UTP, and 0.049 mM CaCl\(_2\), and 0.1 mM EGTA for vesicles loaded with 10 mM Ca\(^{2+}\); 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl\(_2\), 1 mM UTP, and 0.151 mM EGTA for vesicles loaded with 20 mM Ca\(^{2+}\); and 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl\(_2\), 1 mM UTP, and 0.352 mM EGTA for vesicles loaded with 10 mM Ca\(^{2+}\). After dilution, free Ca\(^{2+}\) was 50 μM. The Ca\(^{2+}\)-independent activity was measured in the absence of Ca\(^{2+}\) by diluting aliquots of 0.085 ml containing 20 mM Mops, pH 7.0, 80 mM KCl, and 5 mg/ml SR vesicles in 8.5 ml of medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl\(_2\), and 0.5 mM EGTA.

The reaction was stopped at various times by adding 1-ml samples of reaction mixture to the nitric acid/molybdovanadate reagent. The Ca\(^{2+}\)-independent activity was evaluated in Ca\(^{2+}\)-unloaded vesicles by including 0.5 mM EGTA in the dilution medium and omitting CaCl\(_2\).

UPT hydrolysis was also measured in the second time scale by a radiometric procedure. Experiments were carried out at 25 °C under stirring, in the absence or presence of 5 mM potassium oxalate. SR vesicles (0.4 mg protein/ml) were initially suspended in a medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl\(_2\), 0.5 mM EGTA, and 0.545 mM CaCl\(_2\). In one case, the reaction was started by adding 1 mM [γ-\(^{32}\)P]UTP (~20,000 cpm/nmol) and in the other by adding 1 mM UTP with a pulse of [γ-\(^{32}\)P]UTP (~1 × 10^7 cpm) being delivered 2 min later. Zero time corresponded to the addition of radioactive UTP. In both cases, aliquots of the reaction mixture (0.5 ml) were quenched at different times with 0.5 ml of ice-cold 250 mM perchloric acid plus 4 mM sodium phosphate. The quenched samples were incubated in ice water bath for 5 min. The complete volume (1 ml) was filtered through a 0.45 μm nitrocellulose filter, and the filtrate was collected. The subsequent treatment consisted of removing contaminant [γ-\(^{32}\)P]UTP by charcoal, extraction of 32P phosphomolybdate complex with isobutanol/benzene, and quantification of 32P in the organic phase, according to a previously published procedure (23). The specific activity of standard [γ-\(^{32}\)P]UTP (cpm/nmol) was obtained by correlating the cpm of [γ-\(^{32}\)P]UTP and the chemical concentration of the substrate. When a pulse of [γ-\(^{32}\)P]UTP was added, the UTP concentration after 2 min of reaction (i.e., t = 0) was 0.75 mM, as deduced from the activity data. A 75% yield for the organic extraction of P, and appropriate blank assays were also considered for the transformation of cpm into nmol P. The extraction yield was experimentally measured in 0.5 ml of reaction medium containing 1 m[γ-\(^{32}\)P]UTP. The procedure required chemical hydrolysis of the substrate and further processing as described for the samples.

**Net Ca\(^{2+}\) Uptake**—This was measured at 25 °C under stirring and with the aid of radioactive tracer. Vesicles were first passively loaded with 10, 20, or 40 mM \(^{45}\)Ca\(^{2+}\), and the uptake was initiated by diluting aliquots 100-fold of \(^{45}\)Ca\(^{2+}\)-loaded vesicles, as described for the corresponding UTP hydrolysis experiments. After dilution, the reaction mixture consisted of 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl\(_2\), 0.05 mg/ml of SR protein, 1 mM UTP, and 50 μM free \(^{45}\)Ca\(^{2+}\). Total CaCl\(_2\) and/or EGTA in the dilution medium was adjusted according to the \(^{45}\)Ca\(^{2+}\) concentration used for loading. Loading and dilution media contained \(^{45}\)Ca\(^{2+}\) at the same specific activity (~10,000 cpm/nmol). Full detail is given in the legend of Fig. 3. Ca\(^{2+}\)-unloaded vesicles were subjected to the same protocol. In both cases, aliquots containing 1 ml of reaction mixture were filtered under vacuum at different time intervals, and the filters were then rinsed with 10 ml of ice-cold La\(^{3+}\) medium (20 mM Mops, pH 7.0, and 1 mM LaCl\(_3\)). Radioactivity was measured by liquid scintillation counting after solubilizing the filters. The unspecific Ca\(^{2+}\) retained by the filters was subtracted by performing a blank assay in the absence of UTP.

**Entry of Ca\(^{2+}\)—UTP-dependent Ca\(^{2+}\) entry** was measured in the second time scale as follows: samples of 0.25 ml containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl\(_2\), 0.545 mM \(^{45}\)CaCl\(_2\) (~10,000 cpm/nmol), and 0.5 mM EGTA (50 μM free \(^{45}\)Ca\(^{2+}\)), and 0.4 mg/ml of unloaded vesicles were mixed under continuous vortexing at 25 °C with 1 mM UTP. The reaction was quenched by adding 5 ml of ice-cold La\(^{3+}\) medium (20 mM Mops, pH 7.0, and 1 mM LaCl\(_3\)). Radioactivity was measured by liquid scintillation counting after solubilizing the filters. The unspecific Ca\(^{2+}\) retained by the filters was subtracted by performing a blank assay in the absence of UTP.

** Autoradiographic Detection of EP—This** was studied under two different assay conditions. Phosphorylation in the presence of Ca\(^{2+}\) was performed at 25 °C in a medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl\(_2\), 0.545 mM CaCl\(_2\), 0.5 mM EGTA, 1 mg/ml microsomal protein, and 1 mM [γ-\(^{32}\)P]UTP or 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl\(_2\), 1 mM EGTA, 20% dimethyl sulfoxide, 1 mg/ml microsomal protein, and 1 mM [γ-\(^{32}\)P]UTP. Phosphorylated samples were subjected to polyacrylamide gel electrophoresis in the presence of lithium dodecyl sulfate. Protein staining of samples phosphorylated in the presence (lane 1) or absence of Ca\(^{2+}\) (lane 2) and autoradiograms of samples phosphorylated in the presence (lane 3) or absence of Ca\(^{2+}\) (lane 4) are shown.
sisting of 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 20% dimethyl sulfoxide, and 1 mg of SR protein/ml. Reactions were initiated by adding 0.1 mM [γ-32P]UTP (~1 × 10⁶ cpm/nmol) and stopped by mixing under vortexing 1 ml of reaction mixture with 1 ml of ice-cold 7% trichloroacetic acid plus 10 mM sodium phosphate. The reaction time was 10 s for Ca²⁺-containing samples and 100 s for Ca²⁺-deprived samples. Quenched samples were incubated in ice for 5 min and then sedimented at 4 °C by centrifugation (10,000 rpm for 5 min). The resultant pellets were washed three times with ice-cold quenching solution and once with double distilled water and then dissolved in 0.2 ml of electrophoresis sample buffer (24). Aliquots containing 10 μg of membrane protein were applied to a 7.5% polyacrylamide gel slab in the presence of 0.1% lithium dodecyl sulfate. Electrophoresis was carried out for 1 h at room temperature and 180 volts. (24). Radioactive labels in the dried gel were detected by autoradiography at ~80 °C. The x-ray film and the intensifying screen were Curix RP2 and Curix blue C2, respectively, from Agfa.

**Phosphorylated Intermediate from [γ-32P]UTP—**The levels of radioactive EP at neutral pH and in the presence of K⁺ were measured in a medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl₂, 0.545 mM CaCl₂, 0.5 mM EGTA (50 μM free Ca²⁺), 0.4 mg/ml SR vesicles, and 1 mM [γ-32P]UTP (~20,000 cpm/nmol). Potassium oxalate (5 mM) was also included when indicated. Phosphorylation was maintained for 2 min at 22 °C, and the reaction was stopped by adding 5 ml of ice-cold acid medium (125 mM perchloric acid plus 2 mM sodium phosphate) to 0.5 ml of reaction mixture. The denatured protein was incubated in ice for 5 min before being filtered through a 0.45-μm nitrocellulose filter. The filter, once rinsed with 25 ml of ice-cold acid medium, was solubilized and counted by liquid scintillation technique. A blank was performed by adding the acid solution before the radioactive UTP.

Phosphorylation at alkaline pH and in the absence of K⁺ was measured, as described above, in a medium containing 20 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 0.549 mM CaCl₂, 0.5 mM EGTA (50 μM free Ca²⁺), 0.4 mg/ml SR vesicles, and 1 mM [γ-32P]UTP at ~20,000 cpm/nmol.

**TNP-ATP Fluorescence under Turnover Conditions—**Changes in TNP-ATP fluorescence were measured at 22 °C with a Shimadzu RF-540 spectrofluorimeter in a continuously stirred cuvette. Excitation and emission wavelengths were 420 and 540 nm, respectively. The enzyme content was 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl₂, 0.149 mM CaCl₂, 0.1 mM EGTA (Ca²⁺ free was 50 μM), and 0.1 mM EGTA SR vesicles. Potassium oxalate (5 mM) was present in some experiments. Fluorescence intensity was recorded in the min time scale before and after the sequential addition of 2 μM TNP-ATP and 1 mM UTP. Other measurements were performed in a medium containing 20 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 0.15 mM CaCl₂, 0.1 mM EGTA (50 μM free Ca²⁺) SR vesicles, 2 μM TNP-ATP, and 1 mM UTP. The fluorescence units correspond to a ratio between the intensity after addition of UTP (ΔF) and the intensity after addition of TNP-ATP (F).
**RESULTS**

The Effect of Lumenal Ca$^{2+}$—The hydrolytic activity of the SR Ca$^{2+}$-ATPase protein was evaluated by using 1 mM UTP as phosphorylating substrate. The assay conditions included a buffered medium at neutral pH, 80 mM K$^+$, 5 mM Mg$^{2+}$, and 50 μM free Ca$^{2+}$. Fig. 1 shows that the liberation of inorganic phosphate, in the minute time scale, is characterized by an initial burst followed by a linear phase. The experiments were carried out with native (Ca$^{2+}$-unloaded) SR vesicles. The effect of luminal Ca$^{2+}$ on the Ca$^{2+}$-UTPase activity, measured in the min time scale, was also checked. In this case, the vesicles were initially incubated for 4 h at 30 °C with a Ca$^{2+}$ concentration ranging from 10 to 40 mM. As can be seen, the passive loading of vesicles did not significantly modify the time-dependent appearance of phosphate. Therefore, it was not possible to establish a clear dependence between the rate of UTP hydrolysis and the initial Ca$^{2+}$ load. Furthermore, Ca$^{2+}$-independent UTP hydrolysis displayed a linear time course equivalent to 160 nmol P/min/mg protein, a rate that represented 55% of the steady state rate measured in the presence of Ca$^{2+}$.

The Ca$^{2+}$-dependent and Ca$^{2+}$-independent activities were further investigated by studying the accumulation of phosphorylated intermediate at 25 °C after addition of 0.1 mM [γ-32P]UTP. In one case, SR vesicles were suspended in a standard reaction medium containing 50 μM free Ca$^{2+}$, and in the other, the vesicles were suspended in a Ca$^{2+}$-free medium and 20% dimethyl sulfoxide was added. Phosphorylation in the presence or absence of Ca$^{2+}$ was started by adding radioactive nucleotide and stopped by acid quenching. Once the micro-

Ca$^{2+}$ entry into unloaded vesicles (A, ○) was measured at 25 °C by adding 1 mM UTP to a medium containing 20 mM Mops, pH 7.0, 80 mM KC1, 5 mM MgCl$_2$, 0.149 mM CaCl$_2$, 0.1 mM EGTA (50 μM free Ca$^{2+}$), and 0.055 mg/ml of SR vesicles (i.e., 0.2 μM Ca$^{2+}$-ATPase). The reaction was started (t = 0) by adding 1 mM UTP. The CPA effect was evaluated by adding 0.4 μM CPA at t = 9 min. The CPA effect in leaky vesicles was measured by a similar procedure, although the reaction medium was supplemented with 4 μM A23187. Control assays were performed by not adding CPA. Inorganic phosphate released before and after the CPA addition was evaluated by mixing aliquots of 1 ml of reaction mixture and 1 ml of color reagent at different times (22).

Materials—Radioactive tracers $^{40}$Ca$^{2+}$, and [γ-32P]UTP were obtained from Amersham Pharmacia Biotech. TNP-ATP was from Molecular Probes Europe. CPA from *Penicillium cyclopium* (C 1530) and the liquid scintillation mixture (S 4023) were products of Sigma. A 5 mg/ml stock solution of CPA was prepared in dimethyl sulfoxide. The Ca$^{2+}$-standard solution (Titrisol) was purchased from Merck. A23187 from *Streptomyces chartreusensis* was a Calbiochem product. Nitrocellulose filters with a 0.45-μm pore size were from Millipore. A Hoefer filtration box from Amersham Pharmacia Biotech and a rapid filtration apparatus from Bio-Logic Co. (Clair, France) were used for sample filtration.

Data Presentation—Experimental points represent the average of at least three independent determinations, each performed in duplicate. Standard errors of the mean are also included. The SigmaPlot Graph System from Jandel Scientific was used for data fitting.
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While some vesicles had been thoroughly washed and dissolved, the membrane proteins were subjected to electrophoresis and autoradiography (Fig. 2). The electrophoretic profile of lanes 1 and 2 in Fig. 2 corresponds to samples phosphorylated in the presence or absence of Ca\textsuperscript{2+}, respectively. The samples were stained with Coomassie Blue and presented the characteristic pattern of SR longitudinal tubules with Ca\textsuperscript{2+}-ATPase (116-kDa) as the most abundant protein of the membrane. Moreover, autoradiographic analysis showed a radioactive band at 160-kDa calsequestrin-like proteins (26).

Experiments were also directed at measuring the UTP-dependent Ca\textsuperscript{2+} uptake associated with the release of inorganic phosphate. When unloaded vesicles and 50 \(\mu\text{M}\) free \(\text{45Ca}^{2+}\) were used in the external medium, the addition of 1 mM UTP was followed by the rapid entry of \(\text{45Ca}^{2+}\), which reached asymptotic levels at approximately 80 nmol/mg protein (Fig. 3A). The effect of luminal Ca\textsuperscript{2+} on net uptake was evaluated after a preliminary passive loading of the vesicles with 10–40 mM \(\text{45Ca}^{2+}\) and their subsequent addition to a transport medium containing 50 \(\mu\text{M}\) free \(\text{45Ca}^{2+}\). Caution was taken to ensure that the same \(\text{45Ca}^{2+}\) specific activity existed in both passive loading and active transport media. The net uptake rate and the final accumulation of Ca\textsuperscript{2+} decreased with increasing initial Ca\textsuperscript{2+} loading (Fig. 3A). The passive \(\text{45Ca}^{2+}\) load was 16, 21.5, or 30 nmol/mg of protein, when incubated with 10, 20, or 40 mM \(\text{45Ca}^{2+}\), respectively. At each time point of net uptake, we observed that the sum of the initial passive loading plus the net uptake was independent of the initial Ca\textsuperscript{2+} load (Fig. 3B).

\textbf{Ca\textsuperscript{2+}/P\textsubscript{i} Coupling—}Because Ca\textsuperscript{2+} pumping activity is an energy-dependent process linked to nucleotide hydrolysis, Ca\textsuperscript{2+} transport and UTP hydrolysis were evaluated in the same reaction medium, except that \(\text{45Ca}^{2+}\) was included for evaluating Ca\textsuperscript{2+} transport and \([\gamma\text{-32P}]\text{UTP}\) to measure UTP hydrolysis. The transport process was stopped by La\textsuperscript{3+} quenching and nucleotide hydrolysis by acid/color reagent. Both parameters were measured at the beginning or after 2 min of reaction, i.e. in coupled or uncoupled vesicles. The addition of 1 mM UTP to SR vesicles incubated in the presence of 50 \(\mu\text{M}\) free \(\text{45Ca}^{2+}\) led to the nonlinear active accumulation of \(\text{45Ca}^{2+}\), tending to an asymptotic level (Fig. 4A). These measurements, in the second time scale, were carried out at the beginning of Ca\textsuperscript{2+} pumping. Ca\textsuperscript{2+} entry was also evaluated after 2 min of active Ca\textsuperscript{2+} loading. In this case, 1 mM UTP was added to SR vesicles in the presence of \(\text{40Ca}^{2+}\), followed by a \(\text{40Ca}^{2+}\) pulse 2 min later. Under these conditions there was practically no \(\text{40Ca}^{2+}\) entry (Fig. 4A). Parallel experiments performed in the presence of \([\gamma\text{-32P}]\text{UTP}\) (Fig. 4B) revealed that the rate of \(\text{P}\) release at the beginning of the reaction (430 nmol/min/mg of protein) was somewhat higher than that measured at \(t = 2\) min (320 nmol/min/mg of protein). The effect of time on coupling (Fig. 4B, inset) was revealed by the Ca\textsuperscript{2+}/P\textsubscript{i} ratio of 0.8 at \(t = 1\) s, which showed a tendency to decrease in the following seconds. Total uncoupling of the pump was observed after 2 min of reaction.

The above coupling data prompted us to modify the assay conditions as described for the corresponding experiment in Fig. 4 but including 5 mM potassium oxalate in the initial reaction medium. A, Ca\textsuperscript{2+} entry in unloaded (○) or Ca\textsuperscript{2+}-loaded vesicles (●). B, UTP hydrolysis in unloaded (□) or Ca\textsuperscript{2+}-loaded vesicles (■). Inset of B, coupling ratio of unloaded (△) or Ca\textsuperscript{2+}-loaded vesicles (▲) when measured in the presence of oxalate.
conditions by adding 5 mM oxalate to the reaction medium and maintaining all the other conditions, including the temperature of 25 °C and 50 μM free 45Ca2+. The entry of active 45Ca2+ displayed a linear time course and very similar rates (220 versus 200 nmol Ca2+/min/mg of protein) when measured at the beginning or after 2 min of active 45Ca2+ loading, respectively (Fig. 5B). The corresponding hydrolytic activities measured at the beginning or after 2 min of reaction also showed linear responses. The rates were 330 and 280 nmol P2P/min/mg of protein for unloaded or Ca2+-loaded vesicles, respectively (Fig. 5B). Therefore, the coupling ratio at the beginning of pumping and in the presence of oxalate was 0.7 and did not vary with the Ca2+ loading state of the vesicles (Fig. 5B, inset).

Uncoupling Features—The vectorial translocation of Ca2+ involves the participation of two different EP conformations as outlined in the simplified reaction cycle (Scheme I). Therefore, the identification of accumulated EP species is a critical step for understanding the reaction cycle sustained by UTP. The experiments were performed at 22 °C in a reaction medium containing 50 μM free Ca2+. Total EP was measured by phosphorolysis in the presence of 1 mM [γ-32P]UTP at neutral pH and in the absence of K+ was performed in a medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl2, 0.545 mM CaCl2, 0.5 mM EGTA, 0.4 mg/ml SR vesicles, and 1 mM [γ-32P]UTP. Potassium oxalate (5 mM) was included when indicated. The reaction medium at alkaline pH and in the absence of K+ was performed in a medium containing 20 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 0.549 mM CaCl2, 0.5 mM EGTA, 0.4 mg/ml SR vesicles, and 1 mM [γ-32P]UTP. Levels of EP were measured at 22 °C by a fluorimetric method (closed bars). Measurements at neutral pH and in the presence of K+ were performed in a medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl2, 0.149 mM CaCl2, 0.1 mM EGTA, 0.1 mg/ml SR vesicles, and 2 μM TNP-ATP, and 1 mM UTP. Potassium oxalate (5 mM) was included when indicated. The reaction medium at alkaline pH and in the absence of K+ was performed in a medium containing 20 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 0.15 mM CaCl2, 0.01 mM EGTA, 0.1 mg/ml SR vesicles, 2 μM TNP-ATP, and 1 mM UTP. The fluorescence units, as defined under "Experimental Procedures," are relative to compare the signal under different assay conditions. The accumulation of EP, P-CA2, at neutral pH was inferred from the absence of EP, oxalate.

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Fig. 6. Levels of phosphorylated species under turnover conditions. Total EP was measured at 22 °C by radiometric procedure (open bars). Phosphorolysis at neutral pH and in the presence of K+ was performed in a medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl2, 0.545 mM CaCl2, 0.5 mM EGTA, 0.4 mg/ml SR vesicles, and 1 mM [γ-32P]UTP. Potassium oxalate (5 mM) was included when indicated. Phosphorolysis at alkaline pH and in the absence of K+ was measured in a medium containing 20 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 0.549 mM CaCl2, 0.5 mM EGTA, 0.4 mg/ml SR vesicles, and 1 mM [γ-32P]UTP. Levels of EP were measured at 22 °C by a fluorimetric method (closed bars). Measurements at neutral pH and in the presence of K+ were performed in a medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM MgCl2, 0.149 mM CaCl2, 0.1 mM EGTA, 0.1 mg/ml SR vesicles, 2 μM TNP-ATP, and 1 mM UTP. Potassium oxalate (5 mM) was included when indicated. The reaction medium at alkaline pH and in the absence of K+ was performed in a medium containing 20 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 0.15 mM CaCl2, 0.01 mM EGTA, 0.1 mg/ml SR vesicles, 2 μM TNP-ATP, and 1 mM UTP. The fluorescence units, as defined under "Experimental Procedures," are relative to compare the signal under different assay conditions. The accumulation of EP, P-CA2, at neutral pH was inferred from the absence of EP, oxalate.
nmol/mg of protein. In this case, subsequent flushing with UTP and $^{40}\text{Ca}^{2+}$ induced a decrease of the $^{45}\text{Ca}^{2+}$ associated with the vesicles. The observed $^{45}\text{Ca}^{2+}-^{40}\text{Ca}^{2+}$ exchange amounted to approximately 9 nmol/mg of protein. The mechanism of intramolecular uncoupling was confirmed by using the high affinity inhibitor CPA (31–33) and measuring UTP hydrolysis under the conditions described in Fig. 8. The incubation medium in Fig. 8A included Ca$^{2+}$ ionophore to make the vesicles leaky, and the reaction was started by adding 1 mM UTP. The release of Pi followed a linear time course, equivalent to 700 nmol/min/mg of protein. The inhibitory effect of CPA was demonstrated in another assay by starting the reaction with UTP and then adding CPA at $t = 9$ min. As can be seen, the initial linear accumulation of Pi was clearly stopped by the presence of CPA. In this case the CPA/enzyme molar ratio was 2. However, a protecting role induced by luminal Ca$^{2+}$ was revealed by performing experiments in the absence of Ca$^{2+}$ ionophore. Measurements of UTP hydrolysis in native vesicles (Fig. 8B) showed a linear rate equivalent to 320 nmol P/
min/mg of protein. Furthermore, when the experiments were repeated and CPA was added after 9 min of reaction, the hydrolytic activity was not inhibited. It should be noted that the same CPA/enzyme molar ratio produced complete inhibition in leaky vesicles.

**DISCUSSION**

The Ca²⁺-ATPase reaction cycle was studied in the presence of the nonphysiological substrate of the enzyme UTP in an attempt to understand the uncoupling phenomenon. The rate of UTP hydrolysis was barely affected by the presence of passive Ca²⁺ load in sealed SR vesicles (Fig. 1). The Ca²⁺ load corresponded to lumenal concentrations of 3.2, 4.3, or 6 mM, assuming 5 µg/mg of protein as the volume of vesicles (34). However, the presence of the same passive load reduced the rate of UTP-dependent Ca²⁺ uptake and the final level of accumulated Ca²⁺ (Fig. 3A). Therefore, luminal Ca²⁺ plays a central role in the appearance of uncoupling as occurs in the presence of ATP (8, 16). Taking together the Ca²⁺ transport and the UTP hydrolysis data (Figs. 4 and 5), it is clear that a certain Ca²⁺/Pₐ coupler can be maintained during the reaction as long as the luminal Ca²⁺ remains low, i.e. by including oxalate in the reaction medium. A rise in luminal Ca²⁺ induces complete uncoupling of the pump.

It is common practice to subtract the Ca²⁺⁻independent hydrolytic activity when calculating coupling ratios. However, this is only valid when the Ca²⁺⁻dependent and Ca²⁺⁻independent activities are operating simultaneously, as occurs when they correspond to different proteins. If the total hydrolytic activity is much higher than the Ca²⁺⁻independent activity, as occurs when dealing with ATP, subtraction does not greatly modify the coupling value. In the case of UTP, the Ca²⁺⁻dependent and Ca²⁺⁻independent activities are both related to the same protein (Fig. 2). Moreover, the Ca²⁺⁻independent activity represents about 55% of the total activity (Fig. 1), although it would be expected to be negligible under turnover conditions because of the presence of saturating Ca²⁺ levels in the external (cytoplasmic) medium. Therefore, subtraction of the Ca²⁺⁻independent activity overestimates the coupling, giving values close to 2 (Refs. 11 and 12 and data in this paper), so that it is concluded that the relevant activity for calculating coupling ratios is the total Ca²⁺⁻dependent activity. In the presence of oxalate or at the beginning of the reaction when oxalate is absent, a coupling ratio of 0.7⁻0.8 was measured (insets of Figs. 4B and 5B).

In principle, the uncoupling process can be developed through phosphorylated or unphosphorylated forms of the enzyme. Uncoupling through phosphorylated species involves the release of Pₐ from EₐPₐCaₐ before Ca²⁺ dissociation to the cytoplasmic medium. In other words, EₐPₐCaₐ can be directly hydrolyzed without interconversion into EₐP. Moreover, this uncoupling mechanism will occur without a Ca²⁺ flux (i.e. uptake or exchange). By contrast, uncoupling through unphosphorylated species (EₐPₐ) requires the existence of a Ca²⁺ efflux, i.e. an ADP-independent Ca²⁺ exchange in the absence of any net entry. It should be noted that an ADP-dependent Ca²⁺ exchange does not produce uncoupling because it occurs through the reversal of the ATP phosphorylation reaction.

The experiments on ⁴⁵Ca²⁺ transport measured at the beginning of the reaction (Figs. 4A and 5A) corresponded to a situation of net uptake because the vesicles were initially unloaded. However, at t = 2 min they corresponded to unidirectional ⁴⁵Ca²⁺ influx, when measured in the absence of oxalate (Fig. 4A), because of the presence of lumenal ⁴⁰Ca²⁺ or net ⁴⁴Ca²⁺ uptake when measured in the presence of oxalate (Fig. 5A). A Ca²⁺ influx in the absence of net uptake can be attributed to Ca²⁺ exchange, because influx = uptake + exchange. In the absence of oxalate and after 2 min of reaction there was a negligible component of Ca²⁺ influx (Fig. 4A). Therefore, rapid ⁴⁰Ca²⁺⁻⁴⁵Ca²⁺ exchange does not occur in the presence of UTP. This lends support to the uncoupling mechanism mediated by phosphorylated species. Furthermore, uncoupling through EₐP₂Eₐ in the presence of UTP is unlikely, because activation of Ca²⁺ exchange by this route was only observed when Ca²⁺-loaded vesicles were diluted in the absence of ATP, Mg²⁺, and Ca²⁺ (13, 14).

Further support for our model was provided by the following three observations: (i) There is an absence of accumulated EₐP in vesicles sustaining an active Ca²⁺ load (uncoupled) in the presence of UTP (Fig. 6), an observation that cannot be reconciled with the participation of Eₐ in the uncoupling (mechanism of unphosphorylated forms). (ii) Ca²⁺ can dissociate from EP to the cytoplasmic medium when the vesicles are Ca²⁺⁻loaded (uncoupled) but not at the beginning of the reaction (Fig. 7, A and B). The exchange of ⁴⁵Ca²⁺ bound to EₐP with external ⁴⁰Ca²⁺ when the vesicles are loaded in the presence of UTP is a clear indication that Ca²⁺ release inside the vesicles is blocked and Ca²⁺ dissociation occurs toward the cytoplasmic medium (35). The absence of Ca²⁺ exchange at the beginning of the reaction supports the expected Ca²⁺ accumulation under coupling conditions. (iii) UTP hydrolysis by Ca²⁺⁻loaded vesicles (uncoupled) was not inhibited by CPA (Fig. 8B). The uncoupled state of the enzyme was protected from CPA, an EₐP-directed inhibitor of the enzyme (33). Such inhibition by CPA was patent when the lumenal Ca²⁺ load was relieved by the addition of Ca²⁺ ionophore (Fig. 8A), which may be attributed to the existence of some enzyme turnover through the conventional cycle. The forward operation of the Ca²⁺ pump generates the Eₐ form that is the target of the high affinity inhibitor (32).

Our data indicate that EₐPₐCaₐ is a keystone in the uncoupling mechanism. In this regard, the accumulation of this phosphorylated intermediate will be indistinguishable regardless of whether it is formed from ATP or UTP. The fact that the uncoupling is more easily observed in the presence of UTP suggests that the coupled route, i.e. the EₐPₐCaₐ → EₐP interconversion is favored by ATP but not by UTP. The activating effect of ATP on the phosphoenzyme interconversion step has been described (36, 37).

In experiments with ATP and Ca²⁺⁻loaded vesicles (uncoupled conditions), EₐPₐCaₐ and EₐP are accumulated, and so the uncoupling mechanism is more difficult to study owing to the existence of a rapid ⁴⁰Ca²⁺⁻⁴⁴Ca²⁺ exchange (38, 39) and the presence of Eₐ and EₐP conformations of the enzyme.

All the present observations using the phosphorylating substrate UTP can be explained by Scheme I. The coupled route (Eₐ₁Eₐ₂ cycle) relies on the ordered sequential breakdown of EₐPₐCaₐ. The dissociation of Ca²⁺ inside the vesicles is followed by the release of Pₐ into the cytoplasmic medium. An alteration of this sequence, i.e. the release of Pₐ, followed by the release of Ca²⁺, both to the cytoplasmic medium, produces the uncoupled route (Eₐ cycle). Thus, under low coupling conditions the coexistence of an Eₐ₁Eₐ₂ cycle and Eₐ cycle is to be expected, whereas the total uncoupled state can be explained by the sole operation of the Eₐ cycle. The existence of the uncoupled cycle may account for the different coupling efficiencies reported for different phosphate donor substrates and different experimental conditions.

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J. Biol. Chem. 2000, 275:12521-12529.
doi: 10.1074/jbc.275.17.12521

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