A detailed characterization of p-nitrophenyl phosphate as energy-donor substrate for the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase was undertaken in this study. The fact that p-nitrophenyl phosphate can be hydrolyzed in the presence or absence of Ca\(^{2+}\) by the purified enzyme is consistent with the observed phenomenon of intramolecular uncoupling. Under the most favorable conditions, which include neutral pH, intact microsomal vesicles, and low free Ca\(^{2+}\) in the lumen, the Ca\(^{2+}/P_i\) coupling ratio is 0.6. A rise or decrease in pH, high free Ca\(^{2+}\) in the lumen space, or the addition of dimethyl sulfoxide increase the intramolecular uncoupling. Alkaline pH and/or high free Ca\(^{2+}\) in the lumen potentiate the accumulation of enzyme conformations with high Ca\(^{2+}\) affinity. Acidic pH and/or dimethyl sulfoxide favor the accumulation of enzyme conformations with low Ca\(^{2+}\) affinity. Under standard assay conditions, two uncoupled routes, together with a coupled route, are operative during the hydrolysis of p-nitrophenyl phosphate in the presence of Ca\(^{2+}\). The prevalence of any one of the uncoupled catalytic cycles is dependent on the working conditions. The proposed reaction scheme constitutes a general model for understanding the mechanism of intramolecular energy uncoupling.

It is well established that the free energy released from ATP hydrolysis can be used for the vectorial translocation of cations across cellular membranes (1). In this sense, clear structural and functional similarities among P-type ATPases have been observed (2–4). These similarities include the hydrophobic transmembrane segments containing the porelike region, the extramembranous globular head where the ATP binding site is located, two major conformational states of the enzyme, the acyl phosphate intermediate and the catalytic cycle. Although these common features suggest that ion-transporting ATPases share the same energy transduction mechanism, this idea was challenged when non-nucleotide compounds were used as phosphorylating substrate (5). In fact, it was observed that the erythrocyte membrane Ca\(^{2+}\)-ATPase displays slight Na\(^+\) uptake during the hydrolysis of acetyl phosphate (13), whereas the H\(^+\)-K\(^+\)-ATPase from gastric mucosa cannot actively transport H\(^+\) and K\(^+\) when hydrolyzing acetyl phosphate (5). Thus, it has been recognized that the hydrolysis of non-nucleotide substrates in the absence of transport activity is related to low energy conformations of the enzyme (5, 12, 14), whereas the simultaneous occurrence of both substrate hydrolysis and ion transport is associated with high energy conformations (7, 9, 15).

In an attempt to clarify the role of non-nucleotide substrates in the energy coupling process, we selected pNPP as energy-donor substrate and SR Ca\(^{2+}\)-ATPase as a transduction system. Initially, the enzyme was exposed to conditions that allowed Ca\(^{2+}\) transport at the expenses of pNPP hydrolysis. Then, the assay conditions were modified to induce uncoupling. The use of a purified enzyme preparation (16) and the highly specific inhibitor TG (17, 18) shed light on the hydrolytic activities measured in the presence or absence of Ca\(^{2+}\). The experimental strategy for analyzing the energy uncoupling routes was based on the inhibitory effect of vanadate and TG, namely vanadate interacts selectively with the E\(_2\) conformation of the enzyme whereas TG inhibits the accumulation of E\(_1\) forms.

Our functional model provides a mechanistic description of the intramolecular uncoupling phenomenon when the SR Ca\(^{2+}\)-ATPase hydrolyzes pNPP. This is relevant for understanding the uncoupling of different P-type ATPases in the presence of different phosphorylating substrates and in the absence of a cationic leak.

**EXPERIMENTAL PROCEDURES**

**Materials**—[\(^{45}\text{Ca}\)]CaCl\(_2\) was obtained from Amersham Pharmacia Biotech. The Ca\(^{2+}\) standard solution (Tritisol) was purchased from Merck. Jonophore A23187 from Streptomyces chartreusensis was a product of Calbiochem. Ammonium metavanadate was from Acros Organics. 4-Nitrophenyl phosphate (dissodium salt) was from Roche Molecular Biochemicals. TG was obtained from Molecular Probes Europe. The liquid scintillation mixture (S 4023), deoxycholic acid (sodium salt), and other reagents of analytical grade were from Sigma. HAWP filter units with pore diameter of 0.45 μm were from Millipore. Ca\(^{2+}\)-loaded vesicles were filtered under vacuum with a Hoefer manifold filtration box from Amersham Pharmacia Biotech.

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Sample Preparation—Right side-oriented (intact) vesicles were obtained from the SR membrane of fast twitch rabbit leg muscle as described by Eletr and Inesi (19). Purified Ca$^{2+}$-ATPase was prepared by deoxycholate treatment according to method 2 of Meissner et al. (16). Final pellets were resuspended and stored in frozen aliquots at −80 °C until use. Ca$^{2+}$-dependent activity of Ca$^{2+}$-were obtained by including A23187 in the reaction medium.

Protein Quantitation—The protein concentration was measured by the Lowry et al. (20) procedure using bovine serum albumin as standard.

Ca$^{2+}$ Concentration—Free Ca$^{2+}$ in the external medium was calculated as described by Fabiato (21). The absolute stability constant (22), the pK values for the EGTA protonation (23), pH, and the presence of relevant ligands. Free Ca$^{2+}$ in the lumenal medium was fixed by equilibrating the vesicles in the standard reaction medium but including potassium oxalate in the range of 0.5–5 mM.

Vaduate Solution and Enzyme Inhibition—Stock solutions containing mostly monovanadate were prepared by dissolving ammonium metavanadate in ultrapure water (Milli-Q grade) adjusted at pH 10.0, using mostly monovanadate were prepared by dissolving ammonium metavanadate in ultrapure water (Milli-Q grade) adjusted at pH 10.0, and 50 mM oxalate ensured a low free Ca$^{2+}$ in the lumen. The dependence of the hydrolysis rate on the pNPP concentration measured at pH 7.0 and in the Ca$^{2+}$-containing medium displayed a hyperbolic profile, as shown in Fig. 1A. A reaction medium at pH 6.0 or 8.0 did not significantly modify the hydrolysis rate measured at each pNPP concentration.

The dependence of the Ca$^{2+}$ transport rate on pNPP concentration was also measured. The rate of active Ca$^{2+}$ accumulation was highly dependent on the reaction medium pH (Fig. 1B). A hyperbolic dependence was observed at pH 6.0 or 7.0, even though higher rates were obtained at neutral pH. Negligible rates were observed after a fast and small component of Ca$^{2+}$ transport when the experiments were carried out at pH 8.0.

An analysis of the experimental curves (Table I) revealed that $K_a$ for pNPP hydrolysis in the presence of Ca$^{2+}$ and that for Ca$^{2+}$ transport were in the 2–3 mM range when measured from pH 6.0 to 8.0. Moreover, $V_{\text{max}}$ for pNPP hydrolysis in the presence of Ca$^{2+}$ was around 55 nmol/min/mg of protein, with the pH having only a slight effect whereas $V_{\text{max}}$ for Ca$^{2+}$ transport was clearly affected by pH. The Ca$^{2+}$/IP$_c$ coupling ratio near neutral pH was around 0.6. The coupling ratio decreased to 0.4 when measured at pH 6.0, and complete uncoupling was observed at pH 8.0.

Free Ca$^{2+}$ in the lumen affects to the enzyme coupling ratio when ATP is the substrate (28, 29); therefore, this parameter was also checked. We selected a reaction medium at pH 7.0 containing 20 mM Mg$^{2+}$, 50 μM free Ca$^{2+}$, and 10 mM pNPP that was equilibrated with different concentrations of oxalate. The hydrolysis rate in the presence of Ca$^{2+}$ displayed a small increase from 42 to 50 nmol/min/mg of protein when the oxalate concentration was raised from 0 to 5 mM (Fig. 2A). The rate of Ca$^{2+}$ transport increased as oxalate concentration was raised in the millimolar range, showing a sigmoidal dependence (Fig. 2B). The rate was negligible in the absence of precipitating anion, whereas maximal values of 31 nmol of Ca$^{2+}$/min/mg of protein were obtained in the presence of 5 mM oxalate.

The catalytic properties of the enzyme are sensitive to the presence of Me$_2$SO (30, 31); therefore, we evaluated the organic solvent effect in the standard reaction medium at pH 7.0 containing 50% Me$_2$SO, 10 mM pNPP, and native SR vesicles. The pNPP hydrolysis rate in the presence of Ca$^{2+}$ increased when the organic solvent concentration was raised. Maximal activity was observed at 20% Me$_2$SO, whereas a further increase in the solvent concentration up to 40% induced a progressive decrease in the hydrolysis rate (Fig. 3A). In contrast, the hydrolysis rate measured in the absence of Ca$^{2+}$ increased...
progressively as the Me$_2$SO concentration was raised. Furthermore, maximal Ca$^{2+}$ transport rates were observed in the presence of 10% Me$_2$SO and higher solvent concentrations decreased the transport activity, tending to the zero value (Fig. 4). The dependence of pNPP hydrolysis in the presence of Ca$^{2+}$ and that of Ca$^{2+}$ transport on the Me$_2$SO concentration did not show the same profile. Possible effects of Me$_2$SO on SR vesicle permeability (i.e. in Ca$^{2+}$-transport measurements) were ruled out experimentally using $^{45}$Ca$^{2+}$-loaded vesicles. An active loading was started by adding 10 mM pNPP to the standard reaction medium and stopped after 6 min by adding 5 mM TG. The $^{45}$Ca$^{2+}$-load was ~170 nmol/mg of protein, and a subsequent addition of 40% Me$_2$SO had no effect on the Ca$^{2+}$ content retained by the vesicles (data not shown).

**Effect of Vanadate on Enzyme Activity**—Vanadate is known to interact with the enzyme conformation in the absence of Ca$^{2+}$ by acting as a P$_i$ analog (32, 33). When the reaction medium was at pH 7.0 and contained 20 mM Mg$^{2+}$, 1 mM EGTA (i.e. absence of Ca$^{2+}$), 10 mM pNPP, and 5 mM oxalate, the hydrolysis of pNPP by native SR vesicles was completely inhibited by ~20 μM vanadate (Fig. 4). The inhibition profile in the absence of Ca$^{2+}$ was exactly the same when a preparation of purified Ca$^{2+}$-ATPase was used. Nevertheless, the pNPP hydrolysis rate in a medium containing 50 μM free Ca$^{2+}$ and leaky vesicles required ~100 μM vanadate to induce complete inhibition. A partial inhibition of 45% was observed in the presence of 100 μM vanadate when A23187 was removed (i.e. when intact vesicles were used) and oxalate was included. Similar experiments using SR vesicles were performed at pH 6.0 (Fig. 5). In the absence of Ca$^{2+}$, complete inhibition of the pNPP hydrolysis was now observed in the presence of ~50 μM vanadate. Interestingly, the hydrolysis rate in a Ca$^{2+}$-containing medium and A23187 (leaky vesicles) displayed the same dependence on vanadate concentration. However, when the enzyme activity was measured in the presence of extravesicular Ca$^{2+}$ and oxalate (intact vesicles), the sensitivity to vanadate decreased significantly compared to leaky vesicles. Hydrolysis rates were measured by following the accumulation of the product, p-nitrophenol. The initial reaction medium consisting of: 20 mM Mes, pH 6.0, 80 mM KCl, 20 mM MgCl$_2$, 0.2 mM EGTA, 0.2 mg/ml SR protein, and 5 mM K$^+$-oxalate (●); 20 mM Mops, pH 7.0, 80 mM KCl, 20 mM MgCl$_2$, 0.2 mM EGTA, 0.247 mM CaCl$_2$, 0.2 mg/ml SR protein, and 5 mM K$^+$-oxalate (▼); or 20 mM Tris-HCl, pH 8.0, 80 mM KCl, 20 mM MgCl$_2$, 0.2 mM EGTA, 0.25 mM CaCl$_2$, 0.2 mg/ml SR protein, and 5 mM K$^+$-oxalate (▲). B, Ca$^{2+}$ transport associated with pNPP hydrolysis was measured in parallel experiments by including ~7,000 cpm/nmol $^{45}$Ca$^{2+}$ in the corresponding reaction medium. The reaction was started by the addition of pNPP and stopped at different times by filtering 0.5-ml aliquots of reaction mixture. Filters were processed as described under “Experimental Procedures.” Rates of transport at pH 6.0 (●), pH 7.0 (▼), or pH 8.0 (▲) are shown.

**Table I**

| pH   | K$_{m}$ | V$_{max}$ | K$_{m}$ | V$_{max}$ | Ca$^{2+}$/Pi coupling |
|------|---------|-----------|---------|-----------|----------------------|
| 6    | 1.8     | 52.3      | 1.0     | 20.8      | 0.4                  |
| 7    | 1.9     | 56.5      | 1.8     | 32.5      | 0.6                  |
| 8    | 3.1     | 51.2      | NM$^{a}$| NM$^{a}$  | 0.0                  |

$^{a}$ NM, not measurable (negligible transport).
The initial concentration of oxalate added (34). Thus, the stand-
mM pNPP. Me2SO was also included when indicated (by adding 10 m
presence of Ca2+
medium and A23187 (leaky vesicles) was inhibited by 45% in the
increased by 40% Me2SO and the reaction was started
the absence of Ca2+
M vanadate induced a 35% inhibition of the pNPP hydrolysis rate.
The inhibitor of the enzyme activity in the presence of Ca2+
were measured at 25 °C in the
Inhibition of Purified Enzyme by TG—A key question is to
be attributed to the Ca2+-ATPase protein. Fig. 8 (panels A–C) show data obtained
the enzyme activity in the presence of Ca2+
In any case, the addition of TG to the enzyme in the presence of
Ca2+-ATPase during the hy-
the absence of Ca2+
the effect of TG on these activities were
Fig. 6 shows the effect of lumenal Ca2+
the lumen, there was a
Ca2+-ATPase protein. Fig. 8 (panel
The activation by Ca2+
the lumen, displayed low sensitivity to vanadate.
free Ca2+
Ca2+-ATPase is able to hydrolyze pNPP in the
the effect of TG calculated as
Moreover, TG had no effect on the pNPP hydrolysis rate increased from
was 11.0 nmol/min/mg protein. This value
developed a hyperbolic dependence with respect to vanadate reaching an asymptotic level at ~50 μM
the other case, the reaction medium contained 50 μM free Ca2+, 5 mM oxalate and was also supplemented with
40% Me2SO. The inhibition of the enzyme activity in the presence of
The Me2SO effect on the enzyme activity was also analyzed
by studying vanadate sensitivity (Fig. 7). In one case, the reaction medium at pH 7.0 and in the absence of Ca2+
the presence of Ca2+-ATPase in the absence of Ca2+
ad P (B); 20 mM Mops, pH 7.0, 80 mM KCl, 20 mM MgCl2, 1 mM EGTA, 0.2 mg/ml purified Ca2+-ATPase, and 10 mM pNPP (○); 20 mM Mops, pH 7.0, 80 mM KCl, 20 mM MgCl2, 1 mM EGTA, 0.2 mg/ml purified Ca2+-ATPase, and 10 mM pNPP (○); 20 mM Mops, pH 7.0, 80 mM KCl, 20 mM MgCl2, 1 mM EGTA, 0.247 mM CaCl2, 0.2 mg/ml SR vesicles, 5 mM K+-oxalate, and 10 mM pNPP (■); or 20 mM Mops, pH 7.0, 80 mM KCl, 20 mM MgCl2, 0.2 mM EGTA, 0.247 mM CaCl2, 0.2 mg/ml SR vesicles, 15 μM A23187, and 10 mM pNPP (□). The vanadate effect was studied by including different concentrations in the reaction media.

FIG. 3. Effect of Me2SO when SR vesicles are hydrolyzing pNPP in the presence or absence of Ca2+. A, linear rates of hydrolysis in the presence of Ca2+ were measured at 25 °C in a medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 20 mM mgCl2, 0.2 mM EGTA, 0.247 mM CaCl2, 0.2 mg/ml SR protein, 5 mM K+-oxalate, and 10 mM pNPP. Me2SO was also included when indicated (▲). Enzymatic activity data in the absence of Ca2+ were obtained by omitting the addition of CaCl2 and raising the EGTA concentration up to 1 mM. Me2SO was included when indicated (▲). B, linear rates of Ca2+ transport were measured in the reaction medium described for pNPP hydrolysis in the presence of Ca2+, but supplemented with 45% Ca2+-oxalate (□).

date was lower. A vanadate concentration of 100 μM induced a
60% inhibition of the pNPP hydrolysis rate.
Fig. 5B shows the sensitivity to vanadate when measured at
pH 8.0. The enzymatic activity of SR vesicles in the absence of
Ca2+ was completely inhibited by 10 μM vanadate. Likewise,
the pNPP hydrolysis rate measured in a Ca2+-containing me-
dium and A23187 (leaky vesicles) was inhibited by 45% in the
presence of 100 μM vanadate. The percentage of inhibition
induced by 100 μM vanadate was only 32% when the Ca2+
ionophore was substituted for oxalate (intact vesicles).
Fig. 6 shows the effect of lumenal Ca2+ on vanadate sensi-
tivity. The luminal free Ca2+ was manipulated by modifying
the initial concentration of oxalate added (34). Thus, the stan-
ard reaction medium at pH 7.0 containing 20 mM Mg2+ and 50
μM free Ca2+ in the external medium was equilibrated with
either 0.5 or 5 mM oxalate before the addition of 10 mM pNPP.
Intact vesicles and an oxalate concentration of 5 mM, i.e. a free
Ca2+ in the lumen, displayed low sensitivity to vanadate.
As a reference, 100 μM vanadate produced a 45% inhibition of
the enzyme activity. When the oxalate concentration was cut to
0.5 mM to increase the free Ca2+ in the lumen, there was a
further decrease in vanadate sensitivity. In this case, 100 μM
vanadate induced a 35% inhibition of the pNPP hydrolysis rate.
The Me2SO effect on the enzyme activity was also analyzed
by studying vanadate sensitivity (Fig. 7). In one case, the reaction
medium at pH 7.0 and in the absence of Ca2+ was
supplemented with 40% Me2SO and the reaction was started
by adding 10 mM pNPP. The inhibition of pNPP hydrolysis in

DISCUSSION
The coupling efficiency of SR Ca2+-ATPase during the hy-
drosis of pNPP is clearly dependent on pH (Fig. 1 and Table

FIG. 4. The effect of vanadate on pNPP hydrolysis when measured at neutral pH. The enzyme activity was measured at 25 °C in the
following reaction media: 20 mM Mops, pH 7.0, 80 mM KCl, 20 mM MgCl2, 1 mM EGTA, 0.2 mg/ml SR vesicles, and 10 mM pNPP ( ● ); 20 mM Mops, pH 7.0, 80 mM KCl, 20 mM MgCl2, 1 mM EGTA, 0.2 mg/ml purified Ca2+-ATPase, and 10 mM pNPP ( ○ ); 20 mM Mops, pH 7.0, 80 mM KCl, 20 mM MgCl2, 0.2 mM EGTA, 0.247 mM CaCl2, 0.2 mg/ml SR vesicles, 5 mM K+-oxalate, and 10 mM pNPP (■); or 20 mM Mops, pH 7.0, 80 mM KCl, 20 mM MgCl2, 0.2 mM EGTA, 0.247 mM CaCl2, 0.2 mg/ml SR vesicles, 15 μM A23187, and 10 mM pNPP (□). The vanadate effect was studied by including different concentrations in the reaction media.

The coupling efficiency of SR Ca2+-ATPase during the hy-
drosis of pNPP is clearly dependent on pH (Fig. 1 and Table
It is known that Ca$^{2+}$ binding at equilibrium is a pH-dependent process involving different enzyme conformations and a sequential mechanism (35), as follows.

$$E_1H_4^+ \leftrightarrow E_1H^- \leftrightarrow E_1Ca \leftrightarrow E_1Ca_2$$  

**REACTION 1**

Therefore, acidic pH stabilizes the enzyme in the absence of Ca$^{2+}$ (protonated $E_2$ conformation) and alkaline pH favors Ca$^{2+}$ binding ($E_1Ca_2$ conformation). The activating effect of Ca$^{2+}$ on the pNPP hydrolysis rate in the purified enzyme is also pH-dependent and increases when the H$^+$ concentration decreases (Fig. 8, A–C). This indicates that, under turnover conditions and acidic pH, the protonated $E_2$ conformations will be more abundant, whereas the $E_1$ species will predominate at alkaline pH. Qualitatively similar results were obtained when a preparation of intact vesicles and an oxalate-containing medium were used (data not shown).

The highest sensitivity to vanadate was observed during pNPP hydrolysis in the absence of Ca$^{2+}$ (Figs. 4 and 5). Higher vanadate concentrations were necessary to produce complete inhibition when the H$^+$ concentration was raised. This is to be expected since the target species for vanadate, the protonated $E_2$ forms, will be more abundant at acidic than at alkaline pH. Data obtained with a preparation of purified enzyme (Fig. 4) indicated that pNPP hydrolysis in the absence of Ca$^{2+}$ is associated with the catalytic activity of the Ca$^{2+}$-ATPase protein and not with any other protein.

The effect of vanadate in the presence of Ca$^{2+}$ was also pH-dependent (Figs. 4 and 5). The hydrolysis of pNPP in leaky vesicles and in the presence of Ca$^{2+}$ at pH 6.0 was highly sensitive to vanadate. The inhibition profile was the same for leaky vesicles in a Ca$^{2+}$-containing medium as for intact vesicles in a Ca$^{2+}$-free medium (Fig. 5A). High sensitivity to vanadate is indicative of the predominant accumulation of $E_2$ forms, especially when pNPP is the substrate. The non-nucleotide substrate does not prevent vanadate binding and inhibition, as occurs with mM ATP (33, 36). Moreover, the degree of activation by Ca$^{2+}$ and inhibition by TG in a preparation of purified enzyme was low (Fig. 5A). High sensitivity to vanadate is indicative of the predominant accumulation of $E_1$ forms, whereas low TG inhibition is indicative of a low accumulation of $E_1$ or what is the same, the predominant presence of $E_2$ forms.

Enzyme activity in leaky vesicles and in the presence of Ca$^{2+}$ at pH 8.0 was clearly resistant to vanadate, the inhibition profile being very similar to that displayed by intact vesicles in the presence of Ca$^{2+}$ and oxalate (Fig. 5B). The Ca$^{2+}$-activation and TG inhibition factors at pH 8.0 were high (Fig. 8A). TG selectively inhibits the Ca$^{2+}$-dependent activity (17), and so high inhibition by TG indicates the predominant accumulation of $E_2$ forms, whereas low TG inhibition is indicative of a low accumulation of $E_1$ or what is the same, the predominant presence of $E_2$ forms.

Luminal free Ca$^{2+}$ has profound effects on coupling when the phosphorylating substrate is UTP (39). These data suggest that $E_1$ forms will be the predominant species.
Observations can be explained if Me₂SO favors the reaction cycle. The enzyme was insensitive to TG inhibition (Fig. 8). Sensitivity to vanadate of SR vesicles hydrolyzing pNPP in the presence of 40% Me₂SO. The hydrolysis of pNPP was measured at 25°C in a 50 mM free Ca²⁺-containing medium, i.e., 20 mM Mops, pH 7.0, 80 mM KCl, 20 mM MgCl₂, 0.2 mM EGTA, 0.247 mM CaCl₂, 0.2 mg/ml SR vesicles, 5 mM K⁺-oxalate, 40% (v/v) Me₂SO, and 10 mM pNPP (●), or in a Ca²⁺-free medium, i.e., 20 mM Mops, pH 7.0, 80 mM KCl, 20 mM MgCl₂, 1 mM EGTA, 0.2 mg/ml SR vesicles, 40% (v/v) Me₂SO, and 10 mM pNPP (▲). Vanadate was included in the reaction medium when indicated.

Ca²⁺⁻ATPase Reaction Cycle and p-Nitrophenyl Phosphate

**Figure 7.** Sensitivity to vanadate of SR vesicles hydrolyzing pNPP in the presence of 40% Me₂SO. The hydrolysis of pNPP was measured at 25°C in a 50 mM free Ca²⁺-containing medium, i.e., 20 mM Mops, pH 7.0, 80 mM KCl, 20 mM MgCl₂, 0.2 mM EGTA, 0.247 mM CaCl₂, 0.2 mg/ml SR vesicles, 5 mM K⁺-oxalate, 40% (v/v) Me₂SO, and 10 mM pNPP (●), or in a Ca²⁺-free medium, i.e., 20 mM Mops, pH 7.0, 80 mM KCl, 20 mM MgCl₂, 1 mM EGTA, 0.2 mg/ml SR vesicles, 40% (v/v) Me₂SO, and 10 mM pNPP (▲). Vanadate was included in the reaction medium when indicated.

**Scheme 1.** Catalytic routes of SR Ca²⁺-ATPase in the presence of pNPP and Ca²⁺. The addition of pNPP to the enzyme in the presence of Ca²⁺ (E₁Ca₂) leads to the formation of the phosphorylated intermediate E₁PCa₂. When Ca²⁺ dissociation inside the vesicles takes place before Pₐ release into the external medium, there is an E₁⁻E₂ cycle (solid line). This route yields vectorial translocation of Ca²⁺ from the cytoplasmic to the luminal compartment. When Pₐ release occurs first, Ca²⁺ dissociates to the external medium and there is an E₂ cycle. E₂ generated in a coupled cycle can interact with pNPP producing a phosphorylated intermediate without bound Ca²⁺ (E₂P) and giving rise to the E₁P cycle. Furtile cycles are marked with a dashed line. E₁Ca₂ and E₁PCa₂ are unphosphorylated and phosphorylated conformations, respectively, of the enzyme with high affinity Ca²⁺⁻bound, E₂ and E₂P are unphosphorylated and phosphorylated conformations, respectively, with low Ca²⁺⁻affinity.

The activation by Ca²⁺ and inhibition by TG in a preparation of purified enzyme had different absolute values, but, for each experimental condition tested, the activation and inhibition factors had the same value (Fig. 8). We know that TG produces stabilization of the E₂ conformation (18). Thus, TG inhibits the Ca²⁺⁻dependent activity and allows the hydrolysis of pNPP in the presence of Ca²⁺ through E₂ forms. This points to the existence of a relationship between the Ca²⁺⁻dependent and Ca²⁺⁻independent activities measured in SR vesicles and suggests that both hydrolytic activities are related to the Ca²⁺⁻ATPase protein.

It has been reported that SR Ca²⁺⁻ATPase is phosphorylated by and/or hydrolyzes ATP (40), UTP (39), or even the non-nucleotide substrate furylacryloylphosphate (41) in a Ca²⁺⁻free medium. In the case of pNPP, the rate of phosphorylation, which is much lower than that by ATP (25), does not allow the accumulation of phosphorylated intermediate during the enzyme turnover. The rates of Ca²⁺⁻binding in the presence of pNPP and phosphorylation by TG in the absence of Ca²⁺ must be of similar magnitude in contrast to those observed when ATP is the substrate. This explains why pNPP hydrolysis in a Ca²⁺⁻containing medium can partly occur through E₂ forms.

In conclusion, the low efficiency of Ca²⁺⁻transport sustained by pNPP may be attributed to the coexistence of one coupled (E₁⁻E₂ cycle) and two uncoupled routes (E₁ cycle and E₂ cycle),
as depicted in Scheme I. This means that: (i) alkaline pH favors the operation of the $E_1$ cycle, whereas acidic pH potentiates the $E_2$ cycle; (ii) uncoupling through the $E_1$ cycle is favored by the integrity of the vesicles and occurs even when the free Ca$^{2+}$ in the lumen is low; and (iii) pNPP can be hydrolyzed through the $E_2$ cycle even in a Ca$^{2+}$-containing medium. This study confirms that the catalytic cycle of P-type ATPases does not follow a rigid sequence of reactions and is consistent with the existence of one sole energy transduction mechanism.

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