Lanthanum Inhibits Steady-state Turnover of the Sarcoplasmic Reticulum Calcium ATPase by Replacing Magnesium as the Catalytic Ion*

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LaATP is shown to be an effective inhibitor of the calcium ATPase of sarcoplasmic reticulum because the binding of LaATP to E-Ca$_2$ results in the formation of lanthanum phosphoenzyme, which decays slowly. Steady-state activity of the calcium ATPase in leaky sarcoplasmic reticulum vesicles is inhibited 50% by 0.16 μM LaC13 (15 nM free La$^{3+}$, 21 nM LaATP) in the presence of 25 μM Ca$^{2+}$ and 49 μM MgATP (5 mM MgSO$_4$, 100 mM KCl, 40 mM 4-morpholinepropanesulfonic acid, pH 7.0, 25 °C). However, 50% inhibition of the uptake of $^{45}$Ca and phosphorylation by [γ-32P]ATP in a single turnover experiment requires 100 μM LaCl$_3$ (28 μM free La$^{3+}$) in the presence of 25 μM Ca$^{2+}$; this inhibition is reversed by calcium but inhibition of steady-state turnover is not. Therefore, binding of La$^{3+}$ to the cytoplasmic calcium transport site is not responsible for the inhibition of steady-state ATPase activity.

The addition of 6.7 μM LaCl$_3$ (1.1 μM free La$^{3+}$) has no effect on the rate of dephosphorylation of phosphoenzyme formed from MgATP and enzyme in leaky vesicles, while 6.7 mM CaCl$_2$ slows the rate of phosphoenzyme hydrolysis as expected; 6.7 μM LaCl$_3$ and 6.7 mM CaCl$_2$ cause 95 and 98% inhibition of steady-state ATPase activity, respectively. This shows that inhibition of ATPase activity in the steady state is not caused by binding of La$^{3+}$ to the intravesicular calcium transport site of the phosphoenzyme. Inhibition of ATPase activity by 2 μM LaCl$_3$ (0.16 μM free La$^{3+}$, 0.31 μM LaATP) requires >5 s, which corresponds to ~50 turnovers, to reach a steady-state level of ≥80% inhibition. Inhibition by La$^{3+}$ is fully reversed by the addition of 0.55 mM CaCl$_2$ and 0.50 mM EGTA; this reactivation is slow with $t_1/2$ ~ 9 s. Two forms of phosphoenzyme are present in reactions that are partially inhibited by La$^{3+}$: phosphoenzyme with Mg$^{2+}$ at the catalytic site and phosphoenzyme with La$^{3+}$ at the catalytic site, which undergo hydrolysis with observed rate constants of >4 and 0.05 s$^{-1}$, respectively. We conclude, therefore, that La$^{3+}$ inhibits steady-state ATPase activity under these conditions by replacing Mg$^{2+}$ as the catalytic ion for phosphoryl transfer. The slow development of inhibition corresponds to the accumulation of lanthanum phosphoenzyme. Initially, most of the enzyme catalyzes MgATP hydrolysis, but the fraction of enzyme with La$^{3+}$ bound to the catalytic site gradually increases because lanthanum phosphoenzyme undergoes hydrolysis much more slowly than does magnesium phosphoenzyme. The slow rate of reactivation by Ca$^{2+}$ and EGTA corresponds to the slow decay of lanthanum phosphoenzyme. Co$^{2+}$ inhibits steady-state turnover by binding to the ionophore A23187, which blocks calcium efflux from the vesicles. Lanthanum is the first element of the lanthanide series; the elements of this series show only small changes in chemical properties throughout the series. Ions in the lanthanide series have been used extensively as probes of calcium-binding sites in proteins because of the similar ionic radii and ligand specificities of the lanthanides and calcium (for reviews see Martin and Richardson, 1979; Evans, 1983; Horrocks and Albin, 1984). However, the binding of lanthanides is not restricted to binding sites for calcium; replacement of magnesium and other cations by lanthanides has been reported for several proteins (Martin and Richardson, 1979; Morrison and Cleland, 1983; Giradet et al., 1989). Lanthanides have been shown to bind to the SRV calcium ATPase at the cytoplasmic calcium transport site and at a site that has an association constant of 35–130 M$^{-1}$ for magnesium (Chevallier and Butow, 1971; Highsmith and Head, 1983; Highsmith, 1984; Giradet et al., 1989). Lanthanides are known to inhibit turnover of the SRV calcium ATPase; this inhibition is generally believed to occur by binding of lanthanides to the cytoplasmic site for calcium transport (Krasnow 1977; Stephens and Grisham, 1979; Highsmith and Head, 1983; Highsmith and Murphy, 1984; Scott 1984).

There are at least four different mechanisms by which divalent and trivalent cations inhibit the calcium ATPase: (a) metal ions (Mg$^{2+}$, Mn$^{2+}$) can compete with calcium for the cytoplasmic transport site (Yamada and Tomonoura, 1972; Kalbitzer et al., 1978), (b) metal ions (Ca$^{2+}$) can replace Mg$^{2+}$ as the catalytic ion at the phosphoryl transfer site (Yamada and Tomonoura, 1972; Shigekawa et al., 1983; Oriolusi et al., 1988), (c) metal ions (Ca$^{2+}$, Mg$^{2+}$) can inhibit steady-state turnover by binding to the luminal transport site of the phosphorylated enzyme (Weber, 1966; Ikemoto, 1975; Bishop and Al-Shawi, 1988), and (d) we have found that metal ions (Co$^{2+}$) can bind to ionophore and inhibit the efflux of Ca$^{2+}$ from intact vesicles that is required in order to maintain steady-state turnover. There are at least four different mechanisms by which divalent and trivalent cations inhibit the calcium ATPase: (a) metal ions (Mg$^{2+}$, Mn$^{2+}$) can compete with calcium for the cytoplasmic transport site (Yamada and Tomonoura, 1972; Kalbitzer et al., 1978), (b) metal ions (Ca$^{2+}$) can replace Mg$^{2+}$ as the catalytic ion at the phosphoryl transfer site (Yamada and Tomonoura, 1972; Shigekawa et al., 1983; Oriolusi et al., 1988), (c) metal ions (Ca$^{2+}$, Mg$^{2+}$) can inhibit steady-state turnover by binding to the luminal transport site of the phosphorylated enzyme (Weber, 1966; Ikemoto, 1975; Bishop and Al-Shawi, 1988), and (d) we have found that metal ions (Co$^{2+}$) can bind to ionophore and inhibit the efflux of Ca$^{2+}$ from intact vesicles that is required in order to maintain steady-state turnover.
Inhibition by Lanthanum of the Calcium ATPase

EXPERIMENTAL PROCEDURES

Materials—NaATP (Sonderqualität), NADH, phosphoenoxyribose, pyruvate kinase, and lactate dehydrogenase were purchased from Boehringer Mannheim. MOPS and A21387 were purchased from Calbiochem. KCl and EGTA were purchased from Fluka. LaCl3, THO and CoSO4 were purchased from Aldrich. [γ-32P]ATP (>99% pure) and 44CaCl2 were from Du Pont-New England Nuclear. Chelex 100 was purchased from Bio-Rad and Chelating Sepharose was purchased from Pharmacia. Tris (Ultrapure) was purchased from Schwarz/Mann Biotech. All solutions were made with water treated and stored in polypropylene containers (Nalgene).

Rabbit white back and hind leg skeletal muscle by a slight modification of the method of de Meis and Carvalho (1974). SRV (50 ul of 80 mg/ml) were mixed with 200 ul of 10 mM KCl and 50 mM Tris, pH 9.5, and 200 ul of either 2 mM EGTA, 200 mg of Chelating Sepharose (K+ form), or 200 mg of Chelex 100 (K+ form) and incubated at room temperature for 2 h. Vesicles were then pelleted by addition of ionophore A21387 to the standard ATPase assay failed to give an increase in the rate of steady-state hydrolysis. The leaky vesicles were sedimented through glass wool, which trapped the chelating resins, and the solution was adjusted to pH 7.5 by the addition of 10 μl of 1 M MOPS, pH 6.0.

Methods—Reactions were carried out at 25 °C with 5 mM MgSO4, 100 mM KCl, and 40 mM MOPS at pH 7.0, unless otherwise noted. This standard assay mixture was assayed for Ca2+ contamination by measuring the uptake of 44Ca, Ca2+ by adding 0.1 μCi 44Ca to the assay mixture and for added Ca2+ by measuring the uptake of 44Ca by SRV, which were then incubated in the presence of either 10 μl 44CaCl2 or 100 μl 44CaCl2; the uptake of 44Ca was measured in several experiments established the presence of 1-10 μM Ca2+ contamination. All reported concentrations of Ca2+ were free Ca2+ concentrations, and Ca2+ was added to the assay mixture. Steady-state ATPase activity was assayed spectrophotometrically by coupling ADP production to NADH oxidation with pyruvate kinase and lactate dehydrogenase (Rossi et al., 1979). The pyruvate kinase and lactate dehydrogenase activities were those specified by Boehringer Mannheim. For some experiments, the pyruvate kinase and lactate dehydrogenase activities were reduced to 1/5 of the value specified by Boehringer and Mannheim. 1500 × g for 5 min and 3 ml of the original phase was added to 7 ml of Aquasol-2 (Du Pont-New England Nuclear) for scintillation counting.

Phosphoenzyme levels were measured essentially as described by Verjovski-Almeida et al. (1976) with slight modifications (Hanel and Jencks, 1990). Reaction mixtures were quenched with acid to give a final concentration of 0.5 M HCl and 15 mM K2HPO4; all subsequent manipulations and solutions were at 0-4 °C. H3[35S]orthophosphate and ATP were added to the acid-quenched reaction mixtures to give final concentrations of ~0.3 μCi/ml of total protein and 1 mM ATP, followed by the addition of 100% trichloroacetic acid (w/v) to give a final concentration of ~12% trichloroacetic acid. After 2 ±2 h at 0 °C, the samples were centrifuged at 1500 × g for 15 min and the pellets were resuspended in 5% trichloroacetic acid and 10 mM K2HPO4. The protein was collected on Whatman GF/C glass fiber filters by vacuum filtration and rinsed with 15 ml of the resuspension solution. The radioactivity was measured by liquid scintillation counting in glass vials containing 7 ml of Aquasol-2.

Experiments to measure the uptake of 44Ca were carried out essentially as described previously (Petithory and Jencks, 1988). The dissociation constant of lanthanide ions, in agreement with previous work (Yamada and Murphy, 1984; Gangola and Shamo, 1987; Highsmith and Head, 1983; Scott, 1984). Fig. 1 shows that the inhibition of steady-state turnover by La3+ is linearly dependent on the concentration of SRV, which indicates that La3+ binds to the SRV nonspecifically. The slope of this plot is 10 nmoles of La3+/mg of SRV, which represents the stoichiometry for this binding of La3+ to SRV, and the y intercept is 0.16 μM La3+ (15 nM free La3+), 21 mM LaATP), which represents Kf for La3+ under these conditions extrapolated to zero SRV concentration. Similar stoichiometries for the binding of Tb3+ and Nd3+ to SRV have been reported previously (Highsmith and Head, 1983; Hanel and Jencks, 1990). The binding sites were saturated with La3+ at concentrations that cause only partial inhibition of steady-state turnover; therefore, the binding of La3+ to these sites does not cause inhibition.

Fig. 2 shows that inhibition of steady-state ATP hydrolysis by 8 μM TbCl3 decreases with time; the rate changes from 94
Inhibition by Lanthanum of the Calcium ATPase

**Fig. 1.** The effect of SRV concentration on $K_i$ for inhibition of ATP hydrolysis in the steady-state by LaCl$_3$. Reaction conditions were 25 mM Ca$^{2+}$, 5 mM MgSO$_4$, 100 mM KCl, 50 mM ATP, 1 mM PEP, 0.3 mM NADH, 50 mM pyruvate kinase, 50 mM lactate dehydrogenase, and 40 mM MOPS, pH 7.0, at 25 °C. The values of $K_i$ were calculated from the total concentration of added La$^{3+}$ using the rates observed after the first 20 s of the reaction. The line drawn has a slope of 10 mmol of La$^{3+}$/mg of SRV and a y intercept of 0.16 mM La$^{3+}$.

**Fig. 2.** Inhibition of ATP hydrolysis by 8 mM TbCl$_3$ decreases with time. Reaction conditions were 0.05 mg/ml SRV (made leaky by treatment with Chelex 100), 8 mM TbCl$_3$, 25 mM Ca$^{2+}$, 5 mM MgSO$_4$, 100 mM KCl, 50 mM ATP, 1 mM PEP, 0.3 mM NADH, 50 mM pyruvate kinase, 50 mM lactate dehydrogenase, and 40 mM MOPS, pH 7.0, at 25 °C. The rate of ATP hydrolysis from 0 to 20% inhibition with an approximate $t_0$ of 12 min. This may be caused by a decrease in the concentration of Tb$^{3+}$ as a result of complexation of Tb$^{3+}$ by inorganic phosphate, a product of the reaction. Increases in the rate of ATP hydrolysis with time were also observed with reactions inhibited by La$^{3+}$, Nd$^{3+}$, Sm$^{3+}$, or Y$^{3+}$ (data not shown). The concentration of Pi produced, 0.2 μM, and phosphate, <0.3 mM, result in slow precipitation over ≥5 min; therefore, linear rates of steady-state ATPase activity in the presence of lanthanides were measured at 20-60 s, before a significant amount of lanthanide had precipitated.

Krasnow (1979) has reported previously that inhibition by La$^{3+}$ and Gd$^{3+}$ of ATP hydrolysis and Ca$^{2+}$ uptake with intact SRV decreases with time. This result was interpreted as an indication that La$^{3+}$ and Gd$^{3+}$ might be transported into the vesicles, in a manner analogous to that for Ca$^{2+}$. The finding that leaky (Fig. 2) and intact SRV both show an increase in ATP hydrolysis with time in the presence of lanthanide ions indicates that active transport of these ions is not likely to account for this time-dependent decrease in inhibition.

**Fig. 3.** The effect of calcium on steady-state ATP hydrolysis in the presence and absence of 0.5 mM LaCl$_3$. Reaction conditions were 5 mM MgCl$_2$, 100 mM KCl, 50 mM ATP, 1 mM PEP, 0.3 mM NADH, 25 μg/ml pyruvate kinase, 25 μg/ml lactate dehydrogenase, 2 μM A23187, 25 μg/ml SRV, and 40 mM MOPS, pH 7.0, with 0.5 mM LaCl$_3$ (●) or 0 mM LaCl$_3$ (○) at 25 °C.
The effect of LaCl₃ on steady-state ATPase activity ([C]) and on ⁴⁰Ca uptake ([I], [D]) and phosphoenzyme formation ([Δ]) in a single turnover. All reaction mixtures contained 5 mM MgSO₄, 100 mM KCl, and 40 mM MOPS, pH 7.0, at 25 °C. Reaction conditions for steady-state ATPase ([Δ]) were 30 μg/ml SRV (made leaky by treatment with Chelating Sepharose at pH 9.5), 25 μM Ca²⁺, 50 μM ATP, 10 mM PEP, 0.3 mM NADH, 50 μg/ml pyruvate kinase, and 50 μg/ml lactate dehydrogenase. 100% activity corresponds to 2.1 μmol/min/mg. Mg²⁺ is required to cause a 50% decrease in the steady-state level of phosphoenzyme by La³⁺ is much greater than K₅0ₐ = 0.1 mM for inhibition by La³⁺ of the binding of 25 μM Ca²⁺ to the cytoplasmic transport site (Fig. 4). This is consistent with the much faster rate of Ca²⁺ binding compared with the rate of phosphoenzyme decomposition, as indicated by the high levels of phosphoenzyme observed at low concentrations of La³⁺. Therefore, the binding of Ca²⁺ must be inhibited by >50% to be slower than the decomposition of phosphoenzyme. Domonkos et al. (1989) also reported that La³⁺ added after phosphoenzyme formation from ATP results in inhibition of phosphoenzyme decomposition. The proposed mechanism for this inhibition was the binding of La³⁺ to a low affinity site, which is competitive with respect to Mg²⁺.

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The dashed line in Fig. 5 shows the absence of phosphoenzyme that was observed after phosphorolysis for 55 ms. The solid lines represent the dephosphorylation of phosphoenzyme that was initiated by the addition of 67 μM unlabelled ATP. The lower line was calculated using the scheme from Stahl and Jencks (1987) and is in good agreement with the observed phosphoenzyme level after dephosphorylation for 120 ms in the presence of 6.7 mM CaCl₂ (square), which causes 95% inhibition of steady-state turnover, has no effect on the rate of phosphoenzyme decay.
50 μM Ca2+ (open circle). Inhibition of steady-state turnover by binding of Ca2+ to the intravesicular transport site has been found to follow a Hill slope of ~1 and Kd ~ 1 mM (Khananshvili et al., 1990). The upper line was calculated for inhibition by one Ca2+ ion binding to an intravesicular transport site with Kd = 1 mM and is in good agreement with the data obtained in the presence of 6.7 mM Ca2+ (open triangle). These results confirm that Ca2+ can bind to the intravesicular transport site and inhibit the decay of phosphoenzyme.

Steady-state turnover is inhibited 98% by 6.7 mM Ca2+ under conditions similar to those described in Fig. 1 (Bodley and Jencks, 1987). The difference between the 80% inhibition of dephosphorylation and the 98% inhibition of steady-state turnover by 6.7 mM Ca2+ can be accounted for by phosphorylation of the enzyme by CaATP, which is a second mechanism for inhibition by calcium (Yamada and Tonomura, 1972; Orlowski et al., 1988).

Another possible mechanism of inhibition is binding of La3+ to A23187, which catalyzes the calcium efflux from the vesicles that is required for steady-state turnover with intact cations (Kolber and Haynes, 1981; Shastri et al., 1987; Cader and Horrocks, 1989). La3+ and Co2+ might be expected to cause inhibition by this mechanism because, respectively, they bind 230 and 370 times tighter than Ca2+ to A23187, with dissociation constants in the micromolar range in 80% methanol/water (Chapman et al., 1990). An alternative method of making SRV leaky is incubation with EGTA at pH 9, which has been shown to make the SRV permeable to Ca2+, inulin (Mw = 5,000), and dextran (Mw = 15,000–90,000) (Duggan and Martonosi, 1970). We have found that incubation of SRV with either Chelating Sepharose or Chelex 100 resin at pH 9 also makes the SRV leaky to Ca2+. Since alkaline treatment makes the SRV permeable to both cations and large uncharged molecules, it is unlikely that a cation would block this nonspecific permeability.

Table I shows that the K, for inhibition by LaCl3 of steady-state ATPase activity is comparable for vesicles that were made leaky by treatment with alkali or by the addition of the ionophore A23187; therefore, this inhibition by La3+ does not result from binding of La3+ to the ionophore. However, K, for inhibition by Co2+ is over 1 order of magnitude smaller for ionophore-treated vesicles compared with vesicles that were made leaky by treatment with alkali. Therefore, Co2+ inhibits steady-state ATPase activity of ionophore-treated vesicles by binding to the ionophore A23187, which slows the efflux of Ca2+ out of the vesicles and causes millimolar concentrations of calcium to accumulate in the intravesicular space. It was found that, in the presence of 50 μM Co2+, SRV that were made leaky by treatment with alkali hydrolyzed ATP at the control rate, while SRV that had only been treated with 2 and 4 μM A23187 hydrolyzed ATP at 15% and 17% of the control rate. This result demonstrates that Co2+ inhibits the Ca2+ efflux catalyzed by the ionophore A23187; however, an increase in the concentration of ionophore from 2 to 4 μM did not reverse this inhibition.

Fig. 6 shows that inhibition of ATP hydrolysis by La3+ develops slowly. The steady-state level of >80% inhibition is reached after >5 s, which corresponds to ~50 turnovers. The enzyme was incubated with La3+ and Ca2+ for 30 s before the addition of ATP. The concentration of inorganic phosphate was monitored because the coupled assay system as described above is calculated to have a lag of several seconds between an increase in the rate of ADP production and the attainment of a steady-state rate of NAD production (McCure, 1979).

Fig. 7 shows that enzyme which is inhibited by La3+ is reactivated in a time-dependent process by the addition of CaCl2 and EGTA. The addition of 0.50 mM CaCl2 and 0.50 mM EGTA to vesicles that are hydrolyzing ATP in the presence of 2 μM LaCl3 causes the ATPase rate to increase over several seconds from 0.32 to 1.9 μM min−1 mg−1. The latter rate corresponds to 90% of the ATPase rate in the absence of La3+. Fig. 7B shows the increase in velocity with time for this reactivation process. The solid line in Fig. 7B shows that the kinetics for reactivation can be approximated by a first-order rate constant of 0.08 s−1. The dashed line will be described later.

The lower traces in Fig. 8 show that two different species of phosphoenzyme are formed in the presence of both MgATP and A23187.
and LaATP. There is a rapid disappearance of one species of phosphoenzyme in a burst with a rate constant of \( \geq 4 \) s\(^{-1} \), which is too fast to measure on this time scale, followed by a much slower decay of phosphoenzyme with a rate constant of 0.05 s\(^{-1} \). The slow phase represents the decay of phosphoenzyme with La\(^{3+} \) at the catalytic site after phosphoenzyme formation. The rate constant for the decay of lanthanum phosphoenzyme was found to be 0.05 s\(^{-1} \) that follows a burst phase with an amplitude of \( \leq 0.2 \) s\(^{-1} \) under conditions similar to those described in Fig. 8 (data not shown). Therefore, the phosphoenzyme that undergoes hydrolysis with a rate constant of 0.05 s\(^{-1} \) (Fig. 8) does not have calcium bound at the phosphoryl transfer site.

The rate constant for the decay of lanthanum phosphoenzyme was found to increase from 0.05 to 0.08 s\(^{-1} \) when phosphoenolpyruvate and pyruvate kinase were not included in the reaction (data not shown). This increase in rate can be accounted for by the reaction of lanthanum phosphoenzyme with \( \sim 0.4 \) µM ADP, which is released upon formation of the phosphoenzyme. Lanthanum phosphoenzyme has been shown to react with ADP to regenerate \( [\gamma-\text{P}]ATP \) (Hanel and Jencks, 1990).

The dephosphorylation reaction shown in Fig. 8 was initiated by the addition of 5 mM EGTA, which chelates free La\(^{3+} \) in the solution. Therefore, the slow rate constant for dephosphorylation that is observed in the presence of LaCl\(_3\) results from the binding of LaATP to the phosphoryl transfer site before phosphoenzyme formation, which is essentially irreversible under these conditions (Hanel and Jencks, 1990); it is not caused by binding of La\(^{3+} \) to the intravesicular transport site after phosphoenzyme formation.

The time dependence for reactivation of lanthanum-inhibi-
Inhibition by Lanthanum of the Calcium ATPase

lased enzyme in the presence of 0.55 mM calcium and 0.50 mM EGTA (Fig. 7B) can be accounted for by the slow decay of lanthanum phosphoenzyme. The solid line in Fig. 7B corresponds to reactivation with a rate constant of 0.08 s⁻¹. The rate of reactivation is slightly faster than the hydrolysis rate of lanthanum phosphoenzyme because of the reaction of lanthanum phosphoenzyme with ADP, as described above. The concentration of ADP in the steady state was calculated to increase from 0.2 to 2 μM as the rate of ATP hydrolysis increases, by taking into account the activity of pyruvate kinase. The dashed line in Fig. 7B represents a computer simulation that takes into account the rate constants for the decay of lanthanum phosphoenzyme by hydrolysis and by reaction with ADP, and the activities of pyruvate kinase and lactate dehydrogenase that determine the concentrations of ADP and pyruvate during the course of reactivation (McClure, 1979).

DISCUSSION

If La³⁺ inhibits steady-state turnover of the Ca³⁺ATPase by binding to the cytoplasmic transport site and blocking phosphorylation, as has been generally believed, then (a) low levels of phosphoenzyme would be observed with inhibition by La³⁺, (b) increasing the concentration of Ca³⁺ would increase the activity of enzyme inhibited by La³⁺, and (c) the values of Kᵢ for inhibition by La³⁺ of steady-state turnover and of ⁴⁰Ca uptake would be comparable. However, none of these predictions are consistent with the data reported here. (a) Fig. 8 shows that high levels of phosphoenzyme are observed with enzyme that is inhibited by La³⁺, which agrees with results reported previously (Yamada and Tonomura, 1972). (b) Fig. 3 shows that increasing the concentration of Ca³⁺ does not increase the turnover rate of enzyme inhibited by La³⁺. (c) Fig. 4 shows that the value of Kᵢ for inhibition of ATPase activity in the steady state by La³⁺ is 500-fold lower than Kᵢ for inhibition of ⁴⁰Ca uptake. We conclude that inhibition of steady-state turnover by La³⁺ is not caused by binding of La³⁺ to the cytoplasmic transport site.

The results reported here provide evidence that La³⁺ inhibits steady-state turnover by replacing Mg²⁺ as the catalytic ion for phosphoryl transfer. The upper line in Fig. 8 shows that 'E·Ca₂⁺ is phosphorylated by LaATP to give lanthanum phosphoenzyme, which decays with a rate constant of 0.05 s⁻¹; this is much slower than the rate constant of ~15 s⁻¹ for the decay of magnesium phosphoenzyme (Chiesi and Inesi, 1979; Pickart and Jencks, 1984). The biphasic decay of phosphoenzyme shown in the lower traces of Fig. 8 demonstrates that both lanthanum phosphoenzyme and magnesium phosphoenzyme are present with lanthanum concentrations that inhibit steady-state turnover, but do not significantly inhibit phosphorylation or Ca²⁺ uptake in a single turnover experiment (Fig. 4). Fig. 7B shows that the rate constant for the decay of lanthanum phosphoenzyme through hydrolysis at 0.05 s⁻¹ and reaction with ADP to regenerate ATP; the concentration of ADP was calculated to be 0.2-2.0 μM in the steady state.

The slow development of inhibition by La³⁺ shown in Fig. 6 results from the gradual replacement of Mg²⁺ by La³⁺ at the catalytic site over several seconds, which corresponds to ~50 turnovers. Because the rate constant of 0.05 s⁻¹ for the decay of lanthanum phosphoenzyme is much smaller than the rate constant of ~15 s⁻¹ for the magnesium phosphoenzyme (Hanel and Jencks, 1990; Chiesi and Inesi, 1979; Pickart and Jencks, 1984), LaATP is a very effective inhibitor of the calcium ATPase. Fig. 6 shows that 0.31 μM LaATP causes ≥80% inhibition of steady-state turnover in the presence of 48 μM MgATP. LaATP and MgATP bind to the enzyme with similar second-order rate constants (Hanel and Jencks, 1990); however, the enzyme binds MgATP >100 times more often than LaATP under these conditions because MgATP is in large excess compared with LaATP. At early time points, most of the enzyme binds MgATP, which undergoes rapid turnover. The enzyme binds LaATP infrequently, but when LaATP does bind it forms lanthanum phosphoenzyme, which is very stable and accumulates with time. It is difficult to correlate the effect of La³⁺ on steady-state turnover and on partial reactions that are measured in a single turnover because of this slow development of inhibition. This may account for some of the inconsistencies in the literature concerning the mechanism of inhibition by La³⁺.

The interactions between the sarcoplasmic reticulum calcium ATPase and multivalent metal ions are complex and confusing because there are several different classes of binding sites for cations on the enzyme. Mg²⁺ and Ca²⁺ are required for full activity, yet both inhibit at high concentrations. Mg²⁺ can inhibit by binding to the cytoplasmic calcium transport site (Yamada and Tonomura, 1972) and by binding to the lumenal calcium transport site at pH 8 (Bishop and Al-Shawi, 1988). Ca²⁺ can inhibit by binding to the lumenal transport site (Weber, 1966) or by replacing Mg²⁺ as the catalytic ion for phosphoryl transfer (Shigekawa et al., 1988). These inhibitory effects make it difficult to interpret experiments in which the effects of increases in the concentrations of Ca²⁺ or Mg²⁺ on inhibition of ATPase activity by lanthanide ions are measured.

Lanthanide ions have been reported previously to bind to more than one site on the sarcoplasmic reticulum calcium ATPase. Itoh and Kawakita (1984) have reported the existence of several different classes of lanthanide-binding sites. Gadolinium ion causes inhibition of steady-state ATPase activity and steady-state phosphoenzyme formation at concentrations of ~10⁻⁶ M, but this probably does not occur at the cytoplasmic transport site because 50% inhibition of the binding of 10 μM Ca²⁺ requires ~75 mM Gd³⁺. Terbium ion binds to a site on the enzyme with Kᵢ ~ 10⁻⁴ M that is not competitive with Ca²⁺, but is competitive with Mn²⁺. Terbium fluorescence is enhanced as a result of energy transfer from aromatic amino acid residues to Tb³⁺ bound to this site. It was concluded that gadolinium ion does not inhibit ATPase activity in the steady state by binding to the cytoplasmic transport site; however, the mechanism of inhibition by this lanthanide ion was not identified.

Girardet et al. (1989) have also reported several cases of binding sites for terbium on the sarcoplasmic reticulum calcium ATPase: a magnesium binding site binds terbium with Kᵢ = 10 μM, the Ca²⁺-binding site binds terbium with Kᵢ < 0.1 μM in the absence of Ca²⁺, and the nucleotide-binding site binds terbium formycin triphosphate with Kᵢ = 0.7 μM. The ability of lanthanide ions to bind to several different sites on the enzyme is not surprising because binding sites for both Mg²⁺ and Ca²⁺ on several proteins have been shown to bind lanthanide ions (Martin and Richardson, 1979).

Multivalent cations also can inhibit ATP hydrolysis by interacting with components of the reaction mixture other than the calcium ATPase. The ionophore A23187 is often used to collapse the Ca²⁺ gradient that would form with intact sarcoplasmic reticulum vesicles that are hydrolyzing ATP. Co³⁺ can inhibit ATP hydrolysis by binding to the ionophore A23187 and decreasing the rate of Ca²⁺ efflux out of the vesicles, so that the interior Ca²⁺ concentration increases and...
Inhibition by Lanthanum of the Calcium ATPase

Richard J. Albin, N. Ikemoto, W. D. Horrocks, Jr.

The binding of La³⁺ to the cytoplasmic transport site inhibits ATPase activity. The Kₘ for the inhibition of ATPase activity by cobalt is 3 µM for vesicles treated with ionophore, while it is 300 µM for vesicles that were made leaky by treatment with chelating agents (Table I). Highsmith and Murphy (1984) reported that ATPase activity is inhibited by cobalt with Kᵣ = 3 µM in the presence of 10 µM Ca²⁺, 5 mM Mg²⁺, 2 mM ATP, and 0.96 µM A23187. This inhibition was attributed to binding of Co³⁺ to the calcium transport site with K₋⁰ = 2 x 10⁶ M⁻¹, after correcting for the chelation of Ca²⁺ and Co²⁺ by ATP. They measured fluorescence energy transfer between fluorescein isothiocyanate and 0.8 µM Co³⁺, which they believed to be bound to the cytoplasmic calcium transport site. However, Co³⁺ at this concentration does not inhibit the calcium ATPase directly and is not bound to the calcium transport site, as demonstrated by the value of Kᵣ = 300 µM for inhibition of leaky vesicles by Co³⁺. Therefore, the distance measurement obtained by fluorescence energy transfer does not provide a measure of the distance from the fluorescein isothiocyanate to the calcium transport site.

The concentrations of free La³⁺ and LaATP reported here are not exact because of the large number of equilibria that affect these concentrations. The value of Kᵣ for inhibition of steady-state ATPase activity by LaCl₃ increases with increasing concentrations of SRV, ATP, and Pᵣ because La³⁺ is chelated by these components of the reaction mixture. In order to estimate the concentration of free La³⁺ and LaATP, it is necessary to take into account the concentrations of SRV, ATP, and Pᵣ, as well as the concentration of Ca²⁺ and Mg²⁺, which also bind to these chelators. Sulfate and MOPS have also been reported to bind La³⁺ (Perrin, 1979; Girardet, 1989). The reported dissociation constants for LaATP vary from 3.4 x 10⁻⁸ to 7.0 x 10⁻¹¹ M, when adjusted to pH 7 (Krasnow, 1972; Morrison and Cleland, 1980). The values of Kᵣ for free La³⁺ and LaATP that are reported in this paper refer to the dissociation constant of 7.0 x 10⁻¹⁰ M (Morrison and Cleland, 1983) and the experimental conditions described here.

Lanthanum Does Not Cause Inhibition of Steady-state ATPase Activity by Binding to the Cytoplasmic Transport Site—The high levels of phosphoenzyme that are observed with La³⁺ are not consistent with inhibition by La³⁺ to the cytoplasmic calcium transport site, which is expected to inhibit phosphorylation of the enzyme. High phosphoenzyme levels have been observed previously by Yamada and Tonomura (1972) for enzyme that was inhibited by La³⁺ and Ca²⁺. This result was interpreted in terms of a model in which Ca²⁺ and Mg²⁺ are countertransported. It was concluded that these lanthanide ions inhibited the countertransport of Mg²⁺ that is required for the decomposition of phosphoenzyme, according to this model. The high levels of phosphoenzyme in the steady state demonstrate that the decomposition of the phosphoenzyme is the rate-limiting step for enzyme that is inhibited by La³⁺, i.e., formation of the phosphoenzyme is fast relative to the slow hydrolysis of phosphoenzyme.

Fig. 3 shows that the ATPase activity of enzyme that is inhibited by La³⁺ decreases with increasing concentrations of Ca²⁺. If inhibition were caused by binding of La³⁺ to the cytoplasmic transport site, then Ca²⁺ would be expected to compete with La³⁺ and relieve this inhibition, so that the absolute activity would increase at high concentrations of Ca²⁺. The absence of relief of inhibition with increasing concentrations of Ca²⁺ is not consistent with inhibition by binding of La³⁺ to the cytoplasmic transport site.

Fig. 4 shows that the value of Kᵣ for inhibition of ⁴⁰Ca uptake and phosphoenzyme formation in a single turnover is 500-fold larger than Kᵣ for inhibition of ATPase activity in the steady state. The results of the single turnover experiments demonstrate that Kᵣ is ~100 µM for LaCl₃ that is bound to the cytoplasmic transport site in the presence of 25 µM Ca²⁺. This low affinity binding of La³⁺ to the cytoplasmic transport site under these conditions cannot account for the inhibition of steady-state turnover by La³⁺ with Kᵣ = 0.16 µM.

Lanthanum at the Catalytic Site Inhibits the Decay of Phosphoenzyme—Fig. 8 shows that La³⁺ inhibits ATPase activity by slowing the rate of phosphoenzyme decay. This explains the high levels of phosphoenzyme that are observed in the steady state. It is known that millimolar concentrations of Ca²⁺ are required to inhibit the decay of phosphoenzyme formed from MgATP by binding to the intravesicular transport site (Ikemoto, 1975), but it appeared possible that La³⁺ could bind to this site with a much higher affinity. However, Fig. 5 shows that the addition of 6.7 µM LaCl₃ to leaky vesicles after the formation of phosphoenzyme with MgATP has no effect on the rate of decay of the phosphoenzyme, although this concentration of LaCl₃ causes 95% inhibition of ATPase activity in the steady state. Therefore, La³⁺ does not cause inhibition of ATPase activity in the steady state by binding to the intravesicular transport site.

We conclude that La³⁺ inhibits the decay of phosphoenzyme by replacing Mg²⁺ as the catalytic ion for phosphoryl transfer. The rate constant of 0.05 s⁻¹ for the decay of phosphoenzyme with La³⁺ as the catalytic ion is much smaller than the rate constant of ~15 s⁻¹ for the decay of phosphoenzyme with Mg²⁺ as the catalytic ion; this step is predominantly rate-limiting for the hydrolysis of both LaATP and MgATP (Hanel and Jencks, 1990; Chiesi and Inesi, 1979; Pickart and Jencks, 1984). Fig. 8 shows that lanthanum phosphoenzyme, which decays with k = 0.05 s⁻¹, and magnesium phosphoenzyme, which decays with k = 4 s⁻¹, are both present in enzyme that is partially inhibited by La³⁺; this replacement of Mg²⁺ by La³⁺ is responsible for the inhibition of steady-state ATPase activity.

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T Fujimori and W P Jencks

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