The Metal Sites on Sarcoplasmic Reticulum Membranes That Bind Lanthanide Ions with the Highest Affinity Are Not the ATPase Ca\(^{2+}\) Transport Sites*

(Fernando Henao‡, Stéphane Orlowski, Zalika Merah§, and Philippe Champeil¶)

From the Unité de Recherche 1290 Associée au Centre National de la Recherche Scientifique and Section de Biophysique des Protéines et des Membranes, Département de Biologie Cellulaire et Moléculaire, Centre d'Etudes Nucléaires de Saclay, 91191 Gif-sur-Yvette Cedex, France and the ‡Departamento de Bioquímica y Biología Molecular y Genética, Laboratorio de Bioquímica, Facultad de Ciencias, 06080 Badajoz, Spain

We attempted to establish whether lanthanide ions, when added to sarcoplasmic reticulum (SR) membranes in the absence of nucleotide, compete with Ca\(^{2+}\) for binding to the transport sites of the Ca\(^{2+}\)-ATPase in these membranes, or whether they bind to different sites. Equilibrium measurements of the effect of lanthanide ions on the intrinsic fluorescence of SR ATPase and on \(^{46}\text{Ca}^{2+}\) binding to it were performed either at neutral pH (pH 6.8), i.e. when endogenous or contaminating Ca\(^{2+}\) was sufficient to nearly saturate the ATPase transport sites, or at acid pH (pH 5.5), which greatly reduced the affinity of calcium for its sites on the ATPase. These measurements did reveal apparent competition between Ca\(^{2+}\) and the lanthanide ions La\(^{3+}\), Gd\(^{3+}\), Pr\(^{3+}\), and Tb\(^{3+}\), which all behaved similarly, but this competition displayed unexpected features: lanthanide ions displaced Ca\(^{2+}\) with a moderate affinity and in a noncooperative way, and the pH dependence of this displacement was smaller than that of the Ca\(^{2+}\) binding to its own sites. Simultaneously, we directly measured the amount of Tb\(^{3+}\) bound to the ATPase relative to the amount of Ca\(^{2+}\) and found that Tb\(^{3+}\) ions only reduced significantly the amount of Ca\(^{2+}\) bound after a considerable number of Tb\(^{3+}\) ions had bound. Furthermore, when we tested the effect of Ca\(^{2+}\) on the amount of Tb\(^{3+}\) bound to the SR membranes, we found that the Tb\(^{3+}\) ions which bound at low Tb\(^{3+}\) concentrations were not displaced when Ca\(^{2+}\) was added at concentrations which saturated the Ca\(^{2+}\) transport sites. We conclude that the sites on SR ATPase to which lanthanide ions bind with the highest affinity are not the high affinity Ca\(^{2+}\) binding and transport sites. At higher concentrations, lanthanide ions did not appear to be able to replace Ca\(^{2+}\) ions and preserve the native structure of their binding pocket, as evaluated in rapid filtration measurements from the effect of moderate concentrations of lanthanide ions on the kinetics of Ca\(^{2+}\) dissociation. Thus, the presence of lanthanide ions allowed down the dissociation from its binding site of the first, superficially bound \(^{46}\text{Ca}^{2+}\) ion, instead of specifically preventing the dissociation of the deeply bound \(^{46}\text{Ca}^{2+}\) ion. These results highlight the need for caution when interpreting, in terms of calcium sites,

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‡ Present address: Laboratoire de Biologie de l'Ecole Centrale de Paris, 92295 Châtenay-Malabry Cedex, France.
§ To whom correspondence should be addressed.

Active transport of calcium from the cytoplasm of muscle cells into the lumen of sarcoplasmic reticulum (SR) is mediated by membranous Ca\(^{2+}\)-activated transport ATPase. The cloning and sequencing of this enzyme's cDNA allowed the definition of its amino acid sequence and, combined with low resolution structural data, the prediction of its secondary structure and gross topography relative to the membrane plane (MacLennan et al., 1985). This ATPase was suggested to be composed of three main domains: a globular ATP-binding cytoplasmic domain, a pentahelical stalk, and a transmembrane domain. However, the high affinity Ca\(^{2+}\)-binding sites whose saturation results in ATPase activation and ion transport have not been unambiguously localized on the predicted structure (Brandl et al., 1986; Clarke et al., 1989), and the available ATPase diffraction data obtained with 2D or 3D crystals do not yet provide sufficient resolution to allow such localization (e.g. Stokes and Green, 1990). In the meantime, one reasonable approach to this question is to try to replace Ca\(^{2+}\) with a metal ion with properties allowing distance measurements and triangulation. In this connection, lanthanide ions with magnetic or optical properties have already proved useful as calcium analogs for the study of various soluble calcium-binding proteins (Reuben, 1975; Martin and Richardson, 1979; Rhee et al., 1981; Evans, 1990; Petersheim, 1991). These ions, which have a relatively high atomic mass, are also potentially useful for anomalous x-ray scattering or other x-ray absorption spectroscopy techniques (Powers, 1982; Fairclough et al., 1986).

The effect of lanthanide ions on SR Ca\(^{2+}\)-ATPase has been the subject of several publications during the last few years. Some time ago, these ions were shown to inhibit SR ATPase activity in the presence of ATP (e.g. Yamada and Tonomura, 1972). However, it now appears that this inhibition does not result from competition for occupancy of the calcium transport sites, but rather from the binding of Ln\(^{3+}\) in place of Mg\(^{2+}\) at the catalytic site (Fujimori and Jencks, 1980), or

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1The abbreviations used are: SR, sarcoplasmic reticulum; Ca\(_{46}\)E\(_{n}\), octaethylene glycol monododecyl ether; EGTA, [ethylenebis(oxyethylenenitrito)]tetraacetic acid; NTA, nitrilotriacetic acid; MES, 2-(N-morpholino)ethanesulfonic acid; TES, 1-morpholinepropanesulfonic acid; TES, N-tris(hydroxymethyl) 2-aminoethanesulfonic acid; PIPES, piperazine-N,N'-bis(2-hydroxyethyl) 2-aminoethanesulfonic acid; Ln\(^{3+}\), lanthanide ions; DPA, dipicolinic acid; ANS-maleimide, 2-(4'-maleimidylanilino)naphthalene-6-sulfonic acid.
possibly also from the binding of lanthanide ions to sites distinct from both the transport sites and the catalytic site (Ogurusu et al., 1991). In the absence of ATP, lanthanide ions might nevertheless bind to the ATPase Ca²⁺ transport sites with high affinity. This was indeed suggested, and as a result, fluorescent or paramagnetic lanthanide ions were used to estimate the position of the two calcium-binding sites in the ATPase relative to the membrane surface or to other sites on the ATPase, including the catalytic site and individually labeled amino acids (Stephens and Grisham, 1979; Highsmith and Murphy, 1984; Scott, 1985; Herrmann et al., 1986a; Tereuel and Gómez-Fernández, 1986; Squier et al., 1987, 1990; Jona et al., 1990; see also a discussion in Martonosi et al., 1990). Lanthanide ions have also been used to estimate the distance between the two Ca²⁺-binding sites (Scott, 1985; Herrmann et al., 1986a; Herrmann and Shamo, 1988) as well as their mean hydration (Scott, 1984; Klemens et al., 1986; Gangola and Shamo, 1987; Lockwich and Shamo, 1990). Lastly, resonance x-ray diffraction studies have been performed to detect the bound lanthanide ions (Asturias and Blasie, 1991). The results of some of these last studies, as well as of some of our fluorescence microscopy experiments, suggested that the highest affinity lanthanide-binding sites were located in the stalk domain of the ATPase, where the Ca²⁺-binding sites had originally been predicted to reside (Brandl et al., 1986). However, in subsequent directed mutagenesis experiments, amino acids critical for calcium control of the catalytic events were discovered in the putative transmembrane region of the ATPase and not in the stalk region (Clarke et al., 1989; Green, 1989).

One critical issue is to determine clearly whether or not lanthanide ions bind to the Ca²⁺ transport sites. Several regions in SR membranes are obvious candidates as binding sites for trivalent ions: besides the high affinity Ca²⁺ binding and transport sites, these regions include at least the magnesium-binding site in the catalytic domain of the ATPase, the phospholipid headgroups in the membrane interface region, and the stalk domain of the ATPase, which contains up to 20 negatively charged amino acid residues. A difficulty when trying to design experiments clearly demonstrating true competition between Ca²⁺ and Ln³⁺ for binding to ATPase is that at neutral pH contaminating or endogenous Ca²⁺ is usually sufficient to saturate the ATPase Ca²⁺ transport sites. As no chelating agent is available which would selectively bind Ca²⁺ but not Ln³⁺, it is not a simple matter to study the binding of Ln³⁺ to Ca²⁺-free ATPase. To overcome this difficulty, previous investigators used Ca²⁺-chelating resins for preliminary purification of the membrane and buffer depletion of Ca²⁺ (Chavelli and Butow, 1971; Jona and Martonosi, 1986; Itoh and Kawakita, 1984; Imamura and Kawakita, 1991a).

In the present work, we specifically addressed the question of whether or not lanthanide ions bind to the Ca²⁺ transport sites. To solve the problem created by the saturation of the Ca²⁺ transport sites by contaminating Ca²⁺ at neutral pH, we conducted some of the experiments at acidic pH (pH 5.5), i.e. under conditions in which the ATPase affinity for calcium was so greatly reduced (Verjovsky-Almeida et al., 1977; Watanabe et al., 1981; Guillin et al., 1982) that contaminating or endogenous Ca²⁺ was no longer an obstacle. On the one hand, we performed equilibrium and kinetic measurements of the effect of lanthanide ions on ⁴⁴Ca binding to the ATPase and on the changes in SR intrinsic fluorescence induced by Ca²⁺ (all the lanthanide ions tested behaved similarly), and on the other, we measured directly, by a fluorimetric assay using dipicolinic acid, the amount of Tb³⁺ bound to the ATPase and the effect of Ca²⁺ on this bound Tb³⁺.

Our results show that although the trivalent lanthanide ions did reduce the amount of Ca²⁺ bound to the ATPase, they only did so with a moderate affinity and after a considerable number had been bound, corresponding to 4–10 times the stoichiometry of high affinity Ca²⁺ binding to the transport sites. Moreover, the Tb³⁺ ions which bound to the ATPase first, at low Tb³⁺ concentrations, were not displaced when Ca²⁺ bound to its own sites. In addition, when we studied the effect of lanthanide ions on the kinetics of dissociation of the two Ca²⁺ ions bound to the ATPase, we found that moderate concentrations of these lanthanide ions slowed down the dissociation of the first Ca²⁺ ion to leave its binding pocket, which is clear evidence for binding at a site different from the Ca²⁺-binding sites. We thus conclude that the sites on the ATPase to which lanthanide ions bind with the highest affinity are not the Ca²⁺ transport sites. Even at higher concentrations, Ln³⁺ did not behave like Ca²⁺ because the presence of Ln³⁺ in the dissociation medium did not block dissociation of the second Ca²⁺ ion. These results highlight the need for caution when interpreting experimental data concerning calcium site localization on the ATPase using lanthanide ions.

**EXPERIMENTAL PROCEDURES**

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle, and the protein concentration was determined spectrophotometrically at 280 nm in the presence of 1% sodium dodecyl sulfate, as previously described (Champeil et al., 1986). Lanthanide ions were obtained as chlorides from Aldrich; EGTA and NTA were from Sigma. The dissociation constants for Ca²⁺-EGTA and Mg²⁺-EGTA were assumed to be 0.94 μM and 57 μM, respectively, at pH 6.8 and 380 μM and 0.97 M at pH 5.5. The dissociation constants for Ca²⁺-NTA and Mg²⁺-NTA were assumed to be 400 μM and 4 mM, respectively, at pH 6.8 and 8.5 and 85 mM at pH 5.5. The dissociation constant for complex of NTA with Ln³⁺, Pr³⁺, and Tb³⁺ at pH 6.8 were, respectively, assumed to be 52, 13 and 2.6 μM (Martell and Smith, 1974; Tien and Pozzan, 1979).

Steady-state fluorescence measurements were performed at 20 °C in a continuously stirred cuvette with a Perkin-Elmer Cetus MFP 44A (or a SLM 4000B) fluorimeter, using excitation and emission wavelengths of 290 and 330 nm, respectively. Changes in fluorescence levels were corrected for dilution. Binding of "⁴⁴Ca" was measured in filtration experiments as described by Champeil and Guillain (1986), using [¹⁴C]glucose as a marker of the amount of fluid wetting the filter. Unless otherwise indicated, 0.3 mg/ml protein SR vesicles were preincubated in a medium containing 1 mM [¹⁴C]glucose and either 40 or 100 μM "⁴⁴Ca" (in the form of a CaCl₂ solution). 0.3 mg of protein was layered onto a Millipore HA filter, and the 3H and '⁴⁴Ca radioactivities on the filter were counted by liquid scintillation. As described previously, "⁴⁴Ca" dissociation rates were measured at 20 °C with a Biologic rapid filtration apparatus in which the filters with the vesicles were washed with the filters used for various electronically controlled periods (Orlowski and Champeil, 1991).

Throughout this work we used two main suspension media. The acid "pH 5.5 medium" contained 150 mM MES-Tris, no potassium, and, unless otherwise indicated, 20 mM Mg²⁺ (20°C, pH 5.5 ± 0.1). The pH 6.8 or neutral pH medium contained 50 mM MOPS-KOH, 80 mM KCl, and 10 mM Mg²⁺ (20°C, pH 6.8 ± 0.1). As regards contaminating Ca²⁺ in these media, it is known that the walls of glass vessels adsorb large amounts of Ca²⁺ which under certain conditions may be released into the medium after prolonged storaage. As we found that such release was particularly critical for the experiments performed at pH 5.5, in which we wanted as little contaminating Ca²⁺ as possible, the stock solutions for some of these experiments were stored in plastic vessels.

The lanthanide chloride salts were dissolved in 10 mM HCl and stored at 4 °C in plastic containers at different concentrations, all in the same 10 mM HCl medium (dos Remedios, 1981). The measured absorption properties of the lanthanide stock solutions were as expected from previously published extinction coefficients: for Pr³⁺, C₄₄₃ = 10 cm⁻³ M⁻¹; for Tb³⁺, C₄₄₃ = 3.4 cm⁻³ M⁻¹; for Tb³⁺, t₉₀ = 0.31 M⁻¹ cm⁻¹, and e₉₄₃ = 320 cm⁻¹ M⁻¹ (Brandl et al., 1979). In the case of Tb³⁺, the accuracy of the concentration of the lanthanide stock solution was also confirmed by the fact that the fluorescence of its complex with DPA (dipicolinic acid, see below) was maximal at the
expected stoichiometry of one Tb\(^{3+}\) for three DPA (Barela and Sherry, 1976). In the case of La\(^{3+}\), the concentration of its stock solution was established by observation of its stoichiometric association with NTA or EGTA (see murexide experiments below, Fig. 4B).

Binding of Tb\(^{3+}\) to SR membranes was measured by the difference between the total amount of Tb\(^{3+}\) in the membrane suspension and the amount of Tb\(^{3+}\) not bound to the membranes, using a protocol derived from a previously published one (Fairclough et al., 1986). To separate the membranes from the medium, however, we did not spin but simply filtered the suspension through a Millex GS filter. The Tb(DPA)\(_3\) fluorospectrophotometer cuvettes (Barela and Sherry, 1976) was used to quantify both the Tb\(^{3+}\) concentration added to the filter and its total concentration. Aliquots of the various samples were sequentially added to a cuvette containing 200 \(\mu\)M DPA (the dilution was taken into account for subsequent calculations). Filtering of a control membrane-free Tb\(^{3+}\) sample allowed correction for the small amount of Tb\(^{3+}\) binding to the filter itself.

For example, for the experiment illustrated in Fig. 5B, the initial Tb\(^{3+}\) concentration was 10 \(\mu\)M, and 20\(\mu\)l aliquots of the various samples were added to a 2-mL DPA-containing cuvette. In this particular trace, comparison of C\(_1\) with C\(_5\) showed that after passing 0.5 mL of control membrane-free 10 \(\mu\)M Tb\(^{3+}\) solution through the Millex, 8 \(\mu\)M was recovered in the C\(_5\) filtrate (see legend to Fig. 5A for definition of these samples, C\(_2\), C\(_3\), E\(_5\), and E\(_6\)); similar measurements, performed in duplicates for various Tb\(^{3+}\) concentrations, enabled us to establish a plot allowing Tb\(^{3+}\) binding to the Millex system to be estimated for all Tb\(^{3+}\) concentrations. Similarly, referring again to the legend of Fig. 5B, comparison of C\(_6\) with C\(_7\) showed that Tb\(^{3+}\) filtration through the Millex of a 10 \(\mu\)M Tb\(^{3+}\) solution containing SR membranes (here, 0.1 mg/ml protein was present), the Tb\(^{3+}\) concentration in the filtrate (E\(_0\)) was 5 \(\mu\)M. From the above curve for Tb\(^{3+}\) binding to the Millex system itself, we then deduced that the free concentration of Tb\(^{3+}\) in equilibrium with the SR membranes before filtration was slightly higher, i.e., 6 \(\mu\)M. Therefore, we considered that at a total Tb\(^{3+}\) concentration of 10 \(\mu\)M, the concentration of Tb\(^{3+}\) bound to the membranes was (10 - 6) = 4 \(\mu\)M (±0.4), corresponding to 40 ± 4 of n mole Tb\(^{3+}\) bound/mg of SR protein. To compute this amount of bound Tb\(^{3+}\), the fluorescence observed upon addition of the added Tb\(^{3+}\) was not explicitly taken into account, as it was always consistent with the one observed upon addition of the C\(_7\) sample, although with a slightly reduced amplitude probably due to sample turbidity. Note, in addition, that the trace corresponding to addition of E\(_7\) in Fig. 5B clearly shows that before reacting with DPA, a small fraction of the bound Tb\(^{3+}\) dissociated at a relatively slow rate from the SR membranes.

The Tb(DPA)\(_3\) assay was also used to ascertain whether a significant fraction of the added lanthanide ions bound to the vessel's walls, as such binding reduces the amount of La\(^{3+}\) interacting with the ATPase. By aliquoting a given solution of Tb\(^{3+}\) into different types of vial and then assaying the vial's contents for Tb\(^{3+}\) fluorescence, a small fraction of the added Tb\(^{3+}\) was lost, probably for magnetic bar-containing spectrophotometer cuvettes and at our water quality, conditions of solution storage, protein concentration, etc.) is usually sufficient for virtual saturation of the high affinity SR ATPase transport sites. Lowering the medium pH greatly reduces the affinity of these sites for calcium (Watanabe et al., 1981; Guillain et al., 1982), so that at acidic pH and in the absence of extra Ca\(^{2+}\), most sites can be expected to remain unoccupied. This expectation is confirmed by trace A of Fig. 1, which shows that at pH 5.5 ± 0.1, addition of excess EGTA to SR vesicles only slightly reduced their intrinsic fluorescence level, known to be a reliable index of site occupancy by Ca\(^{2+}\), whereas addition of excess Ca\(^{2+}\) did buffers; as regards 50 mM MOPS, this result fits with the earlier finding that MOPS has no effect on the luminescence lifetime of Eu\(^{3+}\) (Gangola and Shamu, 1987).

RESULTS

Intrinsic Fluorescence Changes Induced by Binding of Ca\(^{2+}\) or La\(^{3+}\) Ions to SR ATPase at Acid pH—At neutral pH, contaminating and endogenous Ca\(^{2+}\) (5–30 \(\mu\)M, depending on water quality, conditions of solution storage, protein concentration, etc.) is usually sufficient for virtual saturation of the high affinity SR ATPase transport sites. Lowering the medium pH greatly reduces the affinity of these sites for calcium (Watanabe et al., 1981; Guillain et al., 1982), so that at acidic pH and in the absence of extra Ca\(^{2+}\), most sites can be expected to remain unoccupied. This expectation is confirmed by trace A of Fig. 1, which shows that at pH 5.5 ± 0.1, addition of excess EGTA to SR vesicles only slightly reduced their intrinsic fluorescence level, known to be a reliable index of site occupancy by Ca\(^{2+}\), whereas addition of excess Ca\(^{2+}\) did

\(^2\) This was not the case for PIPES which did quench Gd\(^{3+}\) fluorescence and vibronic side bands. Using the Tb(DPA)\(_3\) assay, we also found that interaction of Tb\(^{3+}\) with high concentrations of PIPES resulted in the formation of a slowly dissociating complex with unusual binding properties to the cuvette’s walls (not shown). This contrasts with the previous observation that the Gd\(^{3+}\) ESR spectrum did not change in the presence of PIPES (Stephens and Grisham, 1979). However, in agreement with this previous observation, the present measurements of Gd\(^{3+}\) fluorescence confirmed the existence of some interaction between Gd\(^{3+}\) and high concentrations of TES.
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Fig. 2. Titration at acid pH of the effects of Ca\(^{2+}\) and La\(^{3+}\) on the SR ATPase intrinsic fluorescence. The medium contained 150 mM MES-Tris, (pH 5.5, 20 °C), 20 mM Mg\(^{2+}\), and 0.1 mg/ml SR protein. Triangles in panel B refer to similar experiments performed without Mg\(^{2+}\). Panels A and B were deduced from experiments similar to those illustrated in Fig. 1, traces B and C. Panel A: Ca\(^{2+}\) induced changes in SR intrinsic fluorescence, in the absence of La\(^{3+}\) (circles) or in the presence of 0.1 mM La\(^{3+}\) (triangles) or 2 mM La\(^{3+}\) (squares). In these experiments, the concentration of contaminating plus endogenous calcium was estimated to be 15–20 μM (see cross). The fluorescence level in the absence of added Ca\(^{2+}\) was taken as reference (see the cross, and also the dashed line which extends to slightly negative values the curve constructed in the absence of La\(^{3+}\)). Panel B: La\(^{3+}\) dependence of [Ca\(^{2+}\)]\(_i\) in the above plots and other similar ones. The curves in panels A and B were in fact deduced from a series of experiments performed at a slightly higher pH (pH 5.6–5.7 instead of 5.5). Panels C and D were deduced from experiments similar to those illustrated in Fig. 1, trace D. Panel C: La\(^{3+}\) dependence of the SR fluorescence level (the fluorescence level of SR before Ca\(^{2+}\) addition was again taken as 100%), in the presence of four total concentrations of Ca\(^{2+}\): 45 μM (triangles), 125 μM (circles), 225 μM (squares), and 500 μM (diamonds). Panel D: Ca\(^{2+}\) dependence of the fluorescence level (the fluorescence level of SR before Ca\(^{2+}\) addition was again taken as 100%), in the presence of increasing amounts of Ca\(^{2+}\) to the ATPase high affinity sites-Fig. 1 shows that the successive addition of increased amounts of La\(^{3+}\) to SR in the presence of a nearly saturating Ca\(^{2+}\) concentration gradually reduced SR fluorescence. Apparent competition between La\(^{3+}\) and Ca\(^{2+}\) for binding to ATPase sites was suggested by the facts that high Ca\(^{2+}\) concentrations counteracted the La\(^{3+}\)-induced reduction in SR fluorescence and high La\(^{3+}\) concentrations apparently totally reversed the Ca\(^{2+}\)-induced fluorescence change, as shown in panels C and D of Fig. 2. Note that despite the acid pH and the presence of La\(^{3+}\), no irreversible denaturation took place on the time scale of these experiments, since (i) this La\(^{3+}\)-induced reduction in fluorescence was reversed upon addition of the lanthanide chelator NTA and (ii) subsequent addition of EGTA and Ca\(^{2+}\) still allowed monitoring of the previously described Ca\(^{2+}\)-dependent fluorescence changes (see trace D in Fig. 1).

It should be stressed that although lanthanide ions are known to induce aggregation of membranes under certain conditions (Jona and Martonosi, 1986), no turbidity artifact was present in the above experiments. In fact, to our surprise, we found that whereas millimolar concentrations of La\(^{3+}\) or Tb\(^{3+}\) induced large changes in the turbidity of SR suspensions containing KCl, no such change was observed in the absence of KCl (data not shown). Nevertheless, to confirm the intrinsic fluorescence results, we also performed direct measurements of the amount of Ca\(^{2+}\) bound to the ATPase high affinity sites, using \(^{40}\)Ca\(^{2+}\) and filtration methods.

Effect of Various Lanthanides on Equilibrium Binding of \(^{40}\)Ca\(^{2+}\) to the SR ATPase Transport Sites—Fig. 3 shows equilibrium \(^{40}\)Ca\(^{2+}\)-binding measurements performed at pH 5.5 in the presence of 150 mM MES-Tris and 20 mM Mg\(^{2+}\). Either 40 μM \(^{40}\)Ca\(^{2+}\) (triangles) or 100 μM (circles) was added to the assay, which, due to the poor affinity under these conditions, resulted in incomplete site saturation (see Fig. 2A. circles; two \(^{40}\)Ca\(^{2+}\) ions bound/ATPase chain correspond to 10–14 nmol/ mg protein, see Orlowski and Champel, 1991). As illustrated by the closed symbols in panel A of Fig. 3, increasing concentrations of La\(^{3+}\) reduced to zero the amount of bound \(^{40}\)Ca\(^{2+}\). The same was true for all the lanthanide ions tested, i.e. La\(^{3+}\), Pr\(^{3+}\), Gd\(^{3+}\), and Tb\(^{3+}\), all of which had similar affinities (results for Pr\(^{3+}\) are shown in panel B, and those for Tb\(^{3+}\) are shown below in Fig. 6A). The concentrations required to chase half the initially bound \(^{40}\)Ca\(^{2+}\) were 30 and 65 μM La\(^{3+}\) in the presence of 40 and 100 μM added \(^{40}\)Ca\(^{2+}\), respectively, and were consistent with the [La\(^{3+}\)]\(_i\) values deduced from the fluorescence experiments above (compare asterisks to circles in Fig. 2D).

To check that lanthanide ions were added in large excess relative to the number of their binding sites, the experiment...
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FIG. 3. Effect of lanthanide ions on equilibrium binding of \(^{45}\)Ca\(^{2+}\) to the SR ATPase transport sites at acid pH: effect of pH. Main frames in panels A and B: the assay medium contained 20 mM Mg\(^{2+}\) and 150 mM MES-Tris at pH 5.5 (solid line), 40 \(\mu\)M (triangles) or 100 \(\mu\)M (circles) added \(^{45}\)Ca\(^{2+}\) plus about 10 \(\mu\)M contaminating and endogenous \(^{45}\)Ca\(^{2+}\), 0.4 or 1 mM \(\text{[Hglucose, various concentrations of lanthanides, i.e. either La}^{3+}\) (panel A) or Pr\(^{3+}\) (panel B), and for most experiments (closed symbols), 0.3 mg/ml of SR protein. For some experiments (open symbols in panel A), the concentration of SR was only 0.1 mg protein/ml. In all cases, 0.3 mg of protein was layered onto a Millipore HA filter and the amount of \(^{45}\)Ca\(^{2+}\) bound to SR was evaluated by double counting. Insets, the amount of \(^{45}\)Ca\(^{2+}\) bound to SR was measured as described for the main frames, but at a single concentration of added \(^{45}\)Ca\(^{2+}\) (40 \(\mu\)M) and SR (0.3 mg of protein/ml), in the absence (open histograms) or presence (hatched histograms) of 0.1 mM of La\(^{3+}\) (inset to panel A) or Pr\(^{3+}\) (inset to panel B). The medium contained 20 mM Mg\(^{2+}\) and was buffered at different pH with either 150 mM MES-Tris (pH 5.5-6.5) or 150 mM TES-Tris (pH 7.0-8.0). Duplicate measurements are shown. The open and closed circles, which are superposed on the data in the inset to panel A, give the results of similar experiments performed in a medium containing 50 mM of the same buffers plus 100 mM KCl, in the absence of La\(^{3+}\) (open circles) and in its presence (closed circles).

described above in the presence of La\(^{3+}\) (panel A) was also performed using an SR concentration three times lower than the one we used in the previous experiments. Results were similar (open versus closed symbols in panel A), thus excluding the possibility that most of the La\(^{3+}\) ions would bind to membrane components under these conditions (see also below, Figs. 5 and 6). As previously observed on fluorescence titrations (Fig. 2C), there was no evidence for positive cooperative interaction between the La\(^{3+}\) ions that displaced \(^{45}\)Ca\(^{2+}\) from the transport sites. Different ionic media were also tested. The efficiency of La\(^{3+}\) was similar in our standard medium or in the presence of 100 mM KCl and 5 mM Mg\(^{2+}\); in the absence of Mg\(^{2+}\), La\(^{3+}\) was slightly more efficient in chasing \(^{45}\)Ca\(^{2+}\), although more \(^{45}\)Ca\(^{2+}\) was initially bound to the ATPase because of the slightly higher affinity of ATPase for Ca\(^{2+}\) in the absence of Mg\(^{2+}\) (data not shown).

We considered the possibility that the relatively low efficiency with which lanthanide ions displaced \(^{45}\)Ca\(^{2+}\) reflected the rather acidic pH (5.5) of our experiments. We then moved back toward neutrality and for a large range of pH values measured the amount of \(^{45}\)Ca\(^{2+}\) bound to the ATPase after adding 40 \(\mu\)M \(^{45}\)Ca\(^{2+}\) in the presence or absence of 100 \(\mu\)M of the lanthanide ions tested. In the presence of Mg\(^{2+}\), this is shown in Fig. 3 as the inset to panel A for La\(^{3+}\) and as the inset to panel B for Pr\(^{3+}\), and similar results were obtained with Gd\(^{3+}\) and Tb\(^{3+}\) (not shown). In the absence of lanthanide (open histograms), the amount of bound \(^{45}\)Ca\(^{2+}\) increased with the pH up to the value corresponding to two Ca\(^{2+}\) ions/ATPase, as expected from the increased affinity of \(^{45}\)Ca\(^{2+}\) for the ATPase. However, at neutral or alkaline pH, 100 \(\mu\)M of the La\(^{3+}\) or Pr\(^{3+}\) ions was proportionally less efficient than at acidic pH in displacing \(^{45}\)Ca\(^{2+}\). At least for La\(^{3+}\), the same was true of all the media tested (e.g. circles in the inset to panel A). For comparison, we also tested the pH dependence of the efficiency of Sr\(^{2+}\) in displacing bound \(^{45}\)Ca\(^{2+}\) since Sr\(^{2+}\), which binds with a low affinity but can be transported into the SR lumen, is known to be a true competitor of Ca\(^{2+}\) (Berman and King, 1990). In contrast with the results obtained with the lanthanides, Sr\(^{2+}\) proved to be equally efficient at all pH in displacing bound \(^{45}\)Ca\(^{2+}\) (not shown). The above results imply that the apparent ATPase affinity for the lanthanide ions which displace Ca\(^{2+}\) is enhanced by raising the pH to an extent smaller than the ATPase affinity for Ca\(^{2+}\).

What Is the True Efficiency of Free La\(^{3+}\) in Displacing Ca\(^{2+}\) Binding to SR ATPase at Neutral pH? Effect of NTA—To exclude the possibility that the relatively low efficiency of the lanthanide ions in displacing bound \(^{45}\)Ca\(^{2+}\) resulted from a dramatic lowering of their free concentration due to their binding to nonspecific sites, especially under neutral or alkaline conditions, we assessed the effect of adding La\(^{3+}\) on \(^{45}\)Ca\(^{2+}\) binding both in the absence and presence of NTA, a strong chelator for lanthanides but a relatively poor one for Ca\(^{2+}\).

These experiments were performed under a set of conditions commonly used for the study of SR ATPase, i.e. at pH 6.8 in the presence of KCl and Mg\(^{2+}\). As illustrated in panel A of Fig. 4, at 0.1 mM NTA (triangles) or 0.4 mM NTA (squares), addition of La\(^{3+}\) only reduced the amount of bound \(^{45}\)Ca\(^{2+}\) when the concentration of La\(^{3+}\) exceeded that of NTA (compare to the control curve in the absence of NTA, circles in panel A). As is clear from these results and illustrated in the inset to panel A, the efficiency with which La\(^{3+}\) displaced \(^{45}\)Ca\(^{2+}\) only depended on the difference \([\text{La}^{3+}]_{\text{total}} - [\text{NTA}]_{\text{total}}\), and the true potency of free La\(^{3+}\) in our experiments was certainly not in the range of La\(^{3+}\) concentrations which can be precisely buffered with NTA (whose apparent dissociation constant at pH 6.8 in the presence of 10 mM Mg\(^{2+}\) is close to 180 nM). This contrasts with previously reported results (Squier et al., 1987, 1990).

We also considered the possibility that La\(^{3+}\) might bind to the sulfonic acid moiety of the buffer used, which would have reduced the free concentration of La\(^{3+}\) available for binding to the ATPase. When measuring the concentration of free La\(^{3+}\) with murexide, we found that this was not the case (see “Experimental Procedures”). Incidentally, a control experiment in this series, particularly relevant for our study, made it clear that there was no mistake in the evaluation of the relative concentrations of La\(^{3+}\) and NTA in our solutions because in the presence of NTA (or EGTA) murexide only detected the presence of La\(^{3+}\) when the concentration of the latter exceeded that of the chelator (Fig. 4, panel B, triangles and squares, respectively).

Under exactly the same ionic conditions, we also explored, in the presence and absence of NTA, the effect of adding La\(^{3+}\) on SR intrinsic fluorescence in the presence of Ca\(^{2+}\) (data not shown). Here again, the presence of 0.4 mM NTA shifted by

This was also the case in the absence of Mg\(^{2+}\), but in that case analysis was less straightforward because, at neutral or at alkaline pH, \(^{45}\)Ca\(^{2+}\) binding at the Ca\(^{2+}\) concentration used was not restricted to the high affinity transport sites.
ATPase high affinity sites resulted in irreversible ATPase inactivation on a time scale of minutes, concomitantly with a tral medium containing KCl but in the presence of solubilizing similar results were obtained (not shown).

CIZE~ and delipidating concentrations of the nonionic detergent obtained at acid pH, and has already been observed under slow slowness can also be distinguished on drift and turbidity problems (see above). Nevertheless, the distinct feature of the La3+-induced drop was its relatively cont of La3+, in a medium containing 80 mM KCl, 10 mM Me, ATPase was measured at pH 6.8 and absence.

Effect of Solubilization and Partial Delipidation—A series of fluorescence experiments was performed in the same neutral medium containing KCl but in the presence of solubilizing and delipidating concentrations of the nonionic detergent C12E6. At 5 mg/ml of this detergent, removal of Ca2+ from the ATPase high affinity sites resulted in irreversible ATPase inactivation on a time scale of minutes, concomitantly with a large gradual reduction in intrinsic fluorescence intensity (Andersen et al., 1982); the presence of Mg2+ afforded partial protection from this inactivation. We found a similar gradual reduction in fluorescence intensity when 0.3 mM La3+ was added to solubilized ATPase, and this too was tentatively attributed to irreversible ATPase inactivation. It was interesting to observe that the initial rate of this detergent-induced “inactivation” in the presence of La3+ was even faster than the rate noted in the presence of EGTA (data not shown), suggesting that La3+ did not simply act by removing Ca2+ from the ATPase high affinity sites but by binding to one or several other sites. Since the ATPase was largely delipidated at the concentration of detergent used (de Foresta et al., 1989), the site or sites concerned are probably located on the ATPase itself and not on the lipids.

Comparison between Tb3+ Binding to the SR Membranes and the Tb3+-induced Displacement of Bound 45Ca2+—Effect of Ca2+ on Bound Tb3+—In order to make a final check of whether or not massive binding of lanthanide ions to the membrane components was responsible for the low apparent potency of lanthanides in displacing 45Ca2+ at neutral pH, and to correlate the lanthanide-induced displacement of 45Ca2+ with the binding of the lanthanide ion itself, we then also measured, under identical conditions, the amount of bound lanthanide ions. This was previously attempted by using radioactive 153Gd3+ (Krasnow, 1977; Squier et al., 1990) or fluorescent Tb3+ (Fairclough et al., 1986; Sprov and Thomas, 1989). We selected Tb3+ as a prototype lanthanide ion; Tb3+ binding to the membrane was assayed by first passing the suspension through a Millipore filter and then measuring the resulting reduction of the Tb3+ concentration in the filtrate, using the fluorescence Tb(DPA)3 assay to detect Tb3+ (Barello and Sherry, 1976). Panel A in Fig. 5 shows a diagram of our protocol, and panel B shows typical recordings. At moderate Tb3+ concentrations in the pH 6.8 medium containing KCl, the amount of Tb3+ bound to the SR membranes could be reliably measured. At higher concentrations, there was more uncertainty because the measured amount of bound Tb3+ resulted from a difference measurement (Tbbound = Tbtot − Tbfree) and because only a relatively small proportion of Tb3+ ions was bound to the membrane at these high concentrations. Nevertheless, panel C makes it perfectly clear that for Tb3+ concentrations which affected the binding of Ca2+, the binding of Tb3+ to the membrane only reduced the free Tb3+ concentrations moderately (as illustrated by the horizontal bar adjacent to the triangles in panel C of Fig. 5). A first conclusion, therefore, is that the apparent efficiency of lanthanide ions in displacing 45Ca2+ deduced from experiments in which only [La3+]tot was plotted, as in Fig. 4 for La3+, is not far from the true value.

A second, even more important conclusion, is that when the amount of bound Tb3+ (triangles in Fig. 5, panel C, left scale) was compared in quantitative terms to the amount of 45Ca2+ bound to the ATPase (crosses, right scale), the number of bound Tb3+ ions vastly exceeded the number of bound Ca2+ ions (compare triangles and squares, now plotted on the same (left) scale). In addition, under these conditions, overall Tb3+ binding to the membranes occurred with an affinity higher than the one with which Tb3+ displaced 45Ca2+. Note that both the Tb3+ and 45Ca2+ binding measurements were performed under exactly the same conditions, after adding 40 μM 45Ca2+, so that comparison of these affinities is perfectly valid.

Measurements of the amount of bound Tb3+ were repeated under acid conditions at pH 5.5, and the results were again compared to the Tb3+-induced displacement of bound 45Ca2+. The same picture emerged as at neutral pH, i.e., Tb3+ only reduced the amount of 45Ca2+ bound to the ATPase after a considerable number of Tb3+ ions had bound to the mem-

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**FIG. 4. Effect of La3+ on 45Ca2+ binding at neutral pH in the absence or presence of NTA.** Panel A, 45Ca2+ binding to the ATPase was measured at pH 6.8 and 20 °C in the presence of various amounts of La3+, in a medium containing 50 mM KCl, 10 mM Mg2+, 50 μM total Ca2+ and 50 mM MOPS-KOH, in the absence of NTA (circles) or the presence of either 0.1 mM NTA (triangles) or 0.4 mM NTA (squares). 45Ca2+ (40 μM had been added) and [3H]glucose were also present together with 0.3 mg/ml SR protein. The arrow indicates the abcissa corresponding to 0.4 mM La3+. Inset, the same data plotted as a function of the excess of La3+ over NTA. Similar results were obtained when SR intrinsic fluorescence was measured in the presence of various amounts of La3+, without NTA or with 0.4 mM NTA (not shown). Panel B, control experiment, performed with murexide but without SR membranes, showing the change in murexide absorbance (OD410-OD400) as a function of the La3+ added in the absence of NTA (circles) or the presence of 0.4 mM NTA (squares) or EGTA (triangles). The medium contained 5 mM MOPS-Tris (pH 6.8) and 40 μM murexide. Arrow indicates the abcissa corresponding to 0.4 mM La3+.
by Ca²⁺, by simply adding Ca²⁺ at concentrations which, periods between 15 min and 15 min did not modify the amount of bound Tb³⁺. Panel B shows the actual assay, when C₅, C₆, E₅, and E₆ fractions were assayed for Tb³⁺ by adding aliquots to a 2-ml fluorimeter cuvette containing 200 µM DPA (see “Experimental Procedures” for analysis). Panel C shows the results: triangles, Tb³⁺-binding data (left scale); crosses, Ca²⁺-binding data (right scale). Squares refer to the same Ca²⁺ data as the crosses but are plotted here on the same scale (left) as the Tb³⁺-binding data. Free Tb³⁺, not total Tb³⁺, is plotted on the abscissa.

Moreover, under these acid conditions, it was possible to measure the amount of Tb³⁺ bound with or without simultaneous saturation of the transport sites by Ca²⁺, by simply adding Ca²⁺ at concentrations which, according to control fluorescence experiments similar to those shown in Fig. 1 for La³⁺, did saturate the transport sites despite the presence of Tb³⁺ in the medium. It is highly significant that in those experiments addition of Ca²⁺ did not measurably reduce the amount of bound Tb³⁺ (panel B). This agrees with similar results previously reported (Sprowl and Thomas, 1989).

From a theoretical point of view, in an equilibrium situation, one would expect that if the presence of Tb³⁺ reduces the affinity of ATPase for Ca²⁺, the presence of Ca²⁺ would also reduce its affinity for Tb³⁺, at least for the Tb³⁺ ions responsible for displacement of the bound Ca²⁺, leading to a Ca²⁺-induced reduction of the amount of bound Tb³⁺ ions observable under certain conditions. However, due to the relatively modest affinity with which Tb³⁺ (like other lanthanide ions) displaced Ca²⁺, this Ca²⁺-induced reduction of the amount of bound Tb³⁺ ions is probably only observable at relatively high Tb³⁺ concentrations, i.e., when the expected drop in bound Tb³⁺, which is likely to concern only one Tb³⁺ ion/ATPase molecule (see “Discussion”), is smaller than the error bar in our difference measurements (see Fig. 5 or 6). This probably explains why the reduction expected at high Tb³⁺ concentrations could not be resolved in our experiments. Conversely, the clear absence of any significant Ca²⁺-induced reduction of bound Tb³⁺ at low Tb³⁺ concentrations (Fig. 6B) shows that those Tb³⁺ ions which bind to the ATPase first, at low Tb³⁺ concentrations, are not displaced when Ca²⁺ binds to its own sites.

Time-resolved Measurements of Ca²⁺ Dissociation in the Presence of Ln³⁺—The above results for Tb³⁺ binding and Ca²⁺ displacement exclude the possibility of competition between Ca²⁺ ions and the Tb³⁺ ions which bind at the sites of highest affinity. They show that the apparent competition between Tb³⁺ and Ca²⁺ evidenced by the equilibrium measurements reported in Figs. 1–4 is due to Tb³⁺ binding to another class of sites. Even these sites, however, are not necessarily the Ca²⁺ sites. As is well known, equilibrium measurements do not generally permit discrimination between the various molecular mechanisms which lead to apparent competition, only some of which are really based on true competition for a common site at the molecular level. Under favorable conditions, kinetic measurements do allow such discrimination. As we already pointed out, addition of La³⁺ to Ca²⁺-saturated ATPase only reduced its fluorescence level slowly (Fig. 1D), so that kinetic effects could be suspected. We therefore attempted to characterize, by rapid
filtration techniques, the effect of lanthanide ions on the ATPase high affinity Ca\(^{2+}\)-binding sites, using an experimental protocol capable of detecting any true competition between Ca\(^{2+}\) and the lanthanide ions for binding at these sites. Accordingly, \(^{45}\)Ca\(^{2+}\)-equilibrated SR membranes were layered onto a filter in a rapid filtration apparatus and perfused for electronically controlled periods with an appropriate dissociation buffer. In such experiments, the presence of a calcium analog in the perfusion medium is expected to slow down dramatically the rate of dissociation of the second, deeply buried \(^{45}\)Ca\(^{2+}\) ion, whose dissociation is prevented by the binding of the analog to the superficial subsite after the first \(^{45}\)Ca\(^{2+}\) ion has left it, just as the binding of \(^{45}\)Ca\(^{2+}\) would (Dupont, 1984; Inesi, 1987; Petithory and Jencks, 1988; Orlovski and Champel, 1991).

The first functional indication that lanthanides might bind to the ATPase at a location other than the Ca\(^{2+}\) transport sites came from the previous observation that the presence of 10 mM La\(^{3+}\) instead of \(^{45}\)Ca\(^{2+}\) in the perfusion medium considerably reduced the rate of dissociation of the first \(^{45}\)Ca\(^{2+}\) ion (Inesi, 1987). We were able to reproduce this result here (not shown), but it might have been obtained because of the very high La\(^{3+}\) concentration used. We therefore repeated the experiment using lower La\(^{3+}\) concentrations (100 \(\mu\)M or 1 mM), and we also included \(^{40}\)Ca\(^{2+}\) in the perfusion medium so that the conditions during perfusion corresponded to those previously found, at neutral pH, to allow partial displacement of \(^{45}\)Ca\(^{2+}\) (100 \(\mu\)M La\(^{3+}\)) or total displacement (1 mM La\(^{3+}\)), in accordance with the equilibrium measurements made under exactly the same ionic conditions (Fig. 4A). Fig. 7 shows that 100 \(\mu\)M La\(^{3+}\) (open triangles) already reduced the initial dissociation rate of the first, superficially bound \(^{45}\)Ca\(^{2+}\) by more than half (compare to circles) and that the effect of 1 mM La\(^{3+}\) (closed triangles) was still greater. Moderate concentrations of La\(^{3+}\) therefore did perturb the dissociation of the superficially bound \(^{45}\)Ca\(^{2+}\) ion. Similar results were obtained at pH 6, both with La\(^{3+}\) and Tb\(^{3+}\) (data not shown).

Even clearer results were obtained at pH 5.5 (Fig. 8). Here again, \(^{45}\)Ca\(^{2+}\)-equilibrated SR membranes were perfused with various media. Unlike dissociation in a Ca\(^{2+}\)-free medium (squares in panel A), the presence of 100 \(\mu\)M \(^{45}\)Ca\(^{2+}\) in the perfusion medium (open circles in panel A) rendered the \(^{45}\)Ca\(^{2+}\) dissociation kinetics clearly biphasic, although dissociation of the second \(^{45}\)Ca\(^{2+}\) ion was not completely prevented at this concentration, in agreement with the high cooperativity but the poor affinity of the ATPase for Ca\(^{2+}\) at this acid pH (which is responsible for partial site saturation at time zero, see also Fig. 4 in Orlovski and Champel, 1991). In contrast, the presence of 100 \(\mu\)M La\(^{3+}\) instead of \(^{45}\)Ca\(^{2+}\) (open triangles in panel A) did not slow down the dissociation rate of the second \(^{45}\)Ca\(^{2+}\) ion, at least at this pH since the apparent affinity with which La\(^{3+}\) chases Ca\(^{2+}\) at pH 5.5 is in fact higher than the apparent affinity of the ATPase for Ca\(^{2+}\) itself (about 10–30 \(\sim\) versus 50–100 \(\mu\)M, as deduced from extrapolation to zero Ca\(^{2+}\) and La\(^{3+}\) in Fig. 2, panels D and E, respectively). However, this was not the case (compare triangles to circles). Furthermore, although adding 500 \(\mu\)M \(^{45}\)Ca\(^{2+}\) to the initial 100 \(\mu\)M \(^{45}\)Ca\(^{2+}\) in the perfusion medium rendered the biphasic behavior even clearer, as expected (closed circles in panel B), adding 500 \(\mu\)M La\(^{3+}\) instead of \(^{45}\)Ca\(^{2+}\) to the 100 \(\mu\)M \(^{45}\)Ca\(^{2+}\) in the perfusion medium (closed triangles in panel B) again only slowed down the dissociation of the first \(^{45}\)Ca\(^{2+}\) ion without specifically preventing the second from leaving its own subsite. Note that in equilibrium experiments under the latter conditions, 80\% of the bound \(^{45}\)Ca\(^{2+}\) was displaced by La\(^{3+}\) (Fig. 3A).

**DISCUSSION**

The experiments reported here were performed under both neutral and acid conditions. The latter conditions were designed to enable us to obtain easily an ATPase with Ca\(^{2+}\)-depleted binding sites and thus avoid preliminary treatment of the membranes with chelating resins. At pH 5.5 when no Ca\(^{2+}\) was added, the great pH dependence of the ATPase affinity for Ca\(^{2+}\) made it possible to add lanthanide ions to samples of ATPase with unoccupied transport sites. In preliminary fluorescence measurements, we found that lanthanide ions added to Ca\(^{2+}\)-free ATPase slightly raised its intrinsic fluorescence (Fig. 1, trace C), an effect consistent with pre-

![Fig. 7. Effect of La\(^{3+}\) on the kinetics of \(^{45}\)Ca\(^{2+}\) dissociation at pH 5.5. 0.3 mg of SR membrane protein, previously equilibrated in the pH 5.5 medium to which 100 \(\mu\)M \(^{45}\)Ca\(^{2+}\) had been added, was layered onto a filter and perfused for various periods with the pH 5.5 medium, which in addition contained one of the following: 2 mM EGTA (squares), 50 \(\mu\)M total \(^{45}\)Ca\(^{2+}\) (open circles), 50 \(\mu\)M total \(^{45}\)Ca\(^{2+}\) plus 0.1 mM La\(^{3+}\) (open triangles), or 50 \(\mu\)M total \(^{45}\)Ca\(^{2+}\) plus 1 mM La\(^{3+}\) (closed triangles).](image)

![Fig. 8. Effect of La\(^{3+}\) on the kinetics of \(^{45}\)Ca\(^{2+}\) dissociation at pH 5.5. 0.3 mg of SR membrane protein, previously equilibrated in the pH 5.5 medium to which 100 \(\mu\)M \(^{45}\)Ca\(^{2+}\) had been added, was layered onto a filter and perfused for various periods with the pH 5.5 medium, which in addition contained one of the following: 2 mM EGTA (squares in panel A and B), 100 \(\mu\)M total \(^{45}\)Ca\(^{2+}\) (open circles in panel A), 100 \(\mu\)M La\(^{3+}\) (open triangles in panel A), 600 \(\mu\)M total \(^{45}\)Ca\(^{2+}\) (closed circles in panel B), or 100 \(\mu\)M total \(^{45}\)Ca\(^{2+}\) plus 500 \(\mu\)M La\(^{3+}\) (closed triangles in panel B).](image)
vions observations by Jona and Martonosi (1986) and Ima-
mura and Kawakita (1991a) but different from the absence of
effect reported by Ogurusu et al. (1991), who removed endog-
enous Ca\(^{2+}\) from SR membranes but apparently did not elimi-
nate contaminating Ca\(^{2+}\) from the buffers. On the time scale
of minutes used here, our acid treatment did not reduce AT-
Pase stability (see Fig. 1), and the procedure was a con-
venient substitute for the more laborious technique of Ca\(^{2+}\)
depletion with chelating resins (Itoh and Kawakita, 1984;
Jona and Martonosi, 1986; Sprowl and Thomas, 1989; Ima-
mura and Kawakita, 1991a). Under these conditions, we could
then study the apparent competition between Ln\(^{3+}\) and Ca\(^{2+}\)
ions through fluorescence (Fig. 2) and "Ca\(^{2+}\)" binding experi-
ments (Fig. 3). The latter experiments showed that both Ca\(^{2+}\)
ions were displaced from their binding sites by high concen-
trations of Ln\(^{3+}\) ions. However, the apparent competition
between Ln\(^{3+}\) and Ca\(^{2+}\) ions had three puzzling features (Fig.
2); (i) La\(^{3+}\) ions reduced fluorescence without showing any
sign of positive cooperativity between them (panel C in Fig.
2), whereas Ca\(^{2+}\)-induced changes were clearly cooperative, as
is well known (see circles in panel A); (ii) in panels B and D
of Fig. 2, the plots of [Ca\(^{2+}\)]\(e\) versus [La\(^{3+}\)] and [La\(^{3+}\)]
versus [Ca\(^{2+}\)] were not straight lines, but their concavities were
of opposite signs; this, combined with the observation (i) above,
would be compatible with one La\(^{3+}\) ion being responsible for
replacing the two Ca\(^{2+}\) ions from the ATPase-binding sites;
(iii) positive cooperativity between Ca\(^{2+}\) ions was lost or re-
duced\(^{6}\) in the presence of La\(^{3+}\) (compare squares to circles
in panel A of Fig. 2); this observation is not easily reconciled
with true competition for binding at a common site. In addi-
tion, when experiments were performed under various pH
conditions, the pH dependence of the apparent competition
between Ca\(^{2+}\) and lanthanide ions (see insets in Fig. 3) was
different from that of the competition between Ca\(^{2+}\) and Sr\(^{2+}\),
a true analog of Ca\(^{2+}\). Note also that La\(^{3+}\) and Ca\(^{2+}\) have dif-
ferent effects on ATPase intrinsic fluorescence (Fig. 1, C and
D), whereas Sr\(^{2+}\) and Ca\(^{2+}\) have similar effects (Holguin,
1986 and data not shown), which is not in favor of La\(^{3+}\) being
a good analog of Ca\(^{2+}\) for binding to the ATPase transport
sites.

A distinct feature of our measurements both at acid and
neutral pH was that Ln\(^{3+}\) displaced "Ca\(^{2+}\)" with a relatively
modest affinity (see Figs. 3, 4A, 5C, and 6A). It has been
previously suggested that the apparently poor efficiency of
added lanthanide ions in displacing bound Ca\(^{2+}\) was mislead-
ing because it reflected the binding of these ions to mem-
branes, vessels walls, or buffer components (Squier et al.,
1990). In the presence of NTA, a chelator relatively specific
for lanthanide ions, the free La\(^{3+}\) concentrations allowing
displacement of half the bound Ca\(^{2+}\) at neutral pH were
*calculated* to fall within the 15–100 nanomolar range as op-
posed to the 10–100 \(\mu\)M range in terms of total concentra-
tions (Squier et al., 1987, 1990). We devoted much effort to checking
this point but our results did not support this previous sug-
gestion. As stated under "Experimental Procedures," we de-
tected no binding of Tb\(^{3+}\) on the walls of plastic vessels in
our experiments and no complex formation between La\(^{3+}\),
Tb\(^{3+}\), or Gd\(^{3+}\) ions on the one hand and either MOPS or MES
on the other. Although we did observe binding to glassware,
quartz, Teflon, or cellulose esters, it was certainly not respon-
sible for a large change in the free Tb\(^{3+}\) concentration in the
 corresponding experiments (in fact, binding was previously
reported to take place on a time scale of days rather than, as
here, of minutes; see Ellis and Morrison, 1975). In our exper-
iments performed in the presence of NTA, the effects of
lanthanide ions were only observed when their concentration
exceeded that of NTA (this is an unexplained experimental
discrepancy with the results reported by Squier et al. (1987,
1990) and Girardet et al. (1989)), so that a submicromolar
efficiency of lanthanide ions could not be confirmed. Lastly,
Tb\(^{3+}\) binding to either the protein or the lipid constituents of
SR membranes (see Herrmann et al., 1986b) did occur (Figs.
5 and 6) up to high levels, but these levels, which were similar
to those previously reported for Tb\(^{3+}\) and Gd\(^{3+}\) (Krasnow,
1977; Sprowl and Thomas, 1989; Squier et al., 1990), were
certainly not responsible for a drop by several orders of
magnitude in the free Ln\(^{3+}\) concentration during the actual
experiments.

We found that bound Tb\(^{3+}\) only reduced the amount of Ca\(^{2+}\)
bound to the ATPase after a considerable number of Tb\(^{3+}\)
ions had been bound, corresponding to 4–10 times the sto-
ichiometry of high affinity Ca\(^{2+}\) binding to the transport sites
(Figs. 5C and 6A). Moreover, Tb\(^{3+}\) ions bound at low pH,
which bound to the ATPase at low Tb\(^{3+}\) concentrations
were not displaced when Ca\(^{2+}\) bound to its own sites (Fig. 6, panel
B). In a previous work, Sprowl and Thomas already suggested
that measurement of the amount of lanthanide bound in the
absence or presence of Ca\(^{2+}\) was one of the critical experiments
to be done to describe the apparent competition between Ca\(^{2+}\)
and Ln\(^{3+}\) for ATPase-binding sites. These authors, who first
fertilized SR membranes and buffers with a Ca\(^{2+}\)-chelating resin
to reduce their Ca\(^{2+}\) contents, also made parallel measure-
ments of bound "Ca\(^{2+}\)" and Tb\(^{3+}\), but after equilibrium dialysis
under neutral pH conditions (Sprowl and Thomas, 1989).
In our laboratory, we found that an acid pH was suitable for
depleting the ATPase transport sites of Ca\(^{2+}\), and we preferred
filtration because it was a faster way of separating bound and
free Tb\(^{3+}\), thus avoiding possible problems due to lengthy
dialysis in the absence of Ca\(^{2+}\). Both Sprowl and Thomas
and ourselves found that addition of Ca\(^{2+}\) did not displace the
Tb\(^{3+}\) ions bound at low Tb\(^{3+}\) concentrations. The different
approaches by the two groups may have pitfalls, but different
ones, so that the identical results they produced strengthen
our conclusion that among the many sites on SR membranes
to which Tb\(^{3+}\) ions bind, those to which these ions bind with
the highest affinity are not the Ca\(^{2+}\) high affinity binding and
transport sites. Since La\(^{3+}\), Tb\(^{3+}\), Pr\(^{3+}\), and Gd\(^{3+}\) ions all
behave similarly in fluorescence and "Ca\(^{2+}\)" binding meas-
urements (see "Results"), it seems reasonable to assume that
this conclusion can be extended to all lanthanide ions. Indeed,
a similar result has been very recently suggested for Gd\(^{3+}\) by
Imamura and Kawakita (1991a) and Ogurusu et al. (1991),
although in the absence of direct measurements of the amount
of bound lanthanide. Note that it was previously shown that
Mg\(^{2+}\), but not Ca\(^{2+}\), had the capability of displacing Tb\(^{3+}\) from
a particular subset of their binding sites (Highsmith and
Head, 1983).

Obviously, the above conclusion has dramatic implications
for the significance of the various previous attempts (quoted
in the Introduction) to exploit the favorable physical proper-
ties of some of the lanthanide ions to learn something about
the ATPase Ca\(^{2+}\)-transport sites. In those studies, in order to
collect information about a reasonably homogeneous class of
binding sites, most authors added limited amounts of lan-
thanide ions to fairly high concentrations of ATPase, so that
the binding stoichiometry was often below 2 Ln\(^{3+}\) ions/ATP-
ase chain. Under these conditions, only those sites with the
highest affinity for Ln\(^{3+}\) were labeled, and unfortunately they

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\(^{6}\) It is noteworthy that a change in the cooperativity of Ca\(^{2+}\) binding to SR membrane ATPase in the presence of Tb\(^{3+}\) was also reported by Sprowl and Thomas (1989), although in the opposite direction. We do not know the reason for this discrepancy.
do not appear to be the Ca\(^{2+}\) transport sites (Figs. 5 and 6). Irrespective of the controversy already discussed about effective free lanthanide concentrations, a similar situation in fact prevailed in the experiments of Squier et al. (1990), although in that case the total concentration of added Ln\(^{3+}\) greatly exceeded the concentration of ATPase active sites (Squier et al., 1990): in these experiments, 14 nmol of Gd\(^{3+}\) ions/mg protein had to bind to the ATPase before less than 2 nmol of Ca\(^{44}\)+ ions/mg protein could be displaced (pGd = 8 in Fig. 6 of Squier et al., 1990). Therefore, for instance, when Pr\(^{3+}\) was added to ANS-maleimide-labeled SR, it is likely or at least possible, that the energy transfer from covalently bound ANS-maleimide to Pr\(^{3+}\) only monitored the binding of Pr\(^{3+}\) at this lanthanide’s high affinity binding site(s), distinct from the high affinity Ca\(^{2+}\) transport sites. In fact, this energy transfer responded to slightly lower concentrations of Pr\(^{3+}\) than the displacement of bound Ca\(^{44}\)+ (Fig. 8 in Squier et al., 1990).

At pH 6.8, SR membranes displayed a slightly higher overall affinity for Tb\(^{3+}\) than at pH 5.5 (cf. Figs. 5C and 6A). The above considerations about the binding sites with the highest affinity for Ln\(^{3+}\) are based on the assumption that classes of Ln\(^{3+}\) sites with different affinities exist on SR membranes, which is the case at neutral pH when Tb\(^{3+}\) displaced Ca\(^{2+}\) with an apparent affinity poorer than the one for overall Tb\(^{3+}\) binding (Fig. 5) and where Gd\(^{3+}\) enhanced the intrinsic fluorescence of SR ATPase at concentrations lower than those displacing Ca\(^{2+}\) (Imamura and Kawakita, 1991a). At acid pH, however, this is not necessarily the case, based on the virtually indistinguishable affinities for overall binding of Tb\(^{3+}\) and for Ca\(^{44}\)+ displacement (Fig. 6A) and the monotonous reduction by lanthanide ions of the fluorescence of ATPase in the presence of Ca\(^{2+}\) (Figs. 1D and 2C). Nevertheless, as clearly shown by the data in Fig. 6B, even at pH 5.5 the lanthanide ions which displace Ca\(^{2+}\) are only a very small fraction of all the lanthanide ions bound at a given concentration (Fig. 6A; we suspect these ions to be in a 1 to 2 stoichiometry with respect to the bound Ca\(^{2+}\) ions, see above). At this pH too, the contribution of these particular ions is therefore not easy to distinguish when measuring the overall spectroscopic properties of all bound ions.

In addition to these measurements of Tb\(^{3+}\) binding per se, our rapid filtration measurements of the rate of Ca\(^{44}\)+ dissociation (Figs. 7 and 8) showed that at moderate concentrations lanthanide ions bind to sites where they affect the dissociation of Ca\(^{44}\)+ from the transport sites (and also its binding). In other words, such rapid filtration measurements suggest that moderate lanthanide ion concentrations exert allosteric rather than direct competitive effects on Ca\(^{2+}\) binding, a conclusion we were able to reach thanks to the use of kinetic methods and not only equilibrium methods. In addition, after dissociation of the superficially bound Ca\(^{44}\)+ ion, Ln\(^{3+}\) did not specifically prevent the deeply bound Ca\(^{44}\)+ ion from leaving its own subsite (Fig. 8). After completion of our work, experiments along the same rationale were also reported by Ogurusu et al. (1991) with similar results. Our conclusion is that although these results do not strictly exclude that at high concentrations Ln\(^{3+}\) ions might eventually bind to the transmembrane Ca\(^{2+}\)-ATPase (Figs. 5 and 6). However, in the experiment illustrated by this figure, when Ca\(^{44}\)+ dissociation in the presence of Ln\(^{3+}\) was measured, Ca\(^{44}\)+ was present in the perfusion medium as well as the added Ln\(^{3+}\); it is not possible to deduce from this figure that Ln\(^{3+}\) rather than simply Ca\(^{44}\)+ was responsible for the slow dissociation rate observed for the second Ca\(^{2+}\) ion.

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