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# EFFECTS OF LANTHANUM AND GADOLINIUM IONS ON CARDIAC SARCOPLASMIC RETICULUM

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SUMMARY

The tervalent rare earth cations lanthanum ($La^{3+}$) and gadolinium ($Gd^{3+}$) have been studied for their effect on canine cardiac sarcoplasmic reticulum. At dose-related concentrations above 10 $\mu$M, $La^{3+}$ and $Gd^{3+}$ inhibit calcium binding non-competitively, decrease the velocity of calcium uptake (in presence of oxalate), and inhibit the $Ca^{2+}$-activated $Mg^{2+}$-ATPase. The tendency for sarcoplasmic reticulum inhibition by excess $Ca^{2+}$ is accentuated by lanthanides. The effect of the lanthanides is time-dependent, inhibition diminishing during incubation. Since effects were observed in the presence of oxalate, the reactive lanthanide ion concentration is not as predicted from solubility-product considerations.

## INTRODUCTION

The tervalent cation lanthanum ($La^{3+}$) has potent effects in low concentration on the electrical potentials of nerve[1,2] and neuromuscular junction[3], and on force generation of skeletal[4], cardiac[5,6] and smooth muscle[7,8]. These effects seem to arise from interference by $La^{3+}$ with movements of $Ca^{2+}$ across membranes, and studies of mitochondrial[9–15] or artificial phospholipid membranes[16,17] support this general conclusion. Sanborn and Langer[6] found a loss of tension development in cardiac muscle, and from kinetic analysis of the washout rate of perfused labelled calcium, concluded that the $La^{3+}$ effect was on the tissue component responsible for excitation–contraction coupling. However, Entman *et al.*[18] found no effect of $La^{3+}$ on microsomes isolated from the dog heart.

The present study demonstrates that physiologically effective concentrations of added $La^{3+}$ and $Gd^{3+}$ exert significant effects on the binding of calcium ions, and the calcium uptake in the presence of oxalate, by isolated cardiac sarcoplasmic reticulum. Concomitantly, these ions depress the associated $Ca^{2+}$-activated ATPase.

Abbreviation: EGTA, glycol ether diaminetetraacetic acid.

## MATERIALS AND METHODS

*Preparative procedure*: Healthy mongrel dogs (15–22 kg) were anesthetized lightly with intravenous pentobarbital (10 mg/kg) and the heart excised while beating. It was washed, trimmed of gross fat and connective tissue, and 75–100 g of left ventricle were diced into small pieces. All preparative procedures were carried out at 0–4 °C. 50-g portions of muscle were added to 4 vol. of 0.3 M sucrose–10 mM histidine–Tris, pH 7.4, and homogenized in a Waring blender for 35–40 s. The homogenate was centrifuged at 9000 × *g* for 30 min; the supernatant was centrifuged at 38000 × *g* for 60 min; this pellet was resuspended by a Teflon pestle in one-third the original volume of homogenizing medium and then centrifuged at 9000 × *g* for 30 min. The supernatant was centrifuged at 38000 × *g* for 60 min, and the final pellet of microsomes resuspended by a Teflon pestle in a small volume of 0.3 M sucrose–10 mM histidine–Tris, pH 7.4, to a final concentration of protein of 3 to 7 mg/ml. Protein was determined by a biuret reaction, standardized with bovine serum albumin. This fraction of cardiac microsomes was considered to be predominantly sarcoplasmic reticulum since earlier work has shown no effect of sodium azide or other inhibitors of mitochondrial functions on the calcium uptake by these membrane fragments. Electron microscopy of the pellet, performed through the courtesy of Dr J. Stempak, showed small vesicular structures without internal organization or cristae.

### *Calcium binding* and uptake assays*

Binding experiments (oxalate absent) were performed on the day of preparation of vesicles; uptake experiments (oxalate present) and ATPase determinations were performed on the following day after overnight storage at −20 °C. Only a few experiments were performed 2 days after preparation of the vesicles. The yield of active preparations of sarcoplasmic reticulum was about 0.5 mg/g wet tissue.

Binding and uptake of calcium were assayed at 37 °C using 0.45-$\mu$m Millipore filters and positive pressure filtration. The assay medium consisted of 50 mM KCl, 10 mM histidine–Tris, pH 7.0, 5 mM MgATP, and 10–100 $\mu$M $^{45}CaCl_2$. For binding studies, sarcoplasmic reticulum protein concentration was 0.2–0.3 mg/ml; for uptake determinations, sarcoplasmic reticulum protein concentration was 0.05 or 0.10 mg/ml, with 5 mM ammonium oxalate added. $La^{3+}$ or $Gd^{3+}$ were added to the medium prior to initiation of the reaction, in a final concentration of 150–200 $\mu$M for binding and 150–300 $\mu$M for uptake experiments. The reaction was initiated by addition of $^{45}CaCl_2$ to the medium. Aliquots of the assay were filtered at appropriate times, and 0.1 ml of filtrate added to a toluene–alcohol–PPO mixture and counted in a Packard Tri-Carb 3375 liquid scintillation counter. Binding or uptake was calculated as the percentage change in radioactivity of the medium compared to an unfiltered sample, multiplied by the concentration of added unlabelled calcium. Contamination of reagents by calcium was not included.

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* The term calcium "binding" as used here is not meant to define the physical state of the calcium in the vesicles, whether as the free ion in the interior, or bound to the vesicular membrane, or both. The mechanism of calcium accumulation in the absence of oxalate remains undecided[19]. In the presence of oxalate, it has been clearly shown that calcium uptake involves transport into the interior of the vesicle and precipitation as calcium oxalate.

*ATPase assay*

Separate assays were performed, usually simultaneously with the binding or uptake studies. The medium consisted of 50 mM KCl, 10 mM histidine–Tris, pH 7.0, 5 mM MgATP, 50 $\mu$M $CaCl_2$, 0.2 mg sarcoplasmic reticulum protein/ml. In some experiments, glycol ether diaminetetraacetic acid 0.5 mM (EGTA) was added. $La^{3+}$ and $Gd^{3+}$ concentrations were 200 $\mu$M or as specified. Aliquots were taken at 10 and 20 min of incubation at 37 °C, and inorganic phosphorous measured by the Fiske and SubbaRow method.

*Reagents*

Special enzyme-grade sucrose was purchased from Mann Pharmaceuticals; MgATP and Tris buffer were obtained from Sigma Chemical Co. Histidine was obtained from California Biochemical Co. All reagents were of reagent-grade purity. Triply-distilled demineralized water was used throughout.

Statistical significance was determined from the Student t test for paired determinations.

## RESULTS

*Calcium binding*

Vesicles exhibited saturation of calcium binding (Fig. 1A), half-maximum binding occurring at a medium $Ca^{2+}$ of 17 $\mu$M. Binding was inhibited 22.7 % by $La^{3+}$ and to a somewhat lesser extent (17.2 %) by $Gd^{3+}$. Binding inhibition appeared competitive, and double-reciprocal plots show non-intersecting lines. The Scatchard plot (Fig. 1B) was linear, and showed (abscissa intercept) a decrease in the number of calcium-binding sites available in the presence of the lanthanide.

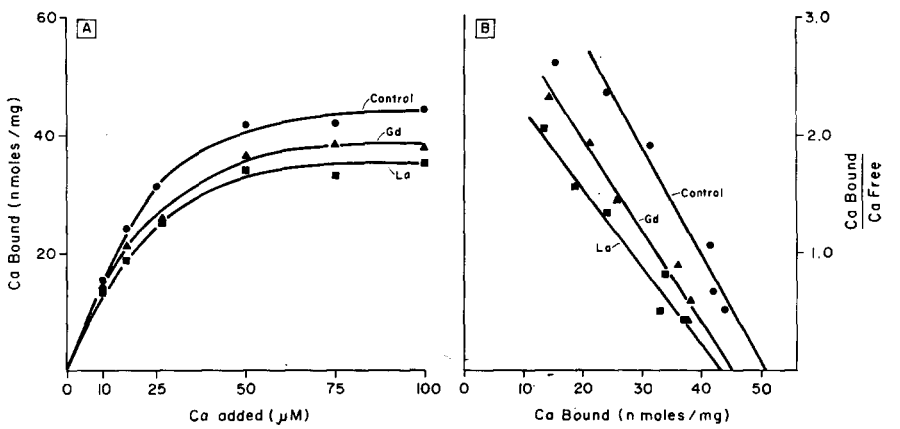


Fig. 1. Linear (A) and Scatchard plots (B) of calcium binding as a function of added $Ca^{2+}$ concentration. Effects of $La^{3+}$ (■) and $Gd^{3+}$ (▲), each 200 $\mu$M. Conditions: 5 mM MgATP, 50 mM KCl, 10 mM histidine–Tris buffer, pH 7.0. Temperature, 37 °C. Binding determined at 2 min incubation, using 0.2 mg/ml sarcoplasmic reticulum protein in a volume of 4 ml. Points are means of 8 experiments.

*Calcium uptake*

*Control studies*

Steady-state uptake of calcium in the presence of 5 mM oxalate was linear with medium $Ca^{2+}$ concentration in control vesicles and was complete (> 99%) by 10 min of incubation. Thus uptake was limited only by calcium availability. As medium $Ca^{2+}$ concentration was increased to 75 or 100 $\mu$M, there was a depression of the initial velocity of uptake (Fig. 2). However, the velocity of uptake increased as

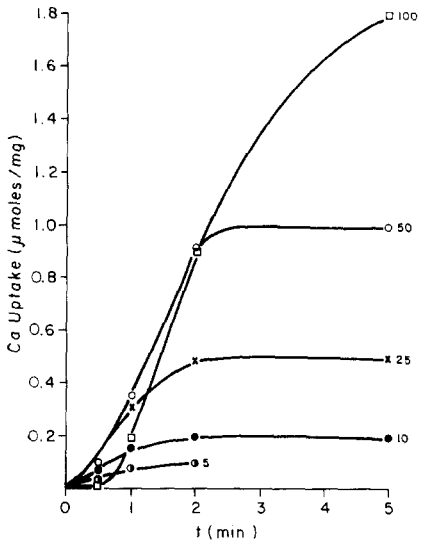


Fig. 2. Calcium uptake at varying $Ca^{2+}$ concentrations. Mean of eight experiments. Numbers along curves refer to medium $Ca^{2+}$ concentration ($\mu$M). Conditions: 5 mM ammonium oxalate, 5 mM MgATP, 50 mM KCl, 10 mM histidine–Tris buffer, pH 7.0, 0.05 mg/ml sarcoplasmic reticulum protein in an assay volume of 4 ml. At 0.5 and 1.0 min of incubation, calcium uptake in a medium $Ca^{2+}$ of 100 $\mu$M is less than uptake in the medium $Ca^{2+}$ of 25 or 50 $\mu$M.

incubation progressed in the high-$Ca^{2+}$ medium, so the final uptake, given sufficient time for the reaction, was proportional to and limited by $Ca^{2+}$ concentration in the medium.

*Lanthanide effects*

The effects of $La^{3+}$ and $Gd^{3+}$ on calcium uptake in the presence of oxalate were demonstrable at concentrations of lanthanide as low as 10 $\mu$M (Fig. 3) and in some experiments at 5 $\mu$M. 50% inhibition of uptake was seen at $La^{3+}$ concentrations of 125 $\mu$M and slightly higher values for $Gd^{3+}$.

The major effect of the lanthanides was to decrease the initial velocity of calcium uptake. As incubation proceeded, the initial depression by lanthanide was overcome, and calcium uptake velocity later in the incubation period increased. Thus the slope of calcium uptake even at 500 $\mu$M $La^{3+}$ concentration was almost equal to the control slope after 1 min of incubation (Fig. 4).

The slowing of the initial velocity of uptake, with later rise in velocity during incubation, is similar to the slowed initial uptake rate seen when control vesicles are loaded heavily with calcium in the absence of $La^{3+}$ or $Gd^{3+}$.

The marked depression of initial velocity decreased the uptake achieved during the given period of incubation, especially when the vesicles were heavily loaded with calcium. The discrepancy between control and lanthanide-treated vesicles diminished as incubation proceeded (Fig. 5) and thus there was no decrease in final uptake by lanthanide treatment.

A double-reciprocal plot of calcium uptake *vs* medium $Ca^{2+}$ concentration at 1 or 2 min of incubation suggested non-competitive inhibition by $La^{3+}$ or $Gd^{3+}$. The slope of the plot decreased with time of incubation, and approached the control, as the lanthanide inhibition diminished in the course of the incubation.

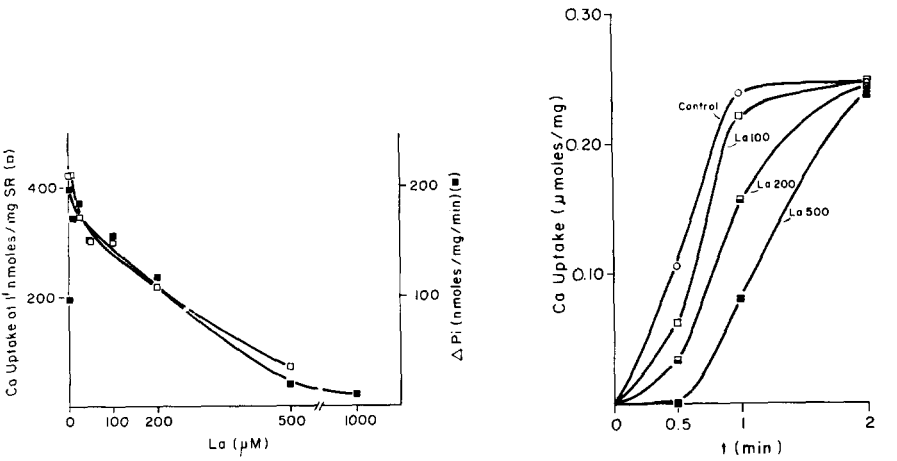


Fig. 3. Inhibition of calcium uptake (□) and ATP hydrolysis (■) as a function of $La^{3+}$ concentration. Calcium uptake determined in the presence of 5 mM oxalate after 1 min incubation. Conditions: for uptake: $^{45}Ca^{2+}$, 50 $\mu$M, sarcoplasmic reticulum protein, 0.05 mg/ml; for ATP hydrolysis: $Ca^{2+}$, 50 $\mu$M; sarcoplasmic reticulum protein, 0.2 mg/ml; no oxalate. Assay medium under Methods. The basal $Mg^{2+}$-ATPase in the absence of $La^{3+}$ or $Ca^{2+}$, with 0.5 mM EGTA present, is indicated (bisected square). SR = sarcoplasmic reticulum protein.

Fig. 4. Time-dependent inhibition of calcium uptake by $La^{3+}$. Medium contained $^{45}Ca^{2+}$, 25 $\mu$M; sarcoplasmic reticulum protein, 0.1 mg/ml; other conditions as in Fig. 2. Numbers refer to concentration of added $La^{3+}$ ($\mu$M).

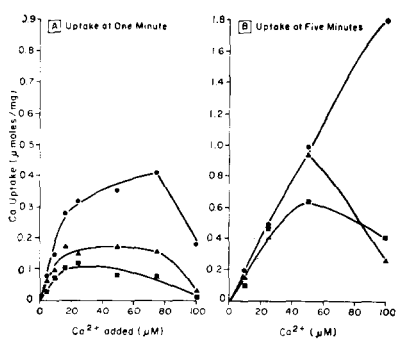


Fig. 5. Decreasing $La^{3+}$ inhibition in the course of incubation. $La^{3+}$ (■), 150–200 $\mu$M; $Gd^{3+}$ (▲), 150–300 $\mu$M; control vesicles (●). Sarcoplasmic reticulum protein, 0.05 mg/ml. Other conditions as in Fig. 2. Points are means of 2–8 experiments. As incubation proceeds, uptake in the presence of lanthanide approaches the control curve, except at high calcium loads, where the duration of incubation may be insufficient. Note change in ordinate scale between A and B.

$K^+$ increased the uptake of control vesicles by 36 %, the maximum effect being seen at 20 mM $K^+$. $Na^+$ (100 mM) could replace $K^+$ almost completely, but was not additive or synergistic. $NH_4^+$ was less effective in replacing $K^+$, and $Li^+$ was only slightly effective. The depression of uptake caused by $La^{3+}$ occurred to the same degree in the presence or absence of $K^+$.

*ATPase activity* (Table I)

In the presence of EGTA 0.5 mM, without added $Ca^{2+}$, basal ATPase activity of vesicle averaged 0.085 ± 0.07 μmole/mg per min. When $Ca^{2+}$ was added to a final free $Ca^{2+}$ concentration of $10^{-5}$ M, ATP hydrolysis increased to 0.153 ± 0.10 μmole/mg per min. The "extra" ATPase constituted therefore 44.5 % of the total. Peak activation occurred at $10^{-5}$ M $Ca^{2+}$ in the absence of lanthanide, with slight depression at $Ca^{2+}$ concentration $10^{-4}$ M. ATP hydrolysis was linear with time in both the $Mg^{2+}$ and ($Mg^{2+}$ + $Ca^{2+}$)-stimulated conditions.

TABLE I

EFFECT OF LANTHANIDES ON ATPase ACTIVITY OF CARDIAC MICROSOMES

Conditions: 50 mM KCl, 10 mM histidine–Tris (pH 7.0), 5 mM MgATP. Temperature, 37 °C. Vesicle concentration, 0.2 mg/ml. $n$ = number of experiments. Values are μmoles $P_i$/mg sarcoplasmic reticulum protein per min.

| *Addition* | *Control* | *$La^{3+}$ (200 μM)* | *$Gd^{3+}$ (200 μM)* |
|---|---|---|---|
| None ($n$ = 2) | 0.121 | 0.077 | 0.107 |
| EGTA, 0.5 mM ($n$ = 8) | 0.085 ± 0.007*** | 0.073 ± 0.009*** | 0.102 ± 0.011*** |
| $Ca^{2+}$, 50 μM ($n$ = 8) | 0.153 ± 0.01 | 0.077 ± 0.012* | 0.106 ± 0.012** |
| "Extra" ATPase (% of total) | 0.068 (44.5 %) | 0.004 (5.2 %) | 0.004 (3.8 %) |

* $P < 0.001$.
** $P < 0.02$.
*** $La^{3+}$ and $Gd^{3+}$ are tightly chelated by EGTA, and values under these conditions represent low $Ca^{2+}$ ($< 10^{-8}$ M) and insignificant $La^{3+}$ and $Gd^{3+}$ concentration.

$La^{3+}$ and $Gd^{3+}$ are tightly chelated by EGTA so their effects on $Mg^{2+}$-ATPase had to be determined in the absence of EGTA or added $Ca^{2+}$. In this circumstance, however, control vesicles showed some activation of the $Ca^{2+}$-ATPase by contaminant calcium from reagents (approximately 5 μM) and the "basal" level of ATP hydrolysis was 0.121 μmole/mg per min *vs* 0.085 in the EGTA assay. $La^{3+}$ and $Gd^{3+}$ depressed ATPase activity (Fig. 3), but not below the level of the basal ATPase activity determined in the presence of EGTA. At high concentration $La^{3+}$ or $Gd^{3+}$ (500–1000 μM), basal $Mg^{2+}$-ATPase is also markedly depressed by $La^{3+}$ (98 %) and moderately by $Gd^{3+}$ (22 %).

Inhibition of $Ca^{2+}$-ATPase by $La^{3+}$ and $Gd^{3+}$ parallel inhibition of calcium uptake (Fig. 3).

## DISCUSSION

The present study indicates that the tervalent cations lanthanum and gadolinium in $10^{-4}$–$10^{-5}$ M concentration block ATP-dependent binding and uptake of calcium by vesicles derived from sarcoplasmic reticulum. This block depended on a slowing of the velocity of uptake, presumably by decreasing the binding sites available

rather than a decrease in capacity for calcium storage. There was a parallel inhibition of the ATP hydrolysis activated by $Ca^{2+}$.

The Scatchard plot of calcium binding indicates that La and Gd decrease the number of available sites for calcium binding (abscissa intercept in Fig. 1B). The equilibrium constant for calcium binding estimated from the ordinate intercept and the number of sites, appears to be decreased by $La^{3+}$ and $Gd^{3+}$, but in view of the large extrapolation required this value has not been calculated.

The data suggest that lanthanide inhibition is non-competitive when applied to the membrane prior to addition of $Ca^{2+}$. The inhibition by $La^{3+}$ in mitochondrial membranes has also been considered non-competitive by Mela[9,10] though Scarpa and Azzone[13] and Selwyn *et al.*[15] dispute this point. Lehninger and Carafoli[11] noted that $La^{3+}$, once bound to mitochondrial membranes, was not displaced by added $Ca^{2+}$.

The effects of lanthanide in slowing the velocity of uptake accentuated a similar slowing seen in the absence of $La^{3+}$ or $Gd^{3+}$ when vesicles were heavily loaded with calcium (approximately 2 $\mu$moles/mg). After a lag period of about 1 min, uptake velocity increased and approached control levels, even in the presence of high $La^{3+}$ concentrations. This time-dependent relief of $La^{3+}$ inhibition has been noted also in mitochondria. Its mechanism is not clear, though it is possible that the $La^{3+}$ or $Gd^{3+}$ might be transported into the vesicles, similar to $Ca^{2+}$, thereby relieving a block at the outer surface of the membrane. The failure of Entman *et al.*[18] to find effects of $La^{3+}$ may be related to the low concentration of La used and the 20-min incubation period in their study.

The free concentration of lanthanide necessary to produce the effects on sarcoplasmic reticulum membranes is not certain. In mitochondria, lanthanide effects occur[9-11] at $10^{-7}$ M. The minimal observed effective concentration of added lanthanide on sarcoplasmic reticular membrane was $10^{-5}$ M, and $2 \cdot 10^{-4}$ M was necessary to demonstrate inhibition of steady-state binding. Since the binding constant of $La^{3+}$ or $Gd^{3+}$ for ATP was determined to be $2.9 \cdot 10^4$ and $9.1 \cdot 10^4$, respectively[14] then free $La^{3+}$ and $Gd^{3+}$ concentration in the presence of 5 mM MgATP are reduced to $10^{-6}$ and $4 \cdot 10^{-7}$ M, respectively. The slightly higher binding constants for $Gd^{3+}$ are consistent with the observed weaker effects of $Gd^{3+}$ compared to $La^{3+}$ at equivalent concentration, since the effective concentration is reduced. Binding to histidine and Tris are minor, but the effects of phosphate and oxalate are significant. The lanthanide salts of these anions are extremely insoluble, and there is sufficient free $PO_4^{3-}$ (approx. $10^{-5}$ M) from spontaneously hydrolyzed ATP even at the start of the incubation period to exceed the solubility product of $10^{-22.4}$ for $LaPO_4$. Also, from the solubility product of lanthanum oxalate of $2 \cdot 10^{-28}$, the addition of $5 \cdot 10^{-3}$ M oxalate should have precipitated $La^{3+}$ in excess of $4 \cdot 10^{-11}$ M concentration. The explanation for the observed data, therefore, is not clear. It may be that the lanthanide forms soluble complexes with the oxalate ion, similar to another rare earth neodymium. These soluble complexes may avoid precipitation of the added $La^{3+}$. However, the free $La^{3+}$ permitted by the oxalate solubility product nevertheless should not be greater than $4 \cdot 10^{-11}$ M, so it is possible that the free $La^{3+}$ concentration which is effective on sarcoplasmic reticular membranes may be considerably lower than previously described. Conceivably, the whole lanthanum oxalate complex may affect the membrane. It was observed by dual-beam spectrophotometry* using murexide as lanthanum indicator

* N. Krasnow and Y. Ogawa, unpublished.

that 1 mM oxalate added to $10^{-4}$ M $La^{3+}$, in the absence of sarcoplasmic reticulum, linearly reduced the lanthanum murexide interaction only by half rather than to undectable levels as predicted by solubility-product calculation.

The present data are consistent with the reported physiological effects of $La^{3+}$ on cardiac muscle. Whereas nerve requires millimolar concentrations of lanthanide to interfere with $Ca^{2+}$ movements[1,2], Sanborn and Langer[6] found that 5–40 $\mu$M $La^{3+}$ reduced tension development in the dog papillary muscle, without effect on cellular action potentials. They concluded from analysis of washout of $^{45}Ca$ in the presence of $La^{3+}$ that the calcium stores displaced by $La^{3+}$ were in the same kinetic compartment as that associated with changes in tension induced by low $Na^+$, heart rate, and temperature change. They suggested the T-tubules and sarcoplasmic reticulum as the locus of this compartment. Whether $La^{3+}$ can develop micromolar concentrations across intact cell membranes to exert such physiologic effect on sarcoplasmic reticulum has not been determined, so an action on the plasma membrane may play a significant role. Weiss suggested that $10^{-6}$ M $La^{3+}$ affects both superficial and deep storage sites for calcium in frog sartorius[4] and mammalian smooth muscles[7,8]. Inhibition by lanthanides of calcium binding by microsomal membranes is in accord with these data. However, since smooth muscle has a poorly developed sarcoplasmic reticulum, significant actions of $La^{3+}$ must occur elsewhere in the intact cell, *e.g.* the plasma membrane. The present study of lanthanide effect on sarcoplasmic reticulum may serve as a model for these other membrane actions.

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