Pre-Steady-State Kinetic Study of the Mechanism of Inhibition of the Plasma Membrane Ca^{2+}-ATPase by Lanthanum†

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ABSTRACT: Lanthanides are known to be effective inhibitors of the $PMCa^{2+}$-ATPase. The effects of $LaCl_3$ on the partial reactions that take place during ATP hydrolysis by the calcium-dependent ATPase from plasma membrane ($PMCa^{2+}$-ATPase) were studied at 37 °C on fragmented intact membranes from pig red cells by means of a rapid chemical quenching technique. $LaCl_3$ added before phosphorylation ($K_{0.5}$ = 2.8 ± 0.2 μM) raised the k_{app} of the $E_2 \rightarrow E_1$ transition from 14 ± 2 to 23 ± 4 s^{-1}. The effect was independent of Ca^{2+} and Mg^{2+}, as if La^{3+} substituted for Mg^{2+} and/or Ca^{2+} in accelerating the formation of E_1 with higher efficiency. At non-limiting conditions, $LaCl_3$ doubled the apparent concentration of E_1 in the enzyme at rest with Ca^{2+} and Mg^{2+}. $LaCl_3$ during phosphorylation ($K_{0.5}$ near 20 μM) lowered the v_o of the reaction from 300 ± 20 to 60 ± 7 pmol/mg of protein/s, a close rate to that in the absence of Mg^{2+}. This effect was reversed by Mg^{2+} (and not by Ca^{2+}), and the $K_{0.5}$ for Mg^{2+} as activator of the phosphorylation reaction increased linearly with the concentration of $LaCl_3$, suggesting that La^{3+} slowed phosphorylation by displacing Mg^{2+} from the activation site(s). If added before phosphorylation, $LaCl_3$ lowered the k_{app} for decomposition of *E*P to 0.8 ± 0.1 s^{-1}, a value which is characteristic of phosphoenzyme without Mg^{2+}. The $K_{0.5}$ for this effect was 0.9 ± 0.5 μM $LaCl_3$ and increased linearly with the concentration of Mg^{2+}. If added after phosphorylation, $LaCl_3$ did not change the k_{app} of 90 ± 7 s^{-1} of decomposition of *E*P, suggesting that La^{3+} displaced Mg^{2+} from the site whose occupation accelerates the shifting of E_1P to E_2P. In medium with 0.5 mM $MgCl_2$, 2 μM $LaCl_3$ lowered rapidly the rate of steady-state hydrolysis of ATP by the $PMCa^{2+}$-ATPase to a value close to the rate of decomposition of *E*P made in medium with $LaCl_3$. Increasing $MgCl_2$ to 10 mM protected the $PMCa^{2+}$-ATPase against inhibition during the first 10 min of incubation. Results show that combination of La^{3+} to the Mg^{2+} (and Ca^{2+}) site(s) in the unphosphorylated $PMCa^{2+}$-ATPase accelerates the $E_2 \rightarrow E_1$ transition and inhibits the shifting $E_1P \rightarrow E_2P$. Since with less apparent affinity La^{3+} slowed but did not impede phosphorylation, it seems that the sharp slowing of the rate of transformation of E_1P into E_2P by displacement of Mg^{2+} was the cause of the high-affinity inhibition of the $PMCa^{2+}$-ATPase by La^{3+}.

Schatzmann and Tschabold (1971) first noticed that lanthanum and praseodymium inhibited active transport of Ca^{2+} across human red cell membranes. Shortly after, Wiener and Lee (1972) reported that 10^{-4} M $LaCl_3$ inhibited about 70% of the Ca^{2+}-ATPase activity from human red cell membranes, leaving unaltered Na^+/K^+-ATPase activity. Quist and Roufogalis (1975) and Larsen et al. (1978) in released ghosts and Sarkadi e al. (1977) in intact erythrocytes reported that at 0.1−0.25 mM, $LaCl_3$ in the extracellular medium blocked the transport of Ca^{2+} and about 50−80% of ATPase activity. Schatzmann and Bürgin (1978) reported that 0.2 mM $LaCl_3$ in the presence of Ca^{2+} increases the amount of *E*P[1] from human red cell membranes. The increase in *E*P by La^{3+} was confirmed in red cells and extended to renal basal-lateral membrane preparations (De Smedt et al., 1983). *E*P formed from lanthanum ATPase is stable, and ADP causes it to decay rapidly. Luterbacher and Schatzmann (1983) reported results suggesting that during inhibition of Ca^{2+}-ATPase activity by La^{3+}, the catalytic cycle is interrupted between conformers E_1P and E_2P with the consequent accumulation of *E*P under the form of the ADP-sensitive E_1P. Such an effect needs $LaCl_3$ to be present before phosphorylation with MgATP takes place. Rossi (1979) studied in our laboratory the inhibition of the Ca^{2+}-ATPase activity on fragmented membranes from human red cells by $LaCl_3$, finding that there exist no interactions in affinity between La^{3+} and ATP and that K_i (1.5 μM $LaCl_3$) was independent of the concentration of Ca^{2+} between 0.005 and 2 mM.

Lanthanide ions have been useful during functional studies of the $SRCa^{2+}$-ATPase. They displaced Ca^{2+} by combining to the high-affinity calcium binding sites (Scott, 1984; Squier et al., 1990). Under such conditions there is phosphorylation, suggesting that lanthanides replace Ca^{2+} as the metal ion responsible for activation of the $SRCa^{2+}$-ATPase for phosphorylation (Squier et al., 1990; Asturias et al., 1994). Phosphorylation is much slower when LaATP substitutes for MgATP as the substrate (Hanel & Jencks, 1990). In addition,

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[1] Abbreviations: $PMCa^{2+}$-ATPase, calcium ion-dependent adenosine triphosphate from plasma membrane; $SRCa^{2+}$-ATPase, calcium ion-dependent adenosine triphosphatase from sarcoplasmic reticulum; *E*P, phosphoenzyme; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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lanthanum phosphoenzyme undergoes hydrolytic cleavage at a slower rate than that formed in the absence of lanthanum (Squier et al., 1990; Fujimori & Jenks, 1990), and this causes inhibition of $SRCa^{2+}$-ATPase activity. After a detailed investigation, Fujimori and Jenks (1990) reached to the conclusion that La^{3+} inhibits steady-state $SRCa^{2+}$-ATPase activity by replacing Mg^{2+}, which is necessary for the rapid splitting of phosphoenzyme.

In view of the information available now both on the actions of La^{3+} in the $SRCa^{2+}$-ATPase and on the requirements of Ca^{2+} and Mg^{2+} of the reaction steps of the catalytic cycle of the $PMCa^{2+}$-ATPase (Adamo et al., 1988, 1990a,b; Herscher et al., 1994), we considered it worthwhile to study the effects of La^{3+} on the partial reactions of the $PMCa^{2+}$-ATPase under pre-steady-state conditions with the aim of understanding the mechanism of inhibition of the steady-state turnover of the enzyme by La^{3+}. We found that the effects of La^{3+} on $PMCa^{2+}$-ATPase activities resemble those found in $SRCa^{2+}$-ATPase (Fujimori & Jencks, 1990) in the sense that La^{3+} and Mg^{2+} seem to compete for the same site(s).

MATERIALS AND METHODS

Pig blood collected on acid/citrate/dextrose solution was used throughout. Red cell membranes depleted of Ca^{2+} and calmodulin were prepared following a modification (Caride et al., 1986) of the procedure of Gietzen et al. (1980), except that EGTA was used for CDTA, and stored at −60 °C until used. [γ-^{32}P]ATP (specific activity = 1 mCi/μmol) was prepared according to Glynn and Chappell (1964) except that no unlabeled orthophosphate was added to the incubation media. [^{32}P]Orthophosphate was provided by Comisión Nacional de Energía Atómica (Argentina). Enzymes and cofactors for the synthesis of [γ-^{32}P]ATP were obtained from Sigma Chemical Co., U.S.A. Salts and reagents were of analytical reagent grade.

Phosphorylation and dephosphorylation experiments were carried out at 37 ± 3 °C in a rapid mixing apparatus adapted for chemical quenching (Intermekron AB, Uppsala, Sweden). Phosphorylation from [γ-^{32}P]ATP was measured as described previously (Adamo et al., 1988). During the experiments, 2 mL of the membrane suspension at 5 mg of protein/mL in Tris-K^+ buffer containing 50 mM Tris-HCl (pH 7.4 at 37 °C), 100 mM KCl plus the concentrations of $CaCl_2$, $MgCl_2$, and $LaCl_3$ given under Results in syringe I, were mixed with an equal volume of the suspending buffer with 20 μM [γ-^{32}P]-ATP in syringe II. Phosphorylation started at the mixing chamber and finished when the reaction mixture was quenched with 5 mL of a solution containing 10% (w/v) trichloroacetic acid, 5 mM ATP, and 50 mM H_3PO_4 at 0 °C. The suspension was centrifuged at 1750*g* for 5 min at 5 °C, and the sediment washed three times with 6 mL of a solution containing 7% (w/v) trichloroacetic acid and 50 mM H_3PO_4. The washed precipitate was dissolved in 0.4 mL of 3% (w/v) sodium dodecyl sulfate solution in 1 M Tris. An aliquot of the solution was put aside for measuring protein, and 0.25 mL was used for measuring radioactivity in a liquid scintillation counter. The amount of *E*P was estimated from the difference between the amount of ^{32}P incorporated into the membrane protein in media with $CaCl_2$ and in media of identical composition to which no $CaCl_2$ was added. When preincubation time was a variable, phosphorylation was carried out using three syringes and two mixing chambers as described previously (Adamo et al., 1990a). During the experiments, 2 mL of the membrane suspension at 5 mg of protein/mL in Tris-K^+ buffer in syringe I were mixed in the first chamber with an equal volume of the suspending buffer containing twice the desired concentration of $CaCl_2$, $MgCl_2$, and $LaCl_3$ in syringe II. Preincubation took place in the lines between the first and the second mixing chambers. Phosphorylation began in the later chamber when 2 mL of the suspending buffer with 30 μM [γ-^{32}P]ATP plus 3 mM EGTA and enough $CaCl_2$ and $MgCl_2$ to reach 0.2 mM Ca^{2+} and 0.5 mM Mg^{2+} final concentrations in syringe III were mixed with the membrane suspension. Dephosphorylation experiments were carried out using three syringes and two mixing chambers as described previously (Herscher et al., 1994).

$PMCa^{2+}$-ATPase activity was estimated from the release of [^{32}P]P_i from [γ-^{32}P]ATP at 37 °C according to Richards et al. (1978), in media of similar composition as those used during phosphorylation and dephosphorylation.

The concentration of protein was estimated by the procedure of Lündahl (1975). To avoid uncertainties in the concentrations of Ca^{2+}, Mg^{2+}, and La^{3+}, unless indicated, media were prepared without EGTA and the concentrations of these metals were considered to be those of the chloride salts added to the buffer, without taking into account contaminant Ca^{2+} and Mg^{2+} brought in by the components of the media. When EGTA was present to chelate all La^{3+}, the concentrations of free Ca^{2+} and Mg^{2+} were calculated assuming the following apparent dissociation constants: $K_{Ca\text{-}EGTA} = 2.6 \times 10^{-7}$ M, $K_{Mg\text{-}EGTA} = 1.34 \times 10^{-2}$ M, $K_{Ca\text{-}ATP} = 5.4 \times 10^{-5}$ M, and $K_{Mg\text{-}ATP} = 4.5 \times 10^{-5}$ M (Adamo et al., 1988), $K_{La\text{-}EGTA} = 2.89 \times 10^{-16}$ M (Luterbacher & Schatzmann, 1983), and $K_{La\text{-}ATP} = 3.3 \times 10^{-7}$ M (Morrison & Cleland, 1980).

Except when stated, the curves drawn and the kinetic parameters ± standard error were those from the theoretical equations that fitted the data by least-squares nonlinear regression using the procedure of Gauss−Newton (Rossi & Garraham, 1985). The independent variable was assumed to be free of error and the variance of the dependent variable was assumed to be constant. In every experiment, the measurements of *E*P level were run at least in duplicate and the individual values differ from the mean by less than 10%.

Experiments presented under Results were chosen as representative from two to four experiments. The concentration of active $PMCa^{2+}$-ATPase varies among membrane preparations, and this is reflected in the quantities measured. For this reason, the control experiments were run simultaneously with those with $LaCl_3$ and on the same membrane preparation.

RESULTS

Effects of La^{3+} before Phosphorylation. Membranes preincubated in media with and without 10 μM $LaCl_3$ during different length of time were phosphorylated during 2.5 ms under similar conditions. Enough EGTA to chelate all La^{3+} was added at the start of phosphorylation. Figure 1 shows the variations of the initial rate of phosphorylation (v_o), measured over the first 2.5 ms, with the duration of preincubation of the membranes. In the absence of $LaCl_3$ during preincubation the rate of phosphorylation increased along an exponential curve up to a maximum of 431 ± 29 pmol/mg of protein/s with $k_{app} = 14 \pm 2$ s^{-1}, as shown previously (Herscher et al., 1996). Membranes that were

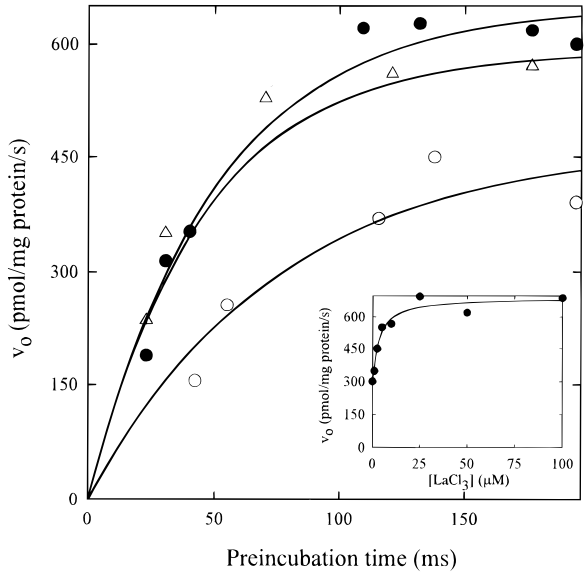


FIGURE 1: Effect of preincubation with $LaCl_3$ on the v_o of phosphorylation. Membranes in Tris-K^+ buffer at 37 °C were mixed with an equal volume of the buffer with either 20 μM $LaCl_3$ (△) or 0.4 mM $CaCl_2$ plus 1 mM $MgCl_2$ with (●) and without (○) 20 μM $LaCl_3$. The membranes were kept in these media for different times in the millisecond range. Phosphorylation was started by mixing 2 volumes of the preincubated membranes with 1 volume of 30 μM [γ-^{32}P]ATP in Tris-K^+ buffer containing 3 mM EGTA and enough $CaCl_2$ and $MgCl_2$ to give final concentrations of 0.2 mM Ca^{2+} and 0.5 mM Mg^{2+} and was carried out for 2.5 ms. v_o was obtained by dividing the total amount of *E*P by 2.5 ms (the duration of the experiment). The equation that fitted the experimental points was $v_o = v_{o(\infty)}(1 - \exp(-kt))$, where $v_{o(\infty)} = v_o$ at infinite preincubation time, k = apparent rate constant, and t = preincubation time. (Inset) Effect of the concentration of $LaCl_3$ during preincubation on the v_o of phosphorylation. Membranes were preincubated 15 min at 37 °C in Tris-K^+ buffer with 0.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, and different concentrations of $LaCl_3$ and phosphorylated for 2.5 ms with 10 μM [γ-^{32}P]ATP in a medium containing 1 mM EGTA, 0.2 mM Ca^{2+}, and 0.5 mM Mg^{2+}. The equation that fitted the experimental points was $v_o = v_{o(max)}[LaCl_3]/(K_{0.5} + [LaCl_3]) + C$, where C is v_o at 0 μM $LaCl_3$.

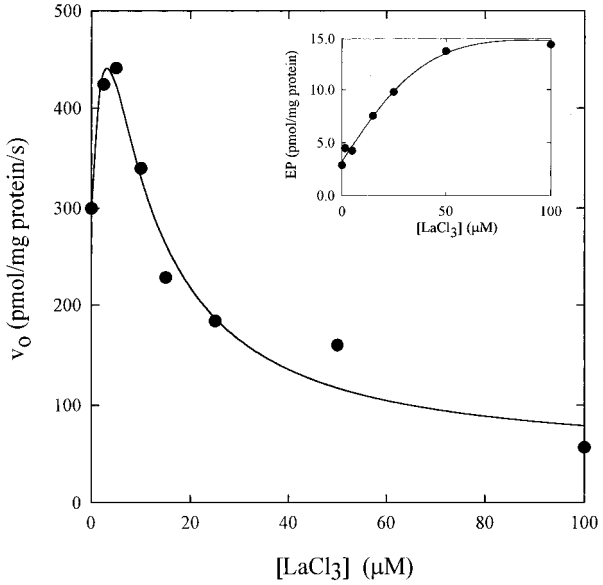


FIGURE 2: Effect of $LaCl_3$ added during phosphorylation. Membranes were preincubated in Tris-K^+ buffer with 0.2 mM $CaCl_2$ and 0.5 mM $MgCl_2$ for 15 min at 37 °C and phosphorylated 2.5 ms in the same medium with 10 μM [γ-^{32}P]ATP plus different concentrations of $LaCl_3$. v_o was the ratio between the amount of *E*P and 2.5 ms. The curve was drawn by eye. (Inset) Effect of $LaCl_3$ on the steady-state level of *E*P. The conditions were identical except that phosphorylation time was 5000 ms.

preincubated with $LaCl_3$ reached a higher rate of phosphorylation of 618 ± 37 pmol/mg of protein/s in a shorter time (k_{app} = 23 ± 4 s^{-1}). Results in Figure 1 also show that the effect of La^{3+} during preincubation was the same regardless of whether or not the preincubation media contained added $CaCl_2$ and $MgCl_2$.

The curves in Figure 1 should have extrapolated to a positive value close to 50 pmol/mg of protein/s on the ordinate due to the small amount of E_1 in the enzyme at rest in the absence of ligands (Adamo et al., 1990a). Experiments such as these in Figure 1 are complex and difficult to perform so that the collected data were not precise enough to set a difference between 0 and 50 pmol/mg of protein/s by extrapolation and the curves were made to start from zero.

The inset to Figure 1 gives results of an independent experiment showing that, under steady-state conditions as a function of the concentration of $LaCl_3$, v_o increased from 310 ± 23 to 657 ± 43 pmol/mg of protein/s along a hyperbolic curve with $K_{0.5}$ = 2.8 ± 0.2 μM.

Effects of La^{3+} Added During Phosphorylation. Membranes in medium without $LaCl_3$ were phosphorylated by the addition of [γ-^{32}P]ATP plus increasing concentrations of $LaCl_3$ with the results shown in Figure 2. As a function of the concentration of $LaCl_3$ v_o increased, passing through a maximum at about 8 μM $LaCl_3$, and then fell to about 20% of the value without $LaCl_3$. If not all, at least part of the high-affinity rise could be ascribed to La^{3+} acting as above on the non-phosphorylated enzyme during the short phosphorylation period. The drop in v_o was half-maximum at about 20 μM $LaCl_3$ and could be attributed either to inactivation of the enzyme or to slower phosphorylation. If the latter were the case, extending phosphorylation time should allow *E*P to reach its highest level at steady-state. Results in the inset to Figure 2 show that 5 s long phosphorylation made $LaCl_3$ increase the concentration of *E*P up to 5-fold the level without $LaCl_3$ with $K_{0.5}$ = 25 ± 1.6 μM. Lack of inhibition of phosphorylation by La^{3+} confirms previous findings by others (Szasz et al., 1978; De Smet et al., 1983; Muallem & Karlish, 1981; Luterbacher & Schatzmann, 1983).

Further information of the action of La^{3+} on the rate of phosphorylation was obtained by measuring v_o of phosphorylation with and without $LaCl_3$ of enzyme that had been preincubated with 0.5 mM $MgCl_2$ and phosphorylated in media with either increasing concentrations of $CaCl_2$ or with 1 mM $CaCl_2$ plus increasing concentrations of $MgCl_2$. Results in Figure 3 show that at 0.5 mM $MgCl_2$ and no added $CaCl_2$ there was phosphorylation due to contaminant Ca^{2+} from the media. Increasing the concentration of $CaCl_2$ made v_o increase by about the same extent both in presence and in absence of $LaCl_3$ during phosphorylation. In contrast with this, increasing the concentration of $MgCl_2$ in the phosphorylation media progressively protected the PMCa^{2+}-ATPase from the effect of La^{3+} until at 25 mM $MgCl_2$ the enzyme phosphorylated at the same rate as in the absence of $LaCl_3$.

The initial rate of phosphorylation was measured at increasing concentrations of either $CaCl_2$ or $MgCl_2$ in media with a fixed concentration of $LaCl_3$ from 0 to 50 μM during phosphorylation. At any concentration of $LaCl_3$, v_o increased with the concentrations of the divalent cations along simple activation hyperbolic curves from which $K_{0.5}$ values were estimated and plotted as in Figure 4. As a function of the

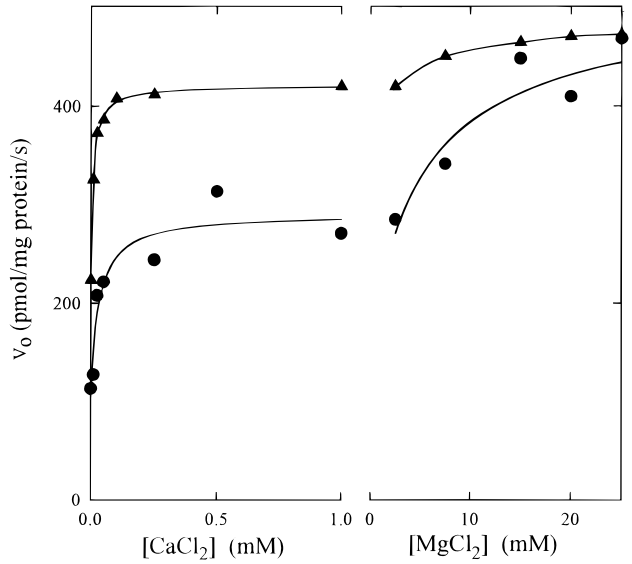


FIGURE 3: Effects of increasing concentrations of $CaCl_2$ and $MgCl_2$ on the lowering of v_o of phosphorylation by La^{3+}. Membranes were preincubated 15 min at 37 °C in Tris-K^+ buffer plus 0.5 mM $MgCl_2$ and then phosphorylated 2.5 ms with 10 μM [γ-^{32}P]ATP in the same medium plus increasing concentrations of $CaCl_2$ and $MgCl_2$ with (▲) and without (●) 30 μM $LaCl_3$. v_o was the ratio between the amount of *E*P and 2.5 ms.

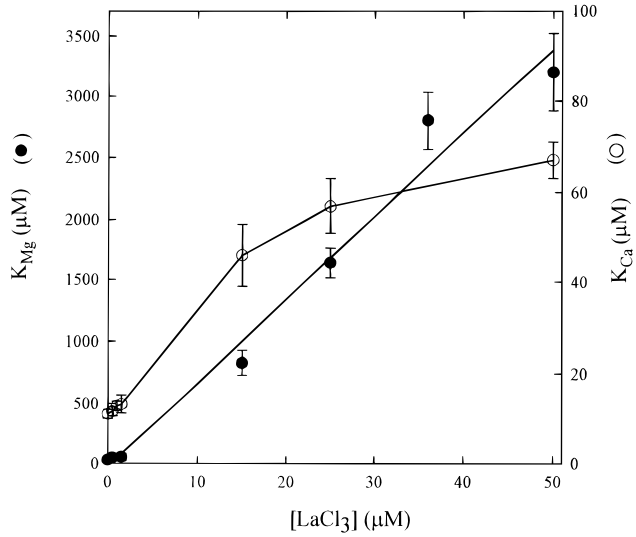


FIGURE 4: Effect of $LaCl_3$ on $K_{0.5}$ values for activation of phosphorylation by $CaCl_2$ and $MgCl_2$. Membranes were preincubated 15 min in Tris-K^+ buffer and phosphorylated 2.5 ms with 10 μM [γ-^{32}P]ATP at increasing concentrations of $CaCl_2$ or $MgCl_2$ plus a fixed concentration of $LaCl_3$. The ratio between the amount of *E*P and 2.5 ms gave v_o values that were plotted against the concentration of the corresponding $CaCl_2$ or $MgCl_2$. $K_{0.5}$ values were estimated on these curves from the concentration of added $CaCl_2$ and $MgCl_2$, without considering the concentrations of contaminant divalent cations in the media.

concentration of $LaCl_3$, $K_{0.5}$ for Ca^{2+} increased up to 6-fold along a curve tending to saturation. In contrast with this, $K_{0.5}$ for Mg^{2+} increased along a straight line up to 54-fold at 50 μM $LaCl_3$.

Dephosphorylation. Results in Figure 5 allow a comparison of the time course of dephosphorylation of *E*P prepared in the presence and absence of $LaCl_3$. As shown previously (Herscher et al., 1994) *E*P prepared without $LaCl_3$ dephosphorylates rapidly along a simple exponential curve with $k_{app} = 90 \pm 7$ s^{-1}. In contrast with this, most of *E*P from $PMCa^{2+}$-ATPase that had been preincubated with $LaCl_3$ underwent a slow decay with $k_{app} = 0.8 \pm 0.1$ s^{-1}.

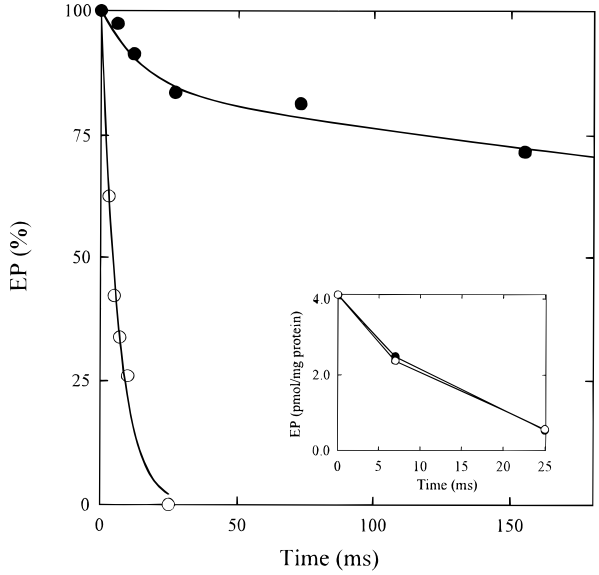


FIGURE 5: Effect on $LaCl_3$ on the time course of dephosphorylation of *E*P. Membranes were phosphorylated in Tris-K^+ buffer, 0.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, plus 75 (●) or 0 μM $LaCl_3$ (○). Dephosphorylation was started by mixing 1 volume of 0.9 mM ATP in the same media with 2 volumes of the phosphorylated membranes. The equations that fitted the experimental points were (○) $[EP] = [EP_{max}]\exp(-kt)$, where $k = 90 \pm 7$ s^{-1} and $[EP_{max}] = 100 \pm 1\%$, and (●) $[EP] = [EP_r]\exp(-k_r t) + [EP_s]\exp(-k_s t)$ (Herscher et al., 1994), where $k_r = 81 \pm 5$ s^{-1}, $k_s = 0.8 \pm 0.1$ s^{-1}, $[EP_r] = 9.7 \pm 0.9\%$, $[EP_s] = 90.3 \pm 1.1\%$. (Inset) Effects of $LaCl_3$ added during dephosphorylation. Membranes were phosphorylated for 700 ms at 37 °C in Tris-K^+ buffer, 0.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, and then dephosphorylated for 7 and 25 ms in the same medium with 0.3 mM ATP plus 300 (○) or 0 μM $LaCl_3$ (●).

To see the effects of $LaCl_3$ added after phosphorylation, *E*P was made in medium with $MgCl_2$ and, after addition of 0.3 mM ATP with and without $LaCl_3$, was allowed to decay for 7 and 25 ms. Results in the inset to Figure 5 show that the phosphoenzyme disappeared at the same rate regardless of whether or not the medium contained $LaCl_3$.

The dependence of the inhibition of dephosphorylation by $LaCl_3$ with the concentration of $MgCl_2$ during phosphorylation was measured with the results shown in Figure 6. The concentration of $LaCl_3$ for half-maximum effect increased linearly with the concentration of $MgCl_2$. Such behavior suggests that Mg^{2+} displaced La^{3+} from the site at which it combined to lower dephosphorylation. Extrapolation gave a value of $K_{0.5}$ of 0.9 ± 0.5 μM $LaCl_3$ for inhibition of dephosphorylation at 0 μM $MgCl_2$.

Effects of La^{3+} on Ca^{2+}-ATPase Activity. Figure 7 shows the effects of increasing concentrations of $LaCl_3$ on Ca^{2+}-ATPase activity measured in medium with 20 μM ATP, 0.5 mM $MgCl_2$, and 0.2 mM $CaCl_2$. $LaCl_3$ lowered the activity in a concentration-dependent fashion with $K_{0.5} = 0.50 \pm 0.04$ μM to a minimum of 5.6 ± 0.7 pmol/mg of protein/s ($n = 3$) at 50 μM $LaCl_3$. Although low, this value of activity was consistently higher than zero.

The inset in Figure 7 shows the effect of 2 μM $LaCl_3$ on the time course of ATP hydrolysis by the $PMCa^{2+}$-ATPase in media with 0.5 or 10 mM $MgCl_2$. ATP was hydrolyzed at contant rates of 150 ± 10 and 42 ± 6 pmol/mg of protein/s in absence and presence of $LaCl_3$, respectively. With 10 mM $MgCl_2$ the rate without $LaCl_3$ was constant but lower (98 ± 10 pmol/mg of protein/s), as expected due to inhibition by Mg^{2+} (Caride et al., 1986). Under these conditions, $LaCl_3$ was with no apparent effect within the first 10 min of

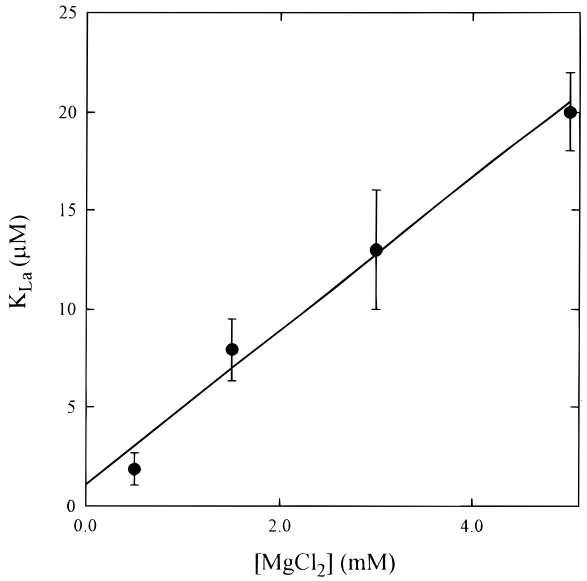


FIGURE 6: Effect of $MgCl_2$ on the $K_{0.5}$ for $LaCl_3$ as inhibitor of dephosphorylation. Membranes were phosphorylated 700 ms at 37 °C in Tris-K^+ buffer, 0.2 mM $CaCl_2$, and different concentrations of $MgCl_2$ and $LaCl_3$. Dephosphorylation took place in media with 0.3 mM ATP. From the amounts of *E*P after 0 and 40 ms dephosphorylation the rates of the reaction were calculated and plotted against the concentration of $LaCl_3$ at fixed concentrations of $MgCl_2$. These plots gave hyperbolic curves from which the values of $K_{0.5}$ for $LaCl_3$ were obtained.

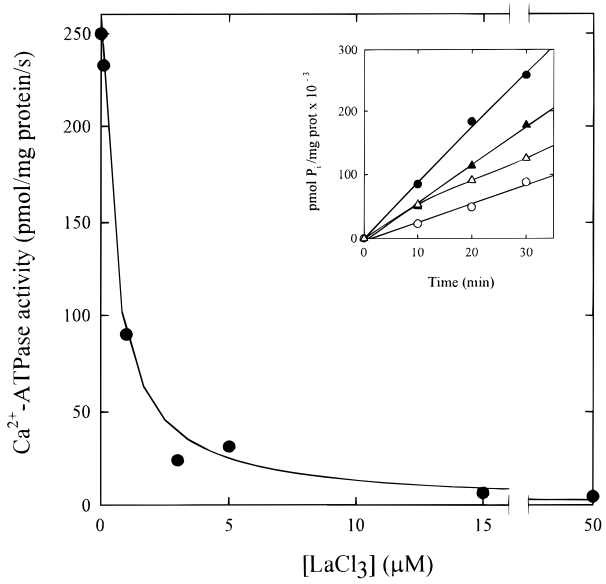
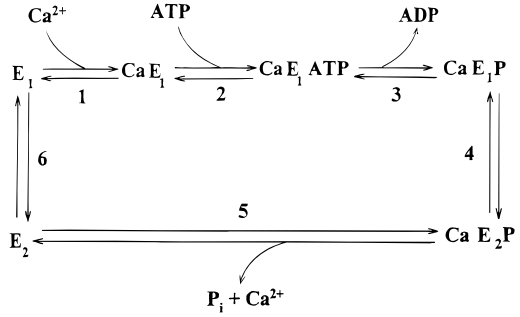


FIGURE 7: Inhibition of the PMCa^{2+}-ATPase activity by $LaCl_3$. Membranes at 0.1 mg of protein/mL were preincubated 10 min at 37 °C without ATP in the same media in which the activity was measured. The PMCa^{2+}-ATPase activity was measured in Tris-K^+ buffer plus 0.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 20 μM ATP, increasing concentrations of $LaCl_3$, and 0.1 mg of protein/mL. (Inset) Effect of $MgCl_2$ on the time course of P_i production. Membranes at 0.1 mg of protein/mL were preincubated 10 min at 37 °C in Tris-K^+ buffer containing 0.2 mM $CaCl_2$, 0.5 (circles) or 10 mM $MgCl_2$ (triangles), and 2 (○, △) or 0 μM $LaCl_3$ (●, ▲). ATPase activity was measured in the same media plus 20 μM ATP.

incubation and then progressively lowered the activity down to 47 ± 5 pmol/mg of protein/s after 20 min incubation. Experiments not shown demonstrated that 1 mM $CaCl_2$ in medium with 0.5 mM $MgCl_2$ and 2 μM $LaCl_3$ did not replace high Mg^{2+} in retarding inhibition of ATP hydrolysis.

To see if inhibition by La^{3+} was reversible, membranes were preincubated at 37 °C in the buffer used for the enzymic

Scheme 1



assay in Figure 7 plus 10 μM $LaCl_3$. After 5 min half of the membranes were washed twice with 5 mM EGTA, 5 mM CDTA, and 50 mM Tris-HCl (pH 7.4 at 37 °C) to remove the $LaCl_3$. The ATPase activity from the membranes that remained in buffer with $LaCl_3$ was assayed in the same buffer while that of the membranes washed with chelators was assayed in buffer without $LaCl_3$. The activities of these membranes were compared with that from membranes that were preincubated, washed with chelators and assayed in media without $LaCl_3$. The activity of the membranes that had been in contact with $LaCl_3$ all along was 8.0 ± 0.7 pmol/mg of protein/s, the activity of those preincubated with $LaCl_3$ and assayed in the absence of $LaCl_3$ was 145 ± 10 pmol/mg of protein/s, while that of membranes preincubated and assayed without $LaCl_3$ was 140 ± 8 pmol/mg of protein/s.

DISCUSSION

Red cell membranes used during this study should bind La^{3+} to sites in their protein components normally occupied by Ca^{2+} or Mg^{2+} and in the lipid components of the membrane (Petersheim & Sun, 1989). For this reason, when possible, the amount of membranes during assays were kept constant.

One way of describing the function of the PMCa^{2+}-ATPase during transport of Ca^{2+} is by assuming that at micromolar ATP concentrations as those used in this study, splitting of ATP takes place through the partial reactions in Scheme 1, where E_1 and E_2 represent conformers of the enzyme.

Previous to phosphorylation La^{3+} produced enzyme that phosphorylated rapidly. Since CaE_1 in Scheme 1 is the only enzyme species that catalyzes phosphorylation from ATP, and phosphorylation is faster than 6 in Scheme 1, the initial rate of phosphorylation should be directly related to the concentration of E_1. If this were so, results in Figure 1 allow the conclusion that $LaCl_3$ increased the k_{app} of the $E_2 \rightarrow E_1$ transition from 14 ± 2 to 23 ± 4 s^{-1}, increasing the concentration of E_1. The $K_{0.5}$ for this change was 2.8 ± 0.2 μM $LaCl_3$. We have proposed previously that Ca^{2+} stabilizes E_1 and that rapid transition of E_2 into E_1 depends on Mg^{2+} (Adamo et al., 1990a). Since $LaCl_3$ during preincubation was equally effective in the absence as in the presence of $MgCl_2$ and $CaCl_2$, it can be concluded that La^{3+} substituted for Mg^{2+} and/or Ca^{2+} in accelerating the formation of E_1 (reaction 6 in Scheme 1) with higher efficiency.

La^{3+} during phosphorylation did not inhibit but lowered the v_o of the reaction ($K_{0.5}$ about 20 μM) to a value close to that observed (Adamo et al., 1990a) in media without Mg^{2+}. Lack of inhibition of phosphorylation confirms previous reports by others (Schatzmann & Bürgin, 1972; Luterbacher & Schatzmann, 1983). The findings that the $K_{0.5}$ for Mg^{2+} as activator of phosphorylation increased linearly with the

concentration of $LaCl_3$ and that the drop in v_o was fully reversed by Mg^{2+} but not by Ca^{2+} suggest rather strongly that during phosphorylation La^{3+} combined to the site from which Mg^{2+} accelerates phosphorylation (Adamo et al., 1990a) without replacing Mg^{2+} in this effect. There are experiments in the literature suggesting that lanthanides in the 10^{-8} to 10^{-7} M concentration range displace calcium from the high-affinity sites of the $SRCa^{2+}$-ATPase from skeletal muscle (Scott, 1984; Squier et al., 1990). Furthermore, under experimental conditions in which lanthanides substitute for all bound calcium, $SRCa^{2+}$-ATPase can be phosphorylated with ATP (Squier et al., 1990). We found that in media with 100 μM $LaCl_3$ plus 0.2 mM $CaCl_2$, the $PMCa^{2+}$-ATPase underwent phosphorylation by ATP. Under such conditions one would expect La^{3+} to occupy most of the Ca^{2+} transport sites in the $PMCa^{2+}$-ATPase and hence that LaE_1 rather than CaE_1 was the phosphorylated species.

Results showed that $LaCl_3$ added before phosphorylation lowered the k_{app} of decay of *E*P to 0.8 ± 0.1 s^{-1}, a rate similar to that observed for decay of phosphoenzyme without magnesium (Herscher et al., 1994). On the basis of the observation that *E*P formed in the presence of $LaCl_3$ is sensitive to ADP, Luterbacher and Schatzmann (1983) advanced the idea that La^{3+} inhibits dephosphorylation by interrupting the cycle between E_1P and E_2P. Our results agree with this idea and suggest that La^{3+} displaced Mg^{2+} from the site from which Mg^{2+} accelerates the transition between conformers (Herscher et al., 1994), without replacing it in this effect. Two observations favor this view. (i) $LaCl_3$ was effective if added before phosphorylation and fully ineffective after, a property accordant with the current information that magnesium bound to *E*P is occluded, and (ii) the concentration of $LaCl_3$ needed for half-maximum inhibition of the dephosphorylation increased linearly with the concentration of $MgCl_2$ before phosphorylation, as if Mg^{2+} displaced La^{3+} from the site at which it combined to impede rapid hydrolysis of *E*P.

Lanthanum lowered the initial rate of phosphorylation to near 60 ± 7 pmol/mg of protein/s and the initial rate of dephosphorylation of the $PMCa^{2+}$-ATPase to 3.5 ± 0.5 pmol/mg of protein/s. Hence, phosphorylation that is faster than dephosphorylation may probably be the cause of the increase in the steady-state concentration *E*P in the lanthanum enzyme.

At 0.5 mM $MgCl_2$ steady-state ATPase activity was inhibited rapidly with high affinity by $LaCl_3$ to a minimum of 5.6 ± 0.7 pmol/mg of protein/s. This value is compatible with the rate of 3.5 ± 0.5 pmol/mg of protein/s for splitting of *E*P made in medium with $LaCl_3$ during the experiment in Figure 5. Since in medium with $LaCl_3$ reaction 6 in Scheme 1 was accelerated and reaction 3 was faster than reaction 4, it seems reasonable to relate the observed inhibition of the ATPase activity with the displacement of Mg^{2+} by La^{3+} before phosphorylation. Further support for this view was provided by the finding that 10 mM $MgCl_2$ protected the Ca^{2+}-ATPase against inhibition by 2 μM $LaCl_3$ during the first 10 min incubation with ATP (inset to Figure 7), as if high $MgCl_2$ shifted the equilbrium between Mg−enzyme and La−enzyme toward the former. On these bases, as proposed by Fujimori and Jencks (1990) for the $SRCa^{2+}$-ATPase, the late onset of inhibition shown in the inset to Figure 7 could be due to the fraction of La−enzyme that would increase because it undergoes turnover at a slower rate to reach a new steady-state of lower activity. Results in this paper therefore agree with the idea that, as it is the case in $SRCa^{2+}$-ATPase (Fujimori & Jencks, 1990), displacement of Mg^{2+} caused inhibition of the $PMCa^{2+}$-ATPase by La^{3+}.

As a byproduct of this study, the number of $PMCa^{2+}$-ATPase units, on the basis of the maximum level of *E*P in the inset to Figure 2, can be estimated to be 9×10^3 per pig red cell.

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