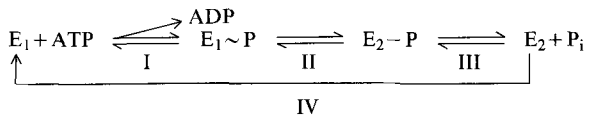
The site of action of La^{3+} in the reaction cycle of the human red cell membrane Ca^{2+}-pump ATPase

S. Luterbacher and H.J. Schatzmann

Department of Veterinary Pharmacology, University of Bern, CH-3000 Bern (Switzerland), August 6, 1982

Summary. Lanthanum (La^{3+}) inhibits the Ca-pump of the red cell by arresting the protein in a phosphorylated form (PI). Similar La^{3+} concentrations are required to increase the amount of PI and to stop PI-decay. In the presence of La^{3+} phosphorylation becomes insensitive to Mg^{2+}. PI made in the presence of Mg^{2+} is not prevented from decaying by subsequent addition of La^{3+}, whereas that made in the absence of Mg^{2+} is. Taken together, these findings seem to indicate that La^{3+} blocks the transition between a 1st and a 2nd form of PI.

ATP hydrolysis by the human red cell Ca^{2+}-pump proceeds under transient phosphorylation of the pump protein (E)[1-7].



Reaction I requires Ca^{2+} in μmolar concentrations[3,4] and from its ATP dependence a K_{ATP}^{app} of 1.5-3 μM (for total ATP) is obtained[3,4]. Reaction II is a conformational change within the protein[3,5] and requires Mg^{2+} for high velocity; dephosphorylation (III) is accelerated by high concentrations of ATP (⩾ 100 μM)[5], possibly in the form of MgATP (at 37 °C and in the presence of calmodulin)[8].

La^{3+} inhibits the Ca^{2+}, Mg^{2+}-ATPase and Ca^{2+} transport completely at 0.1-0.2 mM[9-12]. In its presence, the amount of phosphorylated intermediate (PI ≡ either $E_1 \sim P$, E_2-P or the sum of both) increases[13,14]. It is in a form that can be back-reacted with an excess of ADP[13] but not decomposed by procedures normally inducing forward dephosphorylation (adding ATP + Mg^{2+} with or without EGTA). Thus the point of attack of La^{3+} is beyond reaction I. The present experiments try to determine if La^{3+} interferes with the conformational change (II) or with dephosphorylation (III). They were done with disrupted, calmodulin repleted human red cell membranes, prepared according to Schatzmann[15]. Phosphorylation and dephosphorylation were invariably studied at 0 °C by exposing the membranes to [γ^{32}P]-ATP. For media and techniques see legends to the figures.

Figure 1a describes the effect of increasing La^{3+} concentrations at a fixed, saturating Ca^{2+} and zero Mg^{2+} concentration on the amount of PI found upon exposure of the membranes to [γ^{32}P]-ATP for 45 sec at 0 °C. It shows that La^{3+} brings about a 4-fold increase with a $K_{1/2}$ of 23 μM La^{3+}. Figure 1b is a concentration-effect curve for the inhibition of PI-decay by La^{3+} from which a $K_{1/2}$ of 6 μM can be derived.

The fact that both effects occur in the same range of concentration suggests that the increase in PI is mainly a consequence of the inhibition of decay of PI. This assumption is consistent with the finding that high ADP concentration rapidly decomposes PI made in the presence of La^{3+} by inducing the back reaction (*I*)[13,14], which shows that equilibrium I is not grossly shifted to the right by La^{3+}.

Figure 2 compares the time course of phosphorylation in the presence of Ca^{2+} with or without Mg^{2+} to that observed when, in addition, La^{3+} is present. It may be seen that with Ca^{2+} the effect of Mg^{2+} is to increase the maximal amount of PI and to accelerate its formation. This can be explained without resorting to an additional effect of Mg^{2+} on reaction I with the following assumptions: a) the position of the equilibria is such that $[E_1 \sim P] + [E_2 - P]$ in the steady state is larger than $[E_1 \sim P]$ with the reaction II blocked by lack of Mg^{2+}; and b) the increase in rate reflects the acceleration of the overall reaction by the Mg^{2+} action at reaction II. If Mg^{2+} only acts at reaction II one would expect that phosphorylation becomes Mg^{2+}-insensitive if La^{3+} blocks reaction II. In the presence of La^{3+} the effects of Mg^{2+} indeed vanish (fig. 2).

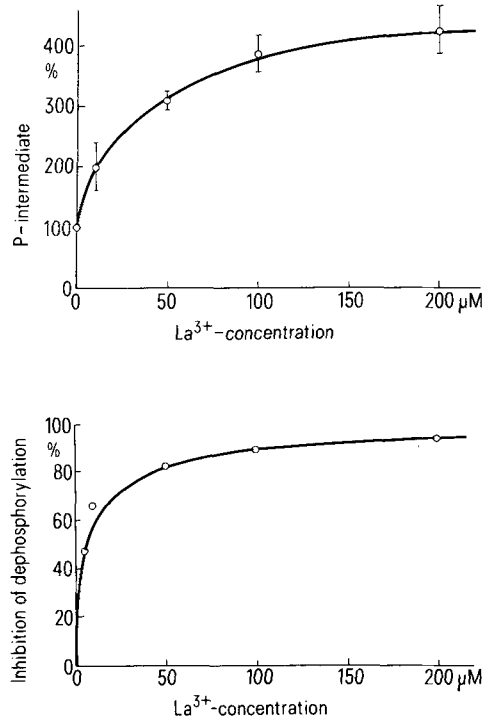


Figure 1. *a* Effect of increasing La^{3+} concentrations on the amount of phosphorylated intermediate (PI). Membranes were prepared according to Schatzmann[15] from pooled blood from 2-3 subjects 1 day after collection of the blood into citrate-phosphate-adenine-glucose solution and stored at −20 °C. Before use the membrane suspension was mixed with an equal volume of a solution containing 240 mM KCl, 60 mM imidazole-Cl (pH 7 at 0 °C) and concentrated to 8 mg protein/ml by centrifugation. Phosphorylation was carried out at 0 °C during 45 sec in the following medium: (mM) KCl 120, imidazole-Cl 30 (pH 7), $CaCl_2$ 0.05 (or tris-EGTA 2), $LaCl_3$ variable, tris-ATP 0.0003 (with approximately 500,000 cpm [γ^{32}P[-ATP (Radiochemical Centre, Amersham); 4 mg membrane protein/ml, vol. 0.5 ml. - Reaction started by adding 50 μl ATP solution, stopped by rapid injection of 1 ml 6% trichloroacetic acid with 50 mM H_3PO_4 and 0.5 mM tris-ATP. The precipitate was washed 4 times with 3 ml of the same solution in the cold and discolved in 0.4 ml 10% sodium dodecylsulfate; an aliquot was counted by liquid scintillation technique. Abscissa: increment of ^{32}P incorporation in Ca^{2+} or $Ca^{2+} + La^{3+}$ containing solution over that in EGTA containing solution. Points: mean ± SE, 3 experiments. *b* Inhibitory effect of increasing La^{3+} concentrations on dephosphorylation of PI made in the presence of La^{3+}. Phosphorylation as described in (a), with 0-0.2 mM $LaCl_3$. At zero time addition of 0.5 mM tris-ATP and 0.5 mM $MgCl_2$. Points: mean of 2 experiments.

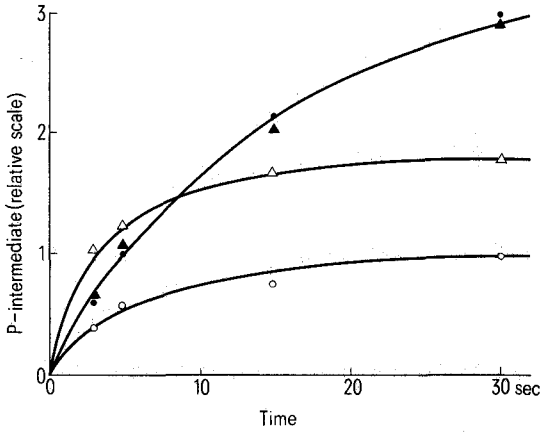


Figure 2. Time course of Ca^{2+} or $Ca^{2+} + La^{3+}$ induced phosphorylation. Technique as in figure 1a. Medium: (mM) KCl 120, imidazole-Cl 30 (pH 7), $CaCl_2$ 0.05, $LaCl_3$ 0.2 (if present), $MgCl_2$ 2 (if present), [γ^{32}P]-tris-ATP 0.0003; membrane protein 4 mg/ml. Ca^{2+} ○; $Ca^{2+} + Mg^{2+}$ △; $Ca^{2+} + La^{3+}$ ●; $Ca^{2+} + Mg^{2+} + La^{3+}$ ▲. Points: mean of 2 experiments.

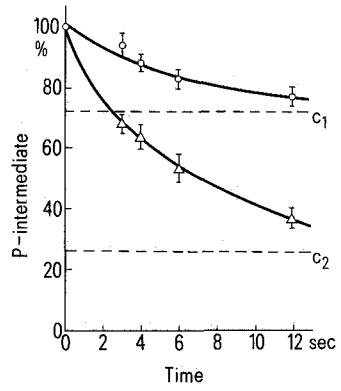


Figure 3. Effect of La^{3+} on dephosphorylation in the presence of $Ca^{2+} + Mg^{2+}$. Phosphorylation as in figure 1a for 30 sec in (mM) KCl 120, imidazole-Cl 30 (pH 7), $CaCl_2$ 0.05, $MgCl_2$ 1 (△), or no Mg^{2+} (○), [γ^{32}P]-tris-ATP 0.0003, membrane protein 4 mg/ml. At 30 sec 0.2 mM $LaCl_3$ was added. At 35 sec (zero time in figure) dephosphorylation was initiated by addition of 0.5 mM tris-ATP (△) or 0.5 mM tris-ATP + 1 mM $MgCl_2$ (○). Thus for dephosphorylation the medium is identical for both curves. Calculation of the asymptote: The experimental points can be approximated by the equation $y = a_i \cdot \exp[-b_i \cdot x] + c_i$; $c_1 = 73\%$, $c_2 = 26\%$ for curves of best fit with $b_1 = b_2$. Points: mean ± SE, 3 experiments.

Figure 3 represents an experiment meant to demonstrate directly the effect of La^{3+} on reaction II. It shows the time course of dephosphorylation in the same medium after phosphorylation has proceeded for 30 sec either with Ca^{2+} or with $Ca^{2+} + Mg^{2+}$ and when La^{3+} was added only after the phosphorylation period (5 sec before dephosphorylation was initiated with 0.5 mM ATP). The result is that PI decays to a low level when Mg^{2+} was present during phosphorylation but not if Mg^{2+} was absent at this stage. The interpretation again is that La^{3+} interrupts the cycle between $E_1 \sim P$ and $E_2 - P$. Without Mg^{2+} most of the PI is in the $E_1 \sim P$ form, not susceptible to ATP, and La^{3+} arrests it in this form. Near the steady state (with Mg^{2+}) most of the PI is in the $E_2 - P$ form, beyond the La^{3+} sensitive step, and is susceptible to ATP-elicited dephosphorylation (that dephosphorylation does not proceed to completion in the 2nd case may reflect some $E_1 \sim P$ formation during the 5 sec in La^{3+} solution).

Thus it seems likely that La^{3+} interferes with the conformational change $E_1 \sim P \rightarrow E_2 - P$; this result constitutes further proof that indeed there is such a transition.

La^{3+} has been shown to be inhibitory not only from the inside (in Ca^{2+}-tight inside-out vesicles[4]) but also from the outside[9,11,12] of the red cell membranes (in intact cells). If the reasonable assumption is made[14] that its binding site is the same in both cases, it becomes probable that La^{3+} binds at or near the Ca^{2+}-transport site which is the part of the protein molecule facing alternatingly the inside and the outside of the cell. $E_1 \sim P$ clearly is the form with the Ca^{2+}-site facing the cell interior (and having high affinity for Ca^{2+}). However, in the presence of La^{3+} the amount of accumulated $E_1 \sim P$ is 4 times the amount of the steady state PI. This implies that when La^{3+} binds to the E_2 form (externally, as it does in experiments with disrupted membranes) it does not prevent the $E_2 \rightarrow E_1$ transition (reaction IV) or that in the steady state most of the protein is in the E_1 form.

It cannot be determined whether La^{3+} replaces Ca^{2+} in the phosphorylation reaction or whether Ca^{2+} is still required because all known Ca^{2+} chelators have a higher affinity for La^{3+} than for Ca^{2+}, which prevents any attempt to study the La^{3+} effect in rigorously Ca^{2+}-free media.

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