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The effect of di- and trivalent cations on the phosphorylation of the Ca^{2+}-ATPase in sarcoplasmic reticulum vesicles

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The steady-state level of phosphorylated intermediate (EP) of (Mg^{2+} + Ca^{2+})-ATPase is influenced by magnesium and calcium concentration in the Ca^{2+}-transporting system of sarcoplasmic reticulum vesicles. At micromolar [Ca^{2+}], the level of EP is increased by Mg^{2+}, depending on its concentration. The effect of Mg^{2+} is less pronounced at lower Ca^{2+} concentration. At low [Mg^{2+}], the EP formation increases at millimolar concentrations of Ca^{2+}, suggesting, in accordance with earlier results, that the substrate may also be CaATP instead of MgATP. $LaCl_3$ (1 mM) enhanced the EP formation at low Mg^{2+} concentration. Surprisingly, 10 μM $LaCl_3$ caused a marked decrease in EP formation at high [Mg^{2+}] and had little or no effect on the level of EP at low Mg^{2+} concentration. The inducing effect of 1 mM $LaCl_3$ on the EP formation at low [Mg^{2+}] and the inhibitory effect of 10 μM $LaCl_3$ at high Mg^{2+} concentration draw attention to the involvement of divalent cation-binding sites with different affinity in phosphorylation and to the particular role of Mg^{2+} in the EP formation and EP decomposition.

Introduction

The calcium transport by sarcoplasmic reticulum vesicles requires optimum concentrations of both calcium and magnesium. 2 mol of calcium are bound with high affinity for each mol of the ATP by the transport sites of the (Mg^{2+} + Ca^{2+})-ATPase on the outside surface of the vesicles; after phosphorylation of the enzyme by ATP, the Ca^{2+} is transported into the intravesicular lumen [1–13] and released from the enzyme before the phosphoenzyme (EP) is hydrolyzed [12–17].

Magnesium ions appear to play a complex role. MgATP is the substrate for the enzyme [3,5–8,18,19]. In addition, Mg^{2+} has been implicated in acceleration of enzyme phosphorylation [14,20], in the hydrolysis of EP [20–24] and in the conversion of the ADP-sensitive phosphoenzyme (E_1P) to ADP-insensitive phosphoenzyme (E_2P) [25]. High Ca^{2+} concentration inhibits the EP hydrolysis [21–23]. The time-course of EP decomposition reveals a fast and a slow kinetic component of EP, with different rate constants. Conversion of EP_{fast} to EP_{slow} is favored by millimolar concentrations of Ca^{2+} and by reduction of Mg^{2+} concentration and vice-versa [26].

The CaATP complex is a potent competitive inhibitor and the sarcoplasmic reticulum membrane displays an affinity for CaATP at least 8-times higher than for MgATP [18]. CaATP can also serve as a substrate for enzyme phosphorylation [21,27,28]. The divalent cation sites on the enzyme that are responsible for the action of Mg^{2+} can be occupied either by Ca^{2+} or by Mg^{2+}, but the rate of phosphorylation was 8–10-times less for CaATP than for MgATP [14]. After the enzyme had been phosphorylated from CaATP,

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calcium remains bound to the enzyme even in the presence of excess EDTA. The EP formed from CaATP turns over slowly because the conversion rate of the E_1P to E_2P is very slow [14]. While 1 mol E_1P formed from MgATP has 2 mol of high-affinity binding sites for Ca^{2+}, E_1P formed from CaATP has 3 mol of binding sites for Ca^{2+}; of these two are transport sites for Ca^{2+}, while the remainder is the acceptor site for Ca^{2+} derived from CaATP [29]. The metal-ATP complex remains bound to the enzyme at least until the E_2P is hydrolyzed [29]. Calcium dissociation from the substrate site is faster than that from the transport sites and it was suggested that on E_1P formed from CaATP, the calcium is released from the transport sites only after the conversion of the phosphoenzyme from E_1P to E_2P [30].

According to Chiesi and Inesi [31], the Mg^{2+} site is distinct from the Ca^{2+}-binding sites involved in the activation of enzyme phosphorylation and Ca^{2+} translocation. Makinose and Boll [19] concluded that the ATPase has a Mg^{2+}-binding site which takes part in the direct activation of the enzyme but is distinct from the substrate site for MgATP. Takakuwa and Kanazawa [32,33] have reported that there exists an extra Mg^{2+} site on the enzyme which is different from the substrate site and is responsible for rapid turnover of the EP. According to Shigekawa et al. [29], the magnesium derived from MgATP is responsible for rapid hydrolysis of the phosphoenzyme intermediate. Further reports [34–36] are also dealing with the binding and dissociation of Mg^{2+}. The results are contradictory and at present the role of Mg^{2+} in the reaction sequence is not clear.

In analyzing the role of divalent cation-binding sites in Ca^{2+} translocation, the investigation of the effects of trivalent cations as Ca^{2+} analogs may be useful. In previous studies an inhibitory effect of lanthanide ions on Ca^{2+} transport in sarcoplasmic reticulum vesicles has been reported [37–41]. In high (10 mM) concentration, $LaCl_3$ quenches both enzyme phosphorylation and EP cleavage. A significant time-lag between the block of EP formation and EP cleavage suggests that the latter effect requires penetration of La^{3+} inside the vesicles [40]. Calcium-binding is competitively inhibited by La^{3+}, with a K_i of 6 μM [42]. Lanthanide ions are assumed to be bound to the Ca^{2+} transport site of the ATPase at lower concentrations [38,41–43]. Highsmith and Head [41] reported high- and low-affinity binding sites for Tb^{3+} which may correspond to the high-affinity Ca^{2+}-binding sites and low-affinity Mg^{2+}-binding sites, respectively. According to Itoh and Kawakita [44] there are three classes of lanthanide ion-binding sites. Binding of Ln^{3+} at 10^{-6} M concentration to the site with the highest affinity was responsible for the inhibition of ATPase activity as well as for that of EP formation and EP decomposition. Binding of these cations at 10^{-5} M concentration to the site with intermediate affinity inhibits the Ca^{2+} binding to the transport site and the Gd^{3+} bound to the high-affinity Ca^{2+}-binding sites do not substitute for Ca^{2+} in the EP-forming reaction. Finally, the binding of Ln^{3+} at 10^{-4} concentration to the site with the low affinity may be equivalent to the Mg^{2+} site [41].

In the present study, we investigated the phosphorylation of (Mg^{2+} + Ca^{2+})-ATPase in sarcoplasmic reticulum membrane at various concentrations of magnesium, calcium and lanthanum.

Methods

The fast-twitch semimembranous muscle of adult rabbits was exclusively used for experiments. After removing the red m. ischio-tibialis located inside of m. semimembranous, the muscle was cut into small pieces and homogenized in 7 vol. of 0.25 M sucrose, 10 mM Tris-maleate buffer of pH 7.0 and 1 mM EGTA in a Potter-Elvehjem homogenizer at 2°C. The further preparation of the microsomal fraction containing the sarcoplasmic reticulum vesicles was performed according to Martonosi et al. [45]. The final pellet was suspended in 0.25 M sucrose and 10 mM Tris-maleate (pH 7.0) and used within some hours for experiment. The assay procedures are described in legends to figures. The free Ca^{2+} concentration below 20 μM was calculated using a stability constant for Ca-EGTA complex of $4.5 \cdot 10^6$ M^{-1} [11,46]. The amount of the phosphorylated intermediate was determined at 0°C on the basis of the ^{32}P incorporation from [γ-^{32}P]ATP into the Ca^{2+} transport protein by the De Meis method [47]. The protein was determined by the method of Lowry et al. [48].

Results

The formation of hydroxylamine-sensitive phosphoenzyme is fast and the steady-state level of EP remains constant for up to 8–10 s at 5 μM ATP at 0°C. The level of EP increases by raising the Mg^{2+} and Ca^{2+} concentration and reaches the highest value at 5 mM $MgCl_2$ and 10–20 μM $CaCl_2$. The level of EP is greater at 5 mM Mg^{2+} than at 50 μM Mg^{2+}. Under 20 μM Ca^{2+}, there is a parallel decrease in Ca^{2+}-dependent and Mg^{2+}-dependent EP. At 5 mM Ca^{2+}, the increase of the steady-state level of EP is striking at low Mg^{2+} concentrations (Fig. 1).

An increase of the EP level occurs if 5 mM $MgCl_2$ is subsequently added to the Mg-free medium in the presence of 20 μM Ca^{2+} (Fig. 2A). The phosphorylated intermediate formed at high Mg^{2+} concentration decays faster after addition of EGTA than that formed at relatively low [Mg^{2+}] (Fig. 2B). These results are in accordance with the observations of Garrahan et al. [20].

At low Mg^{2+} concentration (50 μM), 10 μM La^{3+} causes little or no change in the EP formation, but some increase in the level of EP was observed with 1 mM La^{3+}. At 5 mM Mg^{2+} concentration, 10 μM La^{3+} decreased the EP formation without further change up to 1 mM La^{3+} concentration. 10 mM La^{3+} inhibited the EP formation both at low and at high Mg^{2+} concentration (Fig. 3).

At low Mg^{2+} concentration, $LaCl_3$ added either

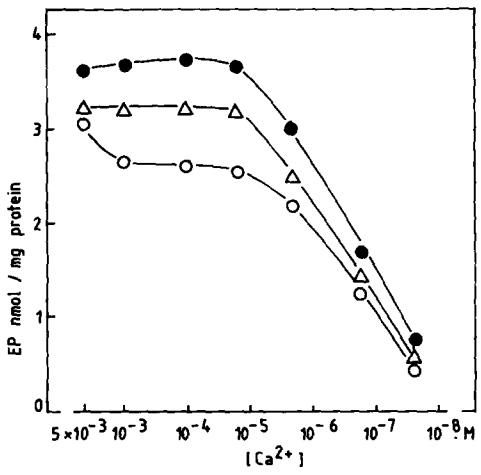

Fig. 1. Dependence of steady-state level of phosphoenzyme formation on magnesium and calcium. Assays were done in solution of 2 ml containing 50 mM KCl, 5 μM [γ-^{32}P]ATP, 25 mM Tris-maleate (pH 7.0), $MgCl_2$ and $CaCl_2$ as indicated. Free Ca^{2+} concentration below 20 μM was adjusted with EGTA (see Methods), 0.1–0.2 mg/ml protein and ●, 5 mM; △, 0.5 mM; ○, 50 μM $MgCl_2$.

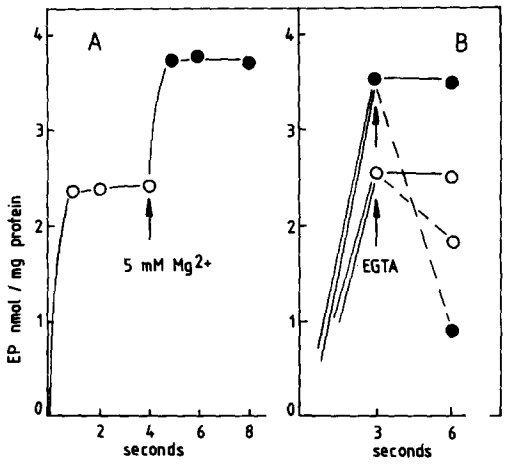

Fig. 2. (A) Effect of magnesium added subsequently to the medium. Assay solution: 50 mM KCl, 5 μM [γ-^{32}P]ATP, 50 μM $MgCl_2$, 20 μM $CaCl_2$, 25 mM Tris-maleate (pH 7.0), 0.1–0.2 mg/ml protein; 0.02 ml of 0.5 M $MgCl_2$ was added (↑) subsequently to the 2 ml medium. (B) Decomposition of phosphoenzyme formed at various Mg^{2+} concentrations. Assay solution: 50 mM KCl, 5 μM [γ-^{32}P]ATP, 20 μM $CaCl_2$, 25 mM Tris-maleate (pH 7.0), 0.1–0.2 mg/ml protein and ●, 5 mM; ○, 50 μM $MgCl_2$. Decomposition of the phosphoenzyme (------) was carried out by addition (↑) of EGTA (final concentration: 1 mM).

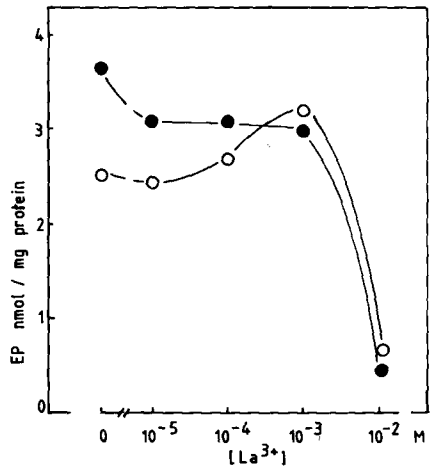

Fig. 3. Effect of $LaCl_3$ on the steady-state level of the phosphoenzyme formation at different Mg^{2+} concentrations. Assay procedure is same as described in Fig. 2B. ●, 5 mM; ○, 50 μM $MgCl_2$.

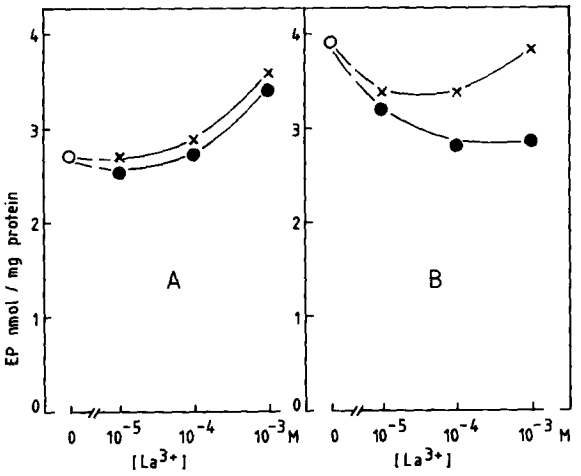

Fig. 4. Effect of $LaCl_3$ added before (●) and following (×) phosphoenzyme formation at 50 μM $MgCl_2$ (A) and 5 mM $MgCl_2$ (B). 0.02 ml of 1, 10 and 100 mM $LaCl_3$ was added to 2 ml medium.

before or after the EP formation produced similar effects (Fig. 4A). At high Mg^{2+} concentration, the level of EP was higher when $LaCl_3$ was added following EP formation (Fig. 4B).

The effect of $LaCl_3$ depends on the time of preincubation of $LaCl_3$ and enzyme. At high Mg^{2+} concentration, the inhibitory effect of 10 μM La^{3+} is immediate and it remains constant up to 6 min, while at higher La^{3+} concentration the inhibitory effect progressively increases during the preincubation time (Fig. 5B).

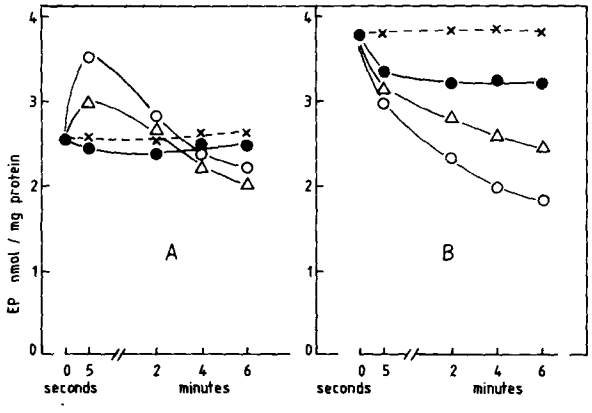

Fig. 5. Time dependence of the preincubation with enzyme and $LaCl_3$ on phosphoenzyme formation at 50 μM $MgCl_2$ (A) and 5 mM $MgCl_2$ (B). Assay procedure is same as described in Fig. 2B. ×------×, Control; ●, 10 μM; △, 100 μM; ○, 1 mM $LaCl_3$.

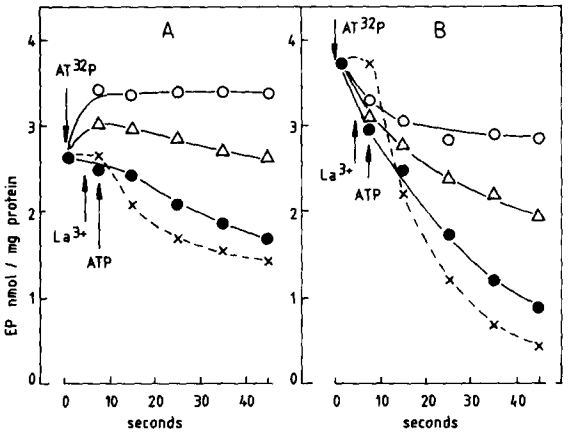

Fig. 6. Decomposition of phosphoenzyme in various concentrations of $LaCl_3$ at 50 μM $MgCl_2$ (A) and 5 mM $MgCl_2$ (B). The decomposition assays were carried out following EP formation with [γ-^{32}P]ATP by addition of $LaCl_3$ and nonradioactive ATP (50 μM final concentration) as indicated (↑ ↑). Composition of phosphorylation solution: 50 mM KCl, 5 μM [γ-^{32}P]ATP, 25 mM Tris-maleate (pH 7.0), 20 μM $CaCl_2$, $MgCl_2$, 0.1–0.2 mg/ml protein. 0.02 ml of 1, 10 and 100 mM $LaCl_3$ and 0.02 ml of 5 mM ATP were subsequently added to 2 ml medium. ×------×, Control; ●, 10 μM; △, 100 μM; ○, 1 mM $LaCl_3$.

At low Mg^{2+} concentration, 10 μM La^{3+} has no effect on the EP formation. At higher La^{3+} concentration, there is an immediate increase in EP formation, followed by a decrease during the preincubation time (Fig. 5A).

The hydrolysis of the phosphoenzyme is progressively inhibited with an increase in La^{3+} concentration, and the extent of this inhibition is greater at low than at high Mg^{2+} concentration (Fig. 6A and B).

Discussion

The role of magnesium and the low-affinity divalent cation-binding site in the Ca^{2+} transport system of sarcoplasmic reticulum vesicles, was investigated. Garrahan et al. [20] have stated that in the presence of Ca^{2+}, ATP phosphorylates the Ca^{2+} pump of sarcoplasmic reticulum at the same site and to the same extent regardless of whether Mg^{2+} is added to the media or not, the main effect of added Mg^{2+} being to increase the rate of phosphorylation. When phosphoenzyme is formed in the presence of Mg^{2+}, it dephosphorylates about 30-times faster than in the absence of Mg^{2+}. It has

been suggested that in the absence of Mg^{2+}, a stable phosphoenzyme is formed [11].

Our results indicate that at saturating [Ca^{2+}] the steady-state level of EP is increased by 5 mM $MgCl_2$. A higher steady-state level of EP is also obtained by addition of 5 mM $MgCl_2$ following phosphorylation of the enzyme at relatively low [Mg^{2+}]. An increase in EP level can also be achieved by increasing the Ca^{2+} concentration at relatively low [Mg^{2+}].

The effects of Mg^{2+} and Ca^{2+} at millimolar concentrations on the steady-state level of EP suggest that EP formation can also be influenced by binding of cations at the low-affinity binding of cations at the low-affinity binding sites and that there is some cooperation between the binding sites of different affinity in the phosphorylation process. The results of the experiments performed with $LaCl_3$ also show this unique role of Mg^{2+} bound at low-affinity binding sites in formation of phosphoenzyme.

10 μM $LaCl_3$ at low [Mg^{2+}] produced little or no change in EP, while in the presence of 5 mM $MgCl_2$ a marked reduction in the steady-state level of EP was observed. 0.1–1 mM La^{3+} increases the EP formation at low [Mg^{2+}], but decreases the higher level of EP formed at high Mg^{2+} concentration.

The results obtained at different Mg^{2+} and La^{3+} concentrations indicate that $LaCl_3$ competitively affects the low-affinity binding sites at 10 μM concentration; 1 mM La^{3+} induces EP formation in place of $MgCl_2$.

The inhibition by 10 μM La^{3+} at high Mg^{2+} concentration on the steady-state level of EP occur immediately after addition of $LaCl_3$ to the medium. When the time of preincubation of the enzyme with 0.1–1.0 mM $LaCl_3$ is increased, the inhibition of EP formation is progressively enhanced.

The results obtained with the EP decomposition also indicate the cooperation between the low- and high-affinity binding sites of the (Mg^{2+} + Ca^{2+})-ATPase. The inhibition of the EP decomposition increases with increasing La^{3+} concentration, and 1 mM $LaCl_3$ completely inhibits the EP decomposition. These results suggest that the binding of La^{3+} in competition with Mg^{2+} at low-affinity sites increases the stability of the phosphoenzyme system and results in the inhibition of the decomposition of Ca^{2+}-dependent EP formed at high-affinity sites.

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