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THE INTERACTION OF MAGNESIUM IONS WITH THE CALCIUM PUMP OF SARCOPLASMIC RETICULUM

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SUMMARY

1. 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 or not to the incubation media, the main effect of added Mg^{2+} being to increase the rate of phosphorylation.

2. When phosphoenzyme is made in Mg^{2+}-containing media it dephosphorylates about 30-times faster than when it is made in the absence of added Mg^{2+}. Addition of Mg^{2+} after phosphorylation is uneffective in accelerating the hydrolysis of phosphoenzyme even in solubilized enzyme, suggesting that phosphorylation of the Ca^{2+} pump results in occlusion of the site at which Mg^{2+} combines to accelerate the release of phosphate.

3. Occlusion of the site for Mg^{2+} can be partially reversed by *trans*-1,2-diaminocyclohexonetetraacetic acid (CDTA). Use was made of this property to demonstrate that for the rapid release of phosphate to occur Mg^{2+} has to be bound to the enzyme.

4. Results seem to indicate that Mg^{2+} combines with the Ca^{2+} pump prior to phosphorylation.

INTRODUCTION

The Ca^{2+} pump of sarcoplasmic reticulum from skeletal muscle is endowed with the property of coupling the hydrolysis of ATP to calcium uptake [1] into sarcoplasmic reticulum vesicles. The hydrolysis of ATP proceeds through a series of steps involving at least a Ca^{2+}-dependent transference of the terminal phosphate of ATP to the Ca^{2+} pump followed by the hydrolysis of the phosphoenzyme with the release of inorganic phosphate [1–8]. Although the requirement for Mg^{2+} of the Ca^{2+}-dependent ATP hydrolysis by the Ca^{2+}-pump in sarcoplasmic reticulum is a

Abbreviations: CDTA, *trans*-1,2-diaminocyclohexanetetraacetic acid; EGTA, ethanedioxybis(ethylamine) tetraacetic acid.

well known fact, a generally accepted mechanism for the role of Mg^{2+} in each of the elementary steps of this reaction is not yet available. In fact, though there seems to be agreement that Mg^{2+} is required for the dephosphorylation reaction [2–8], it is still a matter of discussion whether Mg^{2+} is needed [1, 5–7] or not [2–4] for phosphorylation. The present study was undertaken with the hope of further understanding the role of Mg^{2+} during the hydrolysis of ATP by the Ca^{2+} pump of sarcoplasmic reticulum.

We have reported elsewhere [9] a similar study performed in the Ca^{2+} pump from human red cells. Comparison of these results with those of the present study may help see to what extent a similar mechanism underlines active Ca^{2+} movements in red blood cells and sarcoplasmic reticulum.

METHODS

Preparation of sarcoplasmic reticulum vesicles

Sarcoplasmic reticulum vesicles were prepared from rat skeletal muscle following the procedure described by Martonosi and Feretos [10] for the preparation of the fraction called "Grana I". This preparation was stored overnight at 5 °C in a solution containing: 600 mM KCl, 5 mM histidine. Then 1 mM *trans*-1,2-diaminocyclohexanetetraacetic acid (CDTA) was added to the suspension and after 1 h it was centrifuged at $50\,000 \times g$ for 90 min. The pellet was rinsed with 100 mM KCl, 5 mM histidine, and suspended in more of this solution to give a final concentration of about 1 mg protein/ml. All determinations were performed within 24 h after isolation of the vesicles.

Solubilization

20 μl of Triton X-100 were added to 1 ml of the final suspension of sarcoplasmic reticulum. The mixture was left to stand for 10 min at room temperature. No attempt to remove unsolubilized material was made since under these conditions at least 95 % of the Ca^{2+} pump becomes soluble [11].

Phosphorylation of sarcoplasmic reticulum

The reaction was carried out in 12 ml conical glass centrifuge tubes immersed in an ice-water bath. The reaction mixture contained: 0.6 mM $CaCl_2$, 0.05 mM H_3PO_4, 0.5 mM ethanedioxy-bis(ethylamine)tetraacetic acid (EGTA), 150 mM Tris · HCl, (pH 7.4 at 0 °C); sarcoplasmic reticulum, 0.3 mg protein/ml. When indicated the reaction mixture also contained 5 mM $MgCl_2$. The reaction was started by the addition of enough concentrated [γ-^{32}P]ATP to give a final concentration of 0.05 mM in a reaction volume of 0.3 ml. The reaction was stopped by the quick addition of a freshly prepared solution containing: 300 mM trichloroacetic acid, 1 mM ATP, 50 mM H_3PO_4. After this step 0.1 ml of a solution of 0.02 g/ml of bovine serum albumin was added. The suspension was centrifuged at $1750 \times g$ for 5 min at 5 °C and the sediment was washed 3 times with about 10 ml of the trichloroacetic acid solution. The washed precipitate was suspended in 1 ml of 0.5 M NaOH solution and incubated at 95 °C for 15 min, after which time the radioactivity present was measured in a liquid scintillation counter.

Dephosphorylation was studied measuring the amount of phosphoenzyme at

different time intervals after the addition of 0.1 ml of a chase solution, which in all experiments contained: 10 mM ATP, 2.5 mM EGTA, 150 mM Tris · HCl, (pH 7.4 at 0 °C).

The effect of hydroxylamine was tested as previously described [9].

Measurement of magnesium

Magnesium was measured in an Evans Electroselenium model 140 atomic absorption spectrophotometer fitted with a scale expansion unit. Using the settings recommended by the manufacturer full scale deflection was attained with a 10 μM $MgCl_2$ solution. Samples were incubated at 60 °C for 30 min in a solution containing: 5 mM $LaCl_3$, 0.02 N HNO_3. The precipitate formed was separated by centrifugation at 10 000 $\times g$ for 10 min, and magnesium was measured in the supernatant.

Measurement of Ca^{2+} uptake and Ca^{2+}-dependent ATPase activity

Ca^{2+} uptake was measured following a procedure essentially similar to the millipore method described by Martonosi and Feretos [10]. The media contained: 100 mM KCl, 45 mM Tris · HCl, (pH 7.4 at 37 °C); 5 mM $MgCl_2$, 0.1 mM ^{45}Ca $CaCl_2$, 2 mM potassium oxalate, 4.5 mM ATP (disodium salt). ^{45}Ca was determined by liquid scintillation counting. ATPase activity was measured estimating the P_i released from ATP after incubation of sarcoplasmic reticulum vesicles in media containing: 100 mM KCl, 45 mM Tris · HCl, (pH 7.4 at 37 °C); 5 mM $MgCl_2$, 4.5 mM ATP (disodium salt), 0 or 0.05 mM $CaCl_2$. 0.1 mM EGTA was added to the Ca^{2+}-free medium. The reaction was initiated by the addition of 0.1 ml of a suspension of sarcoplasmic reticulum vesicles (1 mg protein/ml) to 0.9 ml of incubation media. After 5 min incubation at 37 °C, the reaction was stopped by addition of enough concentrated trichloroacetic acid solution to give a final concentration of 5 %. After centrifugation P_i was measured in the supernatant by the procedure of Baginski et al. [12]. Protein was measured by the procedure of Lowry et al. [13].

Sources of materials

[γ-^{32}P]ATP was prepared according to the procedure of Glynn and Chappell [14]. ATP, enzymes and intermediates used for the synthesis of [γ-^{32}P]ATP were from Sigma Chemical Co. (U.S.A.).

X-537A was from Hoffman-La Roche, Nutley, N.J. (U.S.A.). Reagents and salts were of AR grade. All solutions were prepared in doubly glass distilled water. ^{32}P and (^{45}Ca)$CaCl_2$ were provided by the Comisión Nacional de Energía Atómica (Argentina).

RESULTS

The samples of sarcoplasmic reticulum vesicles used in the experiments reported in this paper were treated with *trans*-1,2-diaminocyclohexanetetraacetic acid (CDTA) during preparation. The Ca^{2+}-dependent ATPase activity of the preparation ranged from 68 to 95 μmol P_i/mg protein per h, and was not affected by the exposure to CDTA. Ca^{2+} uptake, on the other hand, was more than 10-times less in CDTA-treated as compared with control vesicles. These results are consistent with the idea that reduction in Ca^{2+} uptake is due to leakage of Ca^{2+} from the vesicles

and hence that treatment with CDTA increases the passive permeability of sarcoplasmic reticulum to Ca^{2+}.

Magnesium content of sarcoplasmic reticulum preparations

Magnesium present in suspensions of sarcoplasmic reticulum was measured in seven different samples. The content of magnesium among the preparations varied from 6 to 14 μmol/g protein. Since no magnesium was measurable in any of the solutions used, it had to be carried by the sarcoplasmic reticulum vesicles. Magnesium was not removed from the vesicles by including either 1 mM EDTA or 1 mM CDTA in all the solutions used to make sarcoplasmic reticulum or by increasing to 10 mM the concn. of CDTA in the KCl solution (see Methods). Table I shows the effects of several treatments on the content of endogenous magnesium of sarcoplasmic reticulum. Results make clear that endogenous magnesium is not accessible to the external solution since its concentration is not lowered by incubation in a 10 mM CDTA solution. Neither is it likely to be dissolved in the intravesicular space since both alkaline pH [15] and the ionophore X-537A [16], which are known to increase the permeability of sarcoplasmic reticulum vesicles to divalent cations, failed to remove endogenous magnesium. Moreover, results in Table I also demonstrate that endogenous magnesium does not exchange with Ca^{2+} in the presence of ATP. Taken together these results rather strongly suggest that endogenous magnesium of the preparations used during these studies exists in a tightly bound state.

TABLE I

THE CONTENT OF MAGNESIUM OF SARCOPLASMIC RETICULUM AFTER DIFFERENT TREATMENTS

Sarcoplasmic reticulum (2 mg protein) was suspended in 2 ml of the different media and incubated with gentle shaking for 10 min at 37 °C. X-537A was added, dissolved in a small volume of ethanol. After incubation the suspension was centrifuged at 31 000 × *g* for 20 min and the pellet suspended in 2 ml of water. An aliquot was put aside for protein determination and then enough volume of a concentrated $LaCl_3/HNO_3$ solution was added to the remaining suspension to measure the magnesium present.

Sarcoplasmic reticulum vesicles incubated in media containing (mM)	pH	Magnesium content (μmol/g protein)
Tris · HCl (150)	7.6	9.2
CDTA (Tris salt), (5)	7.6	8.1
CDTA (5), Tris · HCl (150)	9.3	7.9
X-537A (0.05), Tris · HCl (150)	7.6	8.4
ATP (0.5), $CaCl_2$ (0.5), Tris · HCl (150)	7.6	7.1

The effect of Mg^{2+} on phosphorylation

Fig. 1 shows the amount of ^{32}P transferred from ATP to sarcoplasmic reticulum membranes incubated for various lengths of time in Ca^{2+}-free and Ca^{2+}-containing media with and without $MgCl_2$. In the absence of Ca^{2+} the amount of ^{32}P transferred is very small. When Ca^{2+} is present, phosphorylation increases markedly. Although phosphorylation is much faster in the presence of added Mg^{2+} the steady-

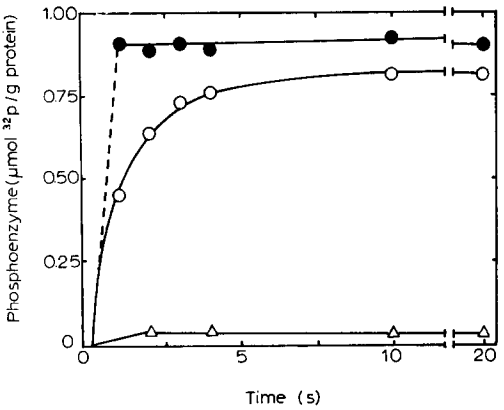

Fig. 1. Time course of the uptake of ^{32}P from [γ-^{32}P]ATP by sarcoplasmic reticulum vesicles in media with (●, △) and without (○) $MgCl_2$ and in the presence (●, ○) and absence (△) of Ca^{2+}.

state level of Ca^{2+}-dependent phosphorylation is the same regardless of the presence or absence of Mg^{2+} in the incubation media. These results agree with findings by others showing that the addition of Mg^{2+} is not required for phosphorylation to reach completion [2–4]. Essentially similar results to those in Fig. 1 were also demonstrated in sarcoplasmic reticulum preparations submitted to the treatments described in Table I, as well as in sarcoplasmic reticulum vesicles solubilized with

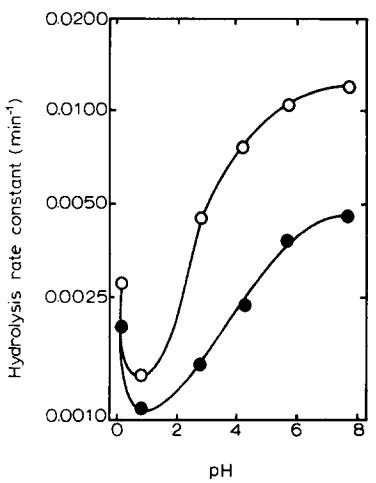

Fig. 2. Hydrolysis of Ca^{2+}-dependent phosphoenzyme in denatured sarcoplasmic reticulum at different pH values. Phosphorylation was carried out in media with (○) and without (●) $MgCl_2$. After precipitation and washing with a trichloroacetic acid solution, about 0.5 mg protein of sarcoplasmic reticulum were suspended in 8 ml of each of the buffers and incubated for 10 min at 40 °C. Incubation was terminated by cooling and by addition of 1 g of trichloroacetic acid. The suspension was centrifuged and washed and the remaining phosphoenzyme measured. The buffers were: pH 0.2, 2 M HCl; pH 0.9, 0.2 M HCl; pH 2.8, 4.3 and 5.7, 0.1 M citric acid adjusted with 0.1 M trisodium citrate; pH 7.8, 0.1 M Tris · HCl. Hydrolysis rate constants were calculated from the difference between the amount of phosphoenzyme before and after incubation, assuming first order kinetics.

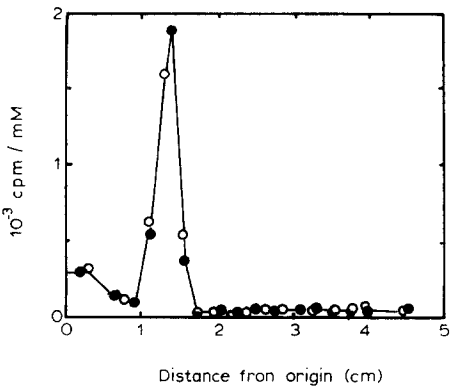

Fig. 3. Distribution of radioactivity in polyacrylamide gels after electrophoresis of sarcoplasmic reticulum phosphorylated in media containing Ca^{2+} (●) and $Ca^{2+}+Mg^{2+}$ (○). Electrophoresis was performed in the presence of sodium dodecyl sulfate following the procedure already described [9]. 0.2 mg protein of sarcoplasmic reticulum were run per gel.

Triton X-100, conditions under which any free Mg^{2+} trapped in sarcoplasmic reticulum vesicles would have been diluted to negligible concentration in the incubation medium.

Comparison of the properties of the phosphoenzyme formed in presence and in the absence of $MgCl_2$

After denaturation with trichloroacetic acid the stability of the phosphoenzyme formed in the presence of $MgCl_2$ upon exposure to solutions of different pH values is similar to the stability of the phosphoenzyme formed in the absence of Mg^{2+} (Fig. 2). Likewise the rate of hydrolysis of the denatured phosphoenzyme in the presence of 50 mM hydroxylamine is the same regardless of whether the phosphoenzyme was prepared in the presence or absence of $MgCl_2$. Gel electrophoresis of sarcoplasmic reticulum phosphorylated with [γ-^{32}P]ATP and Ca^{2+}, results in the appearance of a single band of radioactivity whose mobility is the same regardless of whether Mg^{2+} was added or not to the phosphorylation media (Fig. 3). It seems therefore, that the phosphoenzyme formed in the presence of Ca^{2+} alone is the same as the phosphoenzyme formed in the presence of Ca^{2+} and Mg^{2+}.

Effects of Mg^{2+} on dephosphorylation

Sarcoplasmic reticulum vesicles were phosphorylated in the presence of Ca^{2+} and Mg^{2+} and then dephosphorylation was followed in Mg^{2+}-containing media and in media in which free Mg^{2+} was removed by the addition of a large excess of CDTA. The rate of dephosphorylation under these conditions was compared with that of sarcoplasmic reticulum vesicles phosphorylated in the absence of added Mg^{2+} and dephosphorylated either in Mg^{2+}-containing media or in CDTA-containing media. Results (Fig. 4a) make clear that: (i) the rate of disappearance of the phosphoenzyme formed in the presence of Mg^{2+} is about 30-times larger than that of the phosphoenzyme formed in the absence of Mg^{2+}; (ii) removal of Mg^{2+} after phosphorylation does not change the rate of dephosphorylation; (iii) Mg^{2+} added at the beginning of dephosphorylation is unable to accelerate the rate of dephosphorylation. These

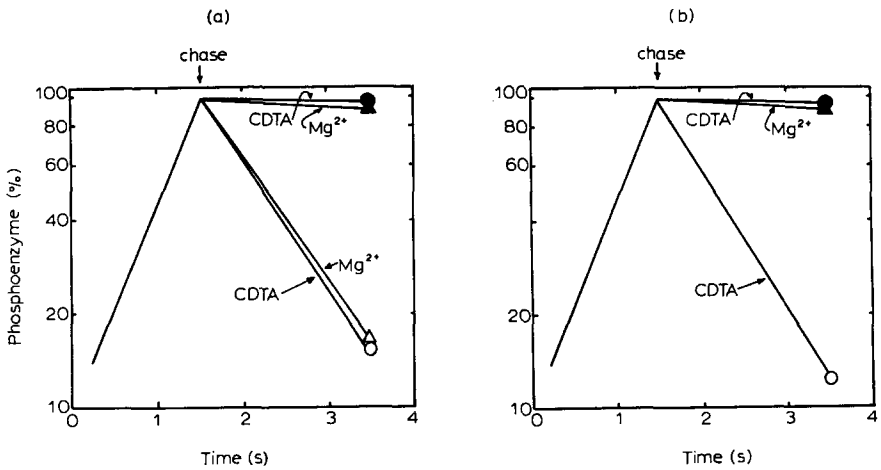

Fig. 4. Hydrolysis of Ca^{2+}-dependent phosphoenzyme in sarcoplasmic reticulum vesicles (a) and in sarcoplasmic reticulum solubilized with Triton X-100 (b). Phosphorylation was initiated adding either ATP (●, ▲) or ATP together with $MgCl_2$ (○, △). Chase solutions (see Methods) contained either 6.6 mM $MgCl_2$ (△, ▲) or 20 mM CDTA (○, ●).

results seem to suggest that the sites at which Mg^{2+} combines to accelerate dephosphorylation drastically reduce their accessibility after reaction of the enzyme with ATP. Identical results to those shown in Fig. 4a were obtained when the effects of Mg^{2+} were tested in sarcoplasmic reticulum vesicles solubilized with Triton X-100 (Fig. 4b). Lack of effect of Mg^{2+} in solubilized membranes is consistent with the idea that unaccessibility of the sites for Mg^{2+} after reaction with ATP is caused by a conformationally-mediated occlusion of these sites rather than by a change in the permeability of the sarcoplasmic reticulum membranes to Mg^{2+}. Our results are in partial agreement with that of Inesi and coworkers [2] who showed that Mg^{2+} accelerated dephosphorylation only after a 2-s long lag. In our case, however, lack of effect of Mg^{2+} persisted even when dephosphorylation was followed for 12 s (experiment not shown).

Results in Figs. 4a and 4b do not show whether occlusion of the sites for Mg^{2+} is a consequence of the formation of the enzyme-ATP complex [8] or a consequence of phosphorylation. To distinguish between these alternatives the rate of dephosphorylation was measured in sarcoplasmic reticulum in which phosphorylation was initiated by the addition of Ca^{2+} and Mg^{2+} to sarcoplasmic reticulum vesicles that had been exposed for 5 s to 100 μM [γ-^{32}P]ATP, to allow Mg^{2+} to react with an enzyme fully occupied by ATP. Results in Fig. 5 show that these conditions lead to the formation of a phosphoenzyme of rapid turnover. Occlusion of the site(s) for Mg^{2+} therefore seems to be caused by phosphorylation. If occlusion of the site for Mg^{2+} is taken for granted, Mg^{2+} would only be able to reach its site(s) in the dephospho state of the enzyme. To test this prediction sarcoplasmic reticulum vesicles were allowed to reach steady-state level of phosphorylation in the absence of added Mg^{2+} and then enough $MgCl_2$ was added to raise Mg^{2+} concentration to 6 mM. Dephosphorylation was started 5 s after the addition of $MgCl_2$. Under these conditions phosphoenzyme decayed at a relatively slow rate the value of which was

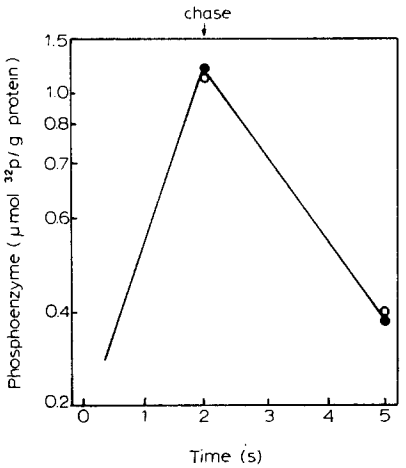

Fig. 5. Effect of the order of addition of Mg^{2+} and ATP on the rate of hydrolysis of Ca^{2+}-dependent phosphoenzyme. Dephosphorylation was measured in phosphoenzyme made by the addition of $Ca^{2+}+Mg^{2+}$ to sarcoplasmic reticulum incubated for 5 s in a [γ-^{32}P]ATP containing medium (●) and by the addition of [γ-^{32}P]ATP to sarcoplasmic reticulum incubated for 5 s in a $Ca^{2+}+Mg^{2+}$-containing medium (○).

in reasonable agreement with that calculated on the assumption that phosphoenzyme of fast hydrolysis rate appears as a consequence of the binding of Mg^{2+} to that fraction of the enzyme that underwent a complete turnover cycle during the 5-s long exposure to Mg^{2+}.

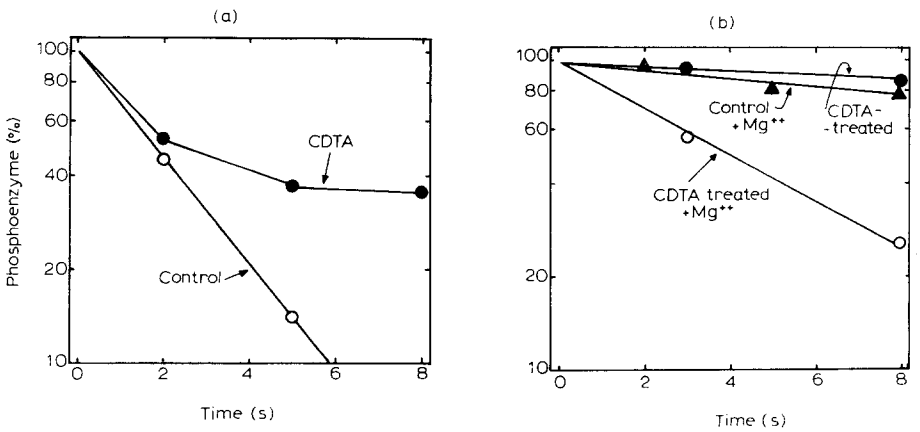

Fig. 6. The effect of CDTA and Mg^{2+} on the time course of hydrolysis of phosphoenzyme. In the experiment of (a), phosphoenzyme was made in a medium containing 0.25 mM $MgCl_2$ and chased in media with (●) and without (○) 20 mM CDTA. In the experiment of (b), phosphoenzyme was made in a Mg^{2+}-free medium and then chased for 5 s in a medium containing 20 mM CDTA. After this period dephosphorylation was followed either in this medium (●) or in a medium to which enough $MgCl_2$ was added to give a free Mg^{2+} concentration of 10 mM (○). The results are plotted taking as 100 % the amount of phosphoenzyme remaining after treatment with CDTA. The control experiment (▲) represents the hydrolysis of the phosphoenzyme not treated with CDTA and chased in a medium containing 10 mM Mg^{2+}.

Effects of CDTA on occlusion of the Mg^{2+} site in phosphoenzyme

In the experiment in Fig. 6a phosphoenzyme made in the presence of added Mg^{2+} was dephosphorylated for 8 s in media with and without CDTA. It can be seen that CDTA has no effect during the first 2 s of dephosphorylation but after this period the rate of dephosphorylation progressively declines tending towards values similar to those of phosphoenzyme made in the absence of Mg^{2+}.

In the experiment in Fig. 6b phosphoenzyme made in the absence of Mg^{2+} was allowed to decay in a medium containing CDTA. After 5 s enough $MgCl_2$ was added to raise Mg^{2+} concentration to 10 mM. Results make clear that, in contrast with the lack of effect of Mg^{2+} on the control preparation, addition of Mg^{2+} to phosphoenzyme previously exposed to CDTA increases the rate of dephosphorylation.

It seems therefore that CDTA, apart from chelating divalent cations, interacts with sarcoplasmic reticulum in a way that leads to a reversal in the occlusion of the sites for Mg^{2+} caused by phosphorylation.

The relation between the rate of dephosphorylation and Mg^{2+} concentration

Sarcoplasmic reticulum vesicles were phosphorylated in media containing from 0 to 5 mM $MgCl_2$ and after 20 s phosphorylation was stopped and the phosphoenzyme allowed to decay for 3 s in a Mg^{2+}-free media (Fig. 7). It is clear that as the concentration of $MgCl_2$ raises, the rate of dephosphorylation markedly increases while the steady-state level of phosphoenzyme remains unchanged. If the effect of 5 mM $MgCl_2$ is taken as maximal, half-maximal stimulation of dephosphorylation would be reached at about 0.150 mM $MgCl_2$. Since the concentration of ATP used in this experiment was 50 μM it seems that the synthesis of "fast" phosphoenzyme can not be attributed solely to the presence of Mg · ATP complex as the substrate for phosphorylation.

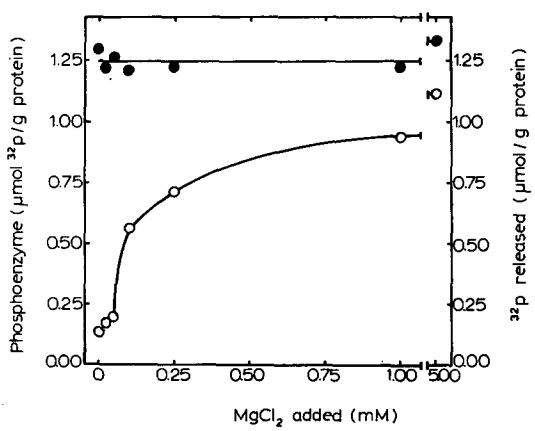

Fig. 7. The effect of magnesium on the steady-state level and on the rate of hydrolysis of Ca^{2+}-dependent phosphoenzyme. Sarcoplasmic reticulum was phosphorylated in media containing the indicated concentrations of $MgCl_2$. For each condition the amount of phosphoenzyme formed in 20 s (●) and the amount of phosphoenzyme remaining after a 3-s long chase were measured, and the P_i released (○) calculated as the difference between the two values.

DISCUSSION

We have shown in this paper that in the absence of added Mg^{2+} the Ca^{2+} pump of sarcoplasmic reticulum is phosphorylated by ATP to almost the same extent and at the same site as when an excess Mg^{2+} is present, the main effect of added Mg^{2+} being to accelerate the rate at which this reaction reaches completion.

Although this result is compatible with the idea that Ca^{2+}-dependent phosphorylation of the Ca^{2+}-pump of sarcoplasmic reticulum does not require the presence of Mg^{2+}, our experiments do not allow us to discard the possibility that the tightly bound Mg^{2+} of sarcoplasmic reticulum plays a role during phosphorylation. Another source of uncertainty lies in the possibility that Mg^{2+} in the media at concentrations below the detection limits of our procedure suffices to drive phosphorylation to completion. In any case however, it must be stressed that neither bound magnesium nor the hypothetical contaminating free Mg^{2+} can account for the requirements of Mg^{2+}, of Ca^{2+}-dependent ATPase or of active Ca^{2+} transport, since neither of these phenomena take place when sarcoplasmic reticulum vesicles are incubated in the absence of added Mg^{2+}.

The properties of the phosphorylation stage of ATP hydrolysis by the Ca^{2+} pump of sarcoplasmic reticulum show a striking resemblance to those of the Ca^{2+} pump of erythrocyte membranes since in this system also, addition of Mg^{2+} is not required but accelerates Ca^{2+}-dependent phosphorylation [9].

In agreement with findings by other authors [2, 5, 8], the Ca^{2+}-dependent phosphoenzyme made in the presence of $MgCl_2$ releases phosphate at a much faster rate than the phosphoenzyme made in the absence of $MgCl_2$. Our results do not however, show if this effect results from the combination of Mg^{2+} at the same site(s) as those at which Mg^{2+} combines to accelerate phosphorylation. The effect of Mg^{2+} on dephosphorylation is consistent with the fact that both phosphorylation of the Ca^{2+} pump by P_i [17] and the $H_2O \rightleftharpoons P_i$ exchange catalyzed by this system [18] only take place in the presence of Mg^{2+}.

One of the salient features of the Mg^{2+}-dependent stimulation of dephosphorylation is that it seems to be exerted by combination at site(s) that after phosphorylation become unable to exchange freely with Mg^{2+} in the bathing solutions. Occlusion of the sites for Mg^{2+} can be partially abolished by CDTA. Treatment with CDTA therefore, makes it possible to add or remove Mg^{2+} from the phosphoenzyme. Experiments of this kind allowed us to demonstrate that Mg^{2+} must remain bound to the enzyme for the rapid transference of phosphate to water. It may be worth commenting at this point that occlusion of sites for cations is an event to be expected if the cation occupying the site, apart from activating catalysis, undergoes translocation [19]. In this respect it may be interesting to mention that occlusion of the sites for K^+ during part of the transport cycle has been shown to occur in the Na^+ pump [20]. Occlusion of the sites for Mg^{2+} during phosphorylation seem to invalidate those schemes for active Ca^{2+} transport that propose that Mg^{2+} enters into the reaction cycle at the phosphorylation stage [5, 18]. Moreover if occlusion persisted under physiological conditions our results seem to favour the idea that separate sites for Ca^{2+} and Mg^{2+} coexist in the dephosphoenzyme and that both have to be occupied to get ATP hydrolysis and active Ca^{2+} movements.

Our results agree with those of Kanazawa and coworkers [5] and of Froehlich

and Taylor [8] in showing that both at low and high concentrations of Mg^{2+} the rate of phosphorylation is considerably larger than the rate of dephosphorylation, this being the reaction rate-limiting in the overall hydrolysis of ATP by the Ca pump. It seems therefore, reasonable to assign the dependence on Mg^{2+} of the Ca^{2+} pump to the effects of Mg^{2+} on the hydrolysis of the phosphoenzyme. If Mg^{2+}-dependent dephosphorylation is in fact rate limiting it becomes easy to explain why, in spite of the increase in the rate of dephosphorylation induced by Mg^{2+}, the steady-state level of phosphoenzyme is not only independent of Mg^{2+} concentration but also almost identical to the total number of phosphorylation sites [7].

We have shown elsewhere [9] that Mg^{2+} is also essential for the rapid dephosphorylation of the Ca^{2+}-dependent ATPase from human red cell membranes. In this case however, there is no occlusion of the Mg^{2+} site and the only effect of Mg^{2+} seems to be acceleration of the transition of the phosphoenzyme from a state of low to a state of high reactivity towards water, Mg^{2+} being apparently not needed for the actual dephosphorylation step. It has been proposed that in sarcoplasmic reticulum vesicles active transport of Ca^{2+} is coupled to the transport of Mg^{2+} [5, 18] while it is known that no such coupling exists during Ca^{2+} transport in red cells [21]. This may perhaps point to a fundamental difference in the role of Mg^{2+} during dephosphorylation and explain why the mechanism of the accelerating effect of Mg^{2+} differs in the two systems.

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