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The reaction of Mg^{2+} with the Ca^{2+}-ATPase from human red cell membranes and its modification by Ca^{2+} *

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(1) Media prepared with CDTA and low concentrations of Ca^{2+}, as judged by the lack of Na^+-dependent phosphorylation and ATPase activity of $(Na^+ + K^+)$-ATPase preparations are free of contaminant Mg^{2+}. (2) In these media, the Ca^{2+}-ATPase from human red cell membranes is phosphorylated by ATP, and a low Ca^{2+}-ATPase activity is present. (3) In the absence of Mg^{2+} the rate of phosphorylation in the presence of 1 μM Ca^{2+} is very low but it approaches the rate measured in Mg^{2+}-containing media if the concentration of Ca^{2+} is increased to 5 mM. (4) The K_{Ca} for phosphorylation is 2 μM in the presence and 60 μM in the absence of Mg^{2+}. (5) Results are consistent with the idea that for catalysis of phosphorylation the Ca^{2+}-ATPase needs Ca^{2+} at the transport site and Mg^{2+} at an activating site and that Ca^{2+} replaces Mg^{2+} at this site. (6) Under conditions in which it increases the rate of phosphorylation, Ca^{2+} is without effect on the Ca^{2+}-ATPase activity in the absence of Mg^{2+} suggesting that to stimulate ATP hydrolysis Mg^{2+} accelerates a reaction other than phosphorylation. (7) Activation of the $E_1P \longrightarrow E_2P$ reaction by Mg^{2+} is prevented by Ca^{2+} after but not before the synthesis of E_1P from E_1 and ATP, suggesting that Mg^{2+} stabilizes E_1 in a state from which Mg^{2+} cannot be removed by Ca^{2+} and that Ca^{2+} stabilizes E_1P in a state insensitive to Mg^{2+}. (8) The response of the Ca^{2+}-ATPase activity to Mg^{2+} concentration is biphasic, activation with a $K_{Mg} = 88$ μM is followed by inhibition with a $K_i = 9.2$ mM. Ca^{2+} at concentration up to 1 mM acts as a dead-end inhibitor of the activation by Mg^{2+}, and Mg^{2+} at concentrations up to 0.5 mM acts as a dead-end inhibitor of the effects of Ca^{2+} at the transport site of the Ca^{2+}-ATPase.

Introduction

We have proposed [1] that under conditions similar to those in an intact cell (0.4 mM Mg^{2+} and 1 to 1.5 mM ATP) ATP hydrolysis by the Ca^{2+}-ATPase from human erythrocyte membranes proceeds through the following pathway:

$$E_1 + ATP \xrightarrow{Ca^{2+}, Mg^{2+}} E_1P + ADP \quad (1)$$

$$E_1P \xrightarrow{Mg^{2+}} E_2P \quad (2)$$

$$E_2P \xrightarrow{ATP} E_2 + P_i \quad (3)$$

$$E_2 \rightarrow E_1$$

where E_1 and E_2 are different conformers of the Ca^{2+}-ATPase. The scheme assumes that only the

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E_2P conformer of the phosphoenzyme undergoes hydrolysis at a significant rate and that the rate of this reaction is accelerated by ATP. In media without added Mg^{2+} reaction [1] takes place slowly but the steady-state level of the phosphoenzyme is similar to that in media with Mg^{2+} [1]. This has been taken as evidence that Mg^{2+} is not essential for phosphorylation. However, as the $K_{0.5}$ for Mg^{2+} during this reaction is in the micromolar range [2], and nominally 'Mg-free' suspensions of membranes in isotonic salt solutions contain in fact 2–4 μM Mg^{2+}, the possibility that contaminant Mg^{2+} is involved in Ca^{2+}-dependent phosphorylation has to be considered. The sources of contaminant Mg^{2+} are the inorganic salts, most of which contain small amounts of magnesium as an impurity and the magnesium that the membranes may carry. The first experiments in this paper were designed to reexamine the requirements of Mg^{2+} for the formation of the phosphoenzyme of the Ca^{2+}-ATPase in conditions under which contaminant Mg^{2+} was reduced to negligible levels.

We have also studied the effects of Mg^{2+} on dephosphorylation. The reaction scheme implies that the only effect of Mg^{2+} on this reaction is to promote the conversion of E_1P into E_2P. Results in this paper show that this effect depends on Ca^{2+} concentration.

It has been reported that the response of Ca^{2+}-ATPase activity to Mg^{2+} concentration is biphasic. There is a small but significant Ca^{2+}-ATPase activity in media containing no added Mg^{2+}. The activity increases as Mg^{2+} concentration raises, reaches a maximum at about 1 mM Mg^{2+} and then drops [3,4]. According to Graf and Penniston [3] activation of the Ca^{2+}-ATPase is exerted by combination of Mg^{2+} at a site in the enzyme, and as proposed by Penniston inhibition of the enzyme is due to displacement of Ca^{2+} [4]. Experiments in this paper also reexamine in detail the degree of dependence on Mg^{2+} of the Ca^{2+}-ATPase and the kinetics of its activation and inhibition by this cation.

Materials and Methods

Mg^{2+}-depleted red cell membranes were obtained following the procedure of Gietzen et al. [5] modified as follows: 1 vol. of packed erythrocytes was mixed at 0°C with 15 vol. of a lysing buffer containing 1 mM CDTA, 15 mM Tris-HCl (pH 7.7 at 20°C) and the suspension was centrifuged at 25 000 × g during 20 min. The pellet was resuspended in 15 vol. of the lysing buffer, collected by centrifugation, and then resuspended in 15 vol. of the same buffer. The suspension was incubated 30 min at 37°C and then centrifuged at 25 000 × g during 20 min at 4°C. The incubation at 37°C was repeated once and then the pellet was resuspended in 15 vol. of 15 mM imidazole-HCl (pH 7.65 at 25°C), 5 μM $CaCl_2$ and the suspension centrifuged at 25 000 × g during 20 min. The final pellet was resuspended in a small volume of 15 mM imidazole-HCl (pH 7.65 at 25°C), 5 μM $CaCl_2$ and stored at −20°C until used. Red cell membranes prepared following the procedure of Garrahan et al. [6] except that 2 mM EGTA replaced 1 mM EDTA in the lysing solution were also used.

$(Na^+ + K^+)$-ATPase was purified from pig kidney by the simpler of the two procedures described by Jørgensen [7]. [γ-^{32}P]ATP was prepared according to the method of Glynn and Chappel [8], except that no orthophosphate was added. [^{32}P]Orthophosphate was provided by Comisión Nacional de Energía Atómica, Argentina. Calmodulin was purified from bovine brain by the procedure of Kakiuchi et al. [9]. Compound 48/80, ATP, ouabain, enzymes and cofactors for the synthesis of [γ-^{32}P]ATP were obtained from Sigma (U.S.A.). Salts and reagents were of analytical reagent grade.

To study activities in Mg^{2+}-free media, contaminant Mg^{2+} was reduced using solutions with low salt concentration and containing 5 mM CDTA. Throughout this paper these solutions will be called 'CDTA media'. Details of their composition are given below and in Results. Ca^{2+}-ATPase activity was measured at 37°C in media with the composition described in Results. Ca^{2+}-ATPase activity was the difference between the activity in these media and the activity in media of identical composition but without $CaCl_2$. $(Na^+ + K^+)$-ATPase activity was measured at 37°C in media containing 50 mM imidazole-HCl (pH 7.4 at 37°C), 5 mM CDTA, 0.01 mM [γ-^{32}P]ATP, 20 mM NaCl, 5 mM KCl, enough $CaCl_2$ for 1 μM

Ca^{2+} (final concentration) with and without 5 mM $MgCl_2$. $(Na^+ + K^+)$-ATPase activity was taken as the difference between the activity in the above-mentioned media and the activity measured in the same media with 0.5 mM ouabain. In all the experiments in which ATPase activities were measured the amount of red cell membranes in the assay medium was the equivalent to 0.1 mg of membrane protein per ml. Activities were estimated from the release of $[^{32}P]P_i$ from $[\gamma\text{-}^{32}P]ATP$ [10], Ca^{2+}-dependent phosphorylation was performed as described previously in media with the composition given in Results. The level of Ca^{2+}-dependent phosphoenzyme was taken as the difference between the amount of ^{32}P incorporated to the membrane protein in media with $CaCl_2$ and in media of identical composition except that $CaCl_2$ was omitted. Na^+-dependent phosphorylation of red cell membranes was performed as described previously. When purified $(Na^+ + K^+)$-ATPase was used, the level of Na^+-dependent phosphoenzyme was measured according to Fukushima and Post [11]. Either the red cell membranes or the purified enzyme were incubated at 0°C during 1 min in 50 mM imidazole-HCl (pH 7.4 at 0°C), 25 mM NaCl, 5 mM CDTA, 0.01 mM $[\gamma\text{-}^{32}P]ATP$ with and without 5 mM $MgCl_2$. $CaCl_2$ was omitted to prevent activation of the Na^+-dependent phosphorylation by Ca^{2+} [11,12]. Na^+-dependent phosphoenzyme was taken as the difference between the level of phosphorylation in the above media and in media of identical composition except that 25 mM KCl replaced NaCl. The amount of red cell membranes in the phosphorylation experiments was the equivalent to 2 mg of membrane protein per ml.

The rate of dephosphorylation was measured by adding to labelled phosphoenzyme in 0.4 ml of incubation media, 0.1 ml of incubation media containing 5 mM unlabelled ATP plus enough of $MgCl_2$, $CaCl_2$ and EGTA to reach the final concentrations of Ca^{2+} and Mg^{2+} that are indicated in Results. The reaction was terminated and the level of phosphoenzyme was measured as described [2]. The values of the rate constants for dephosphorylation were calculated assuming first-order kinetics.

Ca^{2+} (free ionized calcium) concentration in the media was estimated with a Ca^{2+}-selective electrode [13]. Protein concentration was estimated by the procedure of Lündahl [14]. The apparent dissociation constant for the complexes of CDTA, EGTA and ATP with Ca^{2+} or Mg^{2+} under the experimental conditions used during the assays were estimated measuring at 37°C the concentration of Ca^{2+} in solutions of composition similar to those used in the experiments and containing a fixed concentration of chelator and several concentrations of $CaCl_2$ in the absence and in the presence of various concentrations of $MgCl_2$. Calculations were performed adjusting to the experimental points by non-linear regression an equation derived assuming equilibrium among Ca^{2+}, Mg^{2+} and the chelators. The values thus obtained were: $K_{ATP\text{-}MG} = 50\ \mu M$, $K_{ATP\text{-}Ca} = 120\ \mu M$, $K_{EGTA\text{-}Mg} = 13420\ \mu M$, $K_{EGTA\text{-}Ca} = 0.26\ \mu M$ in 100 mM KCl/50 mM imidazole-HCl (pH 7.4 at 37°C) and $K_{ATP\text{-}Ca} = 25\ \mu M$, $K_{CDTA\text{-}Mg} = 1.27\ \mu M$ and $K_{CDTA\text{-}Ca} = 0.022\ \mu M$ in 75 mM imidazole-HCl (pH 7.4 at 37°C).

Using these values, the total concentrations of $CaCl_2$ and $MgCl_2$ necessary to attain a given concentration of free calcium ($[Ca^{2+}]$) and free magnesium ($[Mg^{2+}]$) were calculated by means of the following equations:

$$[CaCl_2] = [Ca^{2+}] + \frac{Q_i}{1 + \frac{K_{Q_iCa}}{[Ca^{2+}]}\left(1 + \frac{[Mg^{2+}]}{K_{Q_iMg}}\right)} \quad (4)$$

$$[MgCl_2] = [Mg^{2+}] + \frac{Q_i}{1 + \frac{K_{Q_iMg}}{[Mg^{2+}]}\left(1 + \frac{[Ca^{2+}]}{K_{Q_iCa}}\right)} \quad (5)$$

where Q_i represents the chelator concentration and K_{Q_iCa} and K_{Q_iMg} the apparent dissociation constants for the complexes chelator-Ca and chelator-Mg, respectively.

Theoretical equations were adjusted to the experimental results by least-squares non-linear regression using the procedure of Gauss-Newton with optional damping [15].

Results

The presence of Mg^{2+} in the reaction media

The first experiments were designed to determine if CDTA-media actually are free of Mg^{2+}.

TABLE I

CONTROL OF THE ABSENCE OF Mg^{2+} IN CDTA-MEDIA ON THE BASIS OF THE Na^+-DEPENDENT PHOSPHORYLATION AND THE ATPase ACTIVITY OF THE $(Na^+ + K^+)$-ATPase

The media used to assay the $(Na^+ + K^+)$-ATPase activity contained 1 μM Ca^{2+}

$MgCl_2$ (mM)	Na^+-dependent phosphorylation (pmol/mg protein)		$(Na^+ + K^+)$-ATPase activity (nmol/mg protein per min)
	Purified enzyme	Red cell membranes	Red cell membranes
0	23 ± 23	0.003 ± 0.031	0.004 ± 0.010
5	1177 ± 58	0.153 ± 0.045	0.386 ± 0.022

With this purpose we measured Na^+-dependent phosphorylation of the $(Na^+ + K^+)$-ATPase by ATP in CDTA-media. This reaction requires Mg^{2+} at micromolar concentrations. Results in Table I show that in these media neither purified $(Na^+ + K^+)$-ATPase from kidney nor Mg^{2+}-depleted red cell membranes are phosphorylated by [γ-^{32}P]ATP unless $MgCl_2$ is added. This suggests that the CDTA medium does not contain Mg^{2+} and that Mg^{2+}-depleted red cell membranes do not add Mg^{2+} to the assay medium.

Since Na^+-dependent phosphorylation was measured in the absence of Ca^{2+}, it could be argued that when $CaCl_2$ is added this salt carries enough contaminant Mg^{2+} and/or displaces chelated Mg^{2+} as to invalidate the conclusion reached above. The Na^+-dependent phosphorylation reaction cannot be used to detect Mg^{2+} in the presence of Ca^{2+} because Ca^{2+} replaces Mg^{2+} as a cofactor for this reaction [11,12]. In addition in red cell membranes Ca^{2+} will also promote phosphorylation of the Ca^{2+}-pump. For this reason we used $(Na^+ + K^+)$-ATPase activity instead of Na^+-dependent phosphorylation to test the presence of Mg^{2+} in Ca^{2+}-containing CDTA-media. Results in Table I show that in CDTA media containing enough $CaCl_2$ as to leave 1 μM free Ca^{2+}, the $(Na^+ + K^+)$-ATPase activity is not significantly different from zero unless $MgCl_2$ is added. In view of this, it seems reasonable to conclude that if Mg^{2+} remained in CDTA-media, its concentration would be below that necessary to produce any detectable effect.

Phosphorylation of the Ca^{2+}-ATPase in the absence of Mg^{2+}

To see if Mg^{2+} is required for the formation of the phosphoenzyme of the Ca^{2+}-ATPase, Ca^{2+}-dependent phosphorylation of red cell membranes by ATP was measured as a function of the reaction time in CDTA-media containing 1 μM Ca^{2+}.

Results in Fig. 1 show that the amount of Ca^{2+}-dependent phosphoenzyme increases with a $t_{1/2}$ of about 1.5 min tending to a value similar to the amount of phosphoenzyme formed in a medium with 5 mM MCl_2 during 1 min, time enough for the phosphoenzyme to reach its maximum level in such medium. In view of this, it can be concluded that although Mg^{2+} accelerates phosphorylation of the Ca^{2+}-ATPase by ATP, it is not an essential cofactor for the reaction.

Effects of Ca^{2+} on the phosphorylation reaction in the presence and absence of Mg^{2+}

Fig. 2 shows the results of an experiment in which the amount of Ca^{2+}-dependent phosphoenzyme was measured as a function of the

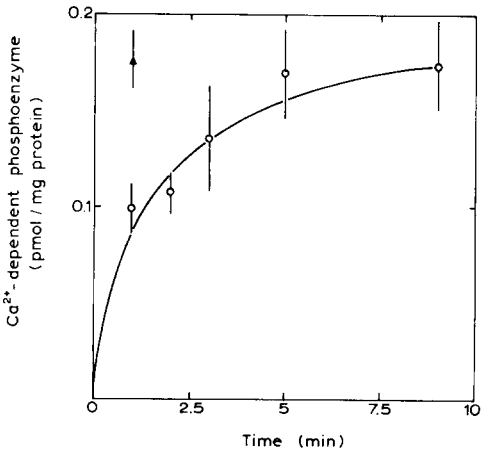

Fig. 1. Time course of Ca^{2+}-dependent phosphorylation of Mg^{2+}-depleted red cell membranes in CDTA-media without added Mg^{2+} (○). For comparison the level of phosphorylation in the presence of 5 mM $MgCl_2$ (370 μM Mg^{2+}) is shown (▲). Phosphorylation was performed at 0°C in media containing 75 mM imidazole-HCl (pH 7.4 at 0°C), 5 mM CDTA, 0.01 mM (γ-^{32}P)ATO, enough $CaCl_2$ for 1 μM Ca^{2+} with and without $MgCl_2$.

concentration of Ca^{2+} in media with and without Mg^{2+}. In the presence of Mg^{2+}, Ca^{2+} increases phosphorylation with a K_{Ca} near 2 μM, a value which is similar to the $K_{0.5}$ for the effects of Ca^{2+} at the transport site of the Ca^{2+}-ATPase while in the absence of Mg^{2+} the level of phosphoenzyme increases with Ca^{2+} along a rectangular hyperbola which is half-maximal at 60 μM Ca^{2+}. To study further the effects of Ca^{2+} on the phosphorylation reaction we have followed the time course for the formation of the Ca^{2+}-dependent phosphoenzyme in media without Mg^{2+} and with either 50 or 5000 μM Ca^{2+}. Results in Fig. 3 show that in the presence of 50 μM Ca^{2+} the Ca^{2+}-ATPase can be phosphorylated by ATP to a maximum level of 1.00 pmol/mg with a $t_{1/2}$ of 13.3 s. On increasing the concentration of Ca^{2+} to 5000 μM the maximum level of phosphoenzyme raises up to 2.2 pmol/mg and $t_{1/2}$ is reduced to 4.6 s. Results (not shown) demonstrated that $t_{1/2}$ for dephosphorylation of phosphoenzyme made in 50 and 5000 μM Ca^{2+} was 3.6 and 3.8 s, respectively. It is clear therefore that increasing the concentration of Ca^{2+} increases the rate of formation and the level of Ca^{2+}-dependent phosphoenzyme.

Effects of Ca^{2+} on dephosphorylation of phosphoenzyme formed in the absence of added Mg^{2+}

The rate of hydrolysis of phosphoenzyme made in media containing no added $MgCl_2$ and various concentrations of Ca^{2+} was measured during 3 s in media with 1 mM ATP, 0.5 mM Mg^{2+} and different concentrations of Ca^{2+} and compared with the rate of hydrolysis of phosphoenzyme made in medium containing 0.5 mM Mg^{2+}. Results in Table II show that phosphoenzyme made in 50 μM Ca^{2+} dephosphorylates at a low rate in media containing 50 μM Ca^{2+}. Phosphoenzyme made in 15 μM Ca^{2+} dephosphorylates at a normal rate in a medium with 10 M Ca^{2+} but at a low rate in medium with 50 μM Ca^{2+}. Table II also shows that phosphoenzyme made in 100 μM Ca^{2+} undergoes rapid hydrolysis in medium with enough EGTA to lower the concentration of Ca^{2+} to less than 1 μM. From these results it seems clear that provided the phosphoenzyme is made in media without added Mg^{2+}, 50 μM Ca^{2+} in the dephosphorylation media blocks the ability of Mg^{2+} to accelerate hydrolysis in a way that is

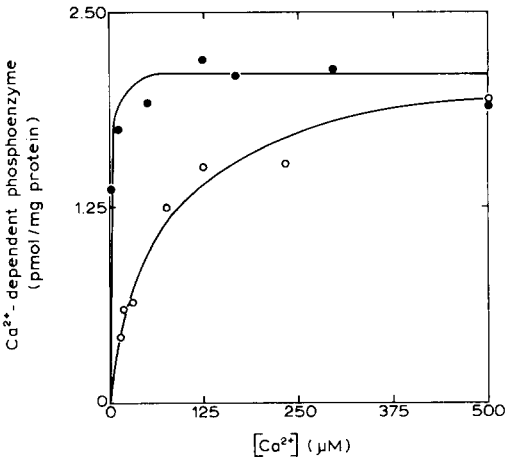

Fig. 2. Ca^{2+}-dependent phosphorylation as a function of Ca^{2+} concentration in the absence (○) and in the presence of 0.5 mM Mg^{2+} (●). Membranes were prepared by the procedure of Garrahan et al. [6] modified as described in Materials and Methods. Phosphorylation was carried out at 0°C during 40 s in media containing 50 mM imidazole-HCl (pH 7.4 at 37°C), 100 mM KCl, 0.5 mM EGTA, 0.01 mM [γ-^{32}P]ATP and enough $CaCl_2$ to obtain the concentrations of Ca^{2+} indicated in the figure with and without $MgCl_2$.

independent of the concentration of Ca^{2+} during phosphorylation. A possible explanation for the observed effect of Ca^{2+} is that in the presence of Ca^{2+} it takes a longer time for Mg^{2+} to accelerate dephosphorylation than in its absence. To see if this is the case, the time course of hydrolysis of phosphoenzyme formed in medium with 50 μM

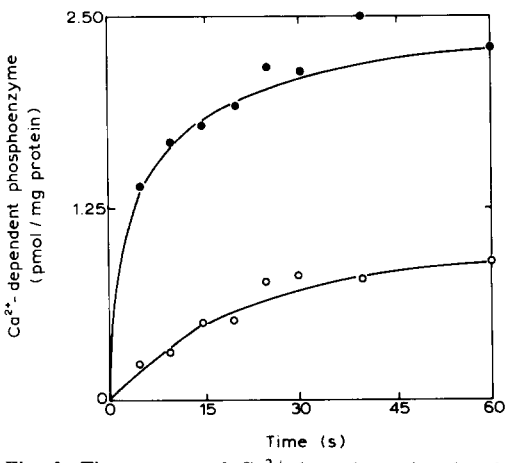

Fig. 3. Time course of Ca^{2+}-dependent phosphorylation at 0°C in the presence of 50 μM (○) or 500 μM (●) Ca^{2+}. Other conditions are those described in the legend to Fig. 2.

TABLE II

EFFECTS OF Ca^{2+} ON THE RATE OF DEPHOSPHORYLATION OF THE PHOSPHOENZYME

Membranes were prepared by the procedure of Garrahan et al. [6] modified as described in Materials and Methods. Phosphorylation wa scarried out as described in the legend to Fig. 2. All dephosphorylation media contained 500 μM Mg^{2+} (* 160 μM $CaCl_2$ plus 1.1 mM EGTA). The rate constant for dephosphorylation of phosphoenzyme made in 50 μM Ca^{2+} and dephosphorylated in medium containing 50 μM Ca^{2+} and no added $MgCl_2$ was 0.230 s^{-1}.

Ca^{2+} concentration (μM)		Rate constant for dephosphorylation (s^{-1})
During phosphorylation	During dephosphorylation	
50	50	0.220
15	10	0.508
15	50	0.257
100	<1 *	0.451
50 plus 500 μM Mg^{2+}	50	0.604

Ca^{2+} was followed during 10 s (instead of 3 s as in Table II) in a medium with 50 μM Ca^{2+} and 0.5 mM Mg^{2+}. Results in Fig. 4 show that in a 10 s period almost 90% of the phosphoenzyme dephosphorylates at a constant and low rate ($k = 0.228 \pm 0.011$ s^{-1}). In view of this finding, the lack of effect of Mg^{2+} on the dephosphorylation reaction in the presence of 50 μM Ca^{2+} can not be attributed to a lag in the effect of Mg^{2+} caused by Ca^{2+}. After this, lack of acceleration by Mg^{2+} under the conditions of Table II could be attributed either to the displacement of Mg^{2+} from the phosphoenzyme by Ca^{2+} or to the inability of the phosphoenzyme to react with Mg^{2+} in media with Ca^{2+}. To discriminate between these alternatives, the rate of dephosphorylation in media with 50 μM Ca^{2+} was measured in the presence of increasing concentrations of Mg^{2+}. Results showed that for any of the conditions used the rate of dephosphorylation remained low, the rate constants for dephosphorylation being 0.227, 0.273, and 0.234 s^{-1} for 0, 0.5 and 19 mM Mg^{2+}, respectively. This suggests that displacement of Mg^{2+} by Ca^{2+} is not the cause of the lack of acceleration by Mg^{2+}. It seems therefore that in the presence of 50 μM Ca^{2+} the phosphoenzyme formed in the absence of Mg^{2+} is unable to react with Mg^{2+} to undergo rapid hydrolysis.

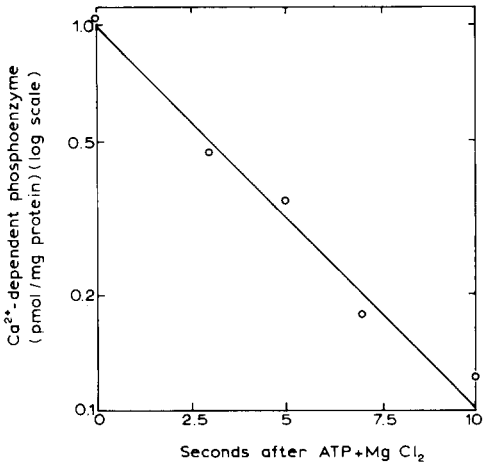

Fig. 4. Time course of dephosphorylation of phosphoenzyme prepared in the absence of added Mg^{2+}. Phosphorylation was carried out at 0°C in the presence of 50 μM Ca^{2+} in a total volume of 0.4 ml as described in the legend to fig. 2. At 40 s dephosphorylation was started adding 0.1 ml of incubation media containing 5 mM ATP and 7.5 mM $MgCl_2$. At the times shown, the reaction was stopped and the level of phosphoenzyme was measured as described previously [2].

Effects of Ca^{2+} on dephosphorylation of the phosphoenzyme formed in media with Mg^{2+}

Phosphoenzyme was made in media containing 0.5 mM $MgCl_2$ plus various concentrations of Ca^{2+} and its rate of hydrolysis was measured with the results shown in Table III. Under these conditions the phosphoenzyme undergoes rapid hydrolysis even when the concentration of Ca^{2+} is raised to 5 mM. These results indicate that in sharp contrast with what happens after, if Mg^{2+} is present before or during phosphorylation Ca^{2+} is unable to impede it to accelerate the hydrolysis of the phosphoenzyme.

The dependence of the rate of dephosphorylation on the Mg^{2+} concentration during phosphorylation

Fig. 5 shows the effect of increasing Mg^{2+} concentration in the phosphorylation media on the rate constant for dephosphorylation. The rate of dephosphorylation increases along a curve that tends to saturation as the concentration of Mg^{2+} raises. The apparent dissociation constant for Mg^{2+} calculated from the equation of the curve

TABLE III

EFFECT OF Ca^{2+} ON THE RATE OF HYDROLYSIS OF THE PHOSPHOENZYME FORMED IN THE PRESENCE OF Mg^{2+}

Membranes were prepared by the procedure of Garrahan et al. [6] modified as described in Materials and Methods. Phosphorylation was carried out as described in the legend to Fig. 2. All the media contained 0.5 mM Mg^{2+}. * The medium contained 0.44 mM $CaCl_2$ plus 10.5 mM EGTA.

Ca^{2+} concentration (μM)		Rate constant for dephosphorylation (s^{-1})
During phosphorylation	During dephosphorylation	
50	very low *	0.762
50	50	0.604
500	500	0.758
5000	5000	0.624

that best fitted the experimental points is 117 ± 47 μM.

Effect of Mg^{2+} on Ca^{2+}-ATPase activity

Fig. 6 represents Ca^{2+}-ATPase activity in the presence of 100 μM Ca^{2+} and 20 μM ATP as a function of the concentration of Mg^{2+} calculated as described in Methods and neglecting the contribution of the samll amounts of Mg^{2+} that may have been brought in as a contaminant. Results show that there is activity in nominally Mg^{2+}-free medium. This confirms for intact membranes findings by Graf and Penniston [3] and Penniston [4] who used purified Ca^{2+}-ATPase from red cell membranes. As the concentration of Mg^{2+} increases, the activity raises reaching a maximum at about 1 mM Mg^{2+}. The inset in Fig. 6 indicates that activation of the Ca^{2+}-ATPase by Mg^{2+} follows a simple hyperbolic curve. Increments in the concentration of Mg^{2+} above 1 mM lower the activity until at 20 mM Mg^{2+} it reaches a value about 70% the maximum. From the equation of the curve in Fig. 6 it can be calculated that the apparent dissociation constant for Mg^{2+} as activator (K_{Mg}) is 87.8 ± 3.7 μM and the apparent dissociation constant for Mg^{2+} as partial inhibitor (K_i) is 9179 ± 979 μM. The fact that $K_{Mg} < K_i$ and that V_r is only half V_m (see legend to Fig. 6) explains why the initial part of the curve shown in the inset of Fig. 6 can be adjusted by an equation like Eqn. 7, neglecting the inhibitory components.

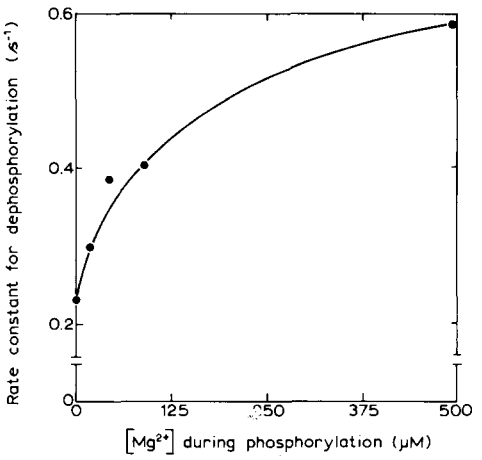

Fig. 5. Rate constant for dephosphorylation as a function of Mg^{2+} concentration in phosphorylation media. Membranes, prepared by the procedure of Garrahan et al. [6] modified as described in Materials and Methods, were phosphorylated as described in the legend to Fig. 2 in media with 50 μM Ca^{2+} and the concentrations of Mg^{2+} indicated in the figure. At 40 s enough $MgCl_2$ and ATP were added to give a final concentration of 0.5 and 1 mM, respectively, and 3 s after, the reaction was stopped. The values of rate constants for dephosphorylation were calculated as it is described in Materials and Methods. The equation that fitted the experimental points was

$$k = \frac{(k_\infty - k_0)}{1 + (K_{Mg}/[Mg^{2+}])} + k_0 \qquad (6)$$

where k_0 is the rate constant for dephosphorylation in the absence of Mg^{2+}, k_∞ is the rate constant for dephosphorylation in the presence of saturating concentration of Mg^{2+} and K_{mg} is the concentration of Mg^{2+} fro half-maximal effect. The values of the parameters ($\pm$S.E.) that gave the best fitting were $k_0 = 0.233 \pm 0.025$ s^{-1}; $k_\infty = 0.659 \pm 0.055$ s^{-1} and $K_{Mg} = 117 \pm 47$ μM.

Ca^{2+}-ATPase activity in the absence of Mg^{2+}

The activity observed at 0 mM added Mg^{2+} in the experiment in Fig. 6 rather than being the expression of the Ca^{2+}-ATPase in the absence of Mg^{2+}, might be attributed to contaminant Mg^{2+}. In fact, from the values of V_m and K_{Mg} in Fig. 6 it can be calculated that 16 μM Mg^{2+} present as contaminant in the incubation medium will give rise to the Ca^{2+}-ATPase activity measured in the medium without added Mg^{2+} in Fig. 6. To solve this uncertainty, we measured Ca^{2+}-dependent

ATP hydrolysis by Mg-depleted red cell membranes suspended in CDTA media. Results showed that under these conditions Ca^{2+}-ATPase activity is 0.089 ± 0.012 nmol/mg protein per min compared with an activity of 0.936 ± 0.048 nmol/mg protein per min in the same medium but containing 5 mM $MgCl_2$.

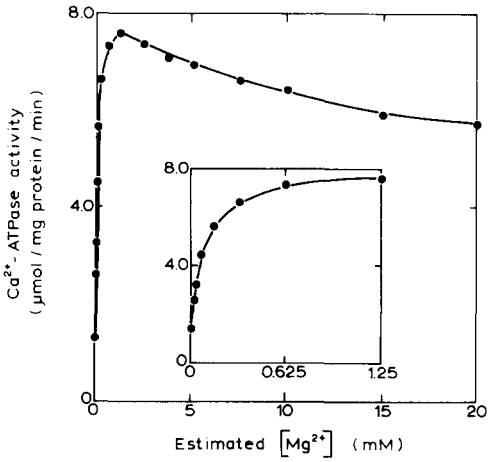

Fig. 6. Ca^{2+}-ATPase activity as a function of Mg^{2+} concentration in the presence of 100 μM Ca^{2+}. Membranes were prepared by the procedure of Garrahan et al. [6] modified as described in Materials and Methods. The incubation media contained 50 mM imidazole-HCl (pH 7.4 at 37°C), 100 mM KCl, 0.5 mM EGTA, 0.5 mM ouabain, 0.02 mM [γ-^{32}P]ATP plus enough $CaCl_2$ and $MgCl_2$ to obtain the final concentrations of Mg^{2+} indicated in the figure. The continuous line is the solution of the following equation.

$$v = V_0 + \frac{(V_m - V_0)}{1 + \frac{K_{Mg}}{[Mg^{2+}]} + \frac{[Mg^{2+}]}{K_i}} + \frac{(V_r - V_0)}{1 + \frac{K_i}{[Mg^{2+}]}\left(1 + \frac{K_{Mg}}{[Mg^{2+}]}\right)} \quad (7)$$

where V_0 is the activity without added $MgCl_2$ (0 mM Mg^{2+} in Fig. 6), V_m is the activity at saturating concentration of Mg^{2+} in the absence of inhibition, V_r is the activity when the concentration of Mg^{2+} tends to infinity, K_{Mg} and K_i are apparent dissociation constants for Mg^{2+} as activator and inhibitor, respectively. The best-fitting values of the parameters ($\pm$S.E.) were:

$V_0 = 1.26 \pm 0.05$ nmol/mg protein/min

$V_m = 8.46 \pm 0.09$ nmol/mg protein/min $\quad K_{Mg} = 87 \pm 3.7\,\mu$M

$V_r = 4.54 \pm 0.11$ nmol/mg protein/min $\quad K_i = 9179 \pm 979\,\mu$M

The inset is the initial part of the curve. The continuous line is the solution of Eqn. 7 neglecting the contribution of the inhibitory component.

Interaction of Mg^{2+} and Ca^{2+} at the Mg^{2+} activating site

Ca^{2+}-ATPase activity was measured in media containing 20 μM ATP plus 50 to 990 μM Ca^{2+} as a function of Mg^{2+} concentration up to 1 mM. As judged by the K_i value calculated from the experiment in Fig. 6, under the conditions chosen the inhibitory effect of Mg^{2+} will be negligible. Results in Fig. 7 show that for all the concentrations of Ca^{2+} used activation by Mg^{2+} follows simple hyperbolic kinetics suggesting that Mg^{2+} activates acting at a single class of non-interacting sites. The inset to Fig. 7 shows that the concentra-

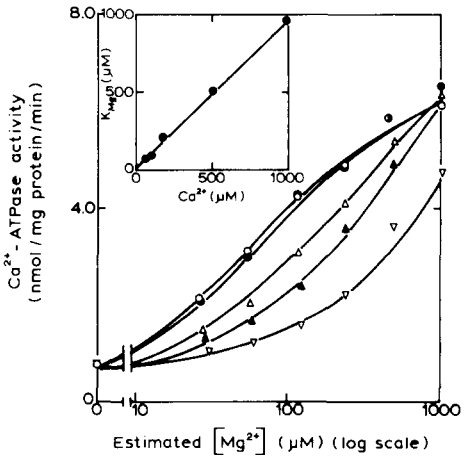

Fig. 7. Ca^{2+}-ATPase activity as a function of Mg^{2+} concentration (log scale) in the presence of 53 (▽), 103 (▲), 170 (△), 443 (●) and 990 (○) μM Ca^{2+}. Membranes were prepared by the procedure of Garrahan et al. [6] modified as described in Materials and Methods. Other conditions are described in the legend to Fig. 6. For each Ca^{2+} concentration the following equation was adjusted by non-linear regression

$$v = V_0 + \frac{(V_m - V_0)}{1 + (K_{Mg}/[Mg^{2+}])}$$

The equation is based on the assumption that there is activity in the absence of Mg^{2+} (V_0) and that the effect of Mg^{2+} is excerted along a Michaelis-Menten-like equation that reaches its half-maximal value when the concentration of Mg^{2+} is equal to K_{Mg}. The inset is a plot of the best-fitting values of K_{Mg} against the concentration of Ca^{2+}.

tion of Mg^{2+} needed for half-maximal activation increases linearly with Ca^{2+}. This suggests that Ca^{2+} is a competitive inhibitor of the activation by Mg^{2+} probably because Ca^{2+} desplaces Mg^{2+} from the activating site without replacing it in its effects. From the intercept at the ordinate the value of K_{Mg} at zero Ca^{2+} concentration can be calculated to be 19.7 μg. The apparent dissociation constant for Ca^{2+} of the site from which Ca^{2+} displaces Mg^{2+} calculated from the intercept at the abscissa is 18.3 μM.

Essentially similar results were obtained when Ca^{2+}-ATPase activity was measured under conditions identical to those used for the experiment in Fig. 7 except that the concentration of ATP was 1000 μM instead of 20 μM (experiment not shown), indicating that combination of Mg^{2+} and its activating site is independent of ATP. It is worth to point out that all the curves in Fig. 7 have the same origin showing that in the absence of Mg^{2+} increasing from 50 to 1000 μM the concentration of Ca^{2+} is without effect on Ca^{2+}-ATPase activity.

Interaction of Ca^{2+} and Mg^{2+} with the transport site

Fig. 8A shows results of an experiment in which Ca^{2+}-ATPase activity was measured as a function of Ca^{2+} concentration in media containing 20 μM ATP and different concentrations of Mg^{2+} calculated as in the experiment of Fig. 6. In a nominally Mg^{2+}-free medium there is a small but significant stimulation of Ca^{2+}-ATPase activity by Ca^{2+} which is maximum at about 10 μM Ca^{2+}. As the concentration of Mg^{2+} raises, the increase in activity due to Ca^{2+} is more apparent and continues beyond 10 μM Ca^{2+}. All curves can be adjusted by Michaelis-Menten-like equations. Fig. 8B shows that the maximum effect of Ca^{2+} increases along a hyperbolic curve which levels off at about 70 μM Mg^{2+}. When the best fitting values of K_{Ca} are plotted against Mg^{2+} concentrations, they fall on a single straight line of positive slope (see Fig. 8B) suggesting competition between Mg^{2+} and Ca^{2+} probably because Mg^{2+} displaces Ca^{2+} from the transport site. By extrapolating the graph to the ordinate, the value of K_{Ca} at 0 mM Mg^{2+} can be calculated to be 1.39 ± 0.11 μM. The apparent dissociation constant for Mg^{2+} calculated from the intercept at the abscissa, is 240 μM. Similar values were obtained at 1000 μM ATP, a result that gives further support to the idea that Mg^{2+} displaces Ca^{2+} from the enzyme rather than from ATP.

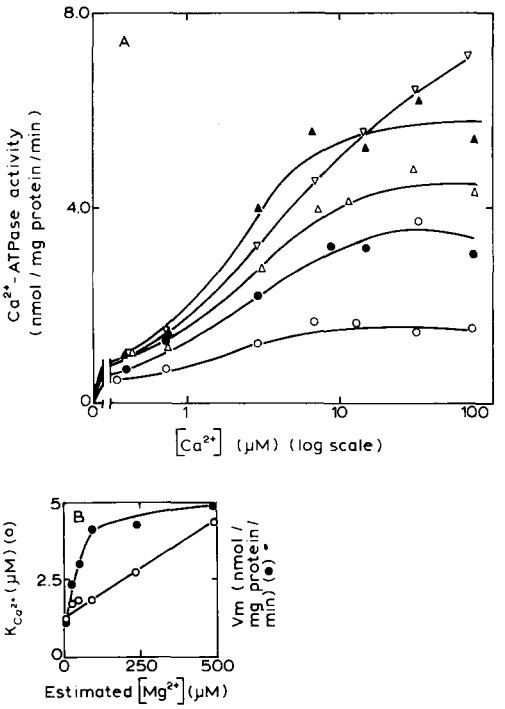

Fig. 8. Ca^{2+}-ATPase activity as a function of Ca^{2+} concentration (log scale) in the presence of 0 (○); 20 (●); 40 (△), 87 (▽) and 480 (▲) μM Mg^{2+}. Membranes were prepared by the procedure of Garrahan et al. [6] modified as described in Materials and Methods. Other experimental conditions as in Fig. 6. For each Mg^{2+} concentration a Michaelis-Menten equation was adjusted by non-linear regression.

Discussion

Most of the reactions catalyzed by the red cell membrane Ca^{2+}-ATPase are activated by Mg^{2+} at concentrations that lie in the micromolar range and are therefore close to the concentration of the Mg^{2+} that is present as a contaminant in suspensions of membranes in isotonic salt solutions. As a consequence of this it is difficult to study the effects of this cation on the enzymatic activities of the Ca^{2+}-ATPase.

In the present study magnesium that might remain associated with the membranes was re-

moved by incubating and washing the membranes in solutions containing 1 mM CDTA. Contaminant Mg^{2+} in the solutions was reduced in part by decreasing the concentration of the inorganic salts, but mainly by the inclusion of a strong chelator as CDTA in the reaction media. The use of chelators to remove Mg^{2+} from solutions that also contain Ca^{2+} is hampered by the fact that there is no chelator that binds Mg^{2+} more strongly than Ca^{2+}. This difficulty was overcome using media containing 5 mM CDTA and just enough of $CaCl_2$ as to give a concentration of free Ca^{2+} of 1 μM. Under these conditions, using the values of dissociation constants given under Materials and Methods, it can be calculated that if contaminant magnesium were 20 μM, the concentration of free-Mg^{2+} will be less than 0.1 μM.

To test the validity of this calculation under our experimental conditions, we looked for the presence of minute amounts of Mg^{2+} in the CDTA-media taking advantage of the known fact that both the overall activity and the Na^+-dependent phosphorylation [16] of the $(Na^+ + K^+)$-ATPase require Mg^{2+} with high apparent affinity ($K_{0.5}$ about 5 μM) [17]. Results showed that in CDTA-media these activities are absent unless $MgCl_2$ is added. This strongly suggests that CDTA-media are indeed free of Mg^{2+}.

Results in this paper showing phosphorylation of the Ca^{2+}-ATPase in CDTA-media might mean either that no site has to be occupied by Mg^{2+} for this reaction to take place or that Mg^{2+} can be replaced with other cation from the incubation media. If the second alternative were taken for granted, Ca^{2+} would seem to be the most likely candidate to substitute for Mg^{2+}. If this were so, when no Mg^{2+} is present phosphorylation would require not only the binding of Ca^{2+} at the transport site with high affinity but also at the Mg^{2+} site with much lower apparent affinity. This view is supported by the experiments, which confirm previous findings by others [18], showing that the apparent dissociation constant for Ca^{2+} during promotion of phosphorylation raises from 2 μM in media with Mg^{2+} to 60 μM in media without Mg^{2+}. Additional evidence for the substitution of Mg^{2+} by Ca^{2+} is provided by the experiments in this paper which show that if the concentration of Ca^{2+} is sufficiently increased, the $t_{1/2}$ for phosphorylation in the absence of Mg^{2+} becomes similar to that observed in the presence of Mg^{2+}. After the findings discussed above, our previous view that Mg^{2+} is not needed for phosphorylation [2] has to be modified in the sense that catalysis of phosphorylation needs the Ca^{2+}-ATPase to bind Ca^{2+} at the transport site and Mg^{2+} at an activating site, and that Ca^{2+} is able to replace Mg^{2+} at this site. Under the conditions that prevail in the cytosol of a normal cell (about 0.4 mM Mg^{2+} and 0.1 μM Ca^{2+}) the catalytically active units of the Ca^{2+}-ATPase will be predominantly those with Ca^{2+} at the transport site and Mg^{2+} at the activating site. The ability of Ca^{2+} to replace Mg^{2+} during phosphorylation is shared by other cation-transport ATPases. In fact, Ca^{2+} replaces Mg^{2+} as a cofactor in the Na^+-dependent phosphorylation of the $(Na^+ + K^+)$-ATPase [11,12,19] and, it is also known that when suspended in media with Mg^{2+}, two Ca^{2+} bind to the transport sites of the Ca^{2+}-ATPases of sarcoplasmic reticulum and that a third Ca^{2+} binds if Mg^{2+} is removed [20]. Since this enzyme is phosphorylated by ATP in the absence of added Mg^{2+} [21], it seems likely that the site for the third Ca^{2+} is the site which normally binds Mg^{2+}. This view is supported by the observation that the third Ca^{2+} is easily exchangeable [22].

A $(Ca^{2+} + Mg^{2+})$-ATPase activity which depends on the association of spectrin with red cell membranes and is unrelated to the Ca^{2+} pump has been recently described [23]. It could be argued that the activities detected in the absence of Mg^{2+} belong to the spectrin-dependent ATPase rather than to the Ca^{2+}-pump. This however does not seem to be the case since the phosphoenzyme made in media prepared with no $MgCl_2$ has the same physicochemical properties as that made in media with $MgCl_2$ [2], and Mg^{2+}-depleted membranes used for the experiments in this paper were prepared using a procedure that involved two 30 min long incubations at 37°C in media containing 1 mM CDTA, a procedure that should release most of the spectrin from the membranes [23]. The CDTA treatment will remove calmodulin from the membranes too. Villalobo et al. [24] have shown that in the absence of Mg^{2+} calmodulin is uneffective in increasing the activity and the affinity for Ca^{2+} of the Ca^{2+}-ATPase. We have confirmed

these results in our laboratory and this is the reason why no exogenous calmodulin was added during the assays reported in this paper.

Previous work from this laboratory [1,2] has shown that Mg^{2+} acting at a site with $K_{0.5} = 80$ μM promotes the transition of the phosphoenzyme from a state with low (E_1P) to a state with high (E_2P) reactivity towards water, In our original proposals we have also provided evidence showing that it sufficed for Mg^{2+} to be present during or after phosphorylation to be fully effective in promoting the $E_1P \rightarrow E_2P$ transition [1]. Results in this paper show that the effects of Mg^{2+} on this reaction depend on the concentration of Ca^{2+} in a way that can be summarized as follows: (a) if Mg^{2+} is added after phosphorylation, 50 μM Ca^{2+} totally blocks the effects of Mg^{2+} on the conformational transition and this effect of Ca^{2+} cannot be surmonted by raising the concentration of Mg^{2+}, while (ii) provided Mg^{2+} is added before phosphorylation, Ca^{2+} in concentrations as high as 5 mM is unable to prevent the activation of the $E_1P \rightarrow E_2P$ transition by Mg^{2+}. These results are consistent with the idea that binding of Mg^{2+} stabilizes the dephosphoenzyme in a state from which Ca^{2+} cannot displace Mg^{2+}, whereas binding of Ca^{2+} to E_1P stabilizes it in a state in which the sites for Mg^{2+} to activate the $E_1P \rightarrow E_2P$ reaction become unaccessible or unreactive to this cation.

Previous experimental evidence [25] and results in this paper show that Ca^{2+} at concentrations that completely inhibit in the conditions described above the activation of dephosphorylation by Mg^{2+}, has no inhibitory effect on the overall Ca^{2+}-ATPase activity. Moreover, from previous studies of this laboratory it is known that the overall activity is inhibited by Ca^{2+} with K_i values (1 and 10 mM for intracellular and extracellular Ca^{2+}, respectively [26]) which are much higher that the concentrations of Ca^{2+} needed to block the effects of Mg^{2+} on dephosphorylation. It would seem therefore that the blockage by Ca^{2+} of the effects of Mg^{2+} on the $E_1P \rightarrow E_2P$ transition does not take place during the overall reaction cycle. The obvious explanation for this is that in media with relatively high concentrations of Ca^{2+}, the binding of Mg^{2+} takes place when the enzyme is dephosphorylated, condition under which, as mentioned above, the interaction is not impeded by Ca^{2+}. It is not yet possible to ascertain whether this is also the case under physiological conditions since the normal cytosolic concentration of Ca^{2+} is well below that needed to impede the interaction of Mg^{2+} with E_1P.

Results show that there is a very slow but significant ATPase activity in the absence of Mg^{2+}. This activity depends on Ca^{2+} with an apparent affinity close to 1 μM making it likely that it is due to interaction of Ca^{2+} with the transport site only. Furthermore, raising the concentration of Ca^{2+} from 50 to 1000 μM, which according with the findings commented above should increase the rate of phosphorylation, has no effect on the hydrolysis of ATP by the ATPase in the absence of Mg^{2+}. This suggests that the phosphorylation reaction is not rate limiting in the absence of Mg^{2+} and that stimulation of the overall reaction of the Ca^{2+}-ATPase by Mg^{2+} has to be attributed to its participation in a reaction different from the phosphorylation of E_1 by ATP.

Observations in this and in previous papers [27,28] indicate that during steady-state ATP hydrolysis there is mutual displacement between Mg^{2+} and Ca^{2+} from their sites in the Ca^{2+} ATPase. These kind of interactions seem at first hand to be in contradiction with the apparent inability of Ca^{2+} to displace Mg^{2+} from the dephosphoenzyme and of Mg^{2+} to displace Ca^{2+} from the phosphoenzyme. Although we cannot discard the possibility that the cause of this apparent contradiction is that the effects reported in this paper take place in elementary steps that are not rate-limiting in the overall reaction, a simpler explanation for the discrepancy is to postulate that the mutual displacement between Ca^{2+} and Mg^{2+} takes place during elementary steps of the overall reaction which are not involved in the partial reactions we measured in this paper.

Stimulation of ATP hydrolysis by Mg^{2+} in media with 100 μM Ca^{2+} takes place along a hyperbolic curve that is half-maximal at 87.8 ± 3.7 μM Mg^{2+}, indicating that the effect of the cation is excerted at a single class of non-interacting sites. For the range of Ca^{2+} concentrations tested (50 to 1000 μM), Ca^{2+} acts as a linear dead-end inhibitor of the effects of Mg^{2+} and the apparent dissociation constant for Mg^{2+} extrapolated to

zero Ca^{2+} concentration is 19.7 ± 7.0 μM, a value close to that reported by Stieger and Luterbacher [29] for the purified enzyme and about six times less than the apparent dissociation constant for the promotion of the $E_1P \rightarrow E_2P$ transition by Mg^{2+}. Available experimental evidence does not allow to decide whether acceleration of phosphorylation, promotion of the $E_1P \rightarrow E_2P$ transition and acceleration of the overall reaction by Mg^{2+} takes place at a single or at two or even three different classes of site. The observed differences between the values of apparent affinity for Mg^{2+} during each of these processes may be caused by the different temperatures used during the assays and/or may express the well known fact that in complex multi-step reactions the apparent affinity for a ligand may vary depending on whether it is studied on the overall reaction or on a single elementary step.

Apart from the effects described above, results in this paper show that Mg^{2+} in a 0 to 500 μM concentration range behaves as a linear dead-end inhibitor of the effects Ca^{2+} at the transport site of the Ca^{2+}-ATPase. The apparent dissociation constant of Mg^{2+} for this effect is sufficiently low (200 μM) as to make it necessary to extrapolate Ca^{2+} activation curves to zero Mg^{2+} concentration in order to get real estimates for the apparent affinity for Ca^{2+}.

Experiments reported here also confirm previous reports [3,4] that the response of the Ca^{2+}-ATPase to Mg^{2+} is biphasic, i.e., as Mg^{2+} concentration raises activation is followed by inhibition. Penniston [4] has proposed that inhibition by high concentrations of Mg^{2+} is caused by competitive displacement of Ca^{2+} from its site in the enzyme. Using the values of the apparent dissociation constants for activation by Ca^{2+} (1.39 μM) and for competition of this effect by Mg^{2+} (200 μm) obtained in the experiments reported in this paper, it can be calculated that in media with 100 μM Ca^{2+}, inhibition by Mg^{2+}, if excerted through the displacement of Ca^{2+}, would take place with a K_i of about 13 mM. This value is not very different from the best fitting value of K_i (9.1 mM) we obtained by nonlinear regression. However, the curve that best fits our data predicts a substantial residual activity when the concentration of Mg^{2+} tends to infinity and this is not compatible with simple linear dead-end inhibition. In view of this we cannot discard the possibility that the observed linear relation between the apparent dissociation constant for Ca^{2+} and the concentration of Mg^{2+} is an approximation which is only valid at relatively low concentrations of Mg^{2+} and that at higher concentrations the relation levels off tending to a constant maximum value. A response like this, which would imply that Mg^{2+} does not displace Ca^{2+} by simple competition but rather by driving the Ca^{2+}-ATPase into a state with lower but not zero affinity for Ca^{2+}, would explain the existence of the residual activity when Mg^{2+} concentrations tends to infinity. Unpublished results by A.J. Caride show that in the presence of *p*-nitrophenyl phosphate, partial inhibition of the Ca^{2+}-ATPase activity by Mg^{2+} is paralleled by activation of the Ca^{2+}-phosphatase activity as if Mg^{2+} were able to drive the Ca^{2+}-pump to a state characterized by low ATPase and maximum phosphatase activities.

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