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ACTIVATION OF PARTIAL REACTIONS OF THE Ca^{2+}-ATPase FROM HUMAN RED CELLS BY Mg^{2+} AND ATP *

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Summary

(1) At ATP concentrations up to 30 μM addition of 0.5 mM $MgCl_2$ in the reaction mixture increases both the rate of formation and the steady-state level of the phosphoenzyme of the Ca^{2+}-ATPase from human red cell membranes. Under these conditions Mg^{2+} has no effect on the rate of dephosphorylation, which remains slow.

(2) In the presence of Mg^{2+} the rate of dephosphorylation is increased 5 to 10 times by high (1 mM) concentrations of ATP.

(3) Provided Mg^{2+} has reacted with the phosphoenzyme, acceleration of dephosphorylation by ATP takes place in the absence of Mg^{2+}. This suggests that the role of Mg^{2+} on dephosphorylation is to convert the phosphoenzyme into a form that, after combination with ATP, reacts rapidly with water.

(4) The results are consistent with the idea that combination of ATP at a non-catalytic site is needed for rapid dephosphorylation of the Ca^{2+}-ATPase.

Introduction

It is now accepted that the hydrolysis of ATP by the Ca^{2+} pump in human red cells proceeds through a series of partial reactions involving the formation of a phosphorylated intermediate [1—3]. We have reported previously that Ca^{2+}-dependent phosphorylation of the Ca^{2+}-ATPase takes place in the absence of Mg^{2+} and that a low concentration of this cation (about 10μM) has no observable effect on phosphorylation [2]. However, Ca^{2+}-dependent ATP hydrolysis measured at ATP concentrations similar to those used for phos-

* Dedicated to the memory of our friend the late Robert Inslee Weed.

Abbreviations: EDTA, ethylene bis(oxythylenenitrilo) tetraacetic acid; CDTA, *trans*-1,2-diaminocyclohexane tetraacetic acid.

phorylation is stimulated by Mg^{2+}, half-maximal stimulation being reached at 0.3 mM [4]. Moreover, full activation of Ca^{2+}-ATPase is only attained in media containing Mg^{2+} and concentrations of ATP well above those necessary for full occupation of the active center of the enzyme, indicating that binding of ATP at a second site is required for maximum activity [4]. In view of these findings it seemed worthwhile to study in more detail the effects of ATP and Mg^{2+} on the partial reactions of the Ca^{2+}-ATPase. The results of such a study are reported here.

Materials and Methods

Materials. Fragmented membranes of human red cells were prepared as described previously [5]. [γ-^{32}P]ATP was prepared according to the procedure of Glynn and Chappell [6] except that no unlabelled orthophosphate was added to the incubation mixture. Orthophosphate labelled with ^{32}P was provided by Comisión Nacional de Energía Atómica, Argentina. ATP, enzymes and cofactors used for the synthesis of [γ-^{32}P]ATP were from Sigma Chemical Co. U.S.A. Salts and reagents were of AR degree.

Methods. Phosphorylation of membranes was carried out at 0—3°C following the procedure already described [2]. The reaction mixture had a volume of 0.4 ml and contained 0.8 mg membrane protein/ml in (mM): ethylene bis(oxyethylenenitrolo) tetraacetic acid (EGTA), 0.100/Tris-HCl, (pH 7.4 at 0°C), 150/[γ-^{32}P]ATP, 0.030. Were indicated in Results the mixture contained $MgCl_2$. If present, $CaCl_2$ was 0.150 mM. Unless otherwise indicated, the reaction was allowed to proceed for 20 s. To measure the rate of dephosphorylation the phosphoenzyme in 0.4 ml of the reaction mixture was 'chased' by the addition of 0.2 ml of Tris-HCl (pH 7.4 at 0°C) containing chelators and salts with and without ATP to give the final concentration indicated in the legends to the figures.

Ca^{2+}-dependent phosphoenzyme is the increment in the amount of ^{32}P bound to the membrane, elicited by 0.150 mM $CaCl_2$.

All experiments were done in quadruplicate. The individual measurements did not differ from the mean more than 15%. Protein was estimated by the method of Lowry et al. [7]. Rate constants for dephosphorylation were calculated assuming first order kinetics.

Results

The effects of magnesium on phosphorylation and dephosphorylation reactions. In the experiment shown in Fig. 1, the time course of formation of Ca^{2+}-dependent phosphoenzyme was followed in media with and without 0.5 mM $MgCl_2$. It can be seen that throughout the experiment the level of phosphoenzyme in the medium with $MgCl_2$ remains higher than that in the medium without $MgCl_2$. Results also show that in the medium with $MgCl_2$, Ca^{2+}-dependent phosphoenzyme reaches steady-state with a half-time of 7 s. It is clear, therefore, that inclusion of 0.5 mM $MgCl_2$ in the incubation medium increases both the rate of formation and the level of Ca^{2+}-dependent phosphoenzyme.

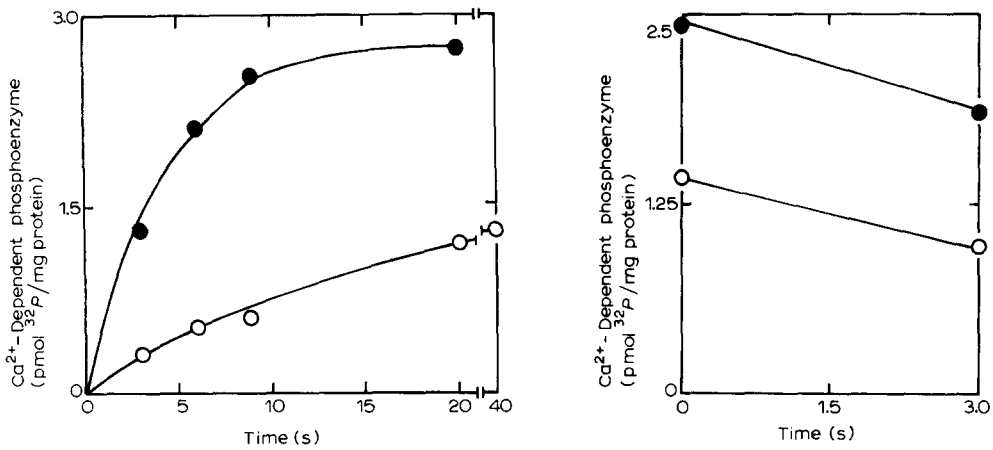

Fig. 1. Time course of the formation of Ca^{2+}-dependent phosphoenzyme in the presence (•) and in the absence (○) of 0.5 mM $MgCl_2$.

Fig. 2. Dephosphorylation of phosphoenzyme prepared in media with (•) and without (○) 0.5 mM $MgCl_2$. Dephosphorylation was performed in media containing 30 mM EGTA (○) and 30 mM EGTA plus 0.5 mM $MgCl_2$ (•). The concentration of ATP during dephosphorylation was 20 μM.

In the experiment shown in Fig. 2, phosphoenzyme was made in media containing 30 μM ATP, with and without 0.5 mM $MgCl_2$. After 20 s, Ca^{2+}-dependent phosphorylation was stopped by the addition of 30 mM EGTA, and after 3 s the amount of remaining phosphoenzyme was measured. Results make clear that, in contrast with the effects of Mg^{2+} on phosphorylation, the rate of hydrolysis of Ca^{2+}-dependent phosphoenzyme is practically the same regardless of the presence of Mg^{2+} in the media. Increasing the concentration of $MgCl_2$ from 0.5 to 7.0 mM was also without observable effect on the hydrolysis of the phosphoenzyme (experiment not shown).

The effects of ATP and magnesium on dephosphorylation. In the experiment shown in Fig. 3, Ca^{2+}-dependent phosphoenzyme was made in the absence of Mg^{2+}. After 20 s the phosphoenzyme was chased by the addition of EGTA in media containing Mg^{2+}. Dephosphorylation was allowed to proceed for 2 s and then half of the tubes received enough of a concentrated solution of unlabelled ATP to give a final concentration of 1 mM ATP. Results show that addition of ATP increases about 7 times (from 0.277 s^{-1} to 1.86 s^{-1}) the rate of dephosphorylation.

In the experiment in Fig. 4, phosphoenzyme made in the absence of Mg^{2+} was chased with EGTA in control media, in media containing either ATP or Mg^{2+} alone and in media with ATP plus Mg^{2+}. The rate of hydrolysis of the phosphoenzyme in media containing either ATP or Mg^{2+} alone, is not much different to that observed under control conditions. Only when ATP and Mg^{2+} are present together a large increase in the rate of dephosphorylation is apparent.

The requirement of Mg^{2+} for rapid dephosphorylation. The rate of dephosphorylation of Ca^{2+}-dependent phosphoenzyme made in the absence of Mg^{2+},

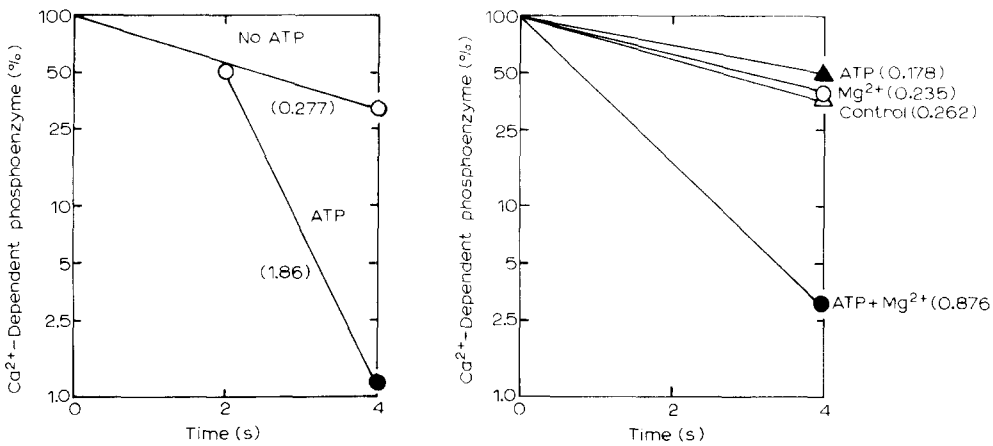

Fig. 3. The effects of ATP in the presence of $MgCl_2$ on the hydrolysis of Ca^{2+}-dependent phosphoenzyme. Phosphoenzyme was made in the absence of $MgCl_2$. Dephosphorylation was performed in media containing 30 mM EGTA and 0.5 mM $MgCl_2$. After 2 s half of the tubes received enough of a concentrated solution of ATP plus $MgCl_2$ to give a final concentration of 1 mM ATP and 1.5 mM $MgCl_2$. In the tubes that did not receive extra ATP the concentration of ATP was 20 μM. The figures in brackets are rate constants for dephosphorylation in s^{-1}, calculated assuming first order kinetics.

Fig. 4. Dephosphorylation of Ca^{2+}-dependent phosphoenzyme made in the absence of $MgCl_2$, in a control medium (△), in a medium containing 1 mM ATP (▲), in a medium with 0.5 mM $MgCl_2$ (○) and in a medium with 1 mM ATP plus 1.5 mM $MgCl_2$ (●). All media contained 30 mM EGTA. The figures in brackets are rate constants for dephosphorylation in s^{-1}, calculated assuming first order kinetics.

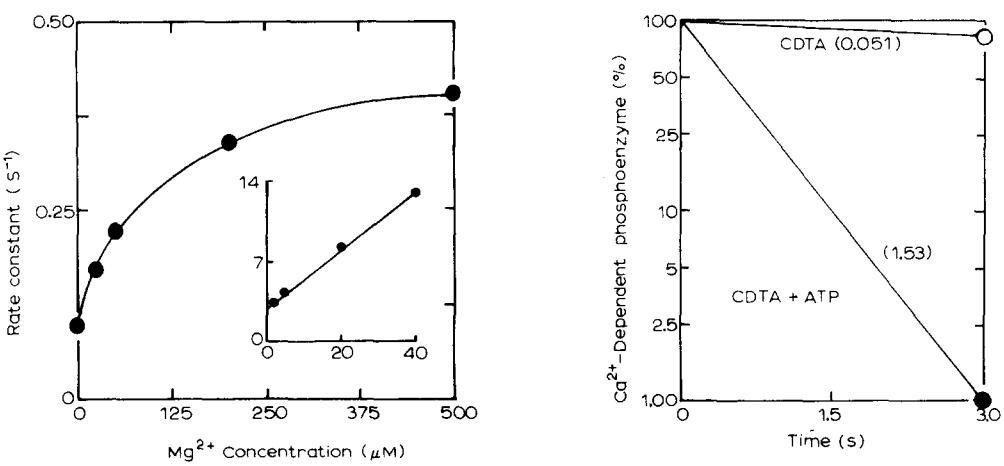

Fig. 5. The rate constant for dephosphorylation of Ca^{2+}-dependent phosphoenzyme in media containing 1 mM ATP as a function of the concentration of free Mg^{2+} calculated assuming that the association constant for the Mg · ATP complex is $3 \cdot 10^4$ M^{-1}. Phosphoenzyme was made in the absence of $MgCl_2$ and chased in media with different concentrations of $MgCl_2$. Rate constants were calculated assuming first order kinetics. The inset is a plot of the reciprocal of the difference between the rate constants in the presence and in the absence of Mg^{2+} as a function of the reciprocal of the concentration of calculated Mg^{2+}.

Fig. 6. The effect of the removal of Mg^{2+} on dephosphorylation in media with and without added ATP. Phosphoenzyme was made in the absence of Mg^{2+}. 5 s before phosphorylation was terminated the concentration of $MgCl_2$ in the medium was raised to 0.5 mM. Dephosphorylation was initiated by the addition of 20 mM CDTA or 20 mM CDTA plus 1 mM ATP. Figures in brackets are rate constants for dephosphorylation calculated assuming first order kinetics.

was measured in media containing 1 mM ATP and different concentrations of $MgCl_2$. Fig. 5 shows the rates of dephosphorylation plotted against the calculated concentration of free Mg^{2+}. It can be seen that the rate of hydrolysis of Ca^{2+}-dependent phosphoenzyme increases with the concentration of free Mg^{2+} along a rectangular hyperbola which is half-maximal at 0.08 mM Mg^{2+}.

In the experiment in Fig. 6 phosphoenzyme made in the absence of Mg^{2+} received enough of a concentrated solution of $MgCl_2$ 5 s before chase to raise the concentration of $MgCl_2$ in the medium to 0.5 mM. Dephosphorylation was measured in media with and without 1 mM ATP and containing 20 mM of *trans*-1,2-diaminocyclohexane tetraacetic acid (CDTA) to chelate all the Mg^{2+} present. It can be seen that if Mg^{2+} is chelated after phosphorylation ATP retains its ability to accelerate dephosphorylation. Under these conditions the effects of ATP cannot be attributed to the formation of an ATP · Mg complex since the medium contains a 20fold excess of a chelator whose affinity for Mg^{2+} is 10^6 times larger than that of ATP [8].

Discussion

Results in this paper show that Mg^{2+} reduces the half-time for steady-state phosphorylation of the Ca^{2+}-ATPase of red cells. A similar effect has also been observed in the Ca^{2+}-ATPase from sarcoplasmic reticulum [9]. In red cells the effect of Mg^{2+} has to be attributed to an increase in the rate of phosphorylation since it is observed under conditions in which Mg^{2+} is without effect on dephosphorylation. The increase in the rate of phosphorylation of the Ca^{2+}-ATPase seems to explain why the steady-state level of phosphoenzyme is higher in Mg^{2+}-containing media. Since an increase in the steady-state level of phosphoenzyme should lead to a higher rate of ATP hydrolysis the effect of Mg^{2+} on phosphorylation is the most likely cause of the stimulation of Ca^{2+}-ATPase by Mg^{2+} at low ATP concentrations [4].

We have shown previously [2] that in the presence of Mg^{2+} the phosphoenzyme undergoes a transition from a state with low reactivity towards water ($E \sim P$) to a state with high reactivity towards water ($E' \sim P$). Results in this paper indicate that rapid hydrolysis of the phosphoenzyme needs not only Mg^{2+} but also ATP and that it suffices for Mg^{2+} to be present during phosphorylation for ATP to be fully effective in promoting rapid dephosphorylation. These results seem to indicate that to react rapidly with water $E' \sim P$ has to combine with ATP. ATP is effective in the absence of Ca^{2+} and at concentrations much higher than those necessary for full occupation of the catalytic center of the Ca^{2+}-ATPase. In view of this, the effects of ATP cannot be attributed to an additional Ca^{2+}-dependent phosphorylation or to occupation of the catalytic center of the Ca^{2+}-ATPase. It seems therefore that ATP binds to $E' \sim P$ at a site different from the catalytic center.

We have shown elsewhere [4] that at concentrations of ATP at which only the phosphorylating site is occupied (K_m 2.5 μM) the Ca^{2+}-ATPase expresses about 10% of its maximum activity. Full activation of the ATPase depends on Mg^{2+} and needs occupation of a second, low-affinity (K_m 145 μM) site for ATP. The properties of the low affinity site of the Ca^{2+}-ATPase make it likely that this is the site at which ATP combines to accelerate the hydrolysis of $E' \sim P$.

TABLE I

EFFECT OF Mg^{2+} AND ATP ON THE PARTIAL REACTIONS AND ON THE ACTIVITY OF THE Ca^{2+}/ATPase

ATP (μM)	Mg^{2+} (μM)	Partial reaction	Rate	E ~ P level	ATPase activity (% of maximum)
30	0	$E + ATP \xleftrightarrow{Ca^{2+}} E{\sim}P + ADP$	low	low	4
		$E{\sim}P + H_2O \longrightarrow E + P_i$	low		
30	500	$E + ATP \xleftrightarrow{Ca^{2+} + Mg^{2+}} E{\sim}P + ADP$	high	high	10
		$E{\sim}P \xleftrightarrow{Mg^{2+}} E'{\sim}P$	high		
		$E'{\sim}P \longrightarrow E' + P_i$	low		
1000	500	$E + ATP \xleftrightarrow{Ca^{2+} + Mg^{2+}} E{\sim}P + ADP$	high	unknown	100
		$E{\sim}P \xleftrightarrow{Mg^{2+}} E'{\sim}P$	high		
		$E'{\sim}P \xrightarrow{ATP} E' + P_i$	high		

Table I shows a list of the partial reactions which the Ca^{2+}-ATPase catalyzes and their expression on the overall reaction of ATP hydrolysis drawn from our results here and previous studies [2,4,10].

It is known that all the effects of ATP and Mg^{2+} on Ca^{2+}-ATPase and on active Ca^{2+} transport are exerted at the inner surface of the cell membrane [11]. In fresh human red cells the intracellular concentration of ATP ranges from 1 to 1.5 mM [12] and that of Mg^{2+} is about 0.7 mM [13]. These concentrations are enough for both Mg^{2+} and ATP to exert fully their effects on the partial reactions of the Ca^{2+} ATPase. It seems likely, therefore, that under physiological conditions the pathway for ATP hydrolysis will be that shown in Table I as occurring in the presence of 1 mM ATP and 0.5 mM Mg^{2+}.

Acknowledgements

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