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Calcium Ion-Dependent Phosphorylation of Human Erythrocyte Membranes

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Summary. Calcium ions promote the rapid transfer of the terminal phosphate of ATP to a protein of human erythrocyte membranes. The concentration of Ca^{2+} for half-maximal effect is 7 μM. At nonlimiting ATP concentrations the level of ^{32}P incorporated by the membranes is independent of the presence or absence of Mg^{2+}. The number of phosphorylating sites in a single erythrocyte membrane is about 700. The influence of pH on the rate of hydrolysis of the bound phosphate and its rapid release on exposure to hydroxylamine are both consistent with an acylphosphate bond. The phosphate in the protein undergoes rapid turnover. Enzymatic splitting of the phosphate is stimulated by Mg^{2+} but not by Ca^{2+}. It is proposed that Mg^{2+} accelerates the splitting of the phosphate by favoring the conversion of the phosphoprotein from a state of low reactivity to a state of high reactivity towards water. The reactions described probably are intermediate steps in the hydrolysis of ATP catalyzed by the Ca^{2+}-dependent ATPase of human erythrocyte membranes.

Human erythrocytes pump calcium ions outward using metabolic energy which comes from the hydrolysis of ATP (Schatzmann & Vincenzi, 1969). In isolated membranes, the system responsible for the pumping of Ca^{2+} remains as a Ca^{2+}-dependent ATPase activity (Schatzmann & Rossi, 1971).

Two different lines of evidence suggest that hydrolysis of ATP by the Ca^{2+}-pump in erythrocyte membranes is a multi-stage process involving intermediates, i.e. (*i*) in the presence of ATP, the Ca^{2+}-pump also catalyzes the hydrolysis of *p*-nitrophenyl phosphate (Rega, Richards & Garrahan, 1973), and (*ii*) in the presence of (^{32}P)ATP, a Ca^{2+}-stimulated phosphorylation of the membranes can be observed (Katz & Blostein, 1973; Knauf, Proverbio & Hoffman, 1974; Rega, Richards & Garrahan, 1974). The ^{32}P incorporated by the membranes shows rapid turnover (Katz & Blostein, 1973; Rega *et al.*, 1974) and is associated to a membrane protein (Knauf *et al.*, 1974).

In this paper we report experiments designed to characterize in detail

the interaction of Ca^{2+} and ATP with human erythrocyte membranes in order to gain information on the reaction mechanism of the Ca^{2+}-dependent ATP hydrolysis catalyzed by the membrane ATPase.

Materials and Methods

Preparation of Erythrocyte Membranes

Erythrocyte membranes were prepared from freshly drawn human blood by the procedure described by Garrahan, Pouchan and Rega (1969). The membranes were fragmented by freezing and thawing and kept in 15 mM Tris-HCl buffer (pH 7.2) at 5 °C. The storage time at low temperature was never more than 60 min.

Phosphorylation of Membranes

The reaction was carried out in 15 ml conical glass centrifuge tubes immersed in an ice-water bath. Unless otherwise indicated in Results, the reaction mixture contained (mM): $MgCl_2$, 0.0125; EGTA (ethanedioxybis(ethylamine)tetra-acetic acid), 0.050; Tris-HCl, 150; (pH 7.4 at 0 °C). When present, $CaCl_2$ was 0.1 mM. The reaction mixture (final volume 0.4 ml) contained 8 mg/ml of membrane protein. The reaction was started by the addition, with vigorous stirring, of enough concentrated (^{32}P)ATP[1] to give a final concentration of 0.0075 mM. The reaction was stopped by the quick addition of 2 ml of a freshly prepared solution of: 60 g per liter, trichloroacetic acid (TCA), 1 mM ATP and 50 mM H_3PO_4 at 0 °C. The suspension was centrifuged at $1,750 \times g$ for 10 min at 5 °C, and the sediment was washed three times with about 10 ml of the TCA solution. The washed precipitate was dissolved in 1 ml of 3% (w/v) sodium dodecylsulfate (SDS) solution. An aliquot of the solution was put aside for measuring protein by the procedure of Lowry, Rosebrough, Farr and Randall (1951) and 0.8 ml were used for measuring radioactivity in a liquid scintillation counter. Experiments were performed in quintuplicate. The individual measurements did not differ from the mean by more than 15%.

Electrophoresis of Labeled Membranes

Labeled erythrocyte membranes precipitated and washed in the TCA solution were solubilized and submitted to electrophoresis in polyacrylamide gels following the procedure of Fairbanks, Steck and Wallach (1971) using 0.8 cm diameter and 9 cm long gels. Electrophoresis was carried out at 7 °C in a cold room. The gels were cut transversely into 2-mm thick slices. The slices were put into scintillation vials containing 0.05% SDS, and incubated 24 hr at 37 °C, after which 5 ml of Bray's (1960) solution were added to each. Since preliminary experiments in which the gels were stained for protein (Fairbanks *et al.*, 1971) showed that all protein bands moved behind the tracking dye (bromophenol blue), gels were sliced up to the band of the tracking dye for radioactivity measurements.

(^{32}P)ATP was prepared according to the procedure of Glynn and Chappell (1964) except that no inorganic phosphate was added to the incubation mixture. With this procedure ATP containing about 10^{15} cpm/mol was obtained. (^{32}P)ATP preparations were submitted to isobutanol extraction as described by Weil-Malherbe and Green (1951). No more than 4% of the total counts passed into the organic phase.

ATP, enzymes and intermediates used for the synthesis of (^{32}P)ATP were from Sigma Chemical Co. (USA). Salts and reagents were of A.R. grade. All solutions were prepared in doubly glass-distilled water. Orthophosphate labeled with ^{32}P was provided by Comisión Nacional de Energía Atómica (Argentina).

1 ATP labeled with ^{32}P in its γ phosphorus atom.

Results

Kinetics of the Incorporation of ^{32}P from (^{32}P)ATP

Fig. 1*a* and *b* shows the time course of the incorporation of ^{32}P into erythrocyte membranes incubated at 0 °C in media containing (^{32}P)ATP. In the absence of divalent cations the uptake of radioactivity is low. When 100 μM Ca^{2+} are present there is a large increase in the amount of ^{32}P taken up by the membranes (Fig. 1*a*) which tends to a steady value of about 1.2 pmol ^{32}P/mg protein after 30 sec of incubation. Fig. 1*b* shows that Mg^{2+} stimulates the uptake of radioactivity by the membranes. This effect is not due to replacement of Ca^{2+} since addition of 100 μM Ca^{2+} in the presence of Mg^{2+} results in a further increase in the radioactivity of the membranes. In the presence of Mg^{2+} the increment due to Ca^{2+} reaches a steady level of 1.2 pmol ^{32}P/mg protein after 20 sec of incubation. Comparison of this value with that from Fig. 1*a* suggests that Mg^{2+} may stimulate the rate of uptake of radioactivity.

Incorporation of (^{32}P)Orthophosphate

As mentioned in Materials and Methods, up to 4% of the total number of counts in the (^{32}P)ATP preparations used during this study was prob-

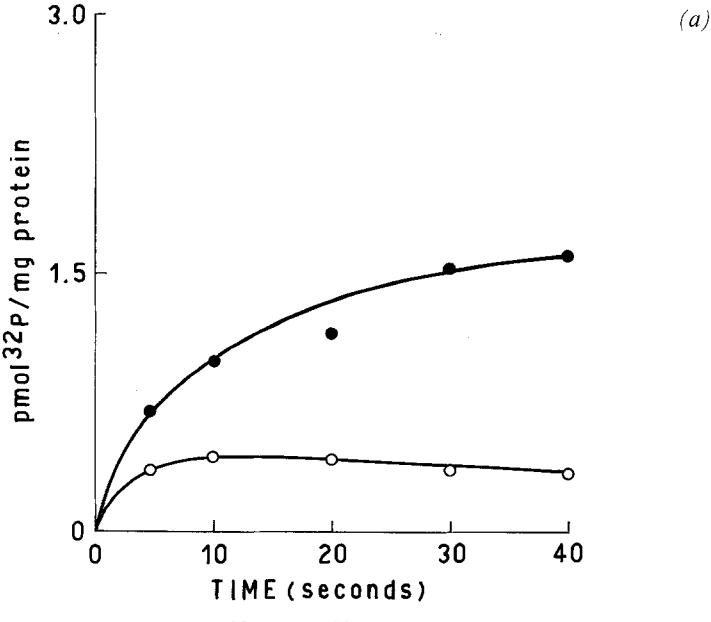

Fig. 1. Time course of the uptake of ^{32}P from (^{32}P)ATP by erythrocyte membranes incubated at 0 °C in media without (*a*) and with (*b*) Mg^{2+} and in the presence (●) and in the absence (○) of Ca^{2+}. The Mg^{2+}-free media contained 0.1 mM ethylenediaminetetracetic acid (EDTA)

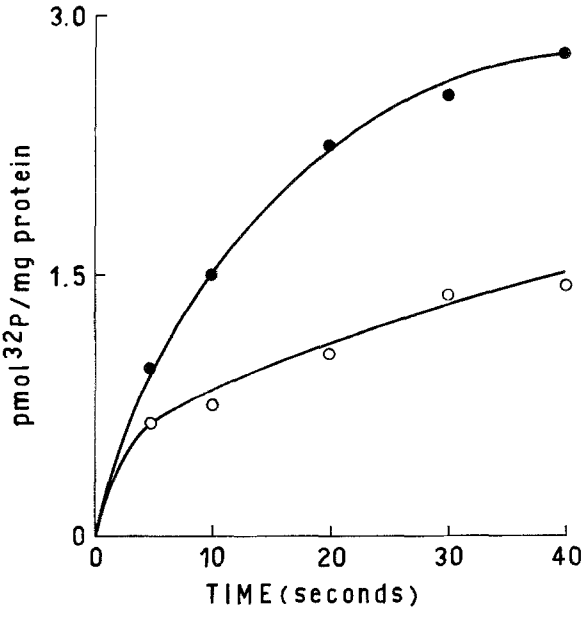

Fig. 1*b*

ably orthophosphate. To eliminate the possibility that the labelling of the membranes in the presence of Ca^{2+} was due to incorporation of ^{32}P from free orthophosphate, erythrocyte membranes were treated under conditions identical to those used during the experiments shown before except that ^{32}P-labeled orthophosphate replaced all the nucleotide. As judged by the amount of label in the pellet after precipitation and washing with TCA, erythrocyte membranes readily incorporate inorganic phosphate (Table 1). Neither Mg^{2+} nor Ca^{2+} however increase labelling of the membranes with orthophosphate.

Requirements of ATP and Ca^{2+}

Fig. 2 shows a reciprocal plot of the effects of different concentrations of (^{32}P)ATP on the Ca^{2+}-dependent uptake of ^{32}P by the membranes

Table 1. Labeling of erythrocyte membranes with ^{32}P-labeled orthophosphate

Membranes phosphorylated in a medium containing	^{32}P in washed membranes (pmol ^{32}P/mg prot)
—	3.18
Mg	3.08
Mg+Ca	2.56

Membranes were phosphorylated as described in Materials and Methods except that ATP was replaced by an equimolar amount of orthophosphate.

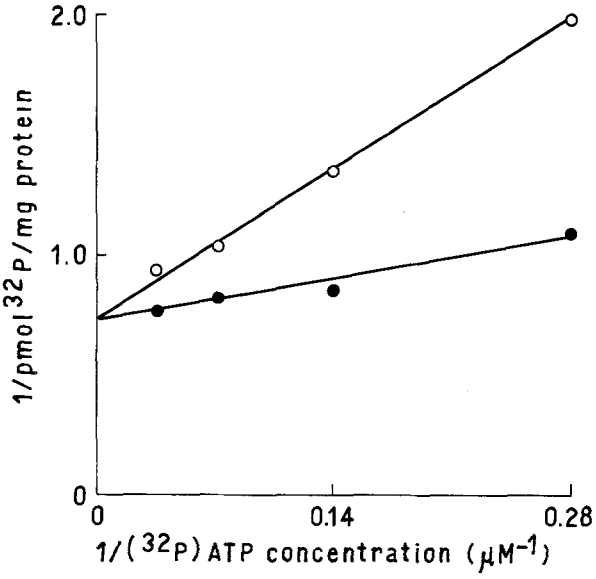

Fig. 2. A Lineweaver-Burk plot of the relation between the steady-state level of Ca^{2+}-dependent phosphorylation and the concentration of (^{32}P)ATP, measured in Mg^{2+}-free (●) and in Mg^{2+}-containing (○) media. Phosphorylation was carried out during 30 sec at 0 °C. The Mg^{2+}-free media contained 0.1 mM EDTA. When phosphorylation was performed in the presence of Mg^{2+}, 0.012 mM $MgCl_2$ was present in the incubation media and (^{32}P)ATP was added together with an equimolar amount of $MgCl_2$. Ca^{2+}-dependent phosphorylation is the amount of ^{32}P bound to membranes after incubation in a medium with Ca^{2+} minus the amount of ^{32}P bound after incubation under identical conditions in medium without Ca^{2+}

incubated 30 sec in media with and in media without Mg^{2+}. It can be seen that Mg^{2+} increases from 1.6 to 6.5 μM the concentration of (^{32}P)ATP needed for half-maximal uptake of ^{32}P. Results also demonstrate that at nonlimiting ATP concentration the phosphorylation attained after incubation during 30 sec is independent of Mg^{2+}.

Fig. 3 shows the relation between increasing Ca^{2+} concentrations and the level of radioactivity in membranes incubated with (^{32}P)ATP. As the concentration of Ca^{2+} is increased, phosphorylation rises following a curve which tends to saturation above 20 μM Ca^{2+}. Half-maximal phosphorylation is reached at 7 μM Ca^{2+}.

Electrophoresis of Erythrocyte Membranes Phosphorylated in the Presence of Ca^{2+}

Knauf *et al.* (1974) have shown that gel electrophoresis of erythrocyte membranes labeled with ^{32}P from ATP in the presence of Ca^{2+} plus Mg^{2+} reveals a major radioactive band having the mobility of a protein

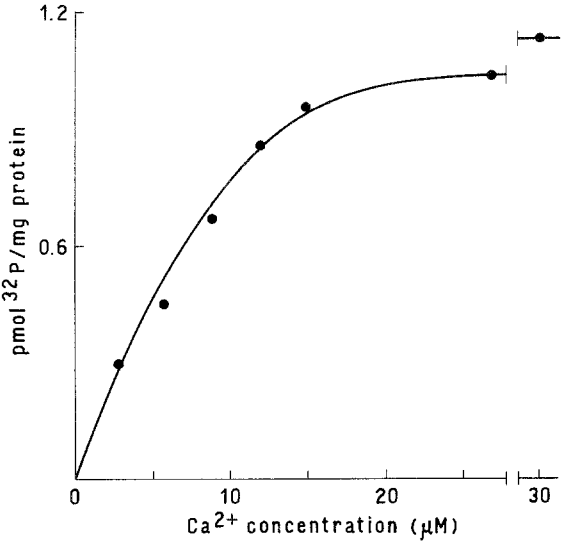

Fig. 3. The relation between the steady-state level of Ca^{2+}-dependent phosphorylation and the concentration of Ca^{2+} in the incubation medium. Phosphorylation was carried out during 20 sec in Mg^{2+}-containing media

with a molecular weight of 150,000. It seemed interesting to compare the electrophoretic behavior of the products of the phosphorylation reaction obtained in medium with Ca^{2+} with those obtained in medium with Ca^{2+} and Mg^{2+}. For this purpose membranes incubated in media with (^{32}P)ATP and Mg^{2+}, Ca^{2+} or $Ca^{2+}+Mg^{2+}$ were solubilized in SDS and electrophoresed in acrylamide gels. Comparison of the patterns in Fig. 4 makes clear that addition of Ca^{2+} results in the appearance of a single band of radioactivity with the same mobility regardless on whether Mg^{2+} was present or not during the incubation period.

Sensitivity of the Phosphoprotein to pH and to Hydroxylamine

In the presence of SDS, membrane proteins exist free from one another and from lipid molecules. Therefore, the results of the electrophoretic run in Fig. 4 suggest that the ^{32}P incorporated by the membrane in the presence of Ca^{2+} is strongly and perhaps covalently bound to a membrane protein. It is known that in other membranes endowed with Ca^{2+}-dependent ATPase activity the first step of ATP hydrolysis is a Ca^{2+}-dependent transfer of the terminal phosphate of ATP to a membrane protein with the formation of an acylphosphate bond (*see* Martonosi, 1968). It is conceivable therefore that a similar process may be responsible for the Ca^{2+}-dependent phosphorylation of erythrocyte membrane protein. This possi-

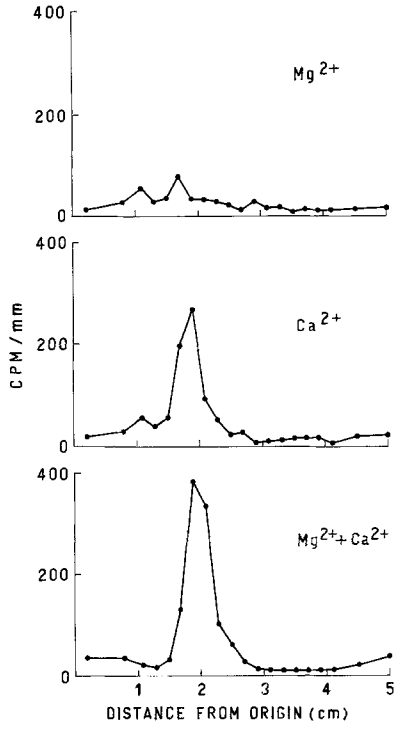

Fig. 4. Distribution of radioactivity in acrylamide gels after electrophoresis of erythrocyte membranes phosphorylated with (^{32}P)ATP in media containing Mg^{2+}, Ca^{2+} or Mg^{2+} plus Ca^{2+}. The total amount of protein (0.5 mg) in each of the three gels was the same. Phosphorylation was carried out during 20 sec at 0 °C. The Mg^{2+}-free media contained 0.1 mM EDTA

bility was tested by studying the effects of pH and of hydroxylamine on the level of radioactivity of phosphorylated membranes treated with TCA. Results in Fig. 5 show that the stability of the phosphorylated protein is maximal near pH 1, the stability being progressively lost as pH rises. Table 2 shows that 150 mM hydroxylamine induces a sevenfold increase in the rate of cleavage of phosphate from the phosphorylated protein. The influence of pH on the rate of hydrolysis of the bound phosphate and its rapid release on exposure to hydroxylamine are both consistent with an acylphosphate bond. Moreover, the above-mentioned properties of the phosphorylated protein make it very unlikely that Ca^{2+}-dependent phosphorylation expresses the binding of (^{32}P)ATP as such to a membrane compound.

Kinetics of Dephosphorylation of ^{32}P Labeled Membranes

Membranes were labeled during 20 sec and then the incorporation of radioactivity was abruptly stopped by the addition of an excess of

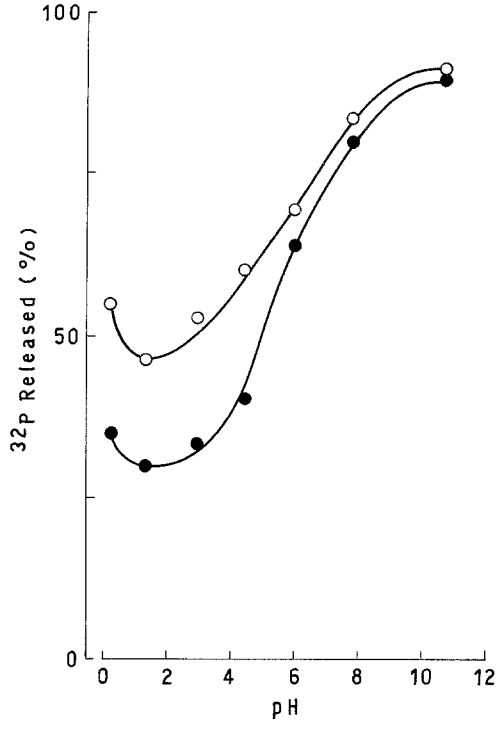

Fig. 5. The release of ^{32}P from TCA-treated membranes after incubation in buffers having different pH values. The membranes were phosphorylated for 20 sec at 0 °C in media with and without Ca^{2+} and with (●) and without (○) Mg^{2+}. After precipitation and washing with TCA, about 3 mg of membrane protein were suspended in 8 ml of each of the buffers and incubated for 30 min at 40 °C. Incubation was terminated by cooling and addition of 1 ml of 100% (w/v) TCA. The suspension was centrifuged and the amount of ^{32}P in the pellet measured as described in Materials and Methods. The buffers were: pH 0.3, 1 N HCl; pH 1.3, 0.1 N HCl; pH levels 3 to 6, 0.1 M citric acid adjusted with 0.1 M trisodium citrate; pH 7.8, 0.1 M Tris-HCl; and pH 10.7, 0.1 M Na_2CO_3. The values represented are the difference between the ^{32}P released by the membranes phosphorylated in the presence and absence of Ca^{2+} expressed as percent of the Ca-dependent phosphorylation before incubation at 40 °C

unlabeled ATP. Fig. 6 shows that after a small initial drop, the amount of radioactivity taken up in Ca^{2+}-free media remains practically unmodified for at least 30 sec after incorporation has ceased. In contrast with this behavior the amount of radioactivity due to Ca^{2+} decays rapidly, reaching with a half-time of about 3 sec a value only slightly higher than that found in the presence of Mg^{2+} alone. It is clear therefore that the ^{32}P incorporated in the presence of Ca^{2+} is capable of undergoing rapid turnover.

Table 2. Effect of hydroxylamine on the stability of the Ca^{2+}-dependent phosphoprotein in TCA-treated membranes

Treatment	^{32}P Released (pmol/mg protein)	
	Membranes phosphorylated in media containing Ca	Membranes phosphorylated in media containing Ca+Mg
Tris-HCl	0.08	0.10
Hydroxylamine	0.66	0.74

The membranes were phosphorylated for 20 sec at 0 °C with (^{32}P)ATP in media with and without Ca^{2+} and with and without Mg^{2+}. After precipitation and washing with TCA about 3 mg of membrane protein were suspended in 2 ml of a medium containing 150 mM hydroxylamine-HCl titrated to pH 6 with Tris base and 150 mM Na acetate titrated to pH 6 with acetic acid. In the control experiments 150 mM Tris-HCl (pH 6) replaced hydroxylamine. The incubation was carried out during 10 min at 20 °C. The values represent the difference between the ^{32}P released by membranes phosphorylated in media containing Ca^{2+} and the ^{32}P released by membranes phosphorylated in the same media but without Ca^{2+}.

Effects of Ca^{2+} on Dephosphorylation

The effect of Ca^{2+} on the rate of loss of ^{32}P from the phosphorylated protein, was studied by measuring the level of phosphorylated protein 3 sec after incubation at 0 °C in media with and without EGTA. To avoid further phosphorylation, enough unlabeled ATP was added at zero time. Fig. 7 shows that the amount of ^{32}P incorporated to the membranes decayed to almost the same level both in EGTA-free and in EGTA-containing media. It seems therefore that, in contrast with the requirement of Ca^{2+} demonstrated for the formation of the phosphoprotein, its rapid breakdown is not dependent on Ca^{2+}. The lack of effect of Ca^{2+} also rules out the possibility that ^{32}P disappears from the protein because of the reversal of the phosphorylation reaction. Thus, rapid breakdown of the phosphoprotein probably is the result of a Ca^{2+}-independent hydrolysis of the acylphosphate bond.

Effects of Mg^{2+} on Dephosphorylation

The effect of Mg^{2+} on the rate of hydrolysis of the Ca^{2+}-dependent phosphoprotein was studied by measuring the amount of ^{32}P that remained bound to the membranes after chasing the label with a solution containing unlabeled ATP with and without Mg^{2+}. These experiments were performed with membranes which had been phosphorylated in the presence and in the absence of Mg^{2+}.

Results in Fig. 8*a* make clear that the addition of Mg^{2+} during dephos-

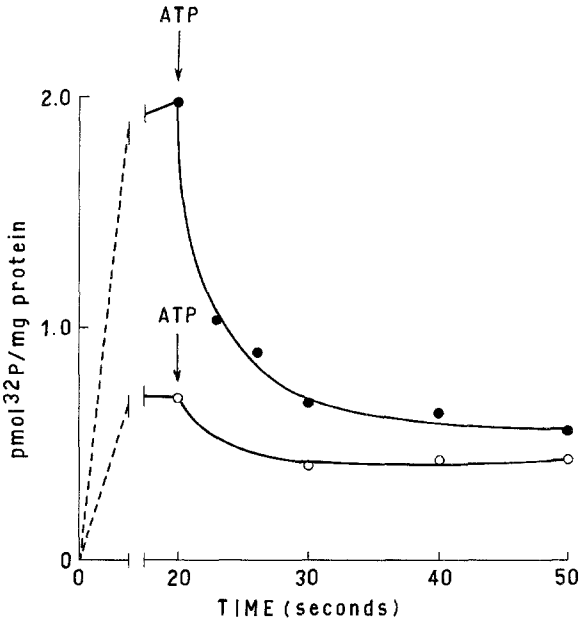

Fig. 6. Time course of the loss of radioactivity from phosphorylated erythrocyte membranes. After 20-sec incubation with (^{32}P)ATP in media with (●) and without (○) Ca^{2+}, 0.5 ml of a solution containing (mM): 2 ATP, 150 Tris-HCl (pH 7.4 at 0 °C) was added. The reaction was stopped as indicated in Materials and Methods. Temperature was 0 °C

phorylation of membranes phosphorylated in the absence of this cation lowers to one-fourth the amount of Ca^{2+}-dependent phosphoprotein remaining at the end of the chase period. The stimulatory effect of Mg^{2+} on the hydrolysis of the phosphoprotein formed in the absence of Mg^{2+} contrasts with the lack of effect of this cation on the splitting of the Ca^{2+}-dependent phosphoprotein formed in the presence of Mg^{2+}. This can be clearly seen in the experiment of Fig. 8*b* which shows that when formed in the presence of Mg^{2+}, almost all the Ca^{2+}-dependent fraction of the phosphoprotein undergoes hydrolysis within 5 sec regardless whether Mg^{2+} was present or not. It is clear therefore that it suffices for Mg^{2+} to be present during phosphorylation to exert its activating effect on the rate of splitting of phosphate from the phosphoprotein.

Discussion

As mentioned in the Introduction, human erythrocyte membranes catalyze a Ca^{2+}-dependent hydrolysis of ATP which is probably coupled

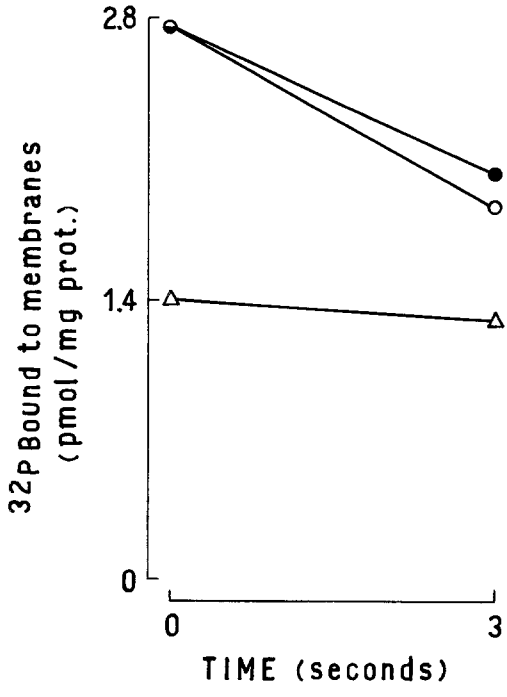

Fig. 7. The effect of chelation of Ca^{2+} on the loss of ^{32}P from phosphorylated membranes. Membranes were phosphorylated with (^{32}P)ATP at 0 °C in media containing Mg^{2+} (△) and Mg^{2+} plus Ca^{2+} (◒). After 20 sec the dephosphorylation was initiated by addition of 0.05 ml of a solution containing 5 mM ATP with (●) and without (○, △) 5 mM EGTA

to the active transport of Ca^{2+}. The results presented in this paper show that provided Ca^{2+} is present, erythrocyte membranes also promote the rapid transfer of the terminal phosphate of ATP to a membrane protein. The concentration of Ca^{2+} for half-maximal effect is 9.3 μM for the ATPase (Rega *et al.*, 1973, but *cf.* Schatzmann, 1973) and 7.0 μM for the phosphorylation. The phosphate in the protein is able to undergo rapid turnover. Splitting of the phosphate bond is enzymatically catalyzed since the phosphoprotein is fairly stable in membranes which have been denaturated with TCA. The sensitivity to pH and to hydroxylamine of the nonenzymatic splitting of the phosphoprotein strongly suggests that the phosphate moiety is attached to the protein by an acylphosphate bond. These findings, together with what is known about other transporting systems which depend on ATP as the energy source (*see* for references, Bastide, Meissner, Fleischer & Post, 1973), make it reasonable to assume that the reactions we have analyzed in this paper are catalyzed by the Ca^{2+}-dependent

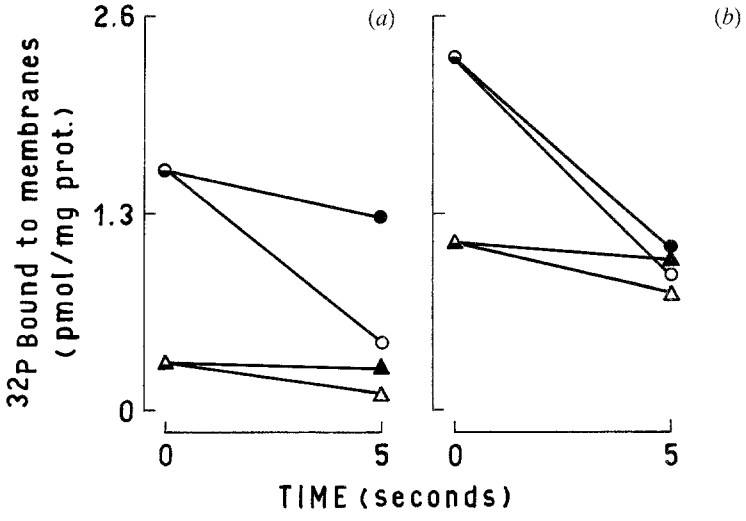

Fig. 8. The effect of Mg^{2+} on the loss of ^{32}P from membranes phosphorylated in the absence (*a*) and in the presence (*b*) of 0.5 mM Mg^{2+} in media with (◒) and without (▲) Ca. During phosphorylation the Mg^{2+}-free media contained 0.1 mM EDTA. Dephosphorylation was initiated after 20 sec incubation in (^{32}P)ATP solutions by the addition of 0.5 ml of a solution containing (mM): 2 ATP, 0.1 EGTA, 150 Tris-HCl, either with (△, ○) or without (●, ▲) 2 mM $MgCl_2$. When phosphorylation was performed in Mg^{2+}-containing media the Mg^{2+}-free chase solutions also contained 30 mM *trans*-1,2-diaminocyclohexanetetracetic acid (CDTA)

ATPase, the phosphoprotein presumably being an intermediate during the hydrolysis of ATP by this system.

From the ratio between the maximum rate of Ca^{2+}-dependent ATP hydrolysis (0.25 μmol/mg protein/hr, Rega *et al.*, 1973) and the level of Ca^{2+}-dependent phosphorylation attainable at nonlimiting ATP concentrations (1.4 pmol/mg prot, *see* Fig. 2) the turnover number of the Ca^{2+}-dependent ATPase from human erythrocytes would be 3×10^3 min^{-1} at 37 °C. This value lies within those for the (Na+K)-ATPase from erythrocyte membranes (1.2×10^4 min^{-1}, Bader, Post & Bond, 1968), and for the Ca^{2+}-dependent ATPase from sarcoplasmic reticulum (10^3 min^{-1}, de Meis, 1972). The maximum level of phosphorylation allows one to estimate that the number of phosphorylating sites is about 700 per cell. From the level of phosphorylated intermediate of the (Na+K)-ATPase (Rega & Garrahan, *unpublished*), the number of phosphorylating sites related to the Na-pump can be estimated to be near 550 per red cell, a value which agrees with the number of glycoside binding sites in red cells (Baker & Willis, 1972). It seems therefore that in human erythrocytes, the number of Ca^{2+}-pumps is close to the number of Na^+-pumps.

If the role of phosphorylation and dephosphorylation in the hydrolysis of ATP by the Ca^{2+}-dependent ATPase from erythrocyte membranes is taken for granted, our results throw some light on the cationic requirements of the intermediate steps of this enzymic activity. Formation, but not hydrolysis, of the phosphorylated intermediate is strictly dependent on Ca^{2+}, suggesting that the requirement of Ca^{2+} for the overall reaction of ATP hydrolysis catalyzed by erythrocyte membranes expresses the requirement of Ca^{2+} at the phosphorylation step. Even in the absence of Mg^{2+}, Ca^{2+} is able to promote the transfer of the terminal phosphate of ATP to the membrane. As judged by its electrophoretic mobility and sensitivity to pH and to hydroxylamine, the phosphoprotein formed in the absence of Mg^{2+} is identical to that formed in the presence of the divalent cation. Mg^{2+} therefore is not required for Ca^{2+}-dependent phosphorylation.

Results presented in this paper show that Mg^{2+} accelerates the enzymatic breakdown of the phosphorylated intermediate. In view of this, the fact that the amount of Ca^{2+}-dependent phosphoprotein is not much lower when made in Mg^{2+}-containing medium than it is when made in Mg^{2+}-free medium is intriguing. Three possible explanations may account for this phenomenon: (*i*) the rate of the overall reaction of ATP hydrolysis may be limited by the dephosphorylation step; (*ii*) Mg^{2+} could accelerate phosphorylation as well as dephosphorylation; and (*iii*) in the presence of Mg^{2+}, the equilibrium of the phosphorylation reaction may be more displaced towards the phosphoprotein than in the absence of the divalent cation *(see below)*. More experimental evidence is necessary to give a definite answer to this question.

Acceleration by Mg^{2+} of the enzymatic hydrolysis of the phosphorylated intermediate, which probably accounts for the dependence on Mg^{2+} of the overall reaction catalyzed by the Ca^{2+}-dependent ATPase, is amenable to two alternative explanations: (*i*) combination of the phosphoprotein with Mg^{2+} makes its phosphate moiety more reactive towards water. In this case the rate of hydrolysis of the phosphoprotein would depend on the presence of Mg^{2+} in the medium in which the hydrolysis takes place; (*ii*) Mg^{2+} is required for the phosphorylated intermediate to undergo a transition from a state of *low* reactivity to a state of *high* reactivity towards water, the equilibrium of this reaction being in favor of the high reactivity state. In this hypothesis, once the transition to the high reactivity state has taken place, removal of Mg^{2+} should not affect the rate of dephosphorylation. Experimental results seem to favor the second

view since we have shown that Mg^{2+} in the medium in which phosphorylation takes place suffices for dephosphorylation to proceed rapidly.

It seems therefore that the relationship among Ca^{2+}, Mg^{2+} and ATPase activity can be described by the following scheme:

$$E + ATP \xrightarrow{Ca^{2+}} EP + ADP \quad (1)$$

$$EP \xrightarrow{Mg^{2+}} E'P \quad (2)$$

$$E'P + H_2O \longrightarrow E + P_i \quad (3)$$

where E is the enzyme and EP and $E'P$ are two different states of the phosphorylated enzyme which differ in their reactivity towards water.

With phosphorylation being independent of Mg^{2+} it is clear that free ATP serves as substrate for this process. Free ATP has also been shown to be a substrate for phosphorylation of sarcoplasmic reticulum (Martonosi, 1969; Panet, Pick & Selinger, 1971). It seems however that the Mg^{2+}-ATP complex can replace free ATP at the active center of the Ca^{2+}-dependent ATPase in human erythrocyte membranes, since in media with enough Mg^{2+} to ensure that all the ATP present will be Mg^{2+}-ATP, the level of both phosphorylation and Ca^{2+}-dependent ATPase is not reduced. The higher concentration of ATP needed for half-maximal phosphorylation in the presence as compared to that required in the absence of Mg^{2+} may indicate that the active center of the Ca^{2+}-dependent ATPase has a higher affinity for ATP than for the Mg^{2+}-ATP complex. Our reaction scheme requires free Mg^{2+} in the reaction media of the Ca^{2+}-ATPase. This is consistent with the finding that optimal Ca^{2+}-dependent ATP hydrolysis is attained when the concentration of Mg^{2+} is about two times the concentration of ATP (D.E. Richards, 1973, *unpublished work*).

We have reported elsewhere (Rega *et al.*, 1974) that in the presence of Mg^{2+} and Ca^{2+}, both Na^+ or K^+ increase the labelling of erythrocyte membranes from (^{32}P)ATP. Studies in relation to this phenomenon are now in progress in our laboratory.

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