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PHOSPHORYLATION AND DEPHOSPHORYLATION OF THE Ca^{2+} PUMP OF HUMAN RED CELLS IN THE PRESENCE OF MONOVALENT CATIONS

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(1) In the presence of calcium ions, K^+ increases the rate and the steady state level of phosphorylation of human red cell membranes by [γ-^{32}P]ATP. The effect of K^+ is mimicked by Rb^+, NH_4^+ and Cs^+. Electrophoresis experiments suggest that the phosphorus taken up by the membranes in the presence of K^+ is bound to the phosphoenzyme of the Ca^{2+}-ATPase. (2) (Ca^{2+} + K^+)-dependent phosphorylation requires Ca^{2+} and ATP with the same apparent affinity as the phosphorylation of the Ca^{2+} pump and the effect of K^+ on phosphorylation is excerted with the same apparent affinity as that for the activation of the Ca^{2+}-ATPase by K^+. (3) The rate of hydrolysis of phosphoenzyme made in the presence of K^+ is higher than that made in its absence and K^+ increases the ratio Ca^{2+}-ATPase activity/Ca^{2+}-dependent phosphoenzyme concentration. (4) Results suggest that monovalent cations activate the Ca^{2+} pump because they increase the level and the turnover of the phosphoenzyme of the Ca^{2+}-ATPase.

Introduction

The very low concentration of Ca^{2+} in human red cells is maintained by active Ca^{2+} extrusion through a Ca^{2+}-pumping ATPase [1]. The splitting of ATP by the Ca^{2+}-ATPase involves the formation and hydrolysis of a phosphoenzyme [2–4]. A simplified scheme of this reaction may be written as follows [5]

$$ATP + E \xrightleftharpoons[\text{ADP}]{Ca^{2+}} E \sim P \xrightarrow{Mg^{2+},ATP} E + P_i$$

where E ~ P is the phosphoenzyme and the cofactors are indicated above the arrows.

Ca^{2+}-dependent ATP [6–8] and *p*-nitrophenylphosphate [9] hydrolysis catalyzed by red cell membranes is stimulated by K^+, NH_4^+, Rb^+ or Na^+. Although is has been suggested that this effect could represent an abnormal behaviour of the (Na^+ + K^+)-ATPase present in the red cell membrane [6,10], its relation with the Ca^{2+}-pump seems now well established since activation by monovalent cations persists in purified preparations of the Ca^{2+}-ATPase [11] and monovalent cations stimulate active Ca^{2+}-transport [12–14]. Furthermore the existence of sites for these cations on the Ca^{2+}-ATPase can be demonstrated by the modulation they exert on the inhibitory effects of *N*-ethylmaleimide [15] and of vanadate [16–17]. Stimulation by monovalent cations has also been observed in the Ca^{2+}-ATPase of sarcoplasmic reticulum in which it has been reported that K^+ accelerates the hydrolysis of the phosphoenzyme (see Ref. 18). We have shown that in the presence of calcium ions, K^+ increases phosphorylation of red cell membranes by ATP [10]. The aim of the present study was to investigate further the effect of K^+ and other monovalent cations on the partial reactions of the Ca^{2+}-ATPase from human red cell membranes. Results show that monovalent cations increase the steady-state level and the turnover of the phosphoenzyme of the Ca^{2+}-ATPase.

Materials and Methods

Materials. Fragmented membranes from human red cells were prepared as described previously [19]. [γ-^{32}P]ATP was prepared according to the procedure of Glynn and Chappell [20] except that no unlabeled orthophosphate was added to the incubation mixture. Orthophosphate labelled with ^{32}P was provided by Comisión Nacional de Energía Atómica, Argentina. ATP disodium salt (substantially vanadium-free), 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 grade.

Methods. Phosphorylation of the membranes was carried out at 0–3°C following the procedure already described [3]. The reaction mixture had a volume of 0.4 ml and contained 0.8 mg membrane protein/ml in (mM): (ethylenebis(oxythylene nitrilo))tetraacetic acid (EGTA), 0.100; Tris-HCl (pH 7.4 at 0°C), 160; [γ-^{32}P]ATP, 0.030. If present, $CaCl_2$ was 0.150 mM. Where indicated in Results the mixture contained 0.5 mM $MgCl_2$. Chloride salts of monovalent cations replaced equimolar amounts of Tris-HCl. To measure the rate of dephosphorylation, phosphorylated membranes in 0.4 ml of incubation media were 'chased' by the addition of 0.25 ml of a solution of composition identical with the incubation media but lacking [γ-^{32}P]ATP and containing enough EGTA to give a final concentration of 30 mM. Ca^{2+}-dependent phosphorylation is the increment in the amount of ^{32}P bound to the membrane elicited by 0.150 mM $CaCl_2$. Ca^{2+}-dependent ATPase activity was measured in media identical with those used for the phosphorylation experiments using [γ-^{32}P]ATP as the substrate according to a procedure already described [21]. All experiments were performed in quadruplicate. Protein was estimated by the method of Lowry et al. [22] using bovine serum albumin as the standard. Gel electrophoresis of phosphorylated membranes was performed following the procedure already described [3]. The amount of phosphoenzyme was estimated assuming that one ^{32}P atom binds per molecule of enzyme.

Results and Discussion

Table I gives the result of an experiment to test the effects of K^+ on phosphorylation of red cell membranes by [γ-^{32}P]ATP. In media with Ca^{2+}, K^+ stimulates phosphorylation both in the absence and presence of 0.5 mM Mg^{2+}. The effect of K^+ is fully dependent on Ca^{2+} since in the absence of this cation, K^+ has no effect on the level of phosphorylation. Mg^{2+} does not substitute for Ca^{2+}. Separate experiments (not shown) demonstrated that the ^{32}P bound to the membranes in the presence of K^+ undergoes rapid turnover since it is released in a few seconds at 0°C upon the addition of an excess either of unlabelled ATP or of ADP.

TABLE I

EFFECT OF K^+ ON PHOSPHORYLATION OF HUMAN RED CELL MEMBRANES BY [γ-^{32}P]ATP

Phosphorylation time was 120 s in media without and 20 s in media with Mg^{2+}. The concentration of K^+ was 100 mM. Values are the mean ±S.E. of four determinations.

Reaction mixture with	Phosphorylation (pmol/mg protein)
–	0.42 ± 0.02
K^+	0.45 ± 0.01
Ca^{2+}	1.63 ± 0.08
Ca^{2+}, K^+	2.32 ± 0.08
Mg^{2+}	3.41 ± 0.13
Mg^{2+}, K^+	3.48 ± 0.17
Mg^{2+}, Ca^{2+}	4.56 ± 0.05
Mg^{2+}, Ca^{2+}, K^+	5.59 ± 0.13

Fig. 1A and B shows the time course of Ca^{2+}-dependent phosphorylation by [γ-^{32}P]ATP in media with and without K^+. In the absence of $MgCl_2$ (Fig. 1A) phosphorylation is low and its half-time is 20 s in the absence and 15 s in the presence of K^+. In media with 0.5 mM $MgCl_2$ (Fig. 1B) phosphorylation is fast and the effect of K^+ on its rate is much more apparent, the half-time for phosphorylation being 3.0 s in the absence and 0.8 s in the presence of K^+. Results in Table I and Fig. 1 make clear that K^+ increases the rate and the steady-state level of Ca^{2+}-dependent phosphorylation of red cell membranes by ATP.

In a previous paper [5] we held the view that Mg^{2+} increases the steady-state level of phosphorylation of the Ca^{2+}-ATPase. This was based on experiments in which phosphorylation was measured after 20 s in K^+-free media. Results in Fig. 1A and B show that this view was wrong since after 20 s incubation in the ab-

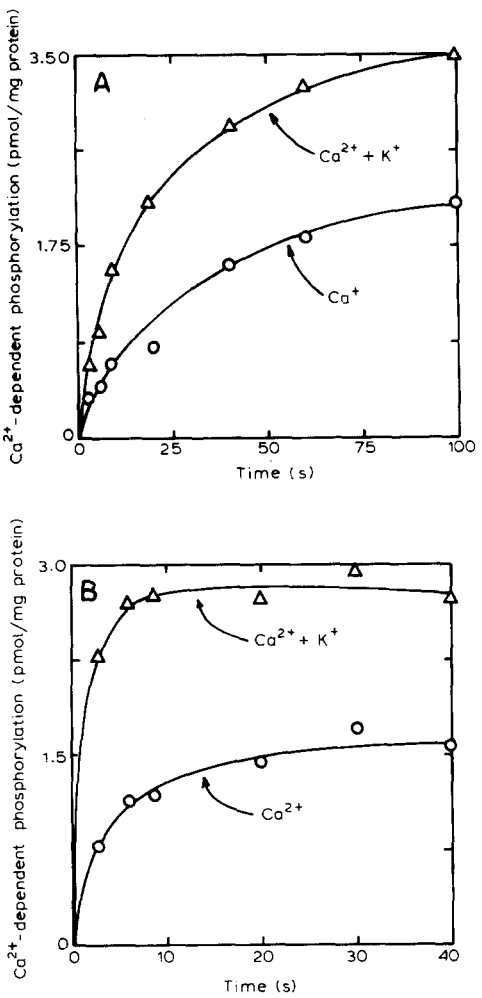

Fig. 1. Time course of Ca^{2+}-dependent phosphorylation of red cell membranes by [γ-^{32}P]ATP in the presence and absence of 100 mM K^+ in media without (A) and with (B) $MgCl_2$.

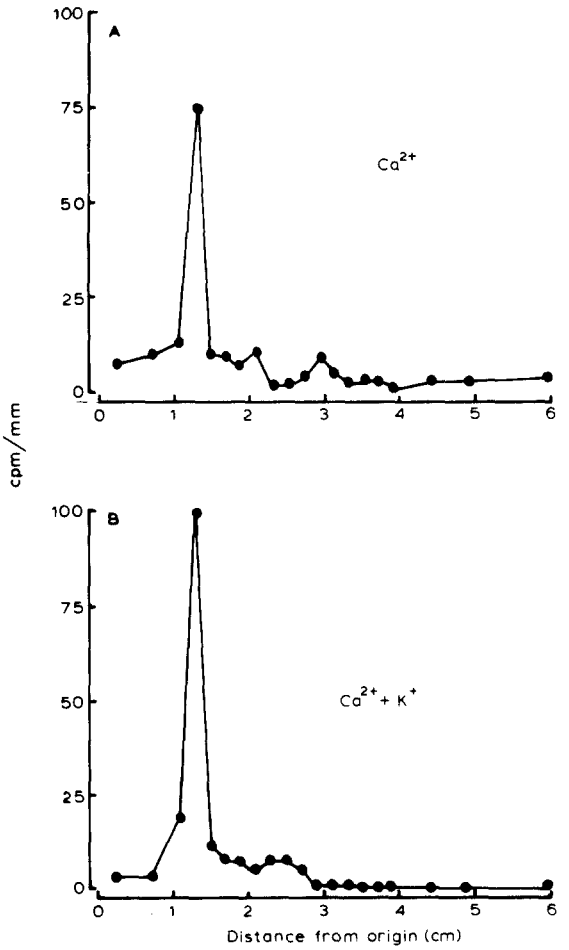

Fig. 2. Distribution of radioactivity in SDS-polyacrylamide gels after electrophoresis of red cell membranes phosphorylated during 20 s with [γ-^{32}P]ATP in media containing Ca^{2+} (A) and Ca^{2+} plus K^+ (B). Each of the gels received 0.5 mg of membrane protein.

sence of Mg^{2+} phosphorylation has not yet reached steady state. In fact, comparison of the time course of phosphorylation without (Fig. 1A) and with (Fig. 1B) Mg^{2+} demonstrates that if incubation time is sufficiently prolonged the level of phosphorylation becomes independent of Mg^{2+}.

Results in Fig. 2 allow to compare the distribution after electrophoresis in sodium dodecyl sulfate (SDS)-polyacrylamide gels of radioactivity in membranes phosphorylated in the presence of Ca^{2+} with that in membranes phosphorylated in the presence of Ca^{2+} plus K^+. A single peak of radioactivity is observed whose mobility is independent on whether K^+ was present or not during phosphorylation. This indicates that in the presence of Ca^{2+}, K^+ stimulates phosphorylation of a membrane protein whose electrophoretic mobility is the same as that of the phosphoenzyme of the Ca^{2+} pump. The peak of radioactivity from the membranes phosphorylated in the medium with K^+ corresponds to a ^{32}P content which is higher than that from membranes phosphorylated in the medium without K^+ by 38%, a value which is close to

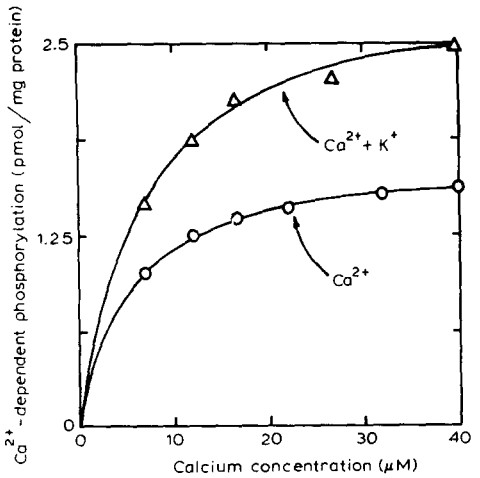

Fig. 3. The relation between the level of Ca^{2+}-dependent phosphorylation and the concentration of Ca^{2+} in media with and without 100 mM KCl. Phosphorylation was carried out during 20 s in the presence of 0.5 mM $MgCl_2$. The curves represent Michaelis equations with $K_{0.5}$ 6.7 μM and maximum effect 3.0 (pmol/mg) for the experiment with K^+ and $K_{0.5}$ 5.5 μM and maximum effect 1.8 (pmol/mg) for the experiment without K^+.

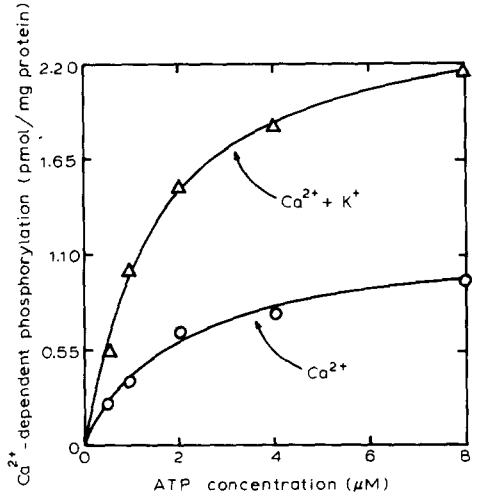

Fig. 4. The relation between Ca^{2+}-dependent phosphorylation and the concentration of [γ-^{32}P]ATP in media with and without 100 mM K^+. Phosphorylation was carried out during 20 s in media with 0.5 mM $MgCl_2$. The curves represent Michaelis equations with K_m 1.98 μM and maximum effect 2.9 (pmol/mg) for the experiment with K^+ and K_m 1.98 μM and maximum effect 1.2 (pmol/mg) for the experiment without K^+.

that expected from the 35% increase in phosphorylation due to K^+ during this particular experiment.

Fig. 3 shows the effect of K^+ on the relation between the level of phosphorylation and the concentration of Ca^{2+}. The increase in phosphorylation by K^+ takes place with little change in the $K_{0.5}$ for Ca^{2+} indicating that the effect of K^+ requires Ca^{2+} at sites with the same high affinity as those from the Ca^{2+} pump.

Fig. 4 shows the effect of K^+ on the level of phosphorylation at different concentrations of ATP. It can be seen that the K_m for ATP of the K^+-dependent phosphorylation is the same as that for phosphorylation of the Ca^{2+} pump.

The close parallelism between the electrophoretic behaviour and the response to Ca^{2+} and ATP of (Ca^{2+} + K^+)-dependent phosphorylation and the phosphorylation of the Ca^{2+}-ATPase strongly suggests that the effect of K^+ is to increase the level of phosphorylation of this enzyme. K^+ could increase phosphorylation by increasing either the level of phosphoenzyme or the number of phosphoryl group per phosphoenzyme. Although experimental evidence in this paper does not allow to distinguish between the two possibilities, data from other transport ATPases [23,24] together with the lack of effect of K^+ on the stoichiometry of the Ca^{2+} pump [14] make unlikely the

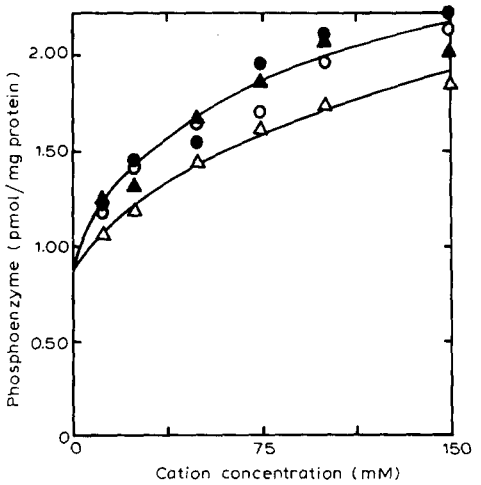

Fig. 5. The effects of increasing concentrations of K^+ (○), NH_4^+ (▲), Rb^+ (●) and Cs^+ (△) in the reaction mixture on the level of Ca^{2+}-dependent phosphoenzyme after 20 s phosphorylation. All media contained 0.5 mM $MgCl_2$. Curves are Michaelis equations whose parameters are given in the text.

idea of the existence of more than one phosphoryl group per phosphoenzyme.

Rb^+, Cs^+, NH_4^+, and Na^+ also stimulate Ca^{2+}-ATPase activity and active Ca^{2+}-transport across red cell membranes [6–8]. In view of this, the effect of K^+ on the level of phosphoenzyme was compared with those of Rb^+, NH_4^+ and Cs^+. Na^+ was not tested because it promotes phosphorylation of the $(Na^+ + K^+)$-ATPase [25] also present in red cell membranes. Results in Fig. 5 show that all the cation tested increase the level of Ca^{2+}-dependent phosphoenzyme. The experimental points of the monovalent cation-dependent fraction of the phosphoenzyme level fit to Michaelis curves with maximum effect 1.70 pmol/mg and $K_{0.5}$ 50 mM for K^+, Rb^+ and NH_4^+ and with maximum effect 1.66 pmol/mg and $K_{0.5}$ 97 mM for Cs^+. These values indicate that at non-limiting concentrations, all the cations tested are equally effective in increasing the level of phosphoenzyme. The ability to promote phosphorylation is in contrast with the low effectiveness of Cs^+ [7–9] as activator of ATP hydrolysis by the Ca^{2+}-ATPase. Although results in Fig. 5 indicate that from all the cation tested Cs^+ is the least reactive, in the light of the experiments in this paper it is not clear why it fails in activating the overall reaction of the Ca^{2+}-ATPase. Results in Fig. 5 also allow to calculate that the amount of phosphoenzyme in the absence of monovalent cations is about one-third that detectable at non-limiting concentrations of monovalent cations. This should be taken into account when estimating the number of Ca^{2+}-ATPase units from measurements of the level of phosphoenzyme.

In the experiment in Fig. 6A and B the effects of K^+ on the level of phosphoenzyme and on the activity of the Ca^{2+}-ATPase were compared. Measurements were performed simultaneously in media of identical composition and at the same temperature. Results shows that the increase by K^+ in phosphoenzyme level and in ATP hydrolysis is half maximal at 94 mM K^+ in the absence (Fig. 6A) and at 62 mM K^+ in the presence of Mg^{2+} (Fig. 6B). Hence, the effect of K^+ on the level of phosphoenzyme has the same apparent affinity as that for the activation by K^+ of the Ca^{2+}-ATPase. This suggests that the same sites for K^+ are involved in both phenomena. This view gets further support from the parallel increase induced by Mg^{2+} in the apparent affinity for K^+ during phosphorylation and activation of ATP hydrolysis. Fig. 6A and B also show that K^+ increases the Ca^{2+}-ATPase activity relatively more than the phosphoenzyme level. As a consequence of this the ratio Ca^{2+}-ATPase activity/phosphoenzyme level increases from 0.021 to 0.031 s^{-1} (Fig. 6A) and from 0.098 to 0.122 s^{-1} (Fig. 6B) as K^+ concentration raises from 0 to 150 mM. K^+ therefore increases not only the level of phosphoenzyme but also the turnover of the Ca^{2+}-ATPase.

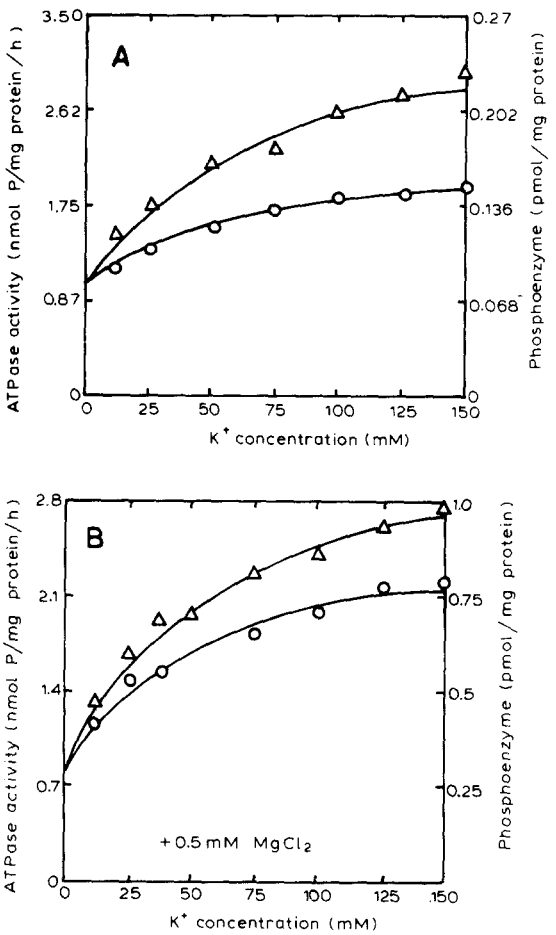

Fig. 6. The effect of K^+ on Ca^{2+}-dependent phosphoenzyme and Ca^{2+}-dependent ATPase activity measured at 0–3°C. The curves represent Michaelis equations with $K_{0.5}$ 94 mM; V 0.23 nmol P/mg protein per h (A) and $K_{0.5}$ 62 mM, V 0.94 nmol P/mg protein per h (B) for the K^+-dependent ATPase activity (△); and with $K_{0.5}$ 94 mM and maximum effect 1.5 pmol/mg protein (A) and $K_{0.5}$ 62 mM and maximum effect 1.96 pmol/mg protein (B) for the K^+-dependent phosphoenzyme (○).

TABLE II

DEPHOSPHORYLATION RATES OF Ca^{2+}-DEPENDENT PHOSPHOENZYME MADE IN THE PRESENCE AND ABSENCE OF K^+ WITH AND WITHOUT Mg^{2+}

Membranes where phosphorylated during 20 s in media with the cations cited and then were allowed to dephosphorylate during 3 s following the procedure described in Methods. The concentration of K^+ was 150 mM. The rate constants for dephosphorylation were calculated assuming first order kinetics. The mean difference ± S.E. were calculated for paired experiments ($n = 4$), P values were calculated using Student's t-test for paired samples.

Reaction mixture with	Dephosphorylation rate	
	s^{-1}	Mean difference ± S.E.
Ca^{2+}	0.121	
Ca^{2+}, K^+	0.213	0.092 ± 0.021 ($P < 0.05$)
Mg^{2+}, Ca^{2+}	0.167	
Mg^{2+}, Ca^{2+}, K^+	0.214	0.064 ± 0.009 ($P < 0.02$)

The effect of K^+ on the dephosphorylation reaction of the Ca^{2+}-ATPase was studied comparing the rate of dephosphorylation of phosphoenzyme made in media with Ca^{2+} plus K^+ with that of phosphoenzyme made in media with Ca^{2+} alone. Results in Table II shows that, both in the presence and absence of Mg^{2+} the phosphoenzyme made with K^+ dephosphorylates at a higher rate than that made without K^+.

It may be worth to point out that the rates of dephosphorylation (Table II) are higher than the ratios ATPase activity/phosphoenzyme concentration (Fig. 6A and B). Dephosphorylation was measured in media containing an excess (30 mM) EGTA. If this does not alter the reaction, the relation mentioned above suggests that dephosphorylation is not rate limiting during ATP hydrolysis by the Ca^{2+}-ATPase.

Results presented in this paper show that combination of K^+, and presumably NH_4^+, Rb^+ and Cs^+ with the Ca^{2+} pump at sites which do not interact with ATP and Ca^{2+}, increases the rate of formation, the steady state level and the rate of hydrolysis of the phosphoenzyme of the Ca^{2+} pump. This is accompanied by an increase in the turnover of the Ca^{2+}-ATPase. The effects of K^+ on phosphoenzyme level and on turnover account for the activation by monovalent cations of ATP hydrolysis by the Ca^{2+}-ATPase measured at 0°C and at low ATP concentrations. It may be possible that the same phenomenon explains the activating effect of monovalent cations on active calcium transport in human red cells.

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