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EFFECTS OF MONOVALENT CATIONS ON THE ($Mg^{2+} + Ca^{2+}$)-DEPENDENT ATPase OF THE RED CELL MEMBRANE

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SUMMARY

1. Stimulatory effects of monovalent cations on the ($Mg^{2+} + Ca^{2+}$)- dependent ATPase of the human red cell membrane are described.

2. This activity was significantly increased by Na^+, K^+, and Rb^+. There was no effect with Cs^+ or Li^+. The cationic specificity of this effect eliminates ionic strength as its source.

3. Stimulation by Na^+ and K^+ followed saturation kinetics, with a concentration of about 10 mM giving a half-maximal effect.

4. Na^+ and K^+ did not alter the apparent affinity of the enzyme for Ca^{2+} but approximately doubled the rate at saturating concentrations of Ca^{2+}.

5. The extra activity appearing in the presence of monovalent cations could be associated either with the ($Mg^{2+} + Ca^{2+}$)-dependent ATPase known to be a part of the Ca^{2+} pump, or with a different ($Mg^{2+} + Ca^{2+}$)-dependent ATPase whose function remains to be established.

INTRODUCTION

Ca^{2+} is not at electrochemical equilibrium in cells, and the characteristically low intracellular levels of free Ca^{2+} must ultimately be maintained by an energy-dependent pump in the plasma membrane (see RASMUSSEN[1] for review). Active extrusion of Ca^{2+} has been demonstrated in the giant squid axon[2,3], in HeLa cells[4], and in the reconstituted human red cell ghost[5–7]. Despite the apparently universal occurrence of this pump, little is known concerning its mechanism or factors which may control its activity.

In the case of the giant squid axon, BLAUSTEIN AND HODGKIN[3] suggested that the energy for Ca^{2+} extrusion could derive, at least in part, from the inward electrochemical gradient for Na^+, with Ca^{2+} efflux coupled to Na^+ influx. Their experiments did not rule out the possibility of a metabolically driven pump, however. In the human red cell, on the other hand, there is good evidence that ATP is the direct energy source for the Ca^{2+} pump, and that the transport system survives in the isolated plasma membrane as a ($Mg^{2+} + Ca^{2+}$)-dependent ATPase activity[5–8]. In this respect

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the Ca^{2+} transport system of the red cell resembles that of the sarcoplasmic reticulum of muscle, which is also manifested as a $(Mg^{2+} + Ca^{2+})$-dependent ATPase in isolated membranes[9].

The present study was undertaken to further characterize the $(Mg^{2+} + Ca^{2+})$-dependent ATPase of the human red cell membrane, and we describe a stimulatory effect of monovalent cations on this activity. Our results extend and confirm an earlier report by SCHATZMAN[10].

METHODS

Preparation of membranes

Human blood was used throughout. The blood was drawn into acid citrate dextrose solution and stored for up to 2 months before use. Cells from 100–200 ml of blood were obtained by centrifugation at 200 × *g* for 10 min at 4°. All subsequent centrifugations were also carried out at this temperature. The cells were washed 3 times in 6 vol. of 0.16 M Tris–HCl (pH 6.8). After the third wash, the buffy coat was removed by suction, and the cells were hemolyzed in 5 vol. of ice-cold 1 mM $(Tris)_4$ EDTA (pH 7.5). The hemolysate was then centrifuged at 18000 × *g* for 20 min. The supernatant fluid was removed by suction, and the packed membranes were washed several times in an ice-cold medium consisting of 1 mM EDTA and 10 mM Tris–HCl (pH 7.5). The washings were continued until the membranes were light pink or nearly white in color. The membranes were then taken up in a medium consisting of 10 mM imidazole which had been adjusted to pH 7.2 with HCl. Enough of this was added to yield a suspension having a fluid consistency. The protein content of these preparations ranged between 0.5 and 1.0 mg per ml. Protein was measured by the method of LOWRY *et al.*[11]. The membranes were stored on ice in a refrigerator, and there was little loss of ATPase activity over a 2–3-week period.

ATPase assay

Di-sodium ATP (Sigma Chemical Co., St. Louis, Mo.) was converted to the Tris salt by passage through a chilled column of Dowex 50X8-100 cation-exchange resin in the Tris form. The effluent was adjusted to pH 7.5 with additional Tris, and the concentration of ATP was calculated from the absorbance at 259 nm.

All cations were reagent grade and were added as their Cl^- salts. The salts of Rb^+ and Cs^+ (99.9 % pure) and Li^+ (99.65 % pure) were purchased from K and K Laboratories, Plainview, N.Y. Tris was obtained from Sigma.

ATPase activity was assayed by measuring the inorganic phosphate produced, using a modification of the method of FISKE AND SUBBAROW[12]. Incubations were carried out in 15 ml plastic centrifuge tubes. The reaction system had the following basic composition: 1.5 mM ATP, 1.7 mM Mg^{2+} and 38 mM Tris–HCl (pH 7.8). Other additions are indicated in the text. The final volume was 2 ml. All ingredients were mixed on ice, and the reaction was started by transferring the tubes to a water-bath shaker at 38°. Blanks were run to correct for non-enzymatic hydrolysis of ATP. Each experimental point represents the average of a duplicate or triplicate determination. After 1–2 h the reaction was stopped by adding 0.5 ml of 1.2 M $HClO_4$. The tubes were then centrifuged at 10000 × *g* for 20 min, and 2 ml aliquots of the supernatants were taken for assay.

RESULTS

Three ATPase activities are found in the red cell membrane: (1) a basal activity which requires only Mg^{2+}, (2) a $(Mg^{2+} + Na^+ + K^+)$-dependent activity which is inhibited by ouabain, and (3) a $(Mg^{2+} + Ca^{2+})$-dependent activity. These activities are compared in Table I. In agreement with the results of WINS AND SCHOFFENIELS[8], the highest activity was found in the presence of Mg^{2+} plus low concentrations of Ca^{2+}. In fresh membranes the specific activity of the $(Mg^{2+} + Ca^{2+})$-dependent ATPase was 2–3 times greater than that of the $(Mg^{2+} + Na^+ + K^+)$-dependent ATPase. It can also be seen from Table I that Na^+ approximately doubled the $(Mg^{2+} + Ca^{2+})$-dependent activity. This effect required the presence of both Mg^{2+} and Ca^{2+}, as reported by SCHATZMAN[10]. This result was found consistently. The fact that ouabain was present eliminates the possibility that Na^+ could have stimulated by generating $(Mg^{2+} + Na^+ + K^+)$-dependent activity due to traces of K^+ (or NH_4^+) introduced along with the Ca^{2+}. In subsequent experiments ouabain was routinely included for this reason.

The stimulatory effect of Na^+ could have resulted from the increase in ionic strength, in which case other monovalent cations would be equally effective at the same concentrations. In order to test this we compared the effects of several monovalent cations in the presence of Mg^{2+} and Ca^{2+} (Fig. 1). This figure summarizes the results of 7 experiments performed on 7 different fresh membrane preparations. In order to facilitate comparisons, the $(Mg^{2+} + Ca^{2+})$-dependent activity measured in the absence of monovalent cations was arbitrarily taken as 100%, and the same activity with a monovalent cation added is given as a multiple of 100%. In the presence of Mg^{2+} and Ca^{2+} there was substantial activation by Na^+, K^+ and Rb^+, while Li^+ and Cs^+ had no effect. None of these cations was stimulatory in the absence of Ca^{2+}. This cationic specificity eliminates ionic strength as well as Cl^- as causative

TABLE I

EFFECT OF CATIONS ON THE ATPase ACTIVITIES OF THE RED CELL MEMBRANE

Mg^{2+} was present in all cases at a concentration of 1.7 mM. The $(Mg^{2+} + Na^+ + K^+)$-dependent activity is the difference between the activity in row 4 and that in row 2. The $(Mg^{2+} + Ca^{2+})$-dependent activity was obtained by subtracting the activity in row 6 from the activities in the presence of Ca^{2+}. Concentrations: Na^+, 120 mM; K^+, 15 mM; Ca^{2+}, 0.1 mM; ouabain, 0.25 mM. Other conditions are given in METHODS. Activity is expressed as μmoles P_i released per h per mg protein. The results of a single representative experiment are shown. Each value is the average of a duplicate measurement.

Cations	*Ouabain*	*Activity*		
		Total	*$(Mg^{2+} + Na^+ + K^+)$-dependent*	*$(Mg^{2+} + Ca^{2+})$-dependent*
1. Mg^{2+}	—	0.26		
2. $Mg^{2+} + Na^+$	—	0.25		
3. $Mg^{2+} + K^+$	—	0.25		
4. $Mg^{2+} + Na^+ + K^+$	—	0.51	0.26	
5. $Mg^{2+} + Na^+ + K^+$	+	0.23		
6. $Mg^{2+} + Na^+$	+	0.20		
7. $Mg^{2+} + Ca^{2+}$	+	0.56		0.36
8. $Mg^{2+} + Na^+ + Ca^{2+}$	+	0.84		0.64

factors, and points to an additional site on the enzyme which binds activating monovalent cations.

When activity in the presence of Mg^{2+} and Ca^{2+} was measured as a function of

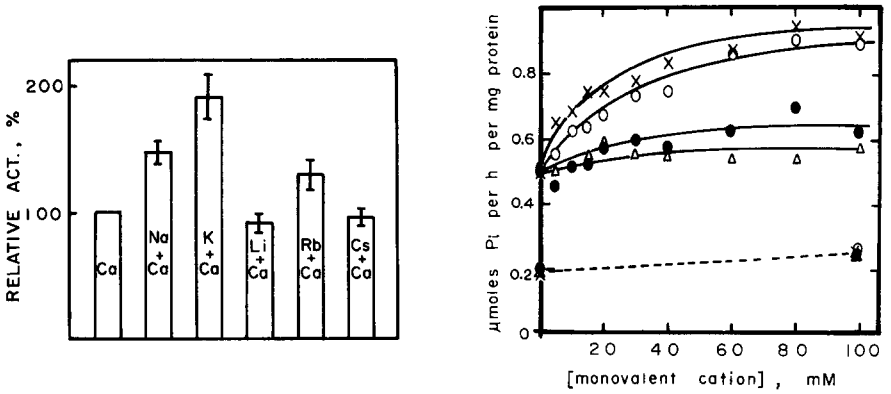

Fig. 1. Effect of monovalent cations on ($Mg^{2+} + Ca^{2+}$)-dependent ATPase activity. Monovalent cation concentrations were 60 mM. Mg^{2+} concn., 1.7 mM; Ca^{2+} concn., 0.1 mM. The ($Mg^{2+} + Ca^{2+}$)-dependent activity in the absence of monovalent cations was taken as 100%. ($Mg^{2+} + Ca^{2+}$)-dependent activity is the difference in the activities of two samples which were identical in all respects except for the presence of Ca^{2+} in one of them. The results of 7 experiments are summarized. Other assay conditions are given in METHODS. Standard errors of the mean are indicated on each bar. Activities that were significantly greater than with Ca^{2+} alone were: $Na^+ + Ca^{2+}$ ($P<0.005$); $K^+ + Ca^{2+}$ ($P<0.005$) and $Rb^+ + Ca^{2+}$ ($P<0.05$). Significance was determined using a one-tailed Student's t test.

Fig. 2. ATPase activity as a function of monovalent cation concentration with and without Ca^{2+}. Solid lines, Ca^{2+} was present. Lower dotted line, Ca^{2+} was not present. Each curve represents a single experiment. Ca^{2+} concn., 0.1 mM; Mg^{2+} concn., 1.7 mM. For other conditions see METHODS. Total ATPase activity is plotted on the ordinate. ×, K^+; ○, Na^+; △, Cs^+; ●, Li^+.

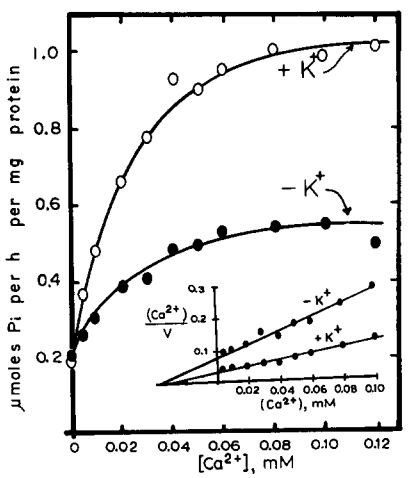

Fig. 3. ATPase activity as a function of Ca^{2+} concentration in the presence and absence of 80 mM K^+. Ordinate: total ATPase activity. The Ca^{2+} concentration given on the abscissa is the concentration added, without correction for binding by ATP or by membranes. The zero Ca^{2+} points may be slightly high, since EGTA (ethyleneglycol-bis(β-aminoethyl ester)-*N*,*N*′-tetraacetic acid) was not added to chelate possible traces of endogenous Ca^{2+}. The inset shows a Woolf plot of the data, from which K_m values were obtained. Other conditions are given in METHODS.

monovalent cation concentration (Fig. 2), it was found that activation by Na^+ and K^+ followed saturation kinetics, with half-maximal stimulation reached at concentrations between 10 and 20 mM. In this experiment Li^+ produced some stimulation, but Fig. 1, which summarizes the results of 7 experiments shows that Li^+ had no consistent effect in most preparations. In other experiments, not shown, no additive effects were noted when Na^+ and K^+ were present together, indicating that these cations act at a single site. SCHATZMAN AND VINCENZI[5] also mentioned the absence of an effect of Na^+ and K^+ in combination.

There was no clear effect of Na^+ or K^+ on the apparent affinity of the enzyme for Ca^{2+}. Their stimulation was due solely to an increase in the maximum velocity. This is shown in Fig. 3, where activity is plotted as a function of Ca^{2+} concentration with and without 80 mM K^+. In both curves the apparent K_m for Ca^{2+} was about 0.03 mM. The enzyme was essentially saturated at 0.1 mM Ca^{2+}, at which point the activity with K^+ present was about twice that in its absence. WINS AND SCHOFFENIELS[8] reported that maximum activity was found at a Ca^{2+} concentration of about 0.3 mM, but in their experiments the concentration of Mg^{2+} was about 2 mM in excess of ATP, and Mg^{2+} was shown to compete with Ca^{2+}.

DISCUSSION

In view of the probable connection between $(Mg^{2+} + Ca^{2+})$-dependent ATPase and Ca^{2+} transport in red cells, it might be expected that the additional activity seen in the presence of monovalent cations should be associated with an increased rate of Ca^{2+} pumping. This appears not to be the case, however, since SCHATZMAN AND VINCENZI[5] reported that Ca^{2+} extrusion from reconstituted human red cells was not reduced by removal of Na^+ and K^+. It is not clear, however, that all traces of these cations were removed in the experiment cited, and this point would bear re-investigation.

The apparent failure of monovalent cations to stimulate Ca^{2+} movement suggests that the monovalent cation-dependent activity may be present only in washed membranes, where it could reflect an altered enzyme no longer coupled to Ca^{2+} pumping. This point could be tested by studying the effect of monovalent cations on the $(Mg^{2+} + Ca^{2+})$-dependent ATPase of reconstituted red cells, where the Ca^{2+} pump is known to be functional.

As an alternative explanation, monovalent cations may stimulate a second $(Mg^{2+} + Ca^{2+})$-dependent ATPase unrelated to Ca^{2+} transport. Support for this possibility is provided in a recent report by BADER[13] that there are two $(Mg^{2+} + Ca^{2+})$-dependent ATPase activities in washed human red cell membranes.

The ATPase activity of skeletal muscle microsomes is increased 1.5- to 4-fold by monovalent cations, with Mg^{2+} as the only divalent cation added[14–16]. This is in contrast to our results with the red cell, where Ca^{2+} in addition to Mg^{2+} was an obligatory requirement for stimulation by monovalent cations (Table I and Fig. 2). LEE *et al.*[14] found the order of effectiveness of monovalent cations in skeletal muscle microsomes to be: $Rb^+ > K^+ > Na^+ > Li^+$. FRATANTONI AND ASKARI[15] reported the sequence: $K^+ > NH_4^+, Rb^+ > Na^+ > Li^+, Cs^+$ (Li^+ and Cs^+ having virtually no effect). DUGGAN[16] found less marked differences in effectiveness. In all these studies, half-maximal activation was found with concentrations of about 10 mM. With the excep-

tion of a requirement for Ca^{2+} in our experiments, these results are in reasonable agreement with ours.

DUGGAN[17] has suggested that the stimulation of skeletal muscle microsomal ATPase is dependent on the presence of endogenous Ca^{2+} in these preparations. In support of this, the effect of monovalent cations was prevented by EGTA (ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid), a Ca^{2+} chelating agent. Furthermore, when care was taken to eliminate all traces of inorganic ions from the microsomes, DUGGAN found that the uptake of Ca^{2+} by microsomal vesicles was stimulated by K^+. Thus it appears that with Ca^{2+} present, K^+ can stimulate both ATP hydrolysis and an associated transport of Ca^{2+} in these preparations.

The Ca^{2+} transport system of the red cell and that of sarcoplasmin reticulum are both manifested as a ($Mg^{2+} + Ca^{2+}$)-dependent ATPase. If DUGGAN'S[17] assumption is correct, this ATPase activity in both systems appears to share the additional feature of stimulation by monovalent cations. In the case of the red cell, however, there is no evidence that monovalent cations stimulate Ca^{2+} transport. Thus despite the similarities between the two systems, the possibility must be considered that monovalent cations act on a second ($Mg^{2+} + Ca^{2+}$)-dependent ATPase in red cells whose function remains to be established.

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