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STIMULATING EFFECTS OF MONOVALENT CATIONS ON ACTIVATOR-DISSOCIATED AND ACTIVATOR-ASSOCIATED STATES OF Ca^{2+}-ATPase IN HUMAN ERYTHROCYTES

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Summary

The additional activation by monovalent cations of the $(Ca^{2+} + Mg^{2+})$-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) in human erythrocyte membranes was studied.

The Ca^{2+}-ATPase occurs in two different states. In the A-state the enzyme is virtually free of protein activator and the kinetics of Ca^{2+} activation is characterized by low apparent Ca^{2+} affinity and low maximum activity. In the B-state the enzyme is associated with activator and the kinetics is characterized by high Ca^{2+} affinity and high maximum activity.

At optimum concentrations of Ca^{2+} the additional activation of the B-state by K^+, NH_4^+, Na^+ and Rb^+ exceeded the corresponding activations of the A-state, and half-maximum activations by K^+, NH_4^+, and Na^+ were achieved at lower concentrations in the B-state than in the A-state. Li^+ and Cs^+ activated the two states almost equally but maximum activation was obtained at lower cation concentrations in the B-state than in the A-state.

The activation of the B-state by the various cations decreased in the order $K^+ > NH_4^+ > Na^+ = Rb^+ > Li^+ = Cs^+$. The A-state was activated almost equally by K^+, Na^+, NH_4^+, and Rb^+ and to a smaller extent by Li^+ and Cs^+.

At sub-optimum concentrations of Ca^{2+} high concentrations of monovalent cations (100 mM) activated the Ca^{2+}-ATPase equally in the A-state and the B-state. In the absence of Ca^{2+} the monovalent cations inhibited the Mg^{2+}-dependent ATPase in both types of membranes. This dependence on Ca^{2+} indicates that the monovalent cations interact with the Ca^{2+} sites in the B-state.

The results suggest that K^+ or Na^+, or both, contribute to the regulation of the Ca^{2+} pump in erythrocytes.

Introduction

$(Ca^{2+} + Mg^{2+})$-dependent ATPase in human erythrocytes is stimulated by low concentrations of Ca^{2+} and is considered to be different from the $(Na^+ +$

K^+)-stimulated ATPase [1]. In the presence of optimum Ca^{2+} concentrations this ATPase is nevertheless stimulated further by monovalent cations, as demonstrated by Schatzmann and Rossi [2] and by Bond and Green [3].

Later investigations have demonstrated the existence of two states of the Ca^{2+}-stimulated ATPase, one state characterized by low Ca^{2+} affinity and low maximum activity (A-state), another state showing high Ca^{2+} affinity and high maximum activity (B-state) [4—7]. The occurrence of two states of Ca^{2+}-stimulated ATPase was recently explained by the existence of a protein activator binding to the enzyme in the presence of Ca^{2+} (B-state) and dissociating in the absence of Ca^{2+} (A-state) [8,9]. These properties make it possible to prepare erythrocyte membranes with the enzyme in A-state and B-state, respectively, and to reveal the kinetics of these states [4,7,10].

The investigations concerning stimulation by monovalent cations were performed with membranes in which the Ca^{2+}-stimulated ATPase appeared to be close to the A-state [2,3], close to the B-state [11], or intermediary to these two states [12].

In the following, the kinetics of activation by alkali and ammonium ions are studied using the two types of membranes which represent the A-state and the B-state, respectively. Differences between the two states analogous to those obtained in case of Ca^{2+} activation are demonstrated.

Methods

Preparation of erythrocyte membranes

Ghosts were prepared from bank blood as described previously [7], centrifuging the membrane suspensions in a Sorvall continuous-flow device which ensures full accessibility of the Ca^{2+}-ATPase [10]. Two types of membranes were prepared.

A-membranes. The erythrocytes were hemolyzed with 6.7 mM sodium phosphate/1.0 mM ethyleneglycol bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA), pH 7.4, and the membranes were washed twice with hypotonic Tris buffer, frozen-thawed, and washed again to obtain membrane-bound Ca^{2+}-ATPase virtually free of protein activator, as described previously [9].

B-membranes. The erythrocytes were hemolyzed in the presence of calcium-nitrilotriacetic buffer and washed twice with Tris to obtain membrane-bound Ca^{2+}-ATPase associated with activator, as described previously [9].

The pretreatment of membranes with ionophore A23187 (a gift from Eli Lilly Co., Denmark) or valinomycin (Sigma) used in some experiments were performed as described previously [10].

Determination of ATPase activity

The activity was assayed [4,10] by measuring P_i liberated at 37°C in a medium of 3 mM Tris · ATP, 4 mM $MgCl_2$, 1 mM EGTA or nitrilotriacetic acid, 0.1 mM *g*-strophantin (ouabain), various concentrations of $CaCl_2$ and chlorides of monovalent cations, 70 mM Tris · HCl (the concentration was not reduced when adding monovalent cation chlorides), 0.8—1.2 g dry membrane per l medium, pH 7.2.

The Ca^{2+}-stimulated ATPase activity was determined as the difference

between (Ca^{2+} + Mg^{2+})-dependent and Mg^{2+}-dependent activity and expressed as $\mu mol \cdot min^{-1} \cdot g^{-1}$, dry membrane exclusive of hemoglobin.

Methods of analysis were performed as previously described [4,10]. The chemicals used were from Merck, analytical grade, unless otherwise stated.

Results

Activation of A-membranes and B-membranes by various concentrations of monovalent cations

In the following the term 'A-membranes' designates membranes in which the Ca^{2+}-stimulated ATPase is predominantly in the A-state, i.e., enzyme is free of protein activator. Correspondingly, 'B-membranes' refers to membranes with the enzyme in the B-state, i.e., enzyme is associated with activator (cf. Methods and ref. 9).

The specific activity of the ATPase at optimum concentrations of Ca^{2+} but in absence of monovalent cations was higher ($P < 0.001$) in the B-membranes than in the A-membranes (cf. legends to Figs. 1 and 2), as shown previously [4,7]. In accordance with previous reports [2,3] the addition of alkali ions caused an additional activation of the ouabain-insensitive ATPase (Figs. 1 and 2).

Figs. 1 and 2 show that the B-membranes were activated more than the A-membranes by K^+, Na^+, and Rb^+. Furthermore, the activation by K^+ exceeded the activation by Na^+ in B-membranes (Fig. 2), contrary to the equal activation by K^+ and Na^+ in A-membranes (Fig. 1). Li^+ and Cs^+ activated the two types of membranes almost equally but maximum activity was obtained at lower concentrations in B-membranes than in A-membranes. At high concentrations of Li^+ and Cs^+ (100 mM) only the A-membranes were activated.

In both types of membranes the activation by ammonium ions was intermediary to the activations by K^+ and Rb^+ (not shown), i.e., the NH_4^+ activation of B-membranes also exceeded that of A-membranes (cf. Fig. 3).

Relative activation by monovalent cations

The activities of Ca^{2+}-stimulated ATPase in the presence of optimum concentrations of both Ca^{2+} and monovalent cations (in the case of A-membranes

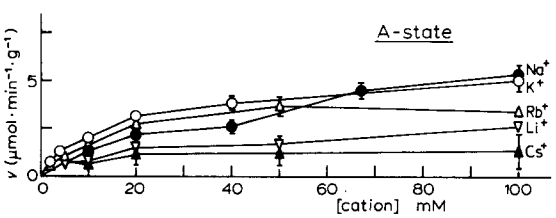

Fig. 1. Additional activation by alkali ions of Ca^{2+}-stimulated ATPase in A-membranes. ATPase medium, 3 mM Tris · ATP/4 mM $MgCl_2$/1 mM EGTA or nitrilotriacetic acid/10^{-4} M g-strophantin/160—180 μM Ca^{2+}/various concentrations of alkali chlorides/70 mM Tris · HCl/0.8—1.2 g dry membrane per l medium, pH 7.2. P_i-production measured. Additional activity (v) is the difference between the activities in presence and absence, respectively, of alkali ions. The Ca^{2+}-stimulated activity (cf. Methods) in absence of alkali ions was 7.3 ± 0.2 $\mu mol \cdot min^{-1} \cdot g^{-1}$ (S.E., 10 experiments). Mean ± S.E. of two or three experiments.

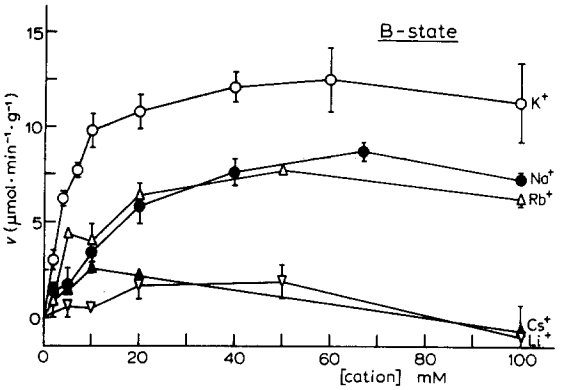

Fig. 2. Additional activation by alkali ions of Ca^{2+}-stimulated ATPase in B-membranes. ATPase assay as in Fig. 1 except for 20—30 μM Ca^{2+} during assay. The Ca^{2+}-stimulated activity in absence of alkali ions was 18.0 ± 0.4 μmol · min^{-1} · g^{-1} (S.E., 14 experiments). Mean ± S.E. of two or three experiments.

at 100 mM of monovalent cations) in relation to the activities in the absence of monovalent cations appear from Table I. The relative activations of A-membranes and B-membranes were equal except for Li^+. The relative activations of B-membranes by Na^+ and Rb^+ showed tendencies (not significant) to be lower than those of A-membranes. In accordance with this tendency the relative activation by K^+ was significantly higher ($P < 0.05$) than the activations by Na^+ and Rb^+ in B-membranes, contrary to a previous report [11], whereas in A-membranes there was no difference between K^+ and Na^+.

The stimulating effect of Li^+ on A-membranes was in agreement with Schatzmann and Rossi [2] but in contrast to other reports [3,13]. The stimulating effects of Cs^+ in both A-membranes and B-membranes were small but significant. In contrast, Bond and Green [3] and Rega et al. [12] found no significant effect of Cs^+.

Apparent affinity for monovalent cations

Lineweaver-Burk plots were made in each individual experiment. Four

TABLE I

RELATIVE ACTIVATION BY MONOVALENT CATIONS OF Ca^{2+}-STIMULATED ATPase IN A-MEMBRANES AND B-MEMBRANES

Conditions as in Figs. 1 and 2. Results of three experiments are shown as mean ± S.E., tested by Student's *t*-test.

Monovalent cation	Relative activation		Significance
	A-membranes	B-membranes	
None	100	100	
Li^+	138 ± 2	111 ± 5	$P < 0.01$
Na^+	188 ± 14	150 ± 4	$P > 0.05$
K^+	181 ± 9	174 ± 7	$P > 0.5$
Rb^+	157 ± 8	137 ± 2	$P > 0.05$
Cs^+	119 ± 6	114 ± 2	$P > 0.5$
NH_4^+	159 ± 5	149 ± 9	$P > 0.5$

TABLE II

MICHAELIS CONSTANT K_d FOR ADDITIONAL ACTIVATION BY MONOVALENT CATIONS OF Ca^{2+}-STIMULATED ATPase IN A-MEMBRANES AND B-MEMBRANES

Conditions as in Figs. 1 and 2. Results of three experiments are shown as mean ± S.E., tested by Student's *t*-test.

Monovalent cation	K_d (mM)		Significance
	A-membranes	B-membranes	
Na^+	36.7 ± 5.6	17.2 ± 2.2	$P < 0.05$
K^+	21.9 ± 3.2	4.5 ± 0.5	$P < 0.01$
Rb^+	12.2 ± 1.3	7.9 ± 1.4	$P > 0.1$
NH_4^+	39.4 ± 0.1	6.7 ± 0.5	$P < 0.001$

of these plots are shown in Fig. 3 and it appears that some of the curves are not straight lines. This makes it difficult to calculate the Michaelis constant, K_d, for the activation by monovalent cations. Consequently, these plots were only used for the determination of maximum velocity, V, whereas K_d was determined from Hill plots, i.e., $\log(v/(V-v))$ vs. log[cation] (not shown). In the Hill plots the observations were fitted by linear regression analysis, and log K_d was determined as the abscissa to $\log(v/(V-v)) = 0$.

The resulting K_d values are shown in Table II. K_d of the B-membranes was significantly lower than K_d of the A-membranes in case of activation by Na^+, K^+, and NH_4^+, indicating a higher affinity of B-membranes for these monovalent cations.

Treatment of the membranes with valinomycin (approx. 1 μmol/g dry membrane, leading to 10^{-6} M during ATPase assay) immediately before the ATPase determination did not alter the different K^+ affinities of A-membranes and B-membranes. Neither did pretreatment of the membranes with the Ca^{2+} ionophore A23187 (approx. 6 μmol/g dry membrane) alter the enzyme activities, in accordance with previous results [10].

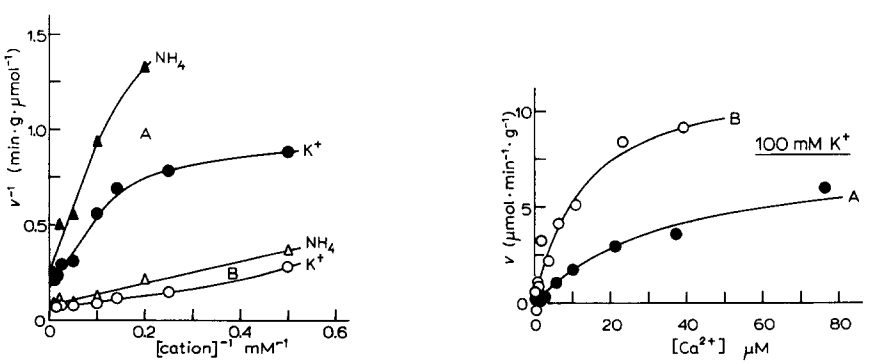

Fig. 3. Lineweaver-Burk plots of additional activity (v) in A-membranes and B-membranes vs. concentration of monovalent cations. ATPase assay as in Figs. 1 and 2 with KCl and NH_4Cl. Single experiment.

Fig. 4. Additional activation by 100 mM KCl of Ca^{2+}-stimulated ATPase in A-membranes and B-membranes vs. Ca^{2+} concentration during ATPase assay. Assay as in Fig. 1 except for various concentrations of $CaCl_2$. Single experiment.

In the B-membranes K_d of Na^+ activation was significantly higher that the K_d values derived from the activation experiments with K^+, Rb^+, and NH_4^+ (Table II). However, in the A-membranes K_d of Na^+ activation was not significantly higher than K_d of activation by K^+ and NH_4^+, but K_d of Rb^+ activation was significantly lower ($P < 0.05$) than the K_d values of the other three ions. These results demonstrate that the order of effectiveness of the monovalent cations was different in the two types of membranes.

Previously, K_d values for Na^+ and K^+ were reported to be 33 and 5.8 mM, respectively (ref. 2, cf. ref. 12), or both values were found to be about 10 mM [3]. These values should mainly be compared with the A-membranes in Table II. However, the discrepancies between the observations may in part be ascribed to differences in membrane preparations, leading to states of the enzyme which are intermediary to the A-state and the B-state (cf. ref. 10).

The curved Lineweaver-Burk plots in Fig. 3 may indicate interactions between more sites, binding monovalent cations. However, calculations of Hill coefficients, n_H, from the Hill plots showed no significant deviations from unity, i.e., no interactions could be detected, except for one case. n_H for K^+ activation of A-membranes was significantly lower than one (0.86 ± 0.02, S.E., 3 experiments), indicating negative cooperativity between two or more K^+-binding sites. The corresponding n_H of B-membranes, however, was approx. 1. By way of comparison it may be mentioned that n_H for Ca^{2+} activation of A-membranes and B-membranes were 0.8 and 1.8, respectively [10].

Interaction between Ca^{2+} and monovalent cations

It was previously shown that the stimulating effect of K^+ or Na^+ on the ATPase was only obtained in the presence of Ca^{2+} [2,3].

The dependence of K^+ activation on the Ca^{2+} concentration during the ATPase determination is illustrated in Fig. 4. The additional activation by 100 mM K^+ increased with increasing Ca^{2+} concentration, reaching half-maximum values at 35 μM Ca^{2+} in A-membranes and at 12 μM Ca^{2+} in B-membranes. The half-maximum value for the A-membranes agrees with the Michaelis constant, K_{Ca}, for Ca^{2+} activation in the absence of K^+, whereas

TABLE III

ADDITIONAL ACTIVATION BY K^+ OF Ca^{2+}-STIMULATED ATPase IN A-MEMBRANES AND B-MEMBRANES DEPENDENT ON Ca^{2+}-ACTIVATION

ATPase assay as in Fig. 1 at different Ca^{2+} concentrations. Additional K^+-activation is the difference between the activities in presence of 100 mM KCl and in absence of KCl, respectively. Ca^{2+}-activation: none, no Ca^{2+} present during assay, i.e., Mg^{2+}-dependent activity. Sub-optimum, Ca^{2+}-stimulated activity at 21 μM Ca^{2+} (A-membranes) and 1.9 μM Ca^{2+} (B-membranes), cf. Results. Optimum, Ca^{2+}-stimulated activity at 184 μM Ca^{2+} (A-membranes) and 21 μM Ca^{2+} (B-membranes). Mean ± S.E. (n observations), tested by Student's t-test.

Ca^{2+} activation	Additional K^+-activation (μmol · min^{-1} · g^{-1})		Significance
	A-membranes	B-membranes	
None	−0.6 ± 0.2 (7)	−1.1 ± 0.3 (7)	$P > 0.2$
Sub-optimum	3.9 ± 0.2 (4)	2.9 ± 0.8 (5)	$P > 0.2$
Optimum	5.4 ± 0.4 (5)	12.7 ± 1.1 (5)	$P < 0.001$

the value for the B-membranes is about ten times higher than K_{Ca} for B-membranes (cf. refs. 9 and 10).

Table III demonstrates the difference between A-membranes and B-membranes more clearly. In the absence of Ca^{2+} during the ATPase assay 100 mM K^+ inhibited the ATPase activity slightly in both A- and B-membranes. At sub-optimum Ca^{2+} concentrations, at which the ATPase activity in the absence of monovalent cations was about 75% of the activity at optimum Ca^{2+} concentrations, the A- and B-membranes were activated equally by 100 mM K^+, contrary to the K^+ activations at optimum Ca^{2+} concentrations (Table III). Consequently, in the B-membranes the relative activations at sub-optimum and optimum Ca^{2+} concentrations, being 122 ± 6 and 179 ± 3, respectively (S.E., 5 experiments), differed significantly ($P < 0.001$). In contrast, the relative activation of the A-membranes was 179 ± 11 (S.E., 4 experiments) at sub-optimum Ca^{2+} concentrations, which do not differ from the relative activation at optimum Ca^{2+} (Table I).

Similar results (not shown) were obtained with the other monovalent cations. The relative activation of A-membranes by 100 mM of cation at sub-optimum (about 25 μM) and at optimum Ca^{2+} concentrations (160—180 μM) were equal, whereas the relative activations of the B-membranes by 100 mM of Na^+, NH_4^+, or Rb^+ were significantly lower at sub-optimum Ca^{2+} concentrations (about 2 μM) than at optimum Ca^{2+} (about 25 μM).

These results suggest that the B-state of Ca^{2+}-ATPase in the presence of monovalent cations needs higher Ca^{2+} concentrations for maximum activation than in the absence of monovalent cations. The explanation might be that in the B-state the monovalent cations enhance the positive cooperativity of the Ca^{2+} sites or that positive cooperativity exists between the Ca^{2+} sites and the binding site(s) for monovalent cations. Positive cooperativity of the Ca^{2+} sites in the B-state was demonstrated previously in the absence of monovalent cations [7]. The need for Ca^{2+} in activation of the A-state, on the contrary, is not changed by the presence of monovalent cations.

Discussion

In previous investigations concerning the additional activation by monovalent cations of Ca^{2+}-stimulated ATPase in erythrocyte membranes it was discussed whether the cations activated the Ca^{2+}-ATPase itself [3], or whether the additional activity was due to the $(Na^+ + K^+)$-ATPase, this being modified by the presence of Ca^{2+} [2,12,14].

The dependence of activation by monovalent cations on the state of Ca^{2+}-ATPase, demonstrated in the present investigation, suggests an effect of the cations directly on the Ca^{2+}-ATPase. This view is shared by Richards et al. [15] and Wolf et al. [11] who recently demonstrated stimulating effects of monovalent cations on a highly purified preparation of Ca^{2+}-ATPase which obviously was in the B-state. Furthermore, the activities of $(Na^+ + K^+)$-ATPase in preparations of erythrocyte membranes, corresponding to A-membranes and B-membranes, respectively, did not differ significantly [4]. However, both the B-state of Ca^{2+}-ATPase (Tables I and II) and the potassium-site

of $(Na^+ + K^+)$-ATPase [16—18] were activated by K^+, NH_4^+, and Rb^+ and to a smaller extent by Li^+ and Cs^+.

The difference between the A-state and the B-state of Ca^{2+}-ATPase with respect to the additional activation by monovalent cations may be compared with the different patterns of Ca^{2+}-activation. The maximum activation of the B-state exceeded that of the A-state both for Ca^{2+} alone [4,5,7,8] and for Na^+, K^+, Rb^+, and NH_4^+ (Figs. 1 and 2). It has to be emphasized that the B-state was activated relatively more by K^+ than by Na^+, contrary to the A-state which was activated equally by K^+ and Na^+ (Table I). The half-maximum activation of the B-state was achieved at lower cation concentrations (K_d) than that of the A-state both with Ca^{2+} alone [4,7,19] and with Na^+, K^+, and NH_4^+ (Table II). These results suggest that the binding of the protein activator to the enzyme, i.e., the conversion from A-state to B-state (cf. ref. 9), causes profound changes of the enzyme molecule.

It has been suggested by Scharff [7] that the A-state and the B-state of the Ca^{2+}-ATPase correspond to a resting and an active state of the Ca^{2+} pump, respectively (cf. ref. 8). Schatzmann and Rossi [2] concluded that the monovalent cations do not participate in the activation of the Ca^{2+} pump. However, the different responses to monovalent cations of the A-state and the B-state of Ca^{2+}-ATPase may indicate that K^+ or Na^+, or both, contribute to the regulation of the Ca^{2+} pump, e.g., by enhancing the regulating role of Ca^{2+} (cf. refs. 7 and 9). This view is supported by the dependence on Ca^{2+} concentration of the additional activation by monovalent cations (cf. Table III), which emphasizes the importance of positive cooperativity for the activation of the B-state.

These phenomena may be compared with the stimulating effects of potassium and other monovalent cations on the Ca^{2+} pump from sarcoplasmic reticulum, described by Duggan [20].

However, further experiments are necessary to show whether the monovalent cations exert their effects on erythrocyte Ca^{2+}-ATPase via intracellular or extracellular sites. In this connection it is noticed that the observed K_d-values for K^+ and Na^+ in B-membranes (Table II) coincide with the plasma concentration of K^+ and the inside concentration of Na^+ in human erythrocytes, respectively (cf. ref. 21). This may be incidental, but on the other hand, if a regulatory function of external K^+ and internal Na^+ exists, this function would be served best if the actual concentration and the K_d did coincide.

Note added in proof (Received June 26th, 1978)

The contribution of Na^+ and K^+ to the regulation of the Ca^{2+} pump, which is suggested above, was recently demonstrated [22] in inside-out vesicles from human erythrocytes.

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