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$(Ca^{2+} + Mg^{2+})$-ACTIVATED MEMBRANE ATPases IN HUMAN RED CELLS AND THEIR POSSIBLE RELATIONS TO CATION TRANSPORT

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SUMMARY

1. Hemoglobin-free membranes were prepared from human red cells by extraction with low-ionic-strength solution and EDTA, made leaky by freezing and thawing and examined with respect to ATPase activity in the presence of Ca^{2+}.

2. In the presence of a few mM Mg^{2+} an ATPase activity is elicited by Ca^{2+} which shows a complex kinetic behaviour. At low Ca^{2+} concentration (up to 10 μM) the results can be fitted by a Michaelis–Menten type curve (high-affinity part). At elevated Ca^{2+} concentration (50–500 μM) the activity exceeds the values on this curve (low-affinity part). This might indicate the existence of two distinct enzymes, one enzyme with two categories of Ca^{2+} acceptor sites or one Ca^{2+} acceptor changing its performance under the influence of Ca^{2+}. A Ca^{2+} concentration above 1 mM is inhibitory.

3. At a fixed Ca^{2+} concentration (0.2 mM) the addition of either Na^+ or K^+ instead of choline further stimulates the ATPase activity. The affinity for K^+ exceeds the affinity for Na^+ as judged by the ATPase activity.

4. None of the ATPases described is inhibited by ouabain (0.1 mg/ml).

5. Evidence is presented, showing that the (Na^+ or $K^+ + Ca^{2+} + Mg^{2+}$)-activated ATPases does not pertain to the Ca^{2+} transport in resealed cells.

6. Comparison of the $(Ca^{2+} + Mg^{2+})$-activated ATPase with Ca^{2+} transport in resealed cells indicates that the low-affinity part or the whole activity, but not the high affinity part alone might be involved in Ca^{2+} transport.

7. The hypothesis is advanced that the ($Na^+ + Ca^{2+} + Mg^{2+}$)-activated ATPase and the ($K^+ + Ca^{2+} + Mg^{2+}$)-activated ATPase are the result of an uncoupling action of Ca^{2+} on the ($Na^+ + K^+ + Mg^{2+}$)-activated ATPase responsible for $Na^+ - K^+$ transport.

INTRODUCTION

It is well established that in the presence of Mg^{2+} isolated red cell membranes display an ATPase activity which is stimulated by Ca^{2+} in the concentration range of 0.5–500 μM, higher Ca^{2+} concentrations being inhibitory[1–3]. The enzymic site

Abbreviation: EGTA, ethylenedioxy-bis-(ethyliminodi(acetic acid)).

obviously faces the cell interior since ATP and Ca^{2+} do not activate the system when present in the medium surrounding intact cells or resealed cells[4]. The available data relating ATPase activity with Ca^{2+} concentration in isolated membranes suggest that the Ca^{2+}-sensitive system is complex. The present work demonstrates that at least three different enzymatic functions are involved. The experiments were undertaken in the hope that they might help to decide whether the whole or only part of the (Ca^{2+} + Mg^{2+})-activated ATPase activity is required for maintaining active Ca^{2+} transport[1-6] from the red cell.

METHODS

Hemoglobin-free red cell membranes were prepared essentially according to GARRAHAN *et al.*[7]. Blood cells were obtained in a small volume of citrated plasma from the Blood Bank Laboratory of the Swiss Red Cross 1 day after collection of the blood. The red cells were washed 4 times in saline at room temperature, the white cells being discarded in the process. The cells were hemolysed at room temperature in a 50-fold or 100-fold volume of 30 mM Tris–HCl solution of pH 7.1 with 1 mM Tris-EDTA added. The ghosts were separated from the bulk fluid with a Szent-Györgyi–Blum continuous-flow device in a refrigerated Sorvall centrifuge at 15000 rev./min and a flow rate of about 1.5 l/h. They were subsequently washed 3–4 times with 15 mM Tris–HCl solution (pH 7.1) under refrigeration and finally frozen once in a small volume of 15 mM Tris–HCl solution (pH 7.1) inside a polyethylene tube in solid CO_2–ethanol, thawed slowly and stored at 2°. The ghost suspension contained on the average 3 mg of protein per ml. In two of these preparations the hemoglobin content was measured and found to be 3 % and 4 % of the total protein, respectively. Electron micrographs showed that the ghosts were not broken up into fragments forming small vesicles but largely preserved the original size of the cell and that a layer of fuzzy material several times the width of the unit membrane was attached to the internal surface (Fig. 1). The ghosts undoubtedly remained right-side-out during the preparation[8].

ATPase activity was assayed by measuring P_i liberated during 60 or 90 min at 37°. Samples of 2.5 ml containing 0.2 ml of ghost suspension and Tris, choline, Na^+, K^+, Cl^-, Ca^{2+}, Mg^{2+}, ethylenedioxy-bis-(ethyliminodi(acetic acid)) (EGTA) or Ca^{2+}–EGTA buffer as required were incubated in glass test tubes. ATP concentration usually was 2 mM ($Na_2ATP \cdot 3H_2O$, Boehringer). When Na^+-free media were necessary 150 μmoles of Na_2ATP were passed over an Amberlite IR-120 column of a capacity of 1500 μequiv in the acid form or the Mg^{2+} form. All ATP solutions were neutralized with Tris base before use. Solutions were made up in glass-distilled water stored in polyethylene containers.

0.5 mM Ca^{2+}–EGTA buffers were used at Ca^{2+} concentrations below 10 μM. For the calculation of the Ca^{2+} concentration in Ca^{2+}–EGTA buffers the complex formation constant of 10^{11} (determined in 0.1 M KCl) was corrected for 37° to $10^{10.65}$ (G. SCHWARZENBACH, personal communication). The experiments were carried out at pH 7.0 (measured at 37°) resulting in a pK' (log of apparent formation constant) of 6.33, and in order to avoid corrections for ionic strength the sum of choline chloride and Tris chloride concentration was set at 100 mM. At pH 7.0 the buffering capacity towards acid of Tris (pK_a 8.24) is low. Only about 3.5 mM out of 60 mM present in

the medium are in the unprotonated form. However, at the maximal hydrolysis rate observed in the present experiments (Fig. 8) 0.45 mM of P_i was produced. In an experiment with a phosphate liberation of 1.5 μmoles per mg protein per h 40 mM Tris was sufficient to keep the pH steady. With pK' 6.33 the dissociation curve was plotted on a log-probit chart, a small correction for 4 mM Mg^{2+} was applied and the ratio Ca^{2+}/EGTA for any intended Ca^{2+} concentration was read on the linear graph. For the calculation of pK' and the Mg^{2+} correction the customary simplifying approximations were applied.

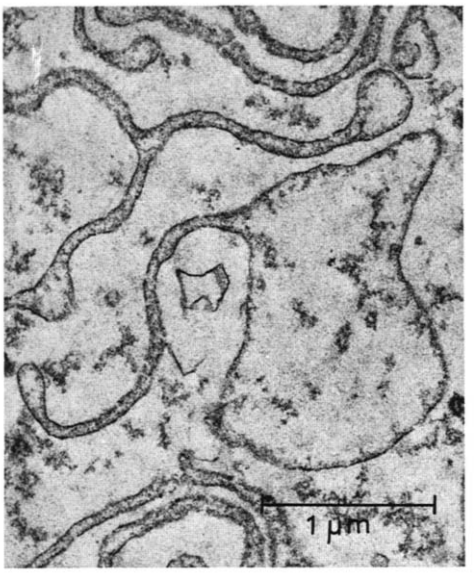


Fig. 1. Electronmicrograph of isolated membranes, prepared as described in text. Fixation: S-collidine buffer (pH 7.2), glutaraldehyde; embedding: Epon; staining: uranyl acetate, lead citrate.

Ca^{2+} and Mg^{2+} stock solutions were titrated with Na_2EDTA using 2-hydroxy-1-(2-hydroxy-4-sulfo-1-naphthylazo)-3-naphthoic acid and eriochrome black as indicators. Ca^{2+} concentrations in media and cells were measured by absorption flame photometry on an EEL instrument using an acetylene–air flame and adding 50 mM lanthanum chloride to samples and standards. All solutions were deproteinized by adding an equal volume of 10% trichloroacetic acid. Na^+ and K^+ were measured without deproteinization in suitable dilutions by emission flame photometry (EEL instrument). P_i was determined according to the method of BERENBLUM AND CHAIN[9] (which, unlike the method of Fiske and SubbaRow, is not disturbed by choline). Protein was measured according to LOWRY *et al.*[10] with the Folin–Ciocalteu reagent (Merck). Hemoglobin attached to the membranes was estimated by the pyridine hemochromogen method (see ref. 11). Ca^{2+} transport was assayed in resealed cells as reported earlier[4].

Chemicals were analytical grade of Merck or Fluka (Buchs). $Na_2ATP \cdot 3H_2O$ was obtained from Boehringer and Na_2EDTA, H_4EDTA and the complexometric indicators were from Siegfried (Zofingen). Ouabain was the DAB product of Merck.

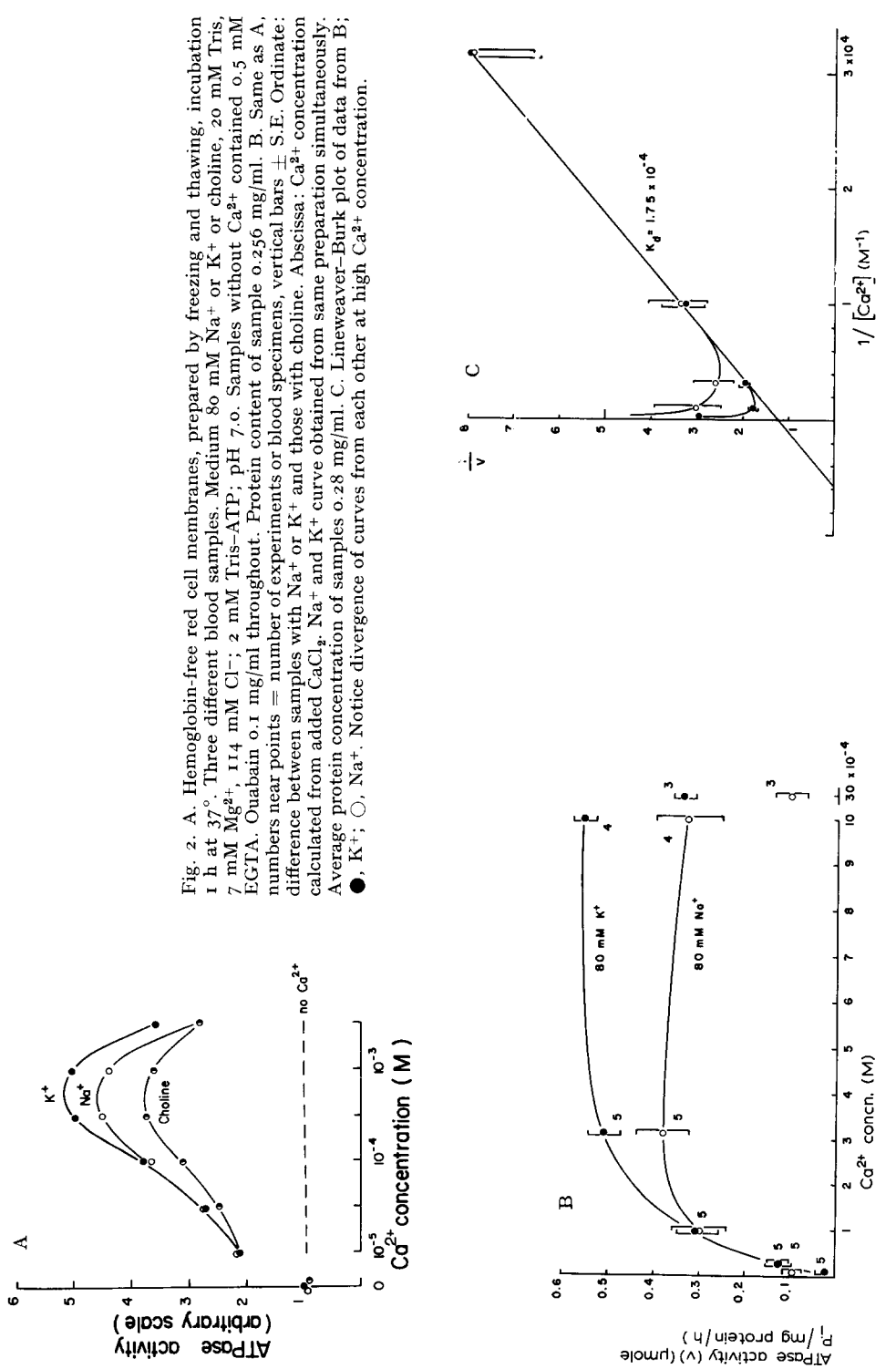


Fig. 2. A. Hemoglobin-free red cell membranes, prepared by freezing and thawing, incubation 1 h at 37°. Three different blood samples. Medium 80 mM Na^+ or K^+ or choline, 20 mM Tris, 7 mM Mg^{2+}, 114 mM Cl^-; 2 mM Tris–ATP; pH 7.0. Samples without Ca^{2+} contained 0.5 mM EGTA. Ouabain 0.1 mg/ml throughout. Protein content of sample 0.256 mg/ml. B. Same as A, numbers near points = number of experiments or blood specimens, vertical bars ± S.E. Ordinate: difference between samples with Na^+ or K^+ and those with choline. Abscissa: Ca^{2+} concentration calculated from added $CaCl_2$. Na^+ and K^+ curve obtained from same preparation simultaneously. Average protein concentration of samples 0.28 mg/ml. C. Lineweaver–Burk plot of data from B; ●, K^+; ○, Na^+. Notice divergence of curves from each other at high Ca^{2+} concentration.

RESULTS

Effect of alkali cations

Fig. 2 shows that, if Ca^{2+} is present in concentrations between 10 μM and 1 mM, either Na^+ or K^+ increases the ATPase activity considerably. These experiments were carried out in the presence of 0.1 mg/ml ouabain, quenching the ($Na^+ + K^+$)-stimulated ATPase completely; considering also Figs. 7B and 7C makes it clear that an activity appears due to the combination of Ca^{2+} with either Na^+ or K^+ which is not affected by ouabain. For comparison the well-known inhibition of the ($Na^+ + K^+$)-activated ATPase by Ca^{2+} (ref. 1) is shown in Fig. 7A. Up to 0.1 mM Ca^{2+} the curves for Na^+ and K^+ of Fig. 2B coincide within the experimental error. Beyond this Ca^{2+}

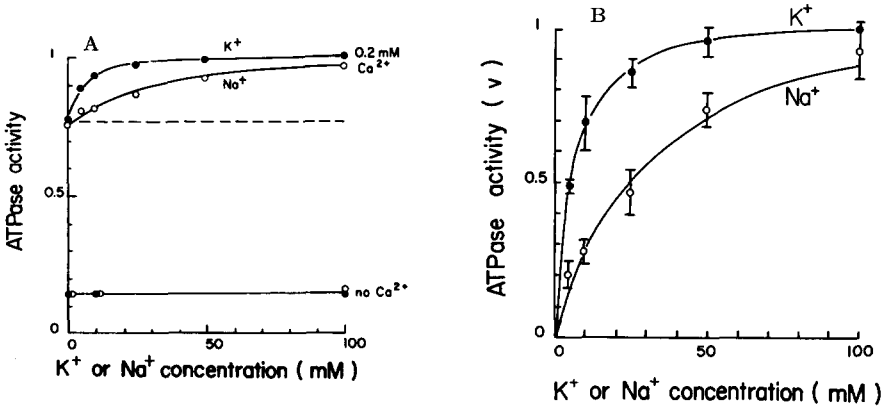


Fig. 3. A. Membranes prepared as in Fig. 2. Four experiments with four different blood specimens. Average protein content of sample suspension 0.228 mg/ml. Incubation 1 h at 37°. Medium (Na^+ or K^+ + choline) = 100 mM (Na^+ or K^+ replaced by choline); Tris, 60 mM; Mg^{2+}, 5 mM; Ca^{2+}, 0.7 mM; EGTA, 0.5 mM (no Ca^{2+} = 0.5 mM EGTA); Cl^-, 171.5 mM; MgATP, 2 mM; pH 7.3; 0.1 mg/ml ouabain throughout. Ordinate: total ATPase activity; 1 corresponds to 2.38 μmoles P_i per mg protein per h liberated. B. Same as A, five experiments with five different blood specimens. Na^+ and K^+ curve obtained from one specimen simultaneously. Vertical bars $\pm$ S.E. Average protein concentration of sample 0.3 mg/ml. Ordinate: ATPase activity due to the presence of 0.2 mM Ca^{2+} + Na^+ or K^+. 1 corresponds to 0.541 μmole P_i per mg protein per h liberated. Drawn curves are lines of best fit of the form $v = v_{max} \cdot [Na]/(K_d + [Na^+])$ or $v = v_{max} \cdot [K^+]/(K_d + [K^+])$, with $v_{max,\,Na^+} = 1.17$, $K_{Na^+} = 32.7$ mM and $v_{max,\,K^+} = 1.07$; $K_{K^+} = 5.8$ mM.

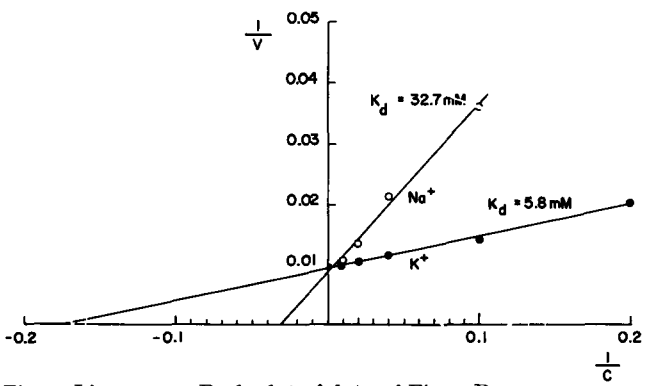


Fig. 4. Lineweaver–Burk plot of data of Fig. 3 B.

concentration, however, 80 mM K^+ elicits a higher activity than 80 mM Na^+. As can be seen from Figs. 3 and 4 this divergence is due to different kinetics of the alkali cation activation.

The activation by Na^+ or K^+ of the ATPase in the presence of Ca^{2+} does not depend on the presence of ouabain. For K^+ this is demonstrated by Fig. 7 and for Na^+ it was found to be equally true. In an experiment with 0.1 mM Ca^{2+} (in a medium of 60 mM Tris–HCl (pH 7.32), 5 mM $MgCl_2$, 2 mM MgATP) the increment over the total activity in 100 mM choline was 20.25 % when 100 mM NaCl replaced choline chloride in the absence and 23.1 % in the presence of 0.1 mg/ml ouabain.

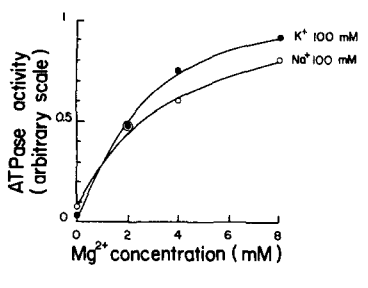
From the Lineweaver–Burk plot (Fig. 4) of the data of Fig. 3 obtained at $[Ca^{2+}] = 0.2$ mM it may be seen that the apparent dissociation constants for Na^+ and K^+ differ by a factor of 5.6 (see also Table I), whereas the maximal rate is similar for both alkali cations. We do not know how the apparent dissociation constants for Na^+ and K^+ depend on Ca^{2+} concentration, because only a 0.2-mM Ca^{2+} concentration was tested. From Figs. 2B and 2C it cannot be concluded with certainty that Ca^{2+}

TABLE I

APPARENT DISSOCIATION CONSTANTS

Type of activation	K_d *for* Ca^{2+} (μM)	K_d *for* Na^+ (mM)	K_d *for* K^+ (mM)
$Ca^{2+} + Mg^{2+}$ (4 mM) (high-affinity component)	4.08		
$Ca^{2} + Mg^{2+}$ (4 mM) (low-affinity component)	Approx. 100		
$Ca^{2+} + Mg^{2+} + Na^+$ (Na^+-dependent component)	175*	33**	
$Ca^{2+} + Mg^{2+} + K^+$ (K^+-dependent component)	175*		5.8**

* Na^+ or K^+, 80 mM; Ca^{2+}, below 0.1 mM; Mg^{2+}, 7 mM.
** Ca^{2+}, 0.2 mM; Mg^{2+}, 5 mM; ATP, 2 mM in all experiments.



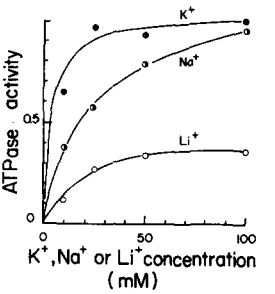


Fig. 5. Single experiment. Conditions as in Fig. 2 but with 2.86 mM Tris–ATP instead of MgATP Ordinate: ATPase activity due to presence of 0.2 mM Ca^{2+} and 100 mM Na^+ or K^+. (Na^+ + K^+-free samples: choline, 100 mM; Na^+, 0.02 mM; K^+, 0.008 mM; Ca^{2+}, 0.2 mM).

Fig. 6. Single experiment. Conditions as in Fig. 3, plot as in Fig. 3B. Ordinate: Na^+, K^+- or Li^+-dependent ATPase activity in the presence of 0.2 mM Ca^{2+}. Notice lower v_{max} in the case of Li^+

concentrations exceeding 0.2 mM would cause the difference in the dissociation constants for Na^+ and K^+ to be larger yet, because it is uncertain whether or not the maximal activity obtained by increasing Na^+ and K^+ concentrations changes at elevated Ca^{2+} concentrations. The different behaviour of Na^+ and K^+ rules out the possibility that choline, used to replace the alkali cations, reduces the activity non-specifically. Fig. 3A in addition shows that Na^+ and K^+ have no effect whatsoever in the absence of Ca^{2+}, indicating that the Mg^{2+}-activated ATPase is neither depressed by choline nor stimulated by Na^+ or K^+. The alkali-cation-stimulated fraction of the Ca^{2+}-activated system also requires Mg^{2+} as demonstrated by the experiment reproduced in Fig. 5. Fig. 6 shows a single experiment comparing Li^+ with Na^+ and K^+. Li^+ seems to differ from Na^+ and K^+ mainly by being less potent an activator at the enzymic site. However, we do not have enough experiments to settle this point conclusively.

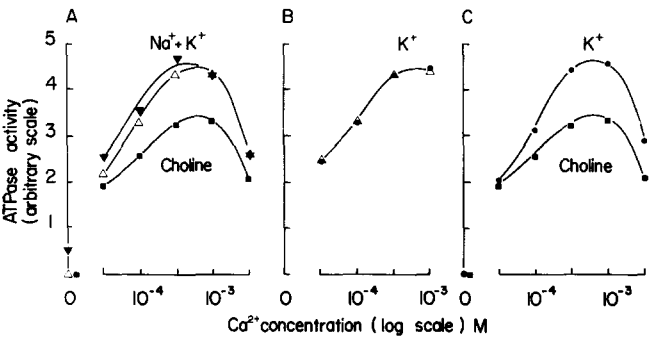


Fig. 7. Membranes prepared as in Fig. 2. Medium: choline, 100 mM, or K^+, 100 mM, or Na^+, 90 mM + K, 10 mM; Mg^{2+}, 7 mM; Tris, 60 mM; Cl^-, 174 mM; pH 7.1, Tris·ATP 2 mM; o Ca^{2+} = 0.5 mM EGTA. Incubation 1 h at 37°. Ordinate: total ATPase activity *minus* activity in samples without Ca^{2+} and alkali cations. 1 corresponds to 0.418 μmole P_i per mg protein per h liberated. Abscissa: Ca^{2+} concentration added, no Ca^{2+} buffering. Δ, with 0.1 mg/ml ouabain; closed symbols: no ouabain. Single experiment, same membrane preparation in A, B and C, but B was done 1 day after A and C. In B results with and without ouabain are nearly identical.

Ca^{2+}-activated ATPases in the absence of Na^+ and K^+

Fig. 3A shows that a large fraction of the Ca^{2+}-activated ATPase persists in the absence of alkali cations. Fig. 8A shows that in a choline–Tris medium of 0.1 ionic strength and pH 7.0 the rate of ATP hydrolysis of this fraction as a function of the Ca^{2+} concentration does not comply with a simple curve of the form $v = v_{max} \cdot [Ca^{2+}]/(K_d + [Ca^{2+}])$ which would reflect dissociation kinetics of one single receptor–Ca^{2+} complex relevant for the enzyme activity. The procedure adopted to resolve the curve of Fig. 8 into its components was as follows: a straight line was fitted to the Lineweaver–Burk plot (Fig. 9) of the results at low Ca^{2+} concentrations. From this the drawn curve of Figs. 8A and 8B is derived (high-affinity system). Computing the deviation of the experimental points from this first curve at higher Ca^{2+} concentrations gave the results shown in Fig. 8C (low-affinity system). This purely formal procedure does not imply any particular chemical mechanism. It is advisable not to interpret the result in terms of two distinct enzymes. It may as well mean that Ca^{2+} binds to and acts on two different categories of sites on the same protein or, in fact, that a single site behaves differently at different Ca^{2+} concentrations.

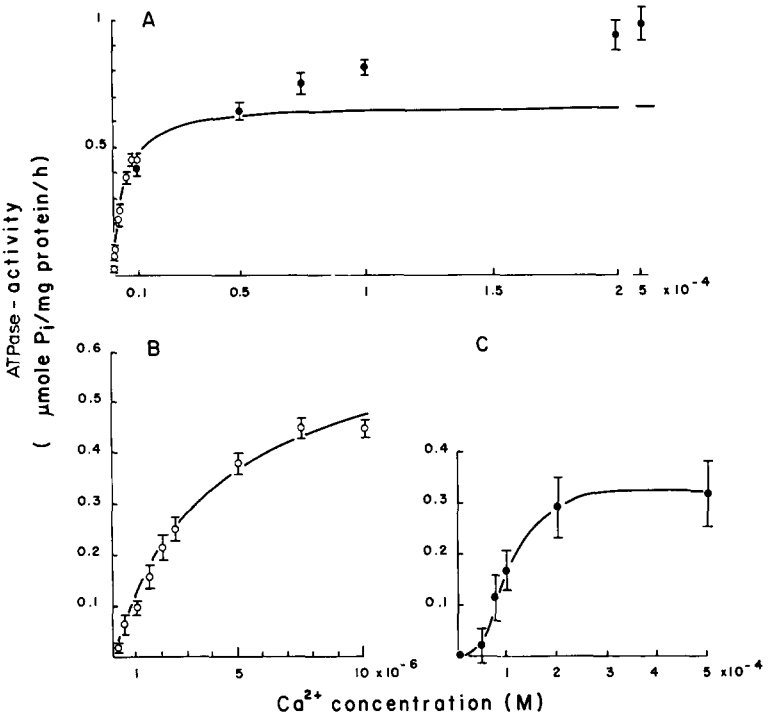


Fig. 8. Membranes prepared as in Fig. 2. Four experiments with different blood specimens. Medium: 60 mM Tris, 40 mM choline, 2 mM Mg^{2+}, 104 mM Cl^-; pH 7.0; 2 mM MgATP. Incubation 90 min at 37°. Mean protein concentration of samples 0.30 mg/ml. Abscissa: ○: $[Ca^{2+}]$ fixed with 0.5 mM Ca–EGTA buffer; ●: $[Ca^{2+}]$ calculated from added $CaCl_2$ without buffer. Vertical bars: ± S.E. A. Ordinate: total ATPase activity *minus* activity in samples without Ca^+ (0.5 mM EGTA). B. Initial part of curve from A on expanded abscissa. Drawn curve in A and B: line of best fit through the experimental points of B of the form $v = v_{max} \cdot [Ca^{2+}]/(K_d + [Ca^{2+}])$, with $v_{max} = 0.666$ µmole/mg/h and $K_d = 4.08$ µM. C. Difference between experimental points and the curve of A. The S-shape of the curve has no significance (see text).

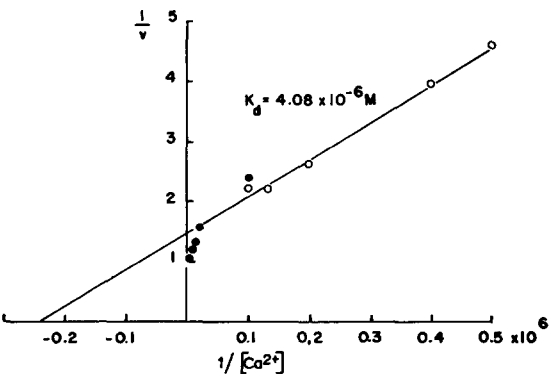


Fig. 9. Lineweaver–Burk plot of Fig. 8A. ○, Ca^{2+} concentration buffered; ● ,Ca^{2+} concentration unbuffered.

The sigmoid shape of the difference curve is not statistically significant (the deviation from zero of the axis-intercept of a straight line through three point in the steep part of the curve was examined). Even if it were statistically significant it might have been caused by some error in determining the low-affinity curve or by a slight depression of the activity at the lowest unbuffered Ca^{2+} concentrations. This depression does in fact exist as seen by the double determination of the activity at 10 μM Ca^{2+} both with and without buffer (Fig. 8A). It might be explained by some complex formation between Ca^{2+} and ATP or by binding of Ca^{2+} to non-enzymatic sites on the membranes, becoming noticeable at very low Ca^{2+} concentrations.

The apparent dissociation constant of the Ca^{2+}–receptor complex is 4 μM for the high-affinity system and approx. 0.1 mM for the low-affinity system (see Table I).

Both these activities require Mg^{2+}. A very small fraction, indeed, seems to be elicited by Ca^{2+} alone. It amounted to 6.5 % of the ($Ca^{2+} + Mg^{2+}$)-stimulated fraction

TABLE II

TOTAL ATPASE ACTIVITY OF ISOLATED MEMBRANES STIMULATED BY Ca^{2+}

Single experiment. Medium: (mM) Tris–HCl, 60; $MgCl_2$, 2.5; MgATP 2; $CaCl_2$, 0.5; KCl as indicated; pH 7.34. Protein content of samples 0.25 mg/ml. Temperature 37°. Incubation time 1 h. The figures are the difference between samples with Ca^{2+} and samples with 0.5 mM Tris–EGTA.

KCl (mM)	*μmoles P_i per mg protein per h*
50	1.45
100	1.61
200	1.60

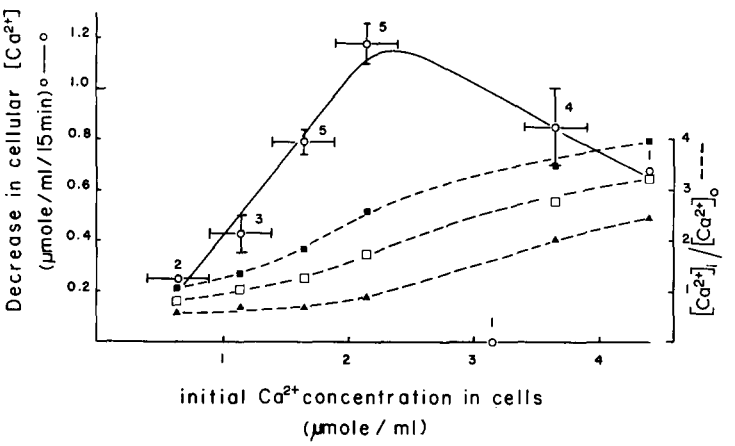


Fig. 10. Summary of five experiments. Cells hemolysed in 5 mM Tris, 2 mM Mg^{2+}, 1 mM Na_2ATP at room temperature. Resealing with KCl. Resealed cells washed immediately in the cold, with 130 mM Na^+, 5 mM K^+, 2 mM Mg^{2+}, 1 mM Ca^{2+} as chlorides; pH 7.3–7.4. Incubation in this medium for 15 min at 25–28°. Left ordinate: ○, rate of Ca^{2+} transport. Abscissa: Ca^{2+} concentration in the cells (per ml cells) at zero time. Vertical bars: ± S.E., horizontal bars: range of Ca^{2+} concentration. Number of experiments near points. Difference between peak rate and rates beyond has a $P = 0.05$. Dashed lines and right ordinate: gradient of Ca^{2+} between cells and medium, ■ at zero time, ▲ at 15 min, □ average.

at 0.2 mM Ca^{2+} in one experiment. Our method did not allow to determine its characteristics. It is conceivable that it reflects some contamination with Mg^{2+}, possibly displaced by Ca^{2+} from the membranes. An ATPase requiring only Ca^{2+} has recently been solubilized by ROSENTHAL *et al.*[12] from red cell membranes; however, its affinity for Ca^{2+} is very low.

The overall activity stimulated by Ca^{2+} is not reduced by adding KCl to the medium in order to increase the ionic strength, as can be seen from Table II. The addition of KCl to an originally K^+-free solution brings forth the alkali-cation-dependent fraction, but going from 50 to 200 mM KCl (or from 0.09 to 0.24 total ionic strength) does not reduce the overall activity in a fashion that comes anywhere near the effect seen with muscle actomyosin ATPase[13].

In Fig. 10 are summarized experiments with Ca- and MgATP-loaded resealed cells. It can be seen that the rate of Ca^{2+}-outward transport increases with increasing intracellular Ca^{2+} concentration up to about 2 mM and decreases beyond that concentration, in spite of the continuously waxing Ca^{2+} gradient from cells to medium.

In experiments not shown here, cells were loaded and incubated in the absence of Na^+ or both Na^+ and K^+, choline replacing the alkali cations. The rate of Ca^{2+} release from the cells was not reduced to any large extent when the Na^+ and K^+ concentration in the suspension was below 0.1 mM as compared with the release in a medium with 135 mM Na^+ and 6.5 mM K^+.

DISCUSSION

The present experiments indicate that the Ca^{2+}-stimulated ATPase activity which is associated with the red cell membrane and was not removed by treatment with low-ionic-strength solutions and EDTA, does not comply with simple kinetics for one binding site. The curve relating activity to Ca^{2+} concentration was resolved rather arbitrarily into two components in order to describe it. This procedure ought not to be interpreted as demonstrating the existence of two distinct enzymes. Preliminary experiments suggested that the curve might show a plateau in the middle portion. It might result from several binding sites for Ca^{2+} on the same enzyme or from a modification of one Ca^{2+} binding site by Ca^{2+} at elevated concentrations (for a discussion see ref. 27). In the presence of Mg^{2+}, Ca^{2+} seems to activate two sites differing in affinity for Ca^{2+} by a factor of about 25 (see Table I). If referred to mg of membrane protein the high-affinity fraction amounts to about 2/3 and the low-affinity fraction to about 1/3 of the total, at maximal activation. If in addition to $Mg^{2+} + Ca^{2+}$, Na^+ or K^+ is present an additional activity appears, which at maximal stimulation amounts to about 1/2 of the sum of the two others. Interestingly, this alkali-cation-stimulated system has higher affinity towards K^+ than towards Na^+ at the Ca^{2+} concentration tested (see Figs. 3 and 4) and activation as a function of Ca^{2+} concentration follows a different curve in the case of Na^+ stimulation as compared with the case of K^+ stimulation (see Fig. 2B). The lower affinity for Na^+ is possibly due to a competitive effect of Ca^{2+}; Figs. 2B and 2C suggest that at low Ca^{2+} concentrations the Na^+-activated and the K^+-activated system respond in the same way to Ca^{2+} but that at increasing Ca^{2+} concentrations the Na^+ system falls below the simple dissociation curve at lower Ca^{2+} concentrations than the K^+ system. Very high

Ca^{2+} concentrations are inhibitory in both systems and the possibility is quite real that the problem is complicated by a Ca^{2+}–Mg^{2+} antagonism[1].

The (Mg^{2+} + Ca^{2+} + (Na^+ or K^+))-activated fraction appears in the range of Ca^{2+} concentrations which inhibit the (Mg^{2+} + Na^+ + K^+)-activated membrane ATPase and reaches a maximum at a Ca^{2+} concentration which completely abolishes the (Mg^{2+} + Na^+ + K^+)-activated ATPase.

None of the Ca^{2+}-activated ATPases is affected by ouabain. This is demonstrated by Fig. 7 comparing the effect of 0.1 g/ml ouabain on the (Mg^{2+} + Na^+ + K^+)-activated ATPase with the effect on the (Mg^{2+} + Ca^{2+} + K^+)-activated ATPase. Whereas in the absence of Ca^{2+} the high concentration of ouabain applied reduces the activity to the same extent as the omission of K^+ and Na^+, the ouabain inhibitable fraction declines with increasing Ca^{2+} concentration, first relatively to the remainder activated by one alkali cation alone and later absolutely.

The ATPases described are of interest in view of their possible connection with cation transport or other function of the intact cell. It has been proposed that red cell membranes might contain a contractile protein[14–16] concerned with the changing distensibility of the membrane[17] or conceivably even with the maintenance of the disc shape of the cell. The large fractions of the Ca^{2+}-activated ATPases here described do not have any similarity to actomyosin ATPase: they require both Ca^{2+} and Mg^{2+} and increasing the ionic strength by adding KCl does not reduce the activity. It seems improbable that the procedure used in preparing the ghosts might have removed an actomyosin-like protein. The possibility remains, of course, that the very small fraction activated by Ca^{2+} alone reflects the presence of such a contractile protein.

The existence of an ATP-dependent, Mg^{2+}-requiring Ca^{2+} extrusion mechanism in red cells[4] must manifest itself by a (Ca^{2+} + Mg^{2+})-activated ATPase in the membrane. It is unlikely that the alkali-cation-stimulated fraction is connected to the Ca^{2+} transport because even an incomplete removal of the alkali cations should drastically slow the Ca^{2+} transport if it were dependent on this ATPase activity. This is clearly not the case. Experiments with Ca- and MgATP-loaded "resealed" cells showed that reducing Na^+ or both Na^+ and K^+ inside and outside the cells below 0.1 mM did not noticeably reduce the rate of Ca^{2+} transport.

Therefore the low- and high-affinity fractions of the activity requiring only Mg^{2+} and Ca^{2+} must be considered. From Fig. 10 it can be seen that the transport rate, much as the (Mg^{2+} + Ca^{2+})-activated ATPase activity, passes through a maximum with increasing Ca^{2+} concentration. It is noteworthy that in the transport experiments of Fig. 10 this reversal occurs in spite of a steady increase in the Ca^{2+} gradient favouring outward Ca^{2+} movement as shown by the dashed lines. However, the rising part of the curve for transport matches the activation curve for the low-affinity system of the ATPase neither in position nor in slope and the same holds true for the comparison of transport with the high affinity system in an even more obvious way. But one must bear in mind that conditions in the transport experiment deviate markedly from those in the enzyme assay: pH and temperature were different and, what is probably more important, in the resealed cells used in the transport studies soluble proteins including hemoglobin were present inside the cells in comparatively high concentrations. These proteins might lower the intracellular ionic Ca^{2+} concentration by binding Ca^{2+}. In addition more Ca^{2+} than expected might be

complexed by ATP in the transport experiments because it is known that higher ATP concentrations are found in the cells after resealing than in the hemolysing medium[4] and Mg^{2+} might also partly be bound to proteins and therefore not be available to occupy ATP. We therefore feel that it is at least possible that the low-affinity system or the total Ca^{2+}-activated ATPase accounts for Ca^{2+} transport, whereas it seems improbable that the high-affinity system alone can be claimed for Ca^{2+} transport. A more explicit statement seems unwarranted before we know whether we deal with two distinct enzymes, one enzyme having two different sites for Ca^{2+} binding or one enzyme with a single site undergoing a modification at elevated Ca^{2+} concentrations.

The activity stimulated by one alkali cation alone appears upon adding Ca^{2+} in concentrations which reduce or abolish the activity requiring $Na^+ + K^+$ and being sensitive towards ouabain. EPSTEIN AND WHITTAM[18] have presented convincing evidence that the inhibition is due to competition between CaATP and MgATP. The $(Na^+ + K^+)$-stimulated system is involved, with a high degree of probability, in the active translocation of Na^+ and K^+. There is little doubt that in this system Na^+ is necessary for the transfer of the terminal phosphate group of ATP to a membrane constituent (phosphorylated intermediate) and that K^+ is instrumental in the hydrolysis of this intermediate.

The present finding of a difference in affinity for Na^+ and K^+ of the system similar to that found in the Na^+–K^+ transport system is compatible with the assumption that we deal with a modified form of this transport system. However, it does not help to prove its correctness, first because the similarity might be accidental (Fig. 2B leaving open the possibility that the difference in affinity for Na^+ and K^+ increases with increasing Ca^{2+} concentration), and secondly because it is compatible with other assumptions as well.

It is tempting to speculate that Ca^{2+} (or CaATP) on the internal surface of the membrane disrupts the joint action of Na^+ and K^+. The mechanism of this disturbance can be conceived of in three different ways: (1) Na^+ or K^+ might continue to function in the normal fashion even if present separately and Ca^{2+} might replace either missing alkali cation to complete the system. Na^+ or K^+ might or might not be transported in the process. (2) Ca^{2+} might abolish the alkali cation specificity completely such that one alkali cation activates both the phosphorylation and the hydrolysis step. (3) Ca^{2+} might uncouple the $Na^+ + K^+$ system in the sense that the Na^+-activated and the K^+-activated moieties may each operate by themselves as a simple ATPase, requiring Na^+ or K^+, respectively, not transferring P_i to a second site and not transporting the alkali cation across a boundary.

POUCHAN and co-workers[19–21] have recently described a Ca^{2+}-dependent *p*-nitrophenylphosphatase activity in the red cell membrane, which requires ATP and K^+ as activators but is insensitive towards ouabain. This system was not activated by Na^+ alone but the K^+-elicited activity was enhanced by Na^+. The latter effect could be abolished by adding 10 mM hydroxylamine. The authors' interpretation is that the K^+-activated nitrophenylphosphatase reflects a modification by Ca^{2+} of the hydrolysing moiety of the Na^+–K^+ transport ATPase, accepting the artificial substrate instead of the phosphorylated intermediate, but yet requiring ATP in order to be active, and some of the intermediate in order to be specific for K^+. Some competition between ATP and nitrophenylphosphate was detected. Since *p*-nitrophenol

was measured, possible ATP hydrolysis may have passed unnoticed and the competition observed might indicate that the modified enzyme accepts and also hydrolyses ATP. If the present observations are interpreted accordingly, we must dismiss possibility (1), because this interpretation postulates that ATP enters the system at the hydrolytic site directly. Simultaneously we also rule out possibility (2) because we know from the experiments by POUCHAN and co-workers that Na^+ has no effect at the hydrolytic site.

Most of the present experiments with Ca^{2+} + (Na^+ or K^+) were performed in the presence of a high concentration of ouabain. However, it must be stressed that ouabain is in no way necessary for the appearance of the (Ca^{2+} + (Na^+ or K^+))-elicited ATPase activity. For K^+ this becomes evident from Fig. 7, and different experiments (see RESULTS) clearly showed it for Na^+.

Ouabain seems to bind to the phosphorylated and unphosphorylated enzyme in the conformational state reached after Na^+ ($+Mg^{2+}$) but before K^+ has acted in the normal cycle of the (Na^+ + K^+)-stimulated ATPase[22,23]. The form occupied by ouabain cannot be rephosphorylated by ATP[22], whereas it can be phosphorylated by P_i (refs. 22 and 24) and dephosphorylated in the absence of K^+ (ref. 22). Recent work[25] has shown that Ca^{2+} is able to replace Na^+ in promoting the process leading to a conformation binding ouabain. Under our experimental conditions, therefore, the part of the machinery undergoing cyclic conformational changes most probably was blocked by ouabain. Consequently, the observed (Ca^{2+} + Na^+)- and (Ca^{2+} + K^+)-activated ATPase does not require the participation of this part. If the hypothesis outlined under heading (3) above is the most adequate interpretation of the Ca^{2+} effect, the present result agrees with the idea that in the normal state phosphate from ATP is bound to a first phosphate acceptor, passes onto the ouabain sensitive second site and leaves the system by way of a third acceptor.

In all the experiments EGTA was present in the same concentration in controls and Ca^{2+}-containing samples. The possibility cannot be ruled out that EGTA might have had a permissive action for the Ca^{2+} effect, for instance by removing heavy metals from the membrane material[26].

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