

Effects of Calcium Ions on the Adenosine-Triphosphatase Activities of Erythrocyte Membrane¹⁾

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Calcium ions were found to stimulate the Na⁺-K⁺-sensitive ATPase as well as Mg²⁺-ATPase (Na⁺-K⁺-insensitive ATPase) activities of human erythrocyte membrane in a concentration between approximately 0.04—0.4 mM, and to inhibit them in a concentration higher than 4 mM. In the presence of Ca ions, the Na⁺-K⁺-ATPase activity does not correspond to the ouabain-sensitive ATPase activity anymore which is apparently inhibited with increasing concentration of Ca ions. The effects of Ca²⁺ on the Mg²⁺-ATPase seems to be dependent on the ratio of Ca²⁺/Mg²⁺ concentrations.

Erythrocyte stroma previously in contact with CaCl₂ and then washed, showed increased Mg²⁺-ATPase activity when assayed in the absence of added Ca²⁺ in the incubation mixture, with the maximal level of the activity almost equivalent to the maximal Ca²⁺-Mg²⁺-ATPase activity. This would mean that the Ca ions absorbed on the outer surface of the erythrocyte membrane is well capable of stimulating the ATPase activity.

Normal values of these ATPase activities of human erythrocyte stroma were established. The ATPase of porcine erythrocyte membrane were similarly stimulated by Ca ions, whereas those of bovine erythrocyte were only slightly stimulated by this divalent cation.

Existence of at least two kinds of ATPase (adenosine-triphosphatase; EC 3.6.1.3 ATP phosphohydrolase) systems in erythrocyte membrane (stroma) has been well established, namely, the so-called Na⁺-K⁺-sensitive ATPase³⁾ which is believed to be concerned with the active transport of sodium and potassium ions, and the Mg²⁺-ATPase or Na⁺-K⁺-insensitive ATPase⁴⁾ of which physiological significance is still unknown. Attempts to isolate the individual enzymes⁵⁾ are in progress.

It has been reported that calcium ions could stimulate Mg²⁺-ATPase activity of erythrocyte membrane^{3b,4b)} and of brain microsomes,⁶⁾ while they inhibited Na⁺-K⁺-sensitive ATPase in disrupted erythrocyte membrane or in certain subcellular fractions of tissue cells.⁷⁾ However, in the course of the experiments with "intact" (undisrupted) stroma preparation obtained from human erythrocytes by hypotonic hemolysis,⁸⁾ it was disclosed by the present

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authors that calcium ions not only stimulate the Mg^{2+} -ATPase activity, but also stimulate the Na^+ - K^+ -sensitive part of the ATPase activity to a considerable extent, and furthermore that in the presence of calcium ions, ouabain inhibition of the latter activity seems to be greatly reduced and consequently the Na^+ - K^+ -sensitive ATPase activity (the difference between Mg^{2+} - Na^+ - K^+ -ATPase and Mg^{2+} -ATPase) is no more equivalent to the "ouabain-sensitive" ATPase activity (the difference of the Mg^{2+} - Na^+ - K^+ -activities in the presence and absence of ouabain). These results are reported in the present paper.

In the following pages, Na^+ - K^+ -insensitive Mg^{2+} -ATPase and Na^+ - K^+ -sensitive ATPase will be simply called "Mg-ATPase" and "Na-K-ATPase", respectively.

Experimental

Preparation of Erythrocyte Stroma—Freshly-drawn blood, with heparin (0.05 mg of Na-salt equivalent to ca 5 heparin units per ml blood) or citrate (4.5 mg trisodium salt per ml blood) added as anticoagulant, was centrifuged at $900 \times g$ for 15 min. After removing the plasma and buffy coat, the red blood cells precipitated were washed three times with physiological saline (0.85% NaCl) and resuspended in the saline. Washed erythrocytes thus prepared were hemolyzed in 20 volumes of hypotonic veronal buffer (30 imOsM, pH 7.4) to obtain hemoglobin-free stroma, according to the method of Dodge, Mitchell and Hanahan.⁸⁾

Assay of ATPase Activity—The assay was performed by a slight modification of the method described by Kielley⁹⁾ and by Nakao, *et al.*¹⁰⁾ The assay system for Mg-ATPase consists of 200 mM histidine-imidazole buffer of pH 7.7, 5 mM $MgCl_2$, 2 mM ATP and stroma suspension (usually 0.3 ml of about 2 mg protein per ml suspension) in a total volume of 1.0 ml. For the assay of Mg- Na^+ - K^+ -ATPase, 140 mM NaCl and 14 mM KCl were added, and when necessary, ouabain in a concentration of 0.1 mM was employed as the inhibitor. $CaCl_2$ was added to this reaction mixture to give desired concentration.

The reaction mixture was incubated at 37° for 30 min. One ml of 5% perchloric acid was added and after allowing to stand at 0° for 5 min, the mixture was centrifuged. With this supernatant, amount of inorganic phosphorus (Pi) liberated from ATP was determined according to the method of Fiske and SubbaRow.¹¹⁾

Determination of Protein—The amount of protein in erythrocyte stroma was determined according to the method of Lowry, *et al.*¹²⁾

Result

Various ATPase Activities of Human Erythrocyte Stroma in the Presence of Different Combinations of Cations and the Effects of Ouabain on Their Activities

In addition to Mg ions which are essential for the manifestation of the ATPase activity,

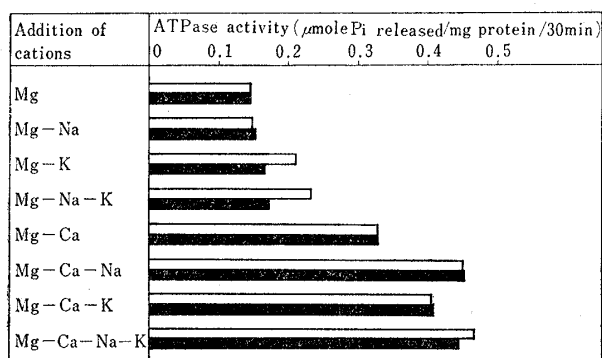


Fig. 1. Effects of Ouabain on Various ATPase Activities of Human Erythrocyte Stroma

Mg 5 mM, Na 140 mM, K 14 mM, Ca 0.4 mM, ATP 2 mM
 □: Ouabain 0 ■: Ouabain 0.1 mM

Na, K and Ca ions individually or in combination were added to the reaction mixture in an optimal concentration previously determined, and the ATPase activities were assayed. The results are graphically indicated in Fig. 1.

As already well recognized, the Mg-ATPase is stimulated by an optimal concentration of K ions and more so by an optimal ratio of Na and K ions, and furthermore it is stimulated even more remarkably by Ca ions in a concentration of 0.4 mM. Fig. 1 clearly indicates that Ca ions exert stimulative effect also on Na-

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K-ATPase activity of the stroma preparation, contrary to the results with fragmented erythrocyte stroma.⁷⁾ Namely, the Mg-Ca-ATPase activity was found to be increased markedly by the addition of Na ions and even more by the addition of both Na and K ions.

With reference to the effects of ouabain on these ATPase activities, an interesting fact was disclosed from these experiments. In the presence of Mg ions alone as divalent cations, ouabain almost completely inhibited the K-sensitive and Na-K-sensitive portions of the ATPase activity, in agreement with the well-known facts.³⁾ In the presence of both Mg and Ca ions, however, not only the Na-sensitive activity is unaffected by ouabain but also the Na-K-sensitive activity is only partially inhibited (approximately 25% inhibition).

TABLE I. Effects of Varying Concentration of Calcium Ion on the Na-K-sensitive and Ouabain-sensitive ATPase Activities^{a)} of Human Erythrocyte Stroma

CaCl ₂ conc. (mM)	(1) Mg-ATPase	(2) Mg-Na-K-ATPase	(3) Mg-Na-K-ATPase with ouabain (10 ⁻⁴ M)	(2)-(1) Na-K-sensitive ATPase	(2)-(3) Ouabain-sensitive ATPase
0	0.117	0.155	0.117	0.038	0.038
0.4	0.180	0.248	0.238	0.068	0.010
4	0.042	0.095	0.090	0.053	0.005
40	0.012	0.012	0.012	0	0

a) The enzyme activities expressed as μ mole Pi liberated per mg stromal protein per 30 minutes under the condition described in the text.

The fact that the ouabain-sensitive ATPase activity does not correspond to the Na-K-sensitive ATPase activity if Ca ions are present in addition to Mg ions, is more clearly explained by the data cited in Table I. At different concentrations of CaCl₂ in the reaction mixture (0–40 mM), Mg-ATPase and Mg-Na-K-ATPase in the presence or absence of 0.1 mM ouabain were assayed respectively, and the Na-K-sensitive and ouabain-sensitive ATPase activities were calculated from these experimental data. It is noted that with increasing concentration of Ca ions, the ouabain-sensitive ATPase activity is reduced, while the Na-K-sensitive activity attained a level higher than the control value in a Ca ion concentration of 0.4–4 mM, though the latter activity is abolished in the presence of 40 mM Ca.

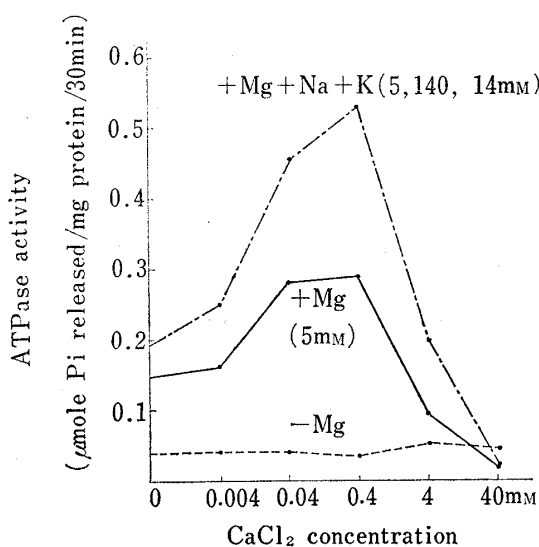


Fig. 2. Effects of Calcium Ion Concentration on ATPase Activities of Human Erythrocyte Stroma

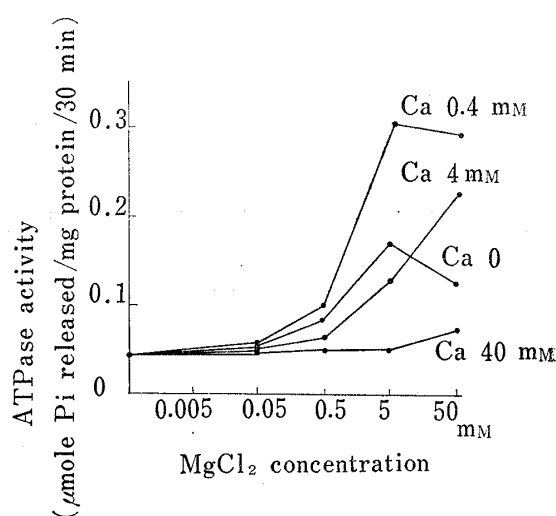


Fig. 3. Combined Effects of Magnesium and Calcium Ion Concentrations on the Mg-ATPase Activity of Human Erythrocyte Stroma

Effects of Ca Ion Concentrations on the ATPase Activities of Human Erythrocyte Stroma

The activities of Mg-ATPase and of Mg-Na-K-ATPase in the presence of different concentrations of Ca ions are graphically shown in Fig. 2. The dotted line indicates the ATP-splitting activity in the absence of Mg ions, which is independent of Ca ion concentration and is almost negligible. It is to be noted that the Mg-ATPase activity is stimulated by Ca ions in the concentration range between approximately 0.004 to 0.4 mM, but is rather inhibited by higher concentration of Ca ions (4–40 mM). Similar results were obtained also in the case of Mg-Na-K-ATPase activity.

It was further demonstrated that such a stimulative effect of Ca ions is not independent of the concentration of the co-existing Mg ions, as shown in Fig. 3. At the Mg concentration of 5 mM, Ca in 4 mM concentration gives inhibitory effect on the Mg-ATPase activity, whereas at the Mg concentration of 50 mM, 4 mM of Ca is definitely stimulative. Although the precise value of the optimal ratio of the Mg/Ca ion concentration for the maximal stimulation of the stromal Mg-ATPase can not be obtained from the present experiment, the value is supposed to be in an order of 10–100.

Effect of Membrane-Bound Ca Ions on the Mg-ATPase Activity of Human Erythrocyte Stroma

The foregoing results are all concerned with the effects of Ca ions present in the reaction mixture. The fact that the Ca ions actually bound to erythrocyte membrane are equally

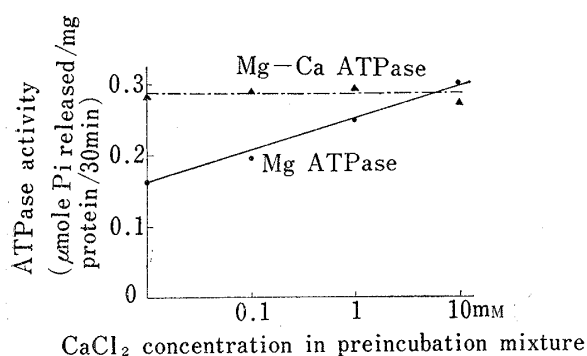


Fig. 4. Effect of Preincubation of Human Erythrocyte Stroma with CaCl₂ Solution on Their ATPase Activities

effective in the stimulating action on the ATPase activity, was confirmed by the following experiment. The stroma were kept in contact (preincubated) with 0.1 to 10 mM of CaCl₂ solution at 4° overnight, and then washed with hypotonic buffer-saline twice. The Mg-ATPase and Mg-Ca-ATPase activities determined on the stroma thus treated and also on the untreated stroma (Ca concentration=0) are indicated in Fig. 4. It is evident from the figure that with increasing concentration of Ca ions in the preincubation mixture, the Mg-ATPase is increased gradually up to the level approximately equivalent to the Mg-Ca-ATPase activity which is not altered by such a treatment

of the stroma.

Normal Values of the Stroma ATPase Activities in Human, Porcine and Bovine Erythrocytes and Their Calcium Activation

As preliminary to such an investigation, possible effect of anticoagulant added to the blood specimen on the stroma ATPase was first checked. From each healthy individual, blood was drawn with heparin as anticoagulant and then with citrate again. From such heparinized or citrated blood, stroma were prepared and their ATPase activities were measured. As shown in Table II, it was observed on the blood specimen from three different individuals that the anticoagulants have no detectable influence on the Mg-ATPase and Na-K-ATPase. Furthermore, Ca ion stimulation of these ATPase activities were similarly unaffected by the kinds of anticoagulant employed.

In order to know the normal range of the ATPase activities in human erythrocyte stroma, the assay was made on the stroma prepared from 24 blood specimen of healthy individuals. Percentage stimulation of the Mg-ATPase and of Na-K-ATPase activity was also determined. All these results are summarized in Table III. It was found that the normal levels of the Mg-ATPase and of Na-K-sensitive ATPase activity, expressed as mean value \pm standard de-

TABLE II. Comparison of ATPase Activities^{a)} between the Human Erythrocyte Stroma Preparations Obtained from Heparinized and Citrated Blood

Blood donor	Anticoagulant added	Mg-ATPase	Ca ²⁺ -Activation of Mg-ATPase (%)	Na-K-sensitive ATPase	Ca ²⁺ -Activation of Na-K-sensitive ATPase (%)
A	heparin	0.174	103	0.075	195
	citrate	0.153	125	0.102	175
B	heparin	0.164	93	0.099	210
	citrate	0.155	113	0.117	190
C	heparin	0.217	91	0.136	122
	citrate	0.213	73	0.094	142

a) The enzyme activity expressed as μ mole Pi liberated per mg stromal protein per 30 minutes under the condition described in the text.

TABLE III. Normal Values of Various ATPase Activities^{a)} in Human, Porcine and Bovine Erythrocyte Stroma and Their Calcium Ion Activation

Species	Mg-ATPase	Mg-Ca-ATPase	Ca-sensitive ATPase	Ca-activation of Mg-ATPase	Mg-Na-K-ATPase	Mg-Ca-Na-K-ATPase	Na-K-ATPase	Ca-Na-K-sensitive ATPase	Ca-activation of Na-K-sensitive ATPase
	(1)	(2)	(2)-(1)	$\frac{(2)-(1)}{(1)} \times 100$	(3)	(4)	(3)-(1)	(4)-(2)	$\frac{(4)-(2)}{(3)-(1)} \times 100$
Human mean ^{a)} \pm S.D.	0.197 \pm 0.028	0.348 \pm 0.039	0.151 \pm 0.030	80 \pm 19	0.307 \pm 0.031	0.550 \pm 0.076	0.110 \pm 0.022	0.188 \pm 0.039	184 \pm 41
Porcine ^{b)}	0.169	0.258	0.089	53%	0.257	0.477	0.088	0.219	249%
Bovine ^{b)}	0.043	0.050	0.007	16%	0.051	0.068	0.008	0.018	23%

a) The enzyme activities expressed as μ mole Pi liberated per mg stromal protein under the condition cited in the text; mean value \pm standard deviation on 24 blood specimen.

b) Average of the duplicate experiments on three different individuals.

viation in terms of the amount of inorganic phosphate liberated in μ mole per mg stroma protein under the experimental condition, are 0.197 ± 0.028 and 0.110 ± 0.022 , respectively. The Ca ion stimulation of the former is $80 \pm 19\%$ and that of the latter is $184 \pm 41\%$.

Similarly, the ATPase activities were determined on the erythrocyte stroma prepared from three healthy pigs and also from three healthy cows, and the results on the duplicate experiments on each sample are cited in Table III. As for the porcine erythrocyte stroma, both the Mg-ATPase and Na-K-ATPase activities are in a level slightly lower than the corresponding activity in human erythrocyte stroma. The enzyme activity was also stimulated by Ca ions to a remarkable extent.

In the case of bovine cells, however, not only the Na-K-ATPase is almost negligibly low, but also the Mg-ATPase activity is very low, being about one-fifth of the activity in human stroma. Furthermore, in the case of bovine enzymes, Ca ions showed only very slight stimulation.

Discussion

From the above-reported experimental results, it now becomes evident that calcium ions exert stimulative effects both on Mg-ATPase (Na-K-insensitive ATPase) and Na-K-sensitive ATPase of human erythrocyte membrane in relatively low concentration while the same ions

inhibit these activities in higher concentrations. It was also disclosed that in the presence of Ca ions, ouabain inhibition of the Na-K-sensitive ATPase activity is greatly reduced.

According to Henn and Sperelakis,¹³⁾ Na-K-sensitive ATPase in cultured heart cells was stimulated and protected from ouabain inhibition by Sr or Ba ions added to the reaction mixture. They considered this effect to be due to competition of these cations with ouabain for the same binding-site on the enzyme molecules. The reverting effect of Ca ions on the ouabain inhibition of the erythrocyte Na-K-ATPase discovered by the present work would be due to the action of Ca ions similar to Sr and Ba.

The stimulative effect of Ca ions on the Na-K-ATPase activity of erythrocyte stroma, demonstrated in the present study, is not in agreement with the results reported by the previous investigators.⁷⁾ The discrepancy would be explained partly by the fact that the erythrocyte stroma employed in the present investigation are the "intact" (unfragmented) stroma with the membrane integrity, whereas those used in the previous studies were the fragmented or disrupted stroma preparations. There seems to be a possibility that with the "intact" stroma, Ca ions may be bound to the outer surface of the membrane and to bring about the conformational changes in the membrane components so as to cause the increased activity of the ATPase, while such a regulatory effect of the membrane-bound Ca ions is lacking in disrupted stroma or isolated enzyme preparation. Or alternatively, with the "intact" stroma Ca ions would not be able to penetrate so easily into the inside of the stroma as Mg ions and to act as a competitive inhibitor in a form of Ca-ATP^{7c)} against Mg-ATP.

Another possible cause of the disagreement will be the difference in the definition of the "Na-K-sensitive ATPase." The previous authors meant this to be the ouabain-sensitive portion of the Mg-Na-K-ATPase activity. However, as already stated, such ouabain-sensitive ATPase activity is very much depressed in the presence of calcium ions and in such a case it does not correspond to the difference between the Mg-ATPase and Mg-Na-K-ATPase activities which is considered to be Na-K-sensitive ATPase activity in the present report.

The normal ranges of these ATPase activities in human erythrocyte stroma were determined in the present work and they will be the basis of the comparison of the enzyme activities in normal and abnormal erythrocytes, which will be reported elsewhere.¹⁴⁾

It is interesting to note that the bovine erythrocyte membrane contains relatively low activity of Mg-ATPase and almost negligible activity of Na-K-sensitive ATPase, and also that these enzymes are only weakly stimulated by the addition of Ca ions, in comparison with those from human or porcine erythrocyte stroma. In the Na⁺-rich erythrocytes¹⁵⁾ such as bovine cells which do not need any Na-pump activity, in contrast to the K⁺-rich human or porcine red blood cells,¹⁵⁾ it is natural that no Na-K-sensitive ATPase is present. The appreciable activity of Mg-ATPase found in such erythrocyte membrane would support the idea that this Mg-ATPase itself might play an important role in the maintenance of the membrane morphology and functions. The reason why the ATPases in bovine erythrocytes are only weakly stimulated by Ca ions, remains to be solved by further studies.

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