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RELATIONSHIP OF Na^+, K^+ AND Mg-ATP BINDING SITES FOR THE HUMAN ERYTHROCYTE STROMAL ($Na^+ + K^+$)-DEPENDENT ATPASE

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SUMMARY

Treatment of human erythrocyte stromata with the endopeptidase trypsin (EC 3.4.4.4) indicates that Na^+ and Mg-ATP binding associated with the ($Na^+ + K^+$)-dependent ATPase (EC 3.6.1.3) are at independent sites in the membrane. Similar treatment with α-chymotrypsin (EC 3.4.4.5) indicates that K^+ and Mg-ATP bind at separate sites. It also appears unlikely that Na^+ or K^+ bind to the phosphorylated intermediate believed to be part of cation-dependent ATPase. The results further indicate, but do not prove, that Na^+ and K^+ also bind at separate sites in the membrane.

INTRODUCTION

Erythrocyte stromata are known to contain a ($Na^+ + K^+$)-dependent ATPase[1] (ATP phosphohydrolase, EC 3.6.1.3) which has been associated with the active transport of these ions. It is not known whether the Na^+ and K^+ binding sites are different or just a single revolving site in the membrane even though Na^+ and K^+ must be inside and outside the cell, respectively, for both the cation-dependent ATPase and the transport system to function in the human erythrocyte[2,3]. The relationship of the binding site of Mg-ATP to those of the cations is not clear. It has recently been shown that microsomes from rat brain can bind ^{22}Na and that the process is ATP-dependent[4,5], but other work has shown that other ions are bound at least as well as Na^+ and that the effect is unrelated to the activity of the cation-stimulated ATPase[6].

The present work was undertaken with the endopeptidases trypsin (EC 3.4.4.4) and α-chymotrypsin (EC 3.4.4.5) with the expectation that treatment of stromata with these proteolytic enzymes would affect the affinity of the ($Na^+ + K^+$)-dependent ATPase for Mg-ATP, Na^+ and K^+ and provide some clue to the relationship of the binding sites. The results indicate that Na^+ and Mg-ATP bind at independent sites in the membrane. It also appears unlikely that Na^+ or K^+ bind to the phosphorylated intermediate believed to be associated with this system. The results further indicate that

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K^+ and Mg-ATP bind at independent sites in the membrane. However, it is still impossible to state definitively that the Na^+ and K^+ binding sites are separate even though the results indicate that this may be the case.

MATERIALS AND METHODS

Outdated human blood was obtained from the blood bank of Middlesex General Hospital, New Brunswick, N.J. The cells were separated from the plasma by centrifugation at 3000 × *g* for 5 min at 2°. The plasma and buffy coat were removed by aspiration, and the cells were washed twice in 0.16 M Tris buffer (pH 7.0) at 2°. The cells were then hemolyzed in 10 vol. of hemolyzing medium containing 1 mM EDTA and 9 mM Tris buffer (pH 7.7) at 2°. The membrane material was separated by centrifugation at 20 000 × *g* for 10–15 min at 2°. The stromata were washed in the above hemolyzing medium until essentially all the hemoglobin was removed and appeared white in color. The membrane material was rapidly frozen and thawed in a solid CO_2–acetone bath to insure complete fragmentation and was stored in the dark at 4° to reduce the loss of activity.

Disodium ATP was obtained from Sigma Chemical Company, St. Louis, Mo. ATP solutions were passed through columns of Dowex 50W-8X in the H-form to remove Na^+ and trace amounts of Ca^{2+}. The solutions were immediately adjusted with 45 mM Tris buffer (pH 7.4).

Trypsin, 2 × crystalline, salt-free, lot No. 9680, was obtained from Nutritional Biochemical. α-Chymotrypsin, 3 × crystalline, salt-free, lot No. 6018-19, was obtained from Worthington Biochemical Corporation. These enzymes were prepared immediately before use in 45 mM Tris buffer (pH 7.4) to minimize autocatalytic digestion. Crystalline soybean trypsin inhibitor and pancreatic trypsin inhibitor were obtained from Worthington Biochemical Corporation. These solutions were prepared in 45 mM Tris buffer (pH 7.4) always at twice the concentration necessary for complete inhibition. All other chemicals were of reagent quality.

Protein concentration of the membrane preparation was determined according to the method of LOWRY *et al.*[7].

ATPase activity was assayed by the determination of P_i released from ATP according to the method of FISKE AND SUBBAROW[8]. Controls for the non-enzymic hydrolysis of ATP were included in all experiments.

RESULTS

The data presented refer only to the (Na^+ + K^+)-dependent ATPase. In all cases the hydrolysis of ATP by the cation-insensitive ATPase in the presence of the appropriate proteolytic enzyme and concentration of Na^+ or K^+ alone has been subtracted from the hydrolysis in the presence of both Na^+ and K^+. No contaminating ion was added to the system by the addition of either protease or of either protease inhibitor since there was no change in either the cation-insensitive or cation-sensitive ATPase activity from that of the controls when, prior to its addition to the reaction mixture, either protease was boiled for 1 h or inhibited by its appropriate inhibitor.

Apparent K_m values for the binding of Mg-ATP by the (Na^+ + K^+)-dependent ATPase are essentially identical for the trypsin-treated and untreated stromata (Fig. 1).

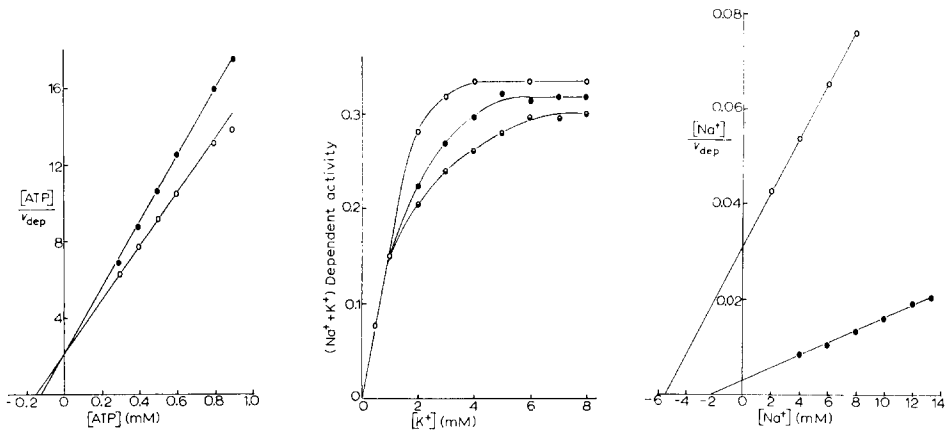

Fig. 1. Effect of $5 \cdot 10^{-3}$ mg trypsin/ml incubation medium on the apparent K_m for Mg-ATP for the ($Na^+ + K^+$)-dependent ATPase. The reaction medium contained: 0.5 mg stroma protein, 45 mM Tris buffer (pH 7.4), 120 mM Na^+ + 20 mM K^+, various concentrations of ATP and Mg^{2+} always in a ratio of 1:1. For the cation-insensitive ATPase the system was the same except for the omission of 20 mM K^+. The results plotted are the difference between these two activities. The final volume was 3 ml at 37°. ○—○, untreated; ●—●, trypsin-treated.

Fig. 2. Effect of various amount of trypsin/ml incubation medium on the apparent K_m value for K^+ measured in the presence of 60 mM Na^+. Proteolytic action of trypsin was stopped after 15 min with twice the amount of soybean trypsin inhibitor necessary for complete inhibition. The reaction medium contained: 0.5 mg stroma protein, 45 mM Tris buffer (pH 7.4), 1.5 mM ATP and 1.5 mM Mg^{2+}, 60 mM Na^+, various concentrations of K^+ indicated. For the cation-insensitive ATPase, the system was the same except for the omission of the K^+. The results plotted are the difference between these two activities. The final volume was 3 ml at 37°. ○—○, untreated; ●—●, $5 \cdot 10^{-3}$ mg trypsin; ◒—◒, $1 \cdot 10^{-2}$ mg trypsin.

Fig. 3. Effect of $5 \cdot 10^{-3}$ mg trypsin/ml incubation medium on the apparent K_m for Na^+ measured in the presence of 10 mM K^+. Stromata were treated for 15 min with $5 \cdot 10^{-3}$ mg trypsin, and its proteolytic action was stopped with twice the amount of soybean inhibitor necessary for complete inhibition. The reaction medium contained: 0.5 mg stroma protein, 45 mM Tris buffer (pH 7.4), 1.5 mM ATP and 1.5 mM Mg^{2+}, various concentrations of Na^+ indicated. For the cation-insensitive ATPase the system was the same except for the omission of Na^+. The results plotted are the difference between these two activities. The final volume was 3 ml at 37°. ●—●, untreated; ○—○, trypsin-treated.

To the degree that K_m values describe enzyme–substrate affinities, the action of trypsin under the conditions of this experiment is not considered to affect the binding site of Mg-ATP.

Since either Na^+ or K^+ can activate this system in the presence of a constant amount of the other cation when saturating concentrations of Mg-ATP are present, the variable ion can be treated as a substrate in the conventional Michaelis–Menten kinetics. A plot for K^+ illustrating this feature is shown in Fig. 2. The apparent K_m for K^+ is in all cases 1.1 mM, a result in excellent accord with the findings of DUNHAM AND GLYNN[9]. Since the inhibition with K^+ is non-competitive, the K^+ binding site is not affected by the trypsin treatment. When the action of Na^+ is analyzed in a similar way, except using a modified Lineweaver–Burk plot for clearer illustration, a mixed inhibition and a significant change in K_m from 2.3 to 5.4 mM is obtained (Fig. 3). Therefore, it seems probable that in this system tryptic action is on Na^+ binding and that the observed inhibition is to be attributed to this action.

TABLE I

EFFECT OF α-CHYMOTRYPSIN ON K^+ AND Mg-ATP. APPARENT K_m VALUES

Experimental conditions for determination of Mg-ATP apparent K_m values same as Fig. 1 except that the amounts of α-chymotrypsin/ml incubation medium indicated were used. Experimental conditions for determination of K^+ apparent K_m values same as Fig. 2 except for the use of α-chymotrypsin.

	K_m values (mM)		
	Control	*$5 \cdot 10^{-3}$ mg α-chymotrypsin/ml incubation medium*	*$1 \cdot 10^{-2}$ mg α-chymotrypsin/ml incubation medium*
K^+	1.1	1.7	2.1
Mg-ATP	0.15	0.11	0.13

The effect of α-chymotrypsin on this system is shown in Table I. The apparent K_m values for Mg-ATP and K^+ were obtained from plots similar to those presented for the action of trypsin. As is evident α-chymotrypsin has no effect on the apparent K_m values for Mg-ATP but does cause a significant change in the K_m value for K^+ from 1.1 mM to 2.1 mM. This indicates that these sites are independent of each other.

DISCUSSION

Since trypsin treatment does affect the binding of Na^+ but does not affect the Mg-ATP binding, it seems likely that these represent two different sites in the membrane. It also appears that the K^+ and Mg-ATP binding sites are independent since α-chymotrypsin affected K^+ binding but not the Mg-ATP binding.

It is reasonable to assume that the phosphorylated intermediate, believed to be part of this system, is associated with the binding site for Mg-ATP; and since trypsin or α-chymotrypsin did not affect the Mg-ATP site, it seems reasonable that the phosphorylated intermediate is also not being affected by either protease. It should also be noted that neither Na^+ nor K^+ could be bound to the phosphorylated intermediate of the cation-stimulated ATPase because Na^+ and K^+ binding were affected by trypsin and α-chymotrypsin, respectively, while it appears that the phosphorylated intermediate was not affected by either protease.

In regard to the Na^+ and K^+ binding sites, it appears that these ions may bind at separate sites since trypsin affected Na^+ binding but not K^+ binding. However, it is quite probable that the same site could have two configurations: one sensitive to trypsin when Na^+ is to be bound and one insensitive to trypsin when K^+ is to be bound. Therefore, it is still impossible to state with certainty that these ions bind at independent places in the membrane even though the above data and the stoichiometry of the transport system, three molecules of Na^+ extruded and two molecules of K^+ accumulated per ATP utilized[10], indicate that this may be the case.

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REFERENCES

1 R. L. POST, C. R. MERRITT, C. R. KINSOLVING AND C. D. ALBRIGHT, *J. Biol. Chem.*, 235 (1960) 1796.
2 R. WHITTAM, *Biochem. J.*, 84 (1962) 110.
3 R. WHITTAM AND M. E. AGER, *Biochem. J.*, 93 (1964) 337.
4 J. JARNEFELT, *Biochem. Biophys. Res. Commun.*, 6 (1961) 285.
5 L. V. VON STEDINGK AND J. JARNEFELT, *Acta Physiol. Scand.*, 57 (1963) 328.
6 K. AHMED AND J. D. JUDAH, *Biochim. Biophys. Acta*, 112 (1966) 58.
7 O. H. LOWRY, N. J. ROSENBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
8 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
9 E. T. DUNHAM AND I. M. GLYNN, *J. Physiol.*, 156 (1961) 274.
10 A. K. SEN AND R. L. POST, *J. Biol. Chem.*, 239 (1964) 345.