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The Effects of Various Experimental Conditions on $Na^{+}-K^{+}$ ATPase and Ca^{++} ATPase Activities of Red Blood Cell Membrane Fragments

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INTRODUCTION

It has been shown that the red cell membrane has an active mechanism for transporting Ca^{++} out of red blood cells (Lee et al, 1969; Olson et al. 1969; Schatzman, 1966; Schatzman et al. 1969). A Ca^{++}-activated ATPase, in the presence of Mg^{++} (Ca^{++} ATPase), appears to provide the energy for the Ca^{++} extrusion process (Schatzman et al, 1969; Vincenzi et al, 1967). On the other hand it is well established that a Na^{+}-K^{+}-activated ATPase (Na^{+}-K^{+} ATPase) is an integral part of the red cell membrane and provides the energy for outward extrusion of Na^{+} (Post et al. 1960; Skou, 1965). In view of the apparent localization of these two ATPases for transport of Na^{+} and Ca^{++} in the cell membrane, it would be of interest to study the relationship between these ATPases.

In the present investigation, the effects of various experimental conditions on Na^{+}-K^{+} ATPase, on the one hand, and the Ca^{++} ATPase, on the other, were studied, employing red blood cell membrane fragments. The results indicate that these two ATPases share many common characteristics. but they are two distinctly different enzyme systems, located in the same cellular membrane.

MATERIALS AND METHODS

Purification of the ATPases of red cell membrane

All procedures were performed at 0-4°C. Citrated human red blood cells (RBC) were washed four times with 6× their volume of 0.9% NaCl solution. The RBC were packed lightly by centrifugation at the end of each washing. The white buffy layer above the RBC after centrifugation was eliminated as completely as possible. After the last washing, one volume of packed RBC was hemolyzed in 5 volumes of a solution containing 1 mM EDTA and 1 mM Tris buffer (pH 7.0). About 15 min after hemolysis, the hemolysate was centrifuged at 20,000 xg for 15 min. The precipitate (ghost cells) was washed twice with 3 volumes of the lysing solution, washed once with 3 volumes of 10 mM EDTA solution, and once again with 3 volumes of the lysing solution. By this time, the precipitate was essentially white. This precipitate was washd once with 3 volumes

of 0.5 M NaCl solution, 4 times with 0.5 mM histidine-imidazole buffer, pH 7.0, and was finally suspended in 0.5 mM histidine-imidazole buffer (pH 7.0). The final suspension, which consisted of RBC membrane fragments (RBCMF) and contained approximately 4 mg protein per ml, was stored at −20°C before use.

Measurement of ATPase activities

The compositions of standard reaction mixtures for ATPase activities are shown in Table 1. Any deviation from the standard compositions is indicated in the text. The procedure of measurement of ATPase activity was as follows: The reaction mixture (total volume, 5 ml) containing all components except ATP, and including RBCMF, was pre-equilibrated at 37°C for 3 min, and the reaction was started by adding ATP to the mixture. The volume of RBCMF suspension in the reaction mixture was 0.5 ml. Thirty minutes after the incubation at 37°C, the reaction was stopped by adding 1 ml of cold 20% TCA or perchloric acid. Then the mixture

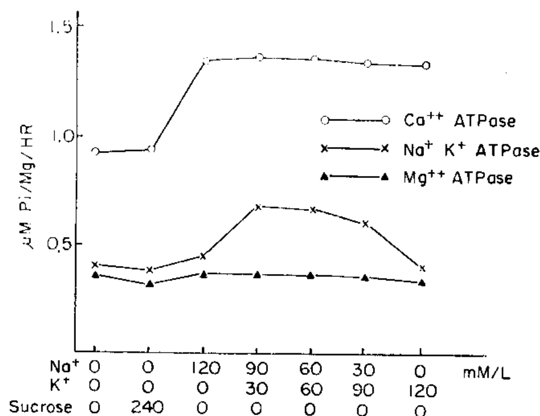

Fig. 1. ATPase activities of RBCMF. Reaction mixtures were standard compositions of Table 1 except concentrations of Na^+ and K^+ which were varied as indicated in the abscissa. An average of 3 experiments.

Table 1. Composition of Reaction Mixtures

Component	Composition of incubation medium		
	for Mg^{++} ATPase	Na^+-K^+ ATPase	Ca^{++}ATPase ATPase
NaCl	100	100	100
KCl	20	20	—
$CaCl_2$	—	—	0.5
EGTA	0.5	0.5	—
Histidine-imidazole buffer, pH 7.0	30	30	30
$MgCl_2$	5	5	5
Ouabain	0.05	—	0.05
ATP	2	2	2

All values in mM.
Total volume, 5 ml, including 0.5 ml of RBCMF suspension.

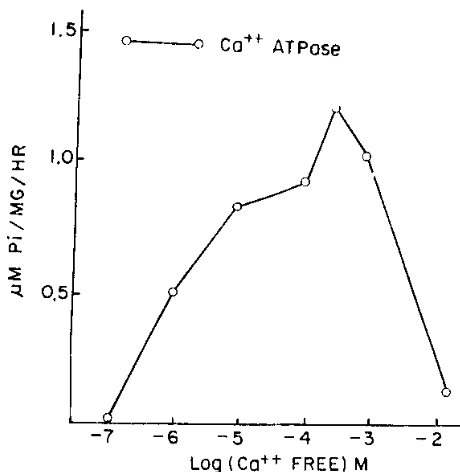

Fig. 2. Effects of free Ca^{++} concentrations on Ca^{++} ATPase activity of RBCMF. An average of 3 experiments.

was centrifuged. and the amount of Pi in the supernatant was measured by the method of Fiske and Sabarrow (Fiske et al, 1929). All ATPase activities are expressed in μ mole Pi liberated per mg protein per hour.

RESULTS AND DISCUSSION

The activities of Mg^{++} ATPase, Na^+-K^+ ATPase and Ca^{++} ATPase during the purif-

Table Ⅱ. ATPase Activity During Purification

Procedure (after)	Preparation	Specific activity of ATPase μ mole Pi/mg protein/hr		
		Mg^{++} ATPase	Na^{+}-K^{+}ATPase	Ca^{++}ATPase
Hemolysis	whole hemolysate	0·004	0.007	0,019
First centrifugation (20,000g 15min)	Supernatant	0.002	0.002	0.003
	Precipitate	0.009	0.020	0.044
2 washings with 1 mM EDTA	Precipitate	0.049	0.114	0.244
Washing with 10 mM EDTA	Precipitate	0.168	0,380	1.6
Final washing	Final RBCMF suspension	0.202	0.430	1.7

Average of 10 experiments.

ication procedure are shown in Table Ⅱ. It was noted that washing of RBCMF with the EDTA solutions increased the specific activity of both Na^{+}-K^{+} ATPase and Ca^{++} ATPase significantly. This probably was due mainly to removal of hemoglobin, since only a trace of hemoglobin was found after washing with 10 mM EDTA. It should be noted that Na^{+}-K^{+} ATPase and Ca^{++} ATPase were purified in parallel fashion, and the specific activity of these ATPases was increased approximately 100 fold over the specific activity of the hemolysate at the end of the purification procedure.

Influence of Na^{+} and K^{+} on ATPase activities

Concentrations on Na^{+} and K^{+} were varied from those in the standard media (see Table 1), and results are shown in Figure 1. It was found that Na^{+}-K^{+} ATPase is activated only in the presence of both Na^{+} and K^{+}. On the other hand, Ca^{++} ATPase does not require Na^{+} or K^{+}, but was increased further in the presence of these monovalent ions without specificity for either Na^{+} or K^{+}.

Influence of Ca^{++} concentrations on Ca^{++} ATPase activity

The Ca^{++} concentrations of medium was varied from that of the standard medium used for Ca^{++} ATPase (Table 1). Ca^{++} concentrations less than 10^{-5}M were adjusted by use of an EGTA-CaEGTA buffer system. The results are shown in Figure 2.

As can be seen in this Figure, the Ca^{++} ATPase activity was maximum at a Ca^{++} concentration of 5×10^{-4}M, and fell off as Ca^{++} concentration was either increased or decreased.

Influence of pH of the reaction mixture

The results of varying the pH of the reaction mixture, with other conditions remaining unchanged, are shown in Figure 3. It was found that both Na^{+}-K^{+} ATPase and Ca^{++} ATPase have the highest activity at pH 7.5.

Effect of preincubation of RBCMF at different temperatures

The RBCMF were exposed for 30 min at various temperatures before measurement of various ATPase activities, and these results are shown in Figure 4. It is noted that up to 40°C Ca^{++} ATPase and Na^{+}-K^{+} ATPase activities are not affected. However, exposure

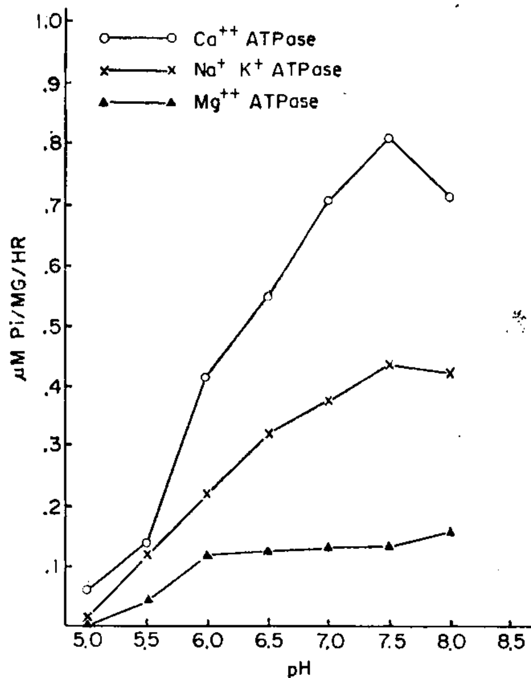

Fig. 3. Effects of varying pH of reaction mixtures on ATPases. An average of 3 experiments.

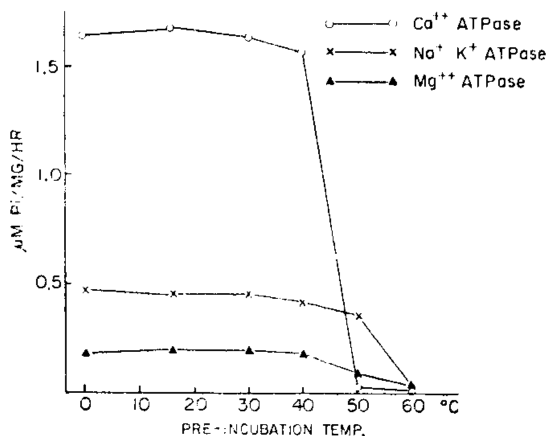

Fig. 4. Effects of pretreatment of RBCMF at various temperatures on ATPases. An average of 3 experiments.

of RBCMF at 50°C for 30 min almost completely destroyed the Ca^{++} ATPase activity without significantly influencing Na^{+}-K^{+} ATPase activity.

The nucleotide specificity

The ATPase activity of RBCMF with various nucleotides is shown in Table Ⅲ. It was observed that ATP was the only specific substrate which was split in the significant amount by RBCMF in the presence of Na^{+}-K^{+} or Ca^{++}. This ATP specificity for Ca^{++} ATPase was in contrast with results obtained previously, which showed Ca^{++}extrusion in released red blood cells in the presence of CTP, GTP and ITP, as well as ATP(Lee et al, 1969).

Table Ⅲ. Substrate Specificity of ATPase Activities of RBCMF

ATPase	Nucleotide				
	ATP	CTP	GTP	ITP	UTP
Mg^{++} ATPase	0.22	0.03	0.03	0.05	0.01
Na^{+}-K^{+} ATPase	0.53	0.09	0.09	0.10	0.05
Ca^{++} ATPase	1.38	0.12	0.08	0.13	0.04

All values in μmole Pi/mg protein/hr.
Average of 3 experiments.
The concentration of all nucleoside triphosphates is 2 mM, and other conditions are standard.

SUMMARY

1. The human red blood cell membrane fragments have both Na^{+}-K^{+} ATPase and Ca^{++} ATPase; and ATP is the specific substrate for both Ca^{++} ATPase and Na^{+}-K^{+} ATPase. The responses of these two ATPases to pH change are similar. However, their responses to alterations of the temperature of the medium are distinctly different. Thus, preincubation at 50°C destroys the Ca^{++} ATPase activity without influencing the Na^{+}-K^{+} ATPase activity.

2. From the above results it is concluded that Na^{+}-K^{+} ATPase and Ca^{++} ATPase are two distinctly different entities located in the cell membrane.

Abbreviations used:

EDTA-ethylenediamine tetraacetate

EGTA-ethylene glycol bis(β-aminoethyl ether)-N, N'-tetraacetic acid
ATP-adenosinetriphosphate
CTP-cytidine triphosphate
ITP-inosine triphosphate
GTP-guanosine triphosphate
UTP-uridine triphospate

—국문초록—

赤血球 細胞膜의 Na^{+}-K^{+} ATPase 및 Ca^{++} ATPase 活性에 關한 硏究

서울대학교 의과대학 약리학교실

朴 贊 雄

赤血球 細胞膜은 칼슘 이온의 能動的 移動機轉을 가지고 있는 것으로 알려져 있다. 한편 各種 細胞膜에서의 物質의 能動的 移動 機轉으로서 Ka^{+}-K^{+} ATPase가 잘 알려져 있다.

本實驗에서는 赤血球 細胞膜에서 Na^{+}-K^{+} ATPase와 Ca^{++} ATPase가 各種 實驗 條件에서 어떠한 影響을 받을 것인지를 檢討하므로써 Na^{+}-K^{+} ATPase와 Ca^{++} ATPase와의 關係를 究明코저 하였다. 實驗結果를 要略하면 다음과 같다.

1) 赤血球 細胞膜은 Na^{+}-K^{+} ATPase 및 Ca^{++} ATPase 活性을 나타내었으며 이들 酵素는 ATP 特有의 것이었다. 이들 ATPase 活性은 反應液의 液性 變化에 別差異를 보이지 않았으나 反應溫度의 變化에 대하여 顯著한 差異를 나타내었다. 即 50°C에서 Na^{+}-K^{+} ATPase 活性의 變化를 別로 볼 수 없는데 反하여 Ca^{++} ATPase 活性은 顯著하게 減退하였다.

2) 以上의 結果에서 Na^{+}-K^{+}ATPase와 Ca^{++} ATPase는 서로 다른 ATP 分解酵素임을 推測할 수 있다.

REFERENCES

1. Fiske, C.H., Sabbarow, Y.: *Phospocreatine. J. Biol. Chem., 81, 629, 1929.*
2. Lee, K.S., Shin, B.C.: *Studies on the active transport of Ca^{++} in human red cells. J. Gen. Physiol., 54, 713, 1969.*
3. Olson, E.J., Cazort, R.J.: *Active calcium and strontium transport in human erythrocyte ghost. J. Gen. Physiol., 53, 311, 1969.*
4. Post, P.L., Merritt, C.R., Kinsolving, C.R., Albright, C.D.,: *Membrane ATPase ase as a participant in the active transport of Na^{+} and K^{+} in human erythrocyte. J. Biol. Chem., 235, 1796, 1960.*
5. Schatzman, H.J.: *ATP-dependent Ca^{++} -extrusion from human red cells. Experientia (Basel), 22, 364, 1966,*
6. Schatzman, H.J., Vincenzi, F.F.: *Calcium movements across the membrane of human red cells. J. Physiol. (London), 201, 369, 1969.*
7. Skou, J.C.: *Enzyme basis for active transport of Na^{+} and K^{+} across cell membrane. Physiol. Rev., 45, 596. 1965.*
8. Vincenzi, F.F., Schatzman, H.J.: *Some properties of Ca^{++}-activated ATPase in human red cell membranes. Helv. Physiol. Acta,* 25, *CR*233. 1967.