THE INHIBITION OF Na,K-ACTIVATED ADENOSINETRIPHOSPHATASE BY A LARGE MOLECULE DERIVATIVE OF p-CHLOROMERCURIBENZOIC ACID AT THE OUTER SURFACE OF HUMAN RED CELL

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SUMMARY

A large mercurial compound was newly synthesized from Aminoethyldextran (mean MW 250,000) and p-chloromercuribenzoic acid with dicyclohexylcarbodiimide. The reagent inhibited Na, K-activated ATPase of the red cell membrane when applied to intact cells, but not the ouabain-insensitive-ATPase, whereas both were inhibited when it was applied to the membrane preparations. The active transport of rubidium in intact cells was inhibited, but was not the passive transport. The reagent may be a useful tool for studying the polarity and orientation of Na,K-ATPase and other proteins in the cell membranes.

INTRODUCTION

Since the molecular weight of Na,K-activated adenosinetriphosphatase (E.C.3.6.1.3.) has been roughly estimated at some hundred thousands (1--4) and it requires three different cations—magnesium and sodium ions inside the cell, and potassium ion outside, it must have a complicated nature. Much attention has been paid to the polarity and orientation of the enzyme protein in situ from the point of view of molecular biology and biochemistry. Relatively small molecules have often been used to modify the enzyme from the outside of the cell only (5-8). However, even a relatively large molecule, a mercurial derivative of sulfobromophthalein (approximately MW 1,000) passed through the red cell membranes (Matsumoto et al. 1969, unpublished), and digitoxin will penetrate bullfrog intestinal wall from the mucosal side, inhibiting the sodium

pump located at the serosal surface of the epithelial cells (9). Therefore, the results obtained with small molecules are difficult to interpret in terms of the polarity of the enzyme in situ.

This paper describes experiments with a high molecular mercurial compound newly synthesized from Dextran T-250 (mean MW 250,000).

MATERIALS, METHODS AND RESULTS

Synthesis of a large molecule mercurial compound: Aminoethyl-dextran T-250 (AED) was prepared from Dextran T-250 (Pharmacia) with 2-aminoethyl hydrogensulphate according to Eldjarn and Jellum(10). Two grams of AED, 50mg of p-chloromercuribenzoic acid (p-CMB) and 5g of dicyclohexylcarbodiimide were mixed with 30ml of dimethylsufoxide and gently stirred at room temperature. After 20 hours, the mixture was exhaustively dialyzed against 90 % acetone, 90 % methanol, and water in succession, each step lasting several days. Mercury content was determined using an atomic absorption spectrophotometer, amino groups with 2,4,6-trinitrobenzene sulfonic acid by the method of Habeeb (13), and Dextran by the anthrone method (14). The results obtained showed Hg : amino group : Dextran in the ratio of 19 : 30 : 1. The p-CMB-AED (PMDT-250) concentration for the half maximal inhibition of the pig brain Na,K-ATPase was 2×10^{-6}M on the basis of the mercury content. The high molecular weight of PMDT-250 and the absence of small mercurial compounds in the reagent were confirmed by gel filtration using a Sephadex G-50 column. A control experiment using a Sepharose 4B column did not show any binding of p-CMB to AED in the absence of dicyclohexylcarbodiimide.

Effect of PMDT-250 on the ATPase activities of the red cell

membranes: Red cells were collected from fresh heparinized blood or from ACD-blood supplied by a blood bank, washed three times with cold saline, once with cold medium A which contained 80mM NaCl, 100mM sucrose, 0.1mM TrisEGTA, 1.1mM glucose and 20mM imidazoleHCl, pH 7.4, and suspended in the same solution.

Red cells or red cell ghosts (0.2ml) were preincubated with or without 55μM PMDT-250 for 60 minutes at 37°, hemolyzed by adding 30ml of cold distilled water and washed three times with 1mM TrisHCl, pH 8.0. The fragmented ghosts were homogenized with a teflon homogenizer and ATPases were assayed by a procedure described previously with minor changes (15).

Table 1 shows the ATPase activities of the red cells and red cell ghosts previously treated with PMDT-250. S and IS denote the activities sensitive and insensitive to 2×10^{-5}M ouabain. In the experiments with intact cells, the S activity was inhibited considerably, whereas the IS activity was not affected by PMDT. On the other hand, the experiments with fragmented ghosts showed a marked contrast to the above results. The IS activity was inhibited as well as the S activity. The inhibition amounted to about 50 % of the control. In the control experiments, neither Dextran T-250 nor AED showed any effect on either of the ATPase activities (data not shown).

Since the IS activity in the intact cells was not affected by PMDT-250, the reagent does not seem to have penetrated the cell membrane. The inhibition of the S-activity in the intact cells may indicate that the ATPase has a group reactive to PMDT outside the cell. Considering the possibility that the translocation of mercuric ion or mercurial derivative occurred during hemolysis, the following two experiments were performed.

Table 1

Effects of PMDT-250 on the ATPase activities of the red cell membrane

a. Experiments in fragmented ghosts

	PMDT -250	IS	S	(IS/S)	relative (IS/S) *
Exp.1	-	0.61	0.87	0.69	1.00
	+	0.28	0.47	0.60	0.87
Exp.2	-	1.07	1.35	0.79	1.00
	+	0.50	1.02	0.49	0.62

b. Experiments in intact red cells

	PMDT -250	IS	S	(IS/S)	relative (IS/S) *
Exp.1	-	0.54	0.47	1.14	1.00
	+	0.61	0.14	4.5	3.94
Exp.2	-	0.77	0.33	2.32	1.00
	+	1.00	0.08	13.3	5.73
Exp.3	-	1.15	0.75	1.54	1.00
	+	0.91	0.27	3.4	2.21

ATPase activities are expressed as umoles Pi liberated/ml red cells/hr

* The IS/S ratio of the tubes containing PMDT-250 relative to that of the tubes without PMDT.

Table 1. Effect of PMDT-250 on the ATPase activities of the human red cell membrane

Red cells or red cell ghosts (0.2 ml) suspended in medium A were preincubated with 55μM PMDT-250 at 37° for 60 minutes. The hematocrit was adjusted to 15 %. In Table 1-a, the ghosts were washed three times with 1mM TrisHCl, pH 8.0, resuspended in 1.6ml of 0.5mM TrisEGTA and homogenized with a teflon homogenizer. The assay medium contained 140mM NaCl, 14mM KCl, 5mM $MgCl_2$, 0.5 mM TrisEGTA, 3mM TrisATP, and 50mM TrisHCl, pH 7.6 with or without 2 x 10^{-5}M ouabain. Incubation was performed for 60 minutes at 37°. Preincubation and incubation were performed both in duplicate. S and IS denote the activities sensitive and insensitive to 2 x 10^{-5}M ouabain. In Table 1-b, the PMDT-treated red cells were hemolyzed by adding 30ml of cold water and ATPase activities were estimated after the same operations as in the Table 1-a.

Red cells were preincubated with 3mM iodoacetamide for 60 minutes at 37° to minimize the number of reactive sites which might cause translocation of PMDT, collected and resuspended in the medium A, followed by the same operations as above. The results obtained were essentially the same as those in Table 1 except that the IS-ATPase in the cells treated with iodoacetamide showed a slight decrease in activity (about 10%) either with or without PMDT. The inhibitory effect of iodoacetamide on the IS activity has already been reported by Garrahan and Glynn (12).

Effect of PMDT-250 on Rb^+ transport in the human red cell:

The second experiment concerned the effect of PMDT-250 on the cation transport in intact cells. Potassium-depleted cells (0.3 ml) suspended in medium A containing 1mM RbCl were preincubated with or without PMDT-250 (55μM) at 0° for 15 minutes prior to the addition of radioactive ^{86}Rb (about 0.3μCi per tube). The incubation was carried out at 37° and stopped by centrifugation at 15,000 x g for 20 minutes. The extent of hemolysis in the control tubes and that in the tubes containing PMDT amounted to about 1 % and 10 % of the complete hypotonic hemolysis, respectively. Neither Dextran T-250 nor AED showed any difference in the extent of hemolysis. The red cells were washed twice with medium A and finally lyzed by adding a small amount of 0.1 % sodium lauryl sulfate at an alkaline pH. The hemolysates were transferred quantitatively on planchets for counting the radioactivity. The active cation uptake was the difference between the values of the tube containing 2 x 10^{-5}M ouabain and the tubes containing no ouabain.

In Fig. 1, the radioactivity of the incorporated ^{86}Rb was

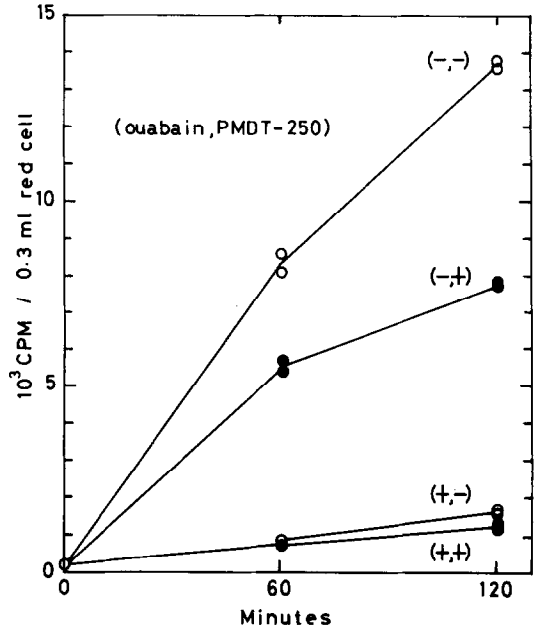

Figure 1. Effect of PMDT-250 on the cation uptake of the human red cell

Potassium-depleted red cells (0.3ml) suspended in medium A containing 1mM RbCl were preincubated with or without 55μM PMDT-250 at 0° for 15 minutes. Final hematocrit was adjusted to 15 %. The incubation was started by adding RbCl (0.3 μCi per tube) at 37°, and stopped by cooling. The red cells were collected once and washed twice with medium A and lyzed by adding small amount of 0.1 % sodium lauryl sulfate at an alkaline pH. The hemolysates were transferred on planchets for counting. The values were corrected for hemolysis.

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plotted against incubation time at 37°, after correcting for hemolysis. The maximum incorporation of Rb^+ amounted to 12 % of the added cation. As had been expected, PMDT-250 greatly reduced the ouabain-sensitive cation uptake of the red cells, whereas the ouabain-insensitive uptake of the cation remained unaffected.

DISCUSSION

The last findings indicate that the high molecule mercury compound outside the cell attacked the Na,K-ATPase *in situ*, and seeing that the passive cation uptake was not affected by

the reagent in spite of the acceleration by various small mercurial compounds, the reagent may neither penetrate nor enter the membrane.

The results clearly indicate that the Na,K-ATPase *in situ* does not only have reactive sites inside the cell, but also has a reactive group or groups at the outer surface of the cell, and this work supports the almost universally accepted view that the enzyme protein constitutes part of cell membrane.

PMDT-250 may be a useful tool for *polar modification* of the proteins located at the cell membrane surface.

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