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A SOLUBLE PROTEIN ACTIVATOR OF ($Mg^{2+}+Ca^{2+}$)-DEPENDENT ATPase IN HUMAN RED CELL MEMBRANES

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SUMMARY

1. ($Mg^{2+}+Ca^{2+}$)-dependent ATPase in human red cell membranes was stimulated about 2-fold by a soluble activator (or activators) in exhaustively dialyzed, membrane-free hemolysates.

2. The Mg^{2+}-dependent ATPase and ($Mg^{2+}+Na^{+}+K^{+}$)-dependent ATPase activities in the membranes were unaffected by the hemolysate.

3. Activation of ($Mg^{2+}+Ca^{2+}$)-dependent ATPase by hemolysate obeyed Michaelis–Menten kinetics, indicating that the activator binds to saturable sites on the enzyme. The activator increased the maximum velocity (V) of the enzyme.

4. When membranes were exposed to *N*-ethylmaleimide or to heat, ($Mg^{2+}+Ca^{2+}$)-dependent ATPase and the hemolysate-dependent component of this activity were inactivated at the same rate, indicating that there is a single ($Mg^{2+}+Ca^{2+}$)-dependent ATPase in the membrane which is activated by hemolysate.

5. Evidence that the activator is a protein is based on the following observations: (a) it is non-dialyzable, (b) it is destroyed by trypsin, and (c) it appears to be heat labile.

6. Human hemoglobin and bovine albumin produced a small activation of the enzyme, but it appears that the activator in hemolysate is probably a protein other than hemoglobin.

INTRODUCTION

The red cell maintains low levels of intracellular Ca^{2+} through an ATP-dependent Ca^{2+} pump which continually extrudes Ca^{2+} from the cell and is manifested in washed membranes as a ($Mg^{2+}+Ca^{2+}$)-dependent ATPase[1–3].

During our studies with this enzyme, we noticed that its activity was greater in whole hemolysates than in the washed membranes recovered from these hemolysates. We have now found that this is due to the presence of one or more soluble proteins in hemolysates which specifically activate ($Mg^{2+}+Ca^{2+}$)-dependent ATPase.

In this paper we describe the kinetics of activation of ($Mg^{2+}+Ca^{2+}$)-dependent ATPase by exhaustively dialysed, membrane-free hemolysate, and present some results bearing on the nature of the activator.

Abbrevation: EGTA, ethyleneglycol-bis-(aminoethyl ether)-*N*,*N*′-tetraacetic acid.

METHODS

Preparation of membranes

Human blood was obtained from the blood bank, and membranes were prepared as described previously[4]. This involved lysis and repeated washing in a medium consisting of 1 mM Tris–EDTA (pH 7.5). The protein content of each preparation was measured by the method of Lowry *et al.*[5].

Freezing and thawing membranes in solid CO_2–acetone had no effect on activity. In addition, results obtained with fresh human blood appeared identical to those obtained with outdated blood.

Preparation of membrane-free hemolysate

After the cells were lysed (1 vol. cells in 4–5 vol. of hemolyzing medium) and packed by centrifugation, the supernatant fluid was carefully decanted and dialyzed against 2 changes of 1 mM Tris–EDTA (pH 7.5) at 0 °C. This was followed by dialysis for at least 1 week against several changes of deionized water. Prolonged dialysis proved necessary to remove traces of contaminating Ca^{2+}.

Exposure of membranes to N-ethylmaleimide and to heat

Membranes were exposed to *N*-ethylmaleimide and to heat, both in the presence and absence of Ca^{2+}, for varying times before assay of ATPase activity, as described previously[6]. When $(Mg^{2+}+Ca^{2+})$-dependent ATPase was measured, the Ca^{2+} content of the assay tubes was adjusted to give the same final concentration in each case. When Mg^{2+}-dependent ATPase was measured, Tris–ethyleneglycol-bis-(aminoethyl ether)-*N*,*N'*-tetraacetic acid (EGTA) was added to the assay tubes to chelate the Ca^{2+}. Exposure to *N*-ethylmaleimide was carried out on ice and the assay tubes contained 2-mercaptoethanol to inactivate the *N*-ethylmaleimide.

Treatment of hemolysate with trypsin

Hemolysate was exposed to 0.5 mg/ml of trypsin for 2 h at room temperature. The exposure was terminated by adding 1.0 mg/ml of trypsin inhibitor. Controls were exposed to a mixture of trypsin and trypsin inhibitor for 2 h. Trypsin (Type III, 2 times crystallized, from bovine pancreas), and trypsin inhibitor (soybean, Type 1-S) were obtained from the Sigma Chemical Co., St. Louis, Mo.

ATPase assay

ATPase activity in washed membranes was assayed by measuring the P_i produced, according to a modification of the method of Fiske and SubbaRow[7], as described previously[4]. The following conditions were common to all experiments: 2 mM Tris–ATP, 2.5 mM Mg^{2+}, 37 mM Tris–HCl (pH 7.8) and about 0.25 mg of membrane protein. In most experiments, $5 \cdot 10^{-4}$ M ouabain was also present. Na^+ (or K^+), Ca^{2+} and hemolysate were generally present at saturating concentrations. Other conditions are described in the text.

Activity is expressed as μmoles P_i/mg protein per h. The protein refers to membrane protein and does not include protein contributed by the hemolysate.

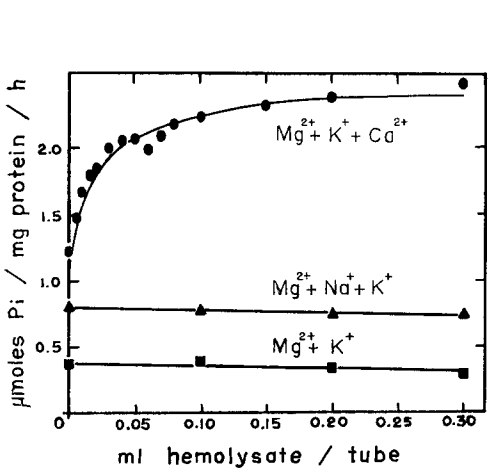
Each point in each experiment was the average of a duplicate determination, and all the results represent the average of at least two experiments which agreed closely.

$(Mg^{2+}+Ca^{2+})$-dependent ATPase is the increment in activity due to the presence of Ca^{2+} and Mg^{2+} (with or without a monovalent cation or hemolysate present) over that with Mg^{2+} alone. Hemolysate-dependent activity is the increment in activity due to the presence of hemolysate in addition to Mg^{2+} and Ca^{2+}. In all experiments, except those of Table I, the total ATPase activity in the presence of Mg^{2+} and Ca^{2+}, or Mg^{2+}, Ca^{2+} and hemolysate is shown, and the above increments have not actually been calculated.

RESULTS AND DISCUSSION

When the three ATPase activities in washed membranes were assayed with increasing concentrations of membrane-free hemolysate, $(Mg^{2+}+Ca^{2+})$-dependent ATPase was activated about 2.5-fold at the highest concentrations of hemolysate, but the Mg^{2+}- and $(Mg^{2+}+Na^{+}+K^{+})$-dependent activities were not affected (Fig. 1). This specificity indicates that hemolysate did not produce any gross change in the morphology of the membranes, because a change of this kind would probably affect more than one membrane-bound enzyme. Activation by hemolysate followed Michaelis–Menten kinetics: a double reciprocal plot of the hemolysate-dependent component of activity *vs* hemolysate concentration was linear. This result is most easily interpreted by assuming that the activator binds to discrete saturable sites on the enzyme.

In the experiment shown in Fig. 1 and in subsequent experiments, ATP and activating cations were present at saturating concentrations. Thus the activator increased the maximum velocity of the reaction.



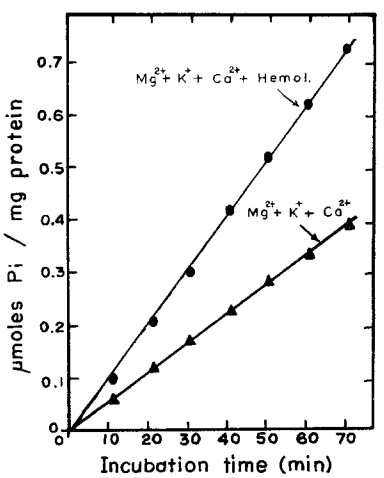


Fig. 1. Effect of hemolysate concentration on Mg^{2+}-, $(Mg^{2+}+Na^{+}+K^{+})$- and $(Mg^{2+}+Ca^{2+})$-dependent ATPase activities. The conditions for assay were as follows: ■—■, 60 mM K^{+} and $2.5\cdot10^{-4}$ M ouabain; ▲—▲, 100 mM Na^{+} and 15 mM K^{+}; ●—●, 60 mM K^{+}, 0.15 mM Ca^{2+} and $2.5\cdot10^{-4}$ M ouabain. Membrane-free hemolysate was added to each tube as shown on the abscissa Other conditions are described in Methods.

Fig. 2. ATPase activity in the presence of Mg^{2+} and Ca^{2+}, with and without hemolysate, as a function of time of incubation. Each tube contained 60 mM K^{+} and 0.15 mM Ca^{2+}. Hemolysate was present at a concentration of 0.20 ml per tube. Other conditions are given in Methods.

Hemolysate alone had no detectable ATPase activity, either with or without Ca^{2+} present. Therefore the extra ATPase activity due to hemolysate appears to reflect an increased activity of the membrane-bound enzyme.

The release of P_i by the enzyme was linear with time of incubation, with or without hemolysate present (Fig.2). Thus the activator does not exert its effects gradually. We also found that activity was linear with enzyme concentration, with or without hemolysate present, indicating that activation by hemolysate does not vary with possible changes in the state of aggregation of the membranes which might occur with changing membrane concentration.

Effect of hemolysate plus monovalent cations on $(Mg^{2+}+Ca^{2+})$-dependent ATPase

All the experiments described so far were carried out in the presence of either Na^+ or K^+. These cations, individually, increase $(Mg^{2+}+Ca^{2+})$-dependent ATPase activity in red cell membranes by about 1.5-fold (refs. 4 and 8). In the experiment shown in Table I, we compared the effect of hemolysate on the enzyme in the absence of monovalent cations and in the presence of either Na^+ or K^+. Hemolysate stimulated to approximately the same extent with or without a monovalent cation and, conversely, stimulation by Na^+ or K^+ was not much affected by hemolysate: the increment in activity due to hemolysate was increased by monovalent cations and *vice versa*. Schatzmann and Rossi[8] have suggested that $(Mg^{2+}+Ca^{2+})$-dependent ATPase and the extra activity which appears with either Na^+ or K^+ might be due to different enzymes. If this is indeed the case, the results of Table I show that both enzymes are activated to about the same extent by hemolysate.

Effect of pre-exposure of membranes to N-ethylmaleimide and to heat on subseuqent activation by hemolysate

As a further test of the possibility that $(Mg^{2+}+Ca^{2+})$-dependent ATPase and

TABLE I

EFFECT OF MONOVALENT CATIONS AND HEMOLYSATE ON $(Mg^{2+}+Ca^{2+})$-DEPENDENT ATPase

Only $(Mg^{2+}+Ca^{2+})$-dependent ATPase activity is shown. This was obtained by subtracting the activity with Mg^{2+} alone from that with Mg^{2+} and Ca^{2+} *plus* any other additions. Assay conditions were as follows: 0.15 mM Ca^{2+}, and, where appropriate, 0.2 ml of hemolysate and 60 mM Na^+ or K^+. Activity is expressed as μmoles P_i/mg protein per h. Δ refers to the increment in activity due to the presence of a monovalent cation or hemolysate compared to that without. The fold-stimulation was calculated by dividing the total activity with a monovalent cation or hemolysate by the corresponding activity without. Other conditions are given in Methods.

Addition	*$(Mg^{2+}+Ca^{2+})$-dependent ATPase activity*			*Fold-stimulation*	
	Total	*Δ due to Na^+ or K^+*	*Δ due to hemolysate*	*By Na^+ or K^+*	*By hemolysate*
—	0.53	—	—	—	—
Na^+	0.88	0.35	—	1.66	—
K^+	0.93	0.40	—	1.75	—
Hemolysate	1.41	—	0.88	—	2.66
Na^++hemolysate	2.16	0.75	1.28	1.53	2.46
K^++hemolysate	2.04	0.63	1.11	1.45	2.20

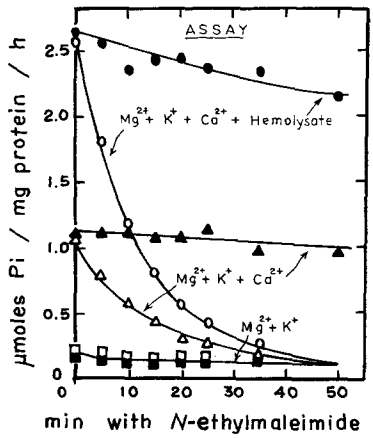


Fig. 3. Inactivation of Mg^{2+}-dependent ATPase and of ($Mg^{2+}+Ca^{2+}$)-dependent ATPase with and without hemolysate during exposure of membranes to *N*-ethylmaleimide. Membranes were exposed to 0.125 mM *N*-ethylmaleimide on ice with 0.1 mM Ca^{2+} (open symbols) and in the absence of Ca^{2+} (closed symbols). The exposure medium also contained 50 mM Tris–HCl (pH 7.8). At the times shown, 1.0-ml aliquots were removed for assay of ATPase activity under the following conditions: ○, ●, 0.20 ml hemolysate, 60 mM K^+ and 0.20 mM Ca^{2+}; △, ▲, 60 mM K^+ and 0.20 mM Ca^{2+} □, ■, 60 mM K^+ and 0.5 mM Tris–EGTA. All tubes contained, in addition, 3 mM mercaptoethanol to inactivate the *N*-ethylmaleimide. Other conditions are given in Methods.

the hemolysate-dependent component of this activity might be due to different enzymes, we studied the relative rates of inactivation of ($Mg^{2+}+Ca^{2+}$)-dependent ATPase with and without hemolysate present, following exposure of membranes to *N*-ethylmaleimide and to heat. These exposures were carried out with and without Ca^{2+}, since we had found previously that Ca^{2+} markedly increases the rate of inactivation of ($Mg^{2+}+Ca^{2+}$)-dependent ATPase by *N*-ethylmaleimide and heat[6]. Fig. 3 shows the results with *N*-ethylmaleimide. ($Mg^{2+}+Ca^{2+}$)-dependent ATPase was inactivated at the same rate with or without hemolysate present: at each time point the percent inactivation was the same in both cases. Furthermore, Ca^{2+} increased the rate of inactivation to the same extent in both cases and both activities disappeared at the same time. Similar results were obtained following heat treatment; therefore these data are not shown. We interpret these results to indicate that the red cell membrane probably contains a single species of ($Mg^{2+}+Ca^{2+}$)-dependent ATPase which is activated by hemolysate; it is improbable that different enzymes would respond so similarly to different inactivating treatments.

It is conceivable that hemolysate might contain a soluble, latent ($Mg^{2+}+Ca^{2+}$)-dependent ATPase, possibly released from the menbranes at the time of hemolysis, which can re-associate with the membranes to become active. The experiments just described argue against this possibility for two reasons: (1) hemolysate-dependent activity was inactivated even though hemolysate itself was not exposed to *N*-ethylmaleimide or to heat, and (2) if exposure of membranes to these treatments prevented a latent enzyme from binding, it is clear that this effect and the inactivation of enzyme already bound to the membranes were equally susceptible to these treatments and were equally affected by the presence of Ca^{2+} during exposure. The likelihood that this should be the case appears remote.

Effect of hemolysate on membranes prepared by other procedures

Most of the experiments described in this paper were carried out with membranes prepared as described in Methods. This involved repeated washings in solutions containing EDTA. It seemed possible that EDTA could have altered the membranes in some way so that the response to hemolysate was an artifact arising out of the method of preparation. Alternatively, the activator might actually be a component of the membrane which is released in soluble form upon exposure to EDTA (see ref. 9). To test these possibilities, we prepared one bath of membranes using only Tris–HCl (pH 7.8) for lysis and washing. A second batch of membranes was given a deliberately prolonged exposure to EDTA by lysing cells in the usual medium and dialyzing the whole hemolysate against 1 mM Tris–EDTA for 3 days before isolating the membranes. Table II shows the effect of hemolysate on these enzymes and on an enzyme prepared by the usual procedure. Hemolysate produced a substantial activation in all cases. If, indeed, the activator is normally a part of the membrane, it appears to be released equally well by Tris–HCl or by EDTA, and prolonged exposure to EDTA releases no more of it than the relatively short exposure employed in the usual preparative procedures. The question of the origin of the activator requires further study, however.

Lack of effect of some low molecular weight ligands on ($Mg^{2+}+Ca^{2+}$)-dependent ATPase

The fact that the activator was found to be non-dialyzable constitutes one line of evidence that it might be a protein. Nevertheless, traces of a number of low molecular weight ligands could have escaped dialysis by virtue of tight binding by proteins. We therefore tested the effect of several cations at a fixed concentration of 1 μM. The following had no effect on ($Mg^{2+}+Ca^{2+}$)-dependent ATPase: Mn^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, and Fe^{2+}. In addition, 100 μM NAD^+ had no effect.

Effect of trypsin on hemolysate

As a more direct test of the activator being a protein, we tested hemolysate for

TABLE II

EFFECT OF HEMOLYSATE ON MEMBRANES PREPARED BY DIFFERENT PROCEDURES

Enzyme A was prepared as described in Methods. Enzyme B was prepared by lysing and washing cells in 25 mM Tris–HCl (pH 7.8). Enzyme C was prepared by lysing cells in the usual medium, dialyzing for 3 days against 1 mM Tris–EDTA (pH 7.5) and then isolating membranes in the usual way. Assay conditions were: 0.2 ml hemolysate, 60 mM Na^+ and 0.15 mM Ca^{2+}. Other conditions are given in Methods.

Enzyme	*ATPase activity*			*Fold stimulation by hemolysate*
	Mg^{2+}	$Mg^{2+}+Ca^{2+}$	$Mg^{2+}+Ca^{2+}+$ *hemolysate*	
A	0.25	0.87	2.30	2.64
B	0.18	0.80	1.77	2.22
C	0.22	0.96	2.42	2.52

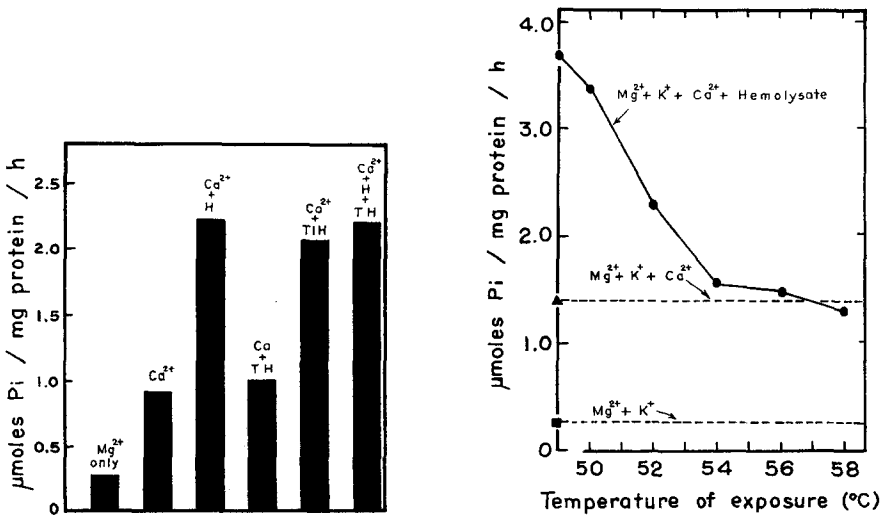


Fig. 4. Effect of trypsin on hemolysate. Hemolysate was exposed to trypsin and to trypsin *plus* trypsin inhibitor as described in Methods. The symbols are: TH, trypsin-treated hemolysate; TIH, hemolysate treated with trypsin *plus* trypsin inhibitor and H, untreated hemolysate. Conditions for assay were as follows: 0.20 mM Ca^{2+}, and 60 mM Na^{+}. 0.20 ml of the appropriate hemolysate was used. Other conditions are given in Methods.

Fig. 5. Effect of heat on hemolysate. Hemolysate was heated for 10 min at the temperatures shown and the precipitate of denatured protein was removed by centrifugation. 0.2 ml of the appropriate supernatant was tested for its effect on ($Mg^{2+}+Ca^{2+}$)-dependent ATPase. Assay conditions were as follows: ●—●, 0.2 ml of appropriate supernatant, 60 mM K^{+} and 0.20 mM Ca^{2+}; ▲—▲, 60 mM K^{+} and 0.2 mM Ca^{2+}; ■—■, 60 mM K^{+} and 0.25 mM Tris–EGTA.

susceptibility to tryptic inactivation (Fig. 4). Trypsin essentially destroyed the ability of hemolysate to activate ($Mg^{2+}+Ca^{2+}$)-dependent ATPase, whereas hemolysate which had been treated with a mixture of trypsin and trypsin inhibitor activated normally. Thus it appears that the activator is susceptible to tryptic attack and is a protein. Nevertheless, it was possible that the activator was not affected by trypsin, but that peptides released from other proteins simply blocked its effect. In order to test this possibility, we studied the effect of a mixture of untreated hemolysate and trypsin-treated hemolysate, and found that the untreated hemolysate activated normally (Fig. 4).

Effect of heat on hemolysate

In the experiment shown in Fig. 5, hemolysates were heated for 10 min at the temperatures indicated. In each tube a precipitate formed which was presumably denatured protein. We removed these precipitates and tested the supernatants for activity. Supernatants from hemolysates exposed to a temperature of 54 °C or higher had no effect on ($Mg^{2+}+Ca^{2+}$)-dependent ATPase. Thus the activator appears to be susceptible to thermal inactivation, and this can be taken as additional evidence that it is a protein.

Effects of hemoglobin and albumin on ($Mg^{2+}+Ca^{2+}$)-dependent ATPase

Since hemoglobin is the most abundant protein in the red cell and can bind to the

plasma membrane[10], it seemed a logical candidate for the activator. However, in the experiment of Fig. 4, hemoglobin did not precipitate; the absorption peaks of oxyhemoglobin at 542 and 576 nm were identical before and after heating. This indicates that hemoglobin is not the activator, but it is possible that the hemoglobin might have been denatured or otherwise altered by heating. We therefore pursued the possibility that hemoglobin might be the activator in two other experiments: (1) we converted the hemoglobin in hemolysates to methemoglobin by treatment with $NaNO_2$, but this did not prevent activation, (2) we tested purified human hemoglobin from commercial sources (which was mostly methemoglobin) as an activator and found a small activation amounting to 1.2- to 1.4-fold at saturating concentrations of hemoglobin.

Bovine albumin also produced an activation of about 1.2-fold at saturating concentrations, but the effects of bovine albumin and human hemoglobin were not additive. Thus it appears that a number of proteins might stimulate ($Mg^{2+}+Ca^{2+}$)-dependent ATPase, but their effects do not add up to that of the activator which we believe to be a protein other than hemoglobin.

Physiological significance

It has been clearly demonstrated that a close functional relationship exists between ($Mg^{2+}+Ca^{2+}$)-dependent ATPase and Ca^{2+} transport in red cells[1–3]. It has been suggested, however, that this enzyme might also be associated with a system of actomyosin-like fibers in the membrane which maintain the flexibility and characteristic biconcave shape of the cell[11,12]. The activator we have described could be related to either or both of these functions, but these points remain to be tested experimentally.

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