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THE INTERACTION OF Ca^{2+}/Mg^{2+} ATPase ACTIVATOR PROTEIN AND Ca^{2+} WITH HUMAN ERYTHROCYTE MEMBRANES

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Summary:
This paper presents the first unambiguous demonstration that a unique protein isolated from the hemolysate of human erythrocytes is responsible for increasing both the apparent Ca^{2+} ion affinity and maximum rate of ATP hydrolysis of the membrane-bound Ca^{2+}/Mg^{2+} ATPase. Unlike previous reports where an unpurified extract from red blood cells was used to activate the ATPase, our results clearly demonstrate that a single protein species, whether initially associated with or added back to the membrane is responsible for the observed changes in ATPase activity.

Introduction:

Following the report of Bond and Clough (1) on the presence of a Ca^{2+}/Mg^{2+} ATPase[1] activator in human erythrocytes and the later partial purification of this component by Luthra et al. (2) a number of studies on the biochemical characteristics of partially purified activator have been reported (3-6). In the course of our studies, we have further purified and characterized this activator. Also, we have noted that EDTA[1] as well as Tris (unpublished observation) were particularly effective in promoting the release of activator molecules from human erythrocyte membrane preparations.

Early reports on the occurence of apparent high and low affinity Ca^{2+} binding sites on the ATPase (ATP phosphohydrolase, EC 3.61.3; Ca^{2+} ATPase) (7-11) attributed to manipulation of the membrane during isolation as the cause for the change in binding states. However, these results can now be interpreted in terms of a specific protein interaction with the membrane. The interface between our observation and the previous reports is that activator

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[1]The abbrevations used are: Ca^{2+}/Mg^{2+} ATPase, Ca^{2+} stimulated/Mg^{2+} dependent ATPase; EDTA, [ethylene dinitrilo]-tetraacetic acid

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protein is capable of being released from the membrane during membrane isolation. The extent of release modulates the expression of either high and/or low Ca^{2+} affinity sites and the rate of ATP hydrolysis.

Experimental Procedures:

The procedures for collecting blood and washing the erythrocytes have been previously described (12). Basically, these consisted of withdrawal of blood, from healthy donors, into heparinized tubes. Plasma and buffy coat were removed by aspiration following sedimentation of the erythrocytes at 1500 x g for 10 minutes. The cells were washed three successive times with 0.155 M NaCl buffered with 5 mM histidine at pH 7.5 (NaCl-His-RBCs).

Membrane Isolation: Two types of membranes were used in these experiments: 1, native membranes and 2, EDTA membranes. They were prepared as follows: native membranes were prepared by hemolysing a 10% cell suspension of NaCl-His-RBCs with 0.01% Ca^{2+}-free saponin (12); for EDTA membranes, disodium EDTA was included in the hemolysing buffer at a 2 mM final concentration. The pH was adjusted to 7.6 before the addition of cells. In either case, the cells were hemolysed for 20 minutes at room temperature. These initial hemolysates were diluted 6 fold in cold NaCl-His and centrifuged for 30 minutes at 39,000 x g at 4°C. The native and the EDTA membrane pellets were washed three times with an equivalent 6 fold volume of NaCl-His buffer. In the case of the EDTA membranes, the first of these washes contained 2 mM EDTA. In either case, the final washed membrane pellets were suspended in a volume of NaCl-His buffer equivalent to the original 10% cell suspension.

Membrane Free Hemolysate: The resulting supernatant from the first hemolysis wash (in the absence of chelator) was termed membrane free hemolysate. This was added back to certain membranes in subsequent reconstitutive experiments and employed as the source for further purification of activator.

Activator Purification: The activator protein was purified from membrane free hemolysate by the procedure of Flynn and Taverna[2]. Briefly the activator was obtained by adsorption to QAE-Sephadex (Pharmacia) followed by elution ultrafiltration using an Amicon PM-30 filter. The ultrafiltrate was dialysed overnight against NaCl-His, pH 7.6. During this procedure, the presence of activator was monitored by the degree of Ca^{2+}/Mg^{2+} ATPase stimulation in native membrane preparations at a single added Ca^{2+} concentration, i.e., 50 μM.

The purity of activator preparations was examined on polyacrylamide gels. The single highly negatively charged band that was seen on 7.5% analytical gels (13) migrated as two bands on 5.6% gels laden with 0.1% sodium dodecyl sulfate (Sigma) (14) (Figure 1, A,B). We have as yet been unable to separate these proteins by additional chromatographic manipulations. The molecular weights on SDS gels of 17,000 and 34,000 plus the difficulty in isolating either highly negatively charged protein suggests they are in a monomer-dimer equilibrium. Further studies exploring this possibility are currently in progress.

Enzyme Assay: The Ca^{2+}/Mg^{2+} ATPase activity was assayed as previously described (12) where 0.1 ml of a 1:1 (v/v) mixture of a 10% membrane suspension (native or EDTA) and either NaCl-His buffer, membrane free hemolysate or purified activator was added to 0.5 ml aliquots of the Ca^{2+}/Mg^{2+} ATPase cocktail. This mixture was incubated for 2 hr at 44°C with occasional shaking. The reaction was terminated by the addition of 1.0 ml ice cold 10% trichloroacetic acid to each tube and ATP hydrolysed was determined by the amount of released inorganic phosphate using the Fiske Subbarow method (15). Hemoglobin was measured by the method of Kachmar (16) and protein concentrations by the Lowry Method (17).

[2]Flynn, D. and Taverna, R. D., manuscript in preparation.

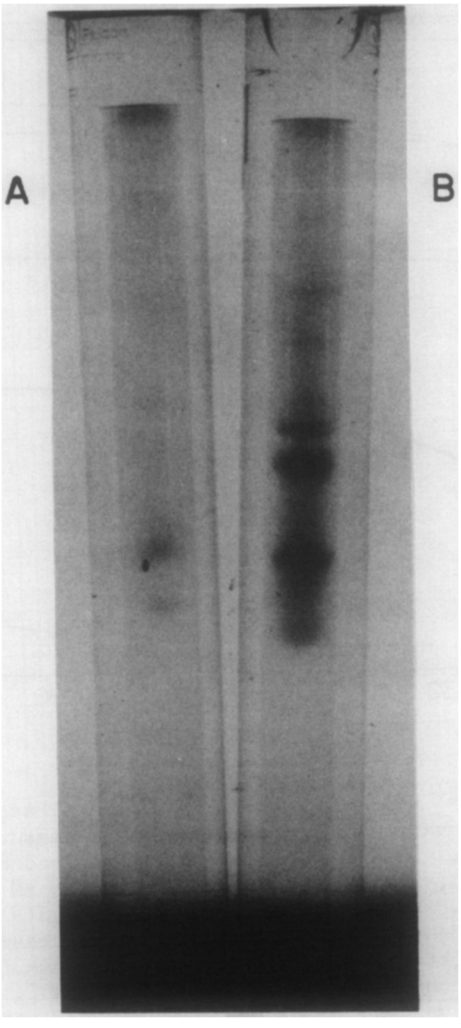

Figure 1. Sodium dodecyl sulfate polyacrylamide gels of activator at different stages of purification. A-Post-PM-30 (Amicon) fraction used in purified activator studies. B-starting material from QAE-Sephadex.

Treatment of Data: The observed rates of ATP hydrolysis at each Ca^{2+} concentration[3] for the type of preparations listed above were plotted as the mean velocity of not less than two experiments. The kinetic parameters, K_m and V_{max}, were obtained using the Wilkinson non-linear regression analysis (18).

[3] $[Ca^{2+}]$ as presented in Figures 1 and 2 and in Table I represents the total calcium ion added to the assay medium. When assay media with and without ATP was titrated for free Ca^{2+} using a calcium ion specific electrode (Radiometer), no detectable difference could be measured below approximately 10 μM added $[Ca^{2+}]$. Beyond this concentration and in the range of the reported high K_m for EDTA membranes the free $[Ca^{2+}]$ was approximately 12% lower than the total Ca^{2+} added. In terms of the free Ca^{2+} ion,, the K_m for EDTA membranes would be 14.5 μM.

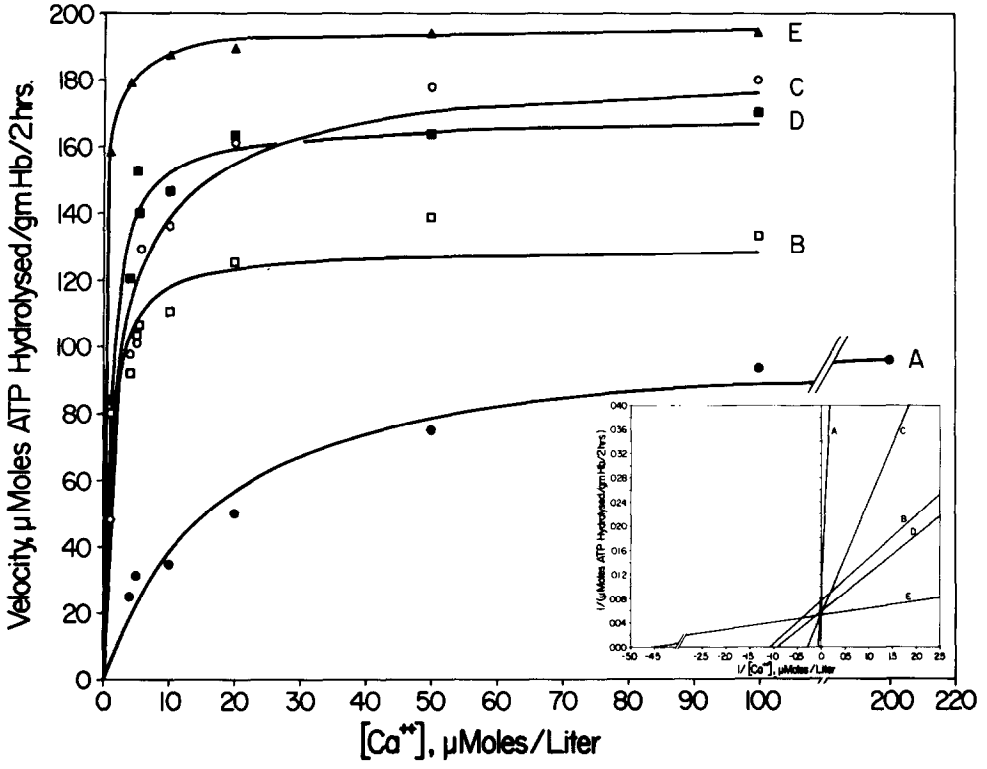

Figure 2. Effect of Ca^{2+} Concentration on Ca^{2+}/Mg^{2+} ATPase Velocity.

Enzyme activity of various membrane samples was measured as described in Experimental Procedures. The reaction cocktail contained 80 mM histidine, 3.6 mM $MgCl_2$, 80 mM NaCl, 33 mM KCl, 2.5 mM Na-ATP, 0.9 mM ouabain, and varying concentrations of $CaCl_2$. The Ca^{2+}/Mg^{2+} ATPase activity is reported as the amount of ATP hydrolyzed upon addition of Ca^{2+} in the presence of Mg^{2+} after subtracting the values obtained for Mg^{2+}-dependent ATPase. Mg^{2+}-ATPase was measured in a cocktail containing 0.5 mM EDTA and no Ca^{2+}. Enzyme activity is expressed as normalized values ± standard deviations as explained in the text. The samples are: A, (●) EDTA membranes; B (□) native membranes; C (○) EDTA membranes plus membrane-free hemolysate; D (■) native membranes plus membrane-free hemolysate; E (▲) EDTA and native membranes plus partially purified activator.

Figure 2 insert. Lineweaver-Burk Plot of Ca^{2+}/Mg^{2+} ATPase Velocity vs. Ca^{2+} Concentration.

The line of best fit, as determined by the Wilkinson non-linear regression analysis (18) was drawn through each set of data points. The legend is as described in Figure 2.

Results and Discussion:

The response of the membrane-bound Ca^{2+}/Mg^{2+} ATPase to varying Ca^{2+} concentrations could be significantly altered by manipulation of the divalent metal ion concentration at the instant of erythrocyte hemolysis. It is evident from Figure 2 that the addition of EDTA to the hemolysing buffer has greatly

TABLE I

Kinetic Parameters of the Ca^{2+} Stimulated/Mg^{2+} Dependent ATPase Activity of Various Membrane Preparations

Membrane Preparation	K_m (± SE)*, μM	V_{max} (± SE)*
A. EDTA	16.5 ± 2.7	103 ± 5
B. Native	0.9 ± 0.3	128 ± 6
C. EDTA + Hemolysate	3.4 ± 0.4	182 ± 5
D. Native + Hemolysate	1.0 ± 0.2	168 ± 5
E. Native or EDTA + Activator	0.2 ± 0.02	192 ± 1

*Expressed as μmoles Pi released/gm Hb/2 hours at 44°C.

(SE) Standard Errors

reduced the apparent binding affinity of Ca^{2+} to the ATPase (compare 2A & 2B). Furthermore, a concomitant lowering of the maximum velocity was observed after EDTA treatment (Table I). Inasmuch as the EDTA membranes were washed to remove the chelating agent, the differences in values of K_m and V_{max} between EDTA and native membranes reflects a loss of membrane associated ATPase activity and not the adventitious chelation of added Ca^{2+} during the assay.

Exposure of the membrane to the chelating agent, EDTA, dramatically changed the kinetic parameters K_m and V_{max} of the ATPase activity. Exhaustive washing of native membranes with isotonic NaCl-His alone similarly changes the kinetic parameters, yet this process is much less efficient than the chelation step (unpublished observation).

In agreement with previous reports (2,3), the addition of membrane free hemolysate to membrane preparations resulted in an increased V_{max} and a pronounced decrease in the Ca^{2+} half-saturation constant for the ATPase activity. This effect is most evident in EDTA membranes (Figures 2C and D)

where at least one component of the hemolysate has been effective in restoring the Ca^{2+} affinity close to that of the native membranes.

To further test the likelihood that the change in ATPase kinetics was a result of the loss of the activator protein species, the response of EDTA and native membrane ATPase activities to purified activator was examined. Inasmuch as no difference could be detected in the kinetic parameters in either case, the data were combined and presented as the averaged values (Figure 2, curve E). The maximim rate of ATP hydrolysis has increased above that observed for all other conditions of reconstitution. The apparent increase in the affinity of the ATPase for Ca^{2+} seen earlier in the presence of various levels of actovator in the medium was significantly raised in the presence of purified activator. These results strongly support the possibility that purified activator protein serves at least two functions on the membrane and these are to promote high affinity Ca^{2+} binding during ATP hydrolysis and to increase the rate of this hydrolysis.

The data presented in this paper have shown that the human erythrocyte Ca^{2+} stimulated/Mg^{2+} dependent ATPase activity is a direct function of membrane preparation. These kinetics were indeed sensitive to conditions that influenced the extent of ATPase activator protein retained by the membrane. As stated above, there have been several different reported values for K_m regarding Ca^{2+} effect upon Ca^{2+}/Mg^{2+} ATPase. The experiments reported here have been designed to offer an explanation for the discrepancy among K_m values reported in the literature (7-11)[4]. Also, we offer the first demonstration that purified activator protein acts in promoting the increased levels of ATPase activity. These data strongly suggest that activator binds reversibly to sites on the membrane via divalent metal cation linkage.

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[4]While this manuscript was in preparation, Scharff and Foder reported (23) findings similar to ours regarding the activator-dependent high and low affinity Ca^{2+} binding sites. They did not however relate the activity to a particular protein.

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