THE RELATIONSHIP BETWEEN CHANGES IN VISCOSITY OF HUMAN ERYTHROCYTE MEMBRANE SUSPENSIONS AND (Mg + Ca)-ATPase ACTIVITY[1]

Eugene E. Quist[2] and Basil D. Roufogalis

Laboratory of Molecular Pharmacology
Faculty of Pharmaceutical Sciences
University of British Columbia
Vancouver, B.C. V6T 1W5 Canada

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SUMMARY: ATP (0.5-4 mM) decreased the viscosity of unsealed erythrocyte membrane suspensions only in the presence of Mg^{2+} and at Ca^{2+} concentrations less than 0.1 μM. Other nucleotides (UTP, ITP, CTP, GTP, ADP) were ineffective. Ca^{2+} increased the viscosity of membranes if both ATP and Mg^{2+} were present, with half-maximal increase at 1-3 μM. Sr^{2+} fully substituted for Ca^{2+}, whereas Ni^{2+} and Ba^{2+} were only partially effective. The dependence of the viscosity changes and the "high affinity" (Mg^{2+} + Ca^{2+})-ATPase on the simultaneous presence of Mg^{2+}, Ca^{2+} and ATP, together with their similar nucleotide and divalent cation specificity, suggest that these events may be associated and may regulate red cell shape or deformability.

INTRODUCTION

Deformability of human erythrocytes is essential for their function and survival against destruction by the spleen (1,2). The deformability of erythrocytes is thought to be reflected by changes in their viscosity (1-3). Loss of erythrocyte deformability appears to be associated with depletion of cellular ATP (2,3). It has been suggested that ATP regulates erythrocyte deformability by chelating intracellular membrane bound Ca^{2+} (3,4) or by activating a membrane bound Ca^{2+}-dependent ATPase (5,6). We recently showed that a (Mg + Ca)-ATPase activity in erythrocyte ghosts was dependent on a group of water soluble proteins (7). In the present study we have compared the effect of Mg^{2+}, Ca^{2+} and ATP, and related nucleotides and cations, on the changes in relative viscosity and (Mg + Ca)-ATPase activity of human erythrocyte membranes.

1. Supported by the Medical Research Council of Canada. The abbreviations used are: (Mg + Ca)-ATPase; Ca^{2+}-activated, Mg^{2+}-dependent adenosine triphosphatase; EGTA, ethylene-glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid.

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MATERIALS AND METHODS

Preparation of Erythrocyte Membranes:

Whole human blood preserved in acid-citrate dextrose solution was obtained from the Canadian Red Cross (1-14 days old). In some experiments fresh heparinized blood was obtained from healthy donors and used immediately. Ghosts were prepared as previously described (7) in the absence of EDTA or EGTA and stored at -20° for 2 to 5 days. The freeze-thawed ghosts were suspended in five volumes of 0.5 mM NaCl and titrated immediately to pH 6.2 with 0.1 N HCl to prevent loss of protein (8,7). The membrane suspension was centrifuged at 20,000 x g for 10 min at 2° and the pellet was resuspensed to the original volume of packed ghosts with 15 mM NaCl and 5 mM Tris-maleate, pH 7.1. Protein was determined by the method of Lowry *et al.* (9).

Viscosity Measurements:

The relative viscosities of erythrocyte membrane suspensions were measured with calibrated Cannon-Fenske viscometers, sizes 25 and 50. Deionized water was used as the reference fluid. The medium contained, unless otherwise indicated, 50 mM Tris-maleate (pH 7.2), 60 mM NaCl, 1 mM EGTA, 6.5 mM $MgCl_2$, 2 mM ATP and various concentrations of $CaCl_2$. The concentration of free Ca ion was calculated according to Katz *et al.* (10) using a CaEGTA stability constant of $10^{10.65}$(7). The concentration of membrane protein was usually 1.8 mg/ml. The suspension was allowed to equilibrate at 37 $\pm$ 0.05° and the ATP was added just before measurement of the flow rate was initiated. The relative viscosities were independent of the size of the viscometer, indicating that they were largely independent of shear rate. The relationship between flow rate and protein concentration was linear up to at least 1.8 mg/ml.

ATPase Assay:

The composition of the assay medium (3 ml) for determining (Mg + Ca)-ATPase activity was identical to that used for the viscosity measurements. The reaction was started with ATP at 37° and was stopped with 1 ml of 20% trichloroacetic acid. Inorganic phosphate (Pi) was linear for at least 20 min with membrane protein up to 1.8 mg/ml. (Mg + Ca)-ATPase activity was corrected for basal Mg-ATPase activity by subtraction.

RESULTS

The suspended membranes were predominantly cup shaped with diameters of 3 to 5 microns as determined by phase contrast miscroscopy. The membranes were unsealed, as a result of disruption of ghosts caused by the freezing and low ionic strength treatment. The membranes did not aggregate during the studies.

Altering the ionic strength with NaCl (0-150 mM) or Tris-maleate (20-80 mM) had no effect on the relative viscosity of the membrane suspensions. $MgCl_2$, however, decreased the relative viscosity from 1.43 to 1.40, the maximal effect occurring at 4mM (not shown). ATP in the presence of Mg^{2+} caused a further decrease in the relative viscosity in a concentration dependent manner (Fig. 1). A significant reduction was manifest at 0.5 mM ATP

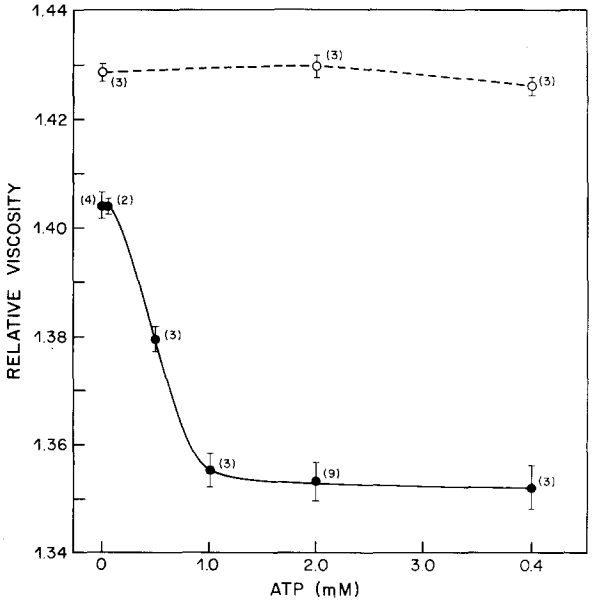

Fig. 1 The effect of ATP on the relative viscosity of membrane suspensions in the presence of 6.5 mM $MgCl_2$ (•) and in the absence of $MgCl_2$ (o). Numbers in parentheses represent the number of separate experiments. Points represent means ± S.E.

with the maximal effect occurring at 1 mM ATP. In the absence of $MgCl_2$ or in the presence of Ca^{2+} alone (5 μM or greater) ATP had no effect on the relative viscosity of the membrane suspensions. In the presence of $MgCl_2$ the decrease in viscosity was specific for ATP. At 2 mM UTP, ITP, CTP, GTP, ADP or Pi were ineffective in the presence or absence of $MgCl_2$.

In the absence of ATP and $MgCl_2$ Ca^{2+} had no effect on the relative viscosity of the membrane suspensions over the concentration range 0 to 500 μM (Fig. 2). However, Ca^{2+} increased the relative viscosity of the suspensions in the presence of Mg^{2+} and ATP (Fig. 2). This effect was manifest above 0.1 μM Ca^{2+} and was maximal at around 5 μM Ca^{2+}. The maximum concentration of Ca^{2+} restored the viscosity to that in the absence of ATP. The viscosity was half maximally increased at 1-3 μM Ca^{2+}. Sr^{2+} (50 μM) was as effective as Ca^{2+} in increasing the viscosity, whereas at this concentration Ni^{2+} and Ba^{2+} were only about 30% as effective (not shown).

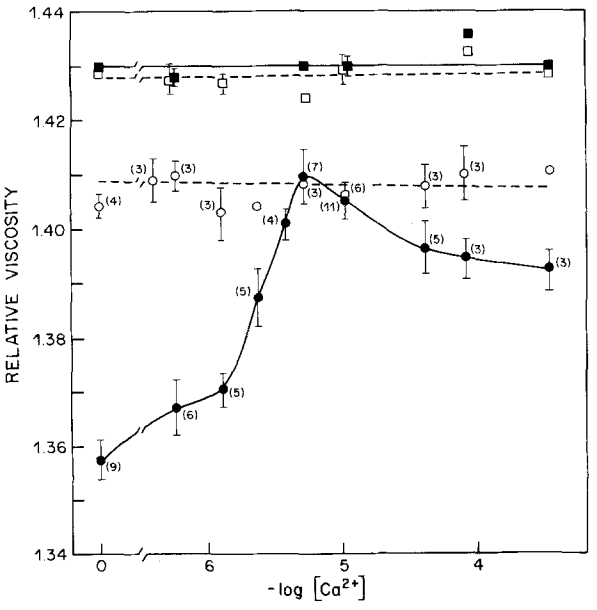

Fig. 2 The effect of Ca^{2+} on the relative viscosity of erythrocyte membrane suspensions in the presence of: 2 mM ATP and 6.5 mM $MgCl_2$ (●); 6.5 mM $MgCl_2$ and no ATP (o); 2 mM ATP and no $MgCl_2$ (■); no ATP or $MgCl_2$ (□). Other conditions are indicated in the Methods. Numbers in parentheses represent number of separate experiments. Points represent means ± S.E.

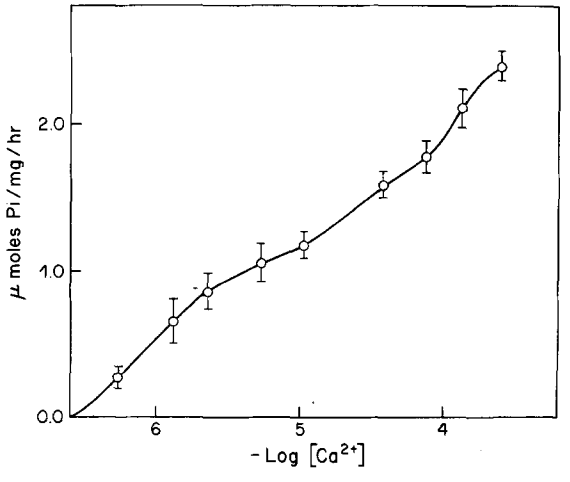

Fig. 3 Ca^{2+} dependence of (Mg + Ca)-ATPase activity in erythrocyte membrane suspensions. Each point represents the mean ± S.E. of at least four separate experiments.

Ca^{2+} activation of ATPase activity in the presence of Mg^{2+} was biphasic (Fig. 3), indicating the presence of "high" and "low" affinity (Mg + Ca)-ATPase activities (12,7). In agreement with the study of Watson et al. (13) neither UTP, ITP, CTP, GTP or ADP were hydrolyzed by the (Mg + Ca)-ATPase (not shown).

DISCUSSION

Depletion of the intracellular levels of ATP in erythrocytes results in changes in shape (14) and rigidity of red cells (3,15), which can be reversed by restoration of cellular ATP levels (3). In the present studies we have prepared human erythrocyte membrane ghosts made leaky by freeze-thawing and measured the changes in their viscosity. It is thought that changes in viscosity of the membranes reflect the deformability of these membranes (1-3) and may help define the molecular mechanism by which ATP, Mg^{2+} and Ca^{2+} interact to regulate the shape and possibly deformability of the red cell.

The addition of ATP to membrane suspensions reduced their viscosity, and hence rigidity, by a mechanism dependent on the presence of Mg^{2+}. This effect was not simply related to ionic strength, and hence changes in hydration or ionic charge of the membrane (16). Although the reduction in viscosity of intact erythrocytes is thought to be related to chelation of membrane-bound Ca^{2+} (4,3), the decrease in membrane viscosity induced by ATP and Mg^{2+} in this study occurred most effectively at Ca^{2+} concentrations less than 0.1 μM (Fig. 2). This implies that reduction in membrane viscosity by ATP and Mg^{2+} occurs independently of Ca^{2+}. In the presence of Mg^{2+} + ATP ghosts appear as thin electron-penetrable structures under electron microscopy (17). The site of interaction of Mg^{2+} and ATP on the membrane is unknown.

The viscosity of membrane suspensions was increased by Ca^{2+} only in the presence of both Mg^{2+} plus ATP. Ca^{2+} increased the viscosity to the level found in the absence of Mg^{2+} and ATP. The increase in membrane viscosity by Ca^{2+} coincides with the formation of electron-dense contracted ghosts (17). The

increase in viscosity and hence "rigidity" of erythrocyte membranes by Ca^{2+} in the presence of Mg^{2+} plus ATP may be the result of their interaction with a group of fibrillar or "contractile-like" proteins loosely associated with the membrane on the inner surface (18) or a membrane (Mg + Ca)-ATPase (5,6). The first possibility has been considered by many workers (3,18,19,20,21). Although a group of water soluble proteins extracted from the erythrocyte ghosts contain high affinity binding sites for Ca^{2+} (22) and perhaps actomyosin-like Ca^{2+}-dependent ATPase activity (5,6,21, 23), neither the formation of aggregated filamentous structures (3,16,19,24, 25) nor the Ca^{2+}-ATPase activities (5,21,23,26) exhibit the simultaneous requirement for Mg^{2+}, Ca^{2+} and ATP found for the viscosity changes observed in this study. On the other hand, we have observed similarities between the nucleotide and cation specificity for the viscosity changes and a membrane-associated (Mg + Ca)-ATPase. Both are Mg^{2+} dependent and have absolute specificity for ATP (13). Both the viscosity increase (Fig. 2) and the "high affinity" (Mg + Ca)-ATPase activities (Fig. 3, 7,12,27) are half-maximally activated by 1-3 μM Ca^{2+}. Sr^{2+} fully substitutes for Ca^{2+} in the activation of (Mg + Ca)-ATPase activity (28) and the increase in membrane viscosity, whereas Ba^{2+} and Ni^{2+} are only partially effective in either case.

The curve for the Ca^{2+}-dependence of the membrane viscosity increase (Fig. 2) coincides with the part of the biphasic Ca^{2+}-activation curve for (Mg + Ca)-ATPase (Fig. 3) generally referred to as "high" affinity ATPase (7,12). We have shown previously that this (Mg + Ca)-ATPase activity is associated with protein(s) readily extracted from the membrane at low ionic strengths (7), but that it may not be involved in the Ca-pump (29). The present results demonstrate a close association between viscosity changes of erythrocyte membranes induced by Ca^{2+} in the presence of Mg^{2+} plus ATP and the "high" affinity membrane-associated (Mg + Ca)-ATPase. However, further work is required to demonstrate a causal relationship between the two phenomena

and to elucidate the physiological significance of the viscosity changes in the regulation of intact erythrocyte shape or deformability.

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