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REGULATION OF ERYTHROCYTE MEMBRANE SHAPE BY Ca^{2+}

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SUMMARY

In the presence of 1.0 mM ATP and $MgCl_2$, the specific viscosity of suspensions of human erythrocyte ghosts decreases 35% in 20 minutes at 22°C. The changes in viscosity are a sensitive index of Mg-ATP dependent shape changes in these membranes. Low concentrations of Ca^{2+} (1 to 5 μM) inhibit Mg-ATP dependent viscosity changes. If ghosts were preincubated with 1 mM Mg-ATP and 20 μM A23187 to produce a maximal decrease in viscosity, addition of 10 μM Ca^{2+} to the preincubated ghosts increased the viscosity to levels observed in ghosts preincubated without ATP. Ca^{2+} (1 to 5 μM) also inhibited Mg^{2+} dependent phosphorylation 30% and stimulated dephosphorylation 25% in ghost membranes. These effects of Ca^{2+} on viscosity and phosphorylation may be due to a membrane bound Ca^{2+} phosphatase activity which dephosphorylates membranes phosphorylated by a Mg^{2+} dependent kinase activity.

INTRODUCTION

It has been known for a number of years that the shape of human erythrocytes is dependent on intracellular ATP and Ca^{2+} concentrations (1,2). Recently, investigators have shown that $MgCl_2$ and ATP transform echinocytic ghosts to discocytic or cup-shaped forms (3,4) and decrease the viscosity, which is an index of membrane shape change, of suspensions of unsealed freeze-thawed erythrocyte membranes (5). Mg-ATP may produce these shape changes in erythrocyte membranes by stimulating a membrane bound Mg^{2+} kinase activity which phosphorylates band 2 of spectrin (6). The mechanism by which Ca^{2+} regulates membrane shape is less clear. Intact discocytic erythrocytes loaded with Ca^{2+} in the presence of the ionophore A23187 are rapidly transformed to echinocytes (7). Weed *et al.* (2) suggested that Ca^{2+} binds to sites on the cytoplasmic surface of the membrane producing sol-gel changes and that ATP regulates membrane shape by removing Ca^{2+} from these sites by chelation. However, Ca^{2+} has no effect on the viscosity or shape of unsealed erythrocyte membranes, but does inhibit Mg-ATP dependent viscosity changes in these membrane suspensions (5).

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These observations indicate that Ca^{2+} may produce shape or viscosity changes by modulating the effect of Mg-ATP. Birchmeirer and Singer (6) suggested that Ca^{2+} inhibited Mg-ATP dependent shape changes by inhibiting a Mg^{2+} kinase activity. In this study, the possibility that Ca^{2+} may inhibit Mg-ATP dependent shape changes by stimulating a Ca^{2+} phosphatase activity was investigated.

MATERIALS AND METHODS

Materials. [γ -^{32}P]-ATP was obtained from New England Nuclear (Boston). A23187 was obtained from Calbiochem (California).

Preparation of ghosts. Whole human blood (1-5 days old) in acid-citrate-dextrose was obtained from the blood bank of a local hospital. Erythrocyte ghosts were prepared as previously described (8) and used within two hours of preparation for the viscosity and phosphorylation experiments described below.

Determination of specific viscosity. The specific viscosity of suspensions of freshly prepared erythrocyte ghosts was determined using Cannon-Manning semi-micro viscometers (size 75). The medium usually contained in a final volume of 1.0 ml; 50 mM Imidazole HCl, pH 7.2, 5.0 mM $MgCl_2$, 60 mM NaCl, 1.0 mM EGTA ± 1 mM ATP and 1.8 mg of membrane protein (0.6 ml of packed ghosts). The concentration of $CaCl_2$ was varied in this medium and the concentration of free Ca^{2+} ion was calculated using a Ca-EGTA stability constant of $10^{10.65}$ as previously described (8). In some experiments, 20 µM A23187 in 0.1% ethanol was included. The tubes were stored at 5°C for subsequent viscosity determinations. Viscometers were washed with 0.4 ml of the sample using an aspirator and the remaining 0.6 ml of the sample was added to the viscometer and the flow rate was determined at various time intervals at 22°C in an accurately controlled temperature water bath. The viscometers were standardized with 0.6 ml of H_2O or buffer. Relative viscosity was determined by dividing the flow time of a sample by the flow time of water or buffer for a given viscometer. Specific viscosity is the relative viscosity minus one (i.e. the viscosity due to H_2O).

Phosphorylation of ghosts. Ghosts were phosphorylated in a final volume of 0.5 ml of medium containing 50 mM Imidazole HCl pH 7.2, 60 mM NaCl, 1 mM EGTA ± 5 mM $MgCl_2$, 1 mM [γ -^{32}P]-ATP and 0.3 mg of membrane protein. The concentration of $CaCl_2$ was varied in this medium and 20 µM A23187 was included in some experiments. Phosphorylation was studied at 22°C. The reaction was stopped with 3 ml of cold solution containing 5% trichloroacetic acid, 0.5 mM K_2HPO_4 and 0.5 mM ATP. The tubes were centrifuged at 4,000 rpm x 15 minutes at 5°C and the pellet was resuspended and washed again with 3 ml of the trichloroacetic acid solution. The pellets were resuspended with 0.5 ml of 1% sodium dodecylsulfate. A 0.4 ml aliquot was counted in 10 ml of Aquasol. Protein was determined in the remainder of the samples according to Lowry et al. (9). The results were corrected for counts in the absence of $MgCl_2$ by subtraction.

RESULTS

Ca^{2+} inhibition of Mg-ATP dependent shape changes of ghost membranes. It was found that the effects of Mg-ATP and Ca^{2+} on the rate of shape changes of ghost membranes could be quantitated more readily and sensitively by measuring the flow rate or viscosity of the membrane suspensions in capillary type visco-

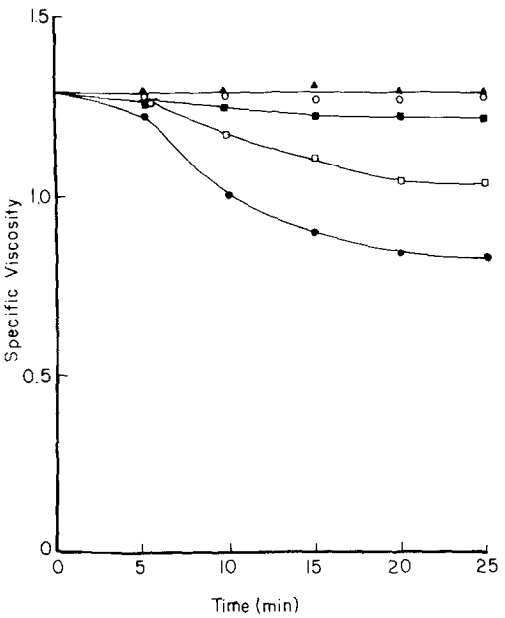

FIG. 1: The effect of Ca^{2+} on the time course of Mg-ATP dependent viscosity changes of erythrocyte ghost suspensions. The studies were done in the presence of 1.0 mM ATP (●), 1 mM ATP + 1.33 μM Ca^{2+} (□), 1 mM ATP + 3.1 μM Ca^{2+} (■) and 1 mM ATP + 5 μM Ca^{2+} (○) and 5 μM Ca^{2+} (▲).

meters than by microscopy. Preliminary experiments also showed that Mg-ATP or Ca^{2+} had no significant effect on the microhematocrit determined by centrifuging the ghost suspensions at 11,000 rpm x 20 minutes and therefore the viscosity changes do not reflect changes in volume as well as shape. Examination of ghost suspensions by phase-contrast microscopy was routinely undertaken in conjunction with viscosity measurements to determine qualitatively the type of shape changes induced by Mg-ATP and Ca^{2+}.

In the presence of 1.0 mM ATP and 5 mM $MgCl_2$, the specific viscosity of freshly prepared ghosts decreased 30 to 35% after 20 minutes at 22°C (Fig. 1). Mg-ATP completely transformed these echinocytic shaped ghosts to discocytic or cup-shaped forms during this incubation (not shown). Unless $MgCl_2$ was included with ATP, the viscosity of these suspensions did not change. Low concentrations of Ca^{2+} (1-5 μM) inhibited the Mg-ATP dependent viscosity decrease of these ghost suspensions. Approximately 1 μM Ca^{2+} half-maximally inhibited and 3-5 μM Ca^{2+} completely inhibited the Mg-ATP dependent viscosity decrease. In the ab-

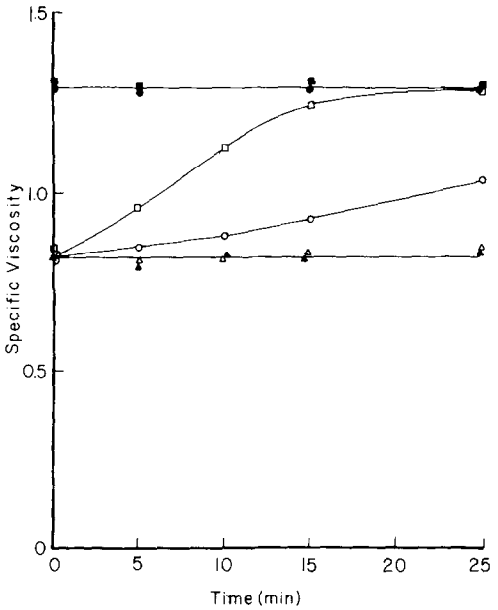

FIG. 2: Reversal of Mg-ATP dependent viscosity changes by Ca^{2+}. Ca^{2+} (10 μM) was added to ghosts preincubated for 30 minutes at 22°C under the following conditions; 20 μM A23187 (■), 1 mM ATP + 20 μM A23187 (□), no additions (●) and 1 mM ATP (○). Other conditions are indicated in the Methods. Ca^{2+} was not added to ghosts preincubated with 1 mM ATP (▲) and 1 mM ATP + 20 μM A23187 (△).

sence of either ATP or $MgCl_2$, these low concentrations of Ca^{2+} had no effect on the viscosity or shape of these ghost membranes.

Reversal of Mg-ATP dependent viscosity or shape changes by Ca^{2+}. In order to determine if Ca^{2+} could reverse Mg-ATP dependent shape changes, ghosts were preincubated for 30 minutes at 22°C in the presence of 1 mM ATP and 5 mM $MgCl_2$. In some experiments 20 μM of the ionophore A23187 was included in the incubation mechanism because the ghosts rapidly reseal under these experimental conditions and the ionophore enables added Ca^{2+} to penetrate to the cytoplasmic surface of these resealed ghosts. Addition of 10 μM Ca^{2+} to ghosts preincubated with Mg-ATP and 20 μM A23187 resulted in a gradual increase in the viscosity of the ghosts to the same level observed in membranes preincubated without ATP (Fig. 2). Concommitant with the increase in viscosity, Ca^{2+} completely transformed the preincubated discocytic ghosts to echinocytic forms after 15 minutes of incubation. Addition of 10 μM Ca^{2+} to ghosts preincubated with ATP but without A23187, produced a slow and incomplete increase in viscosity indicating that

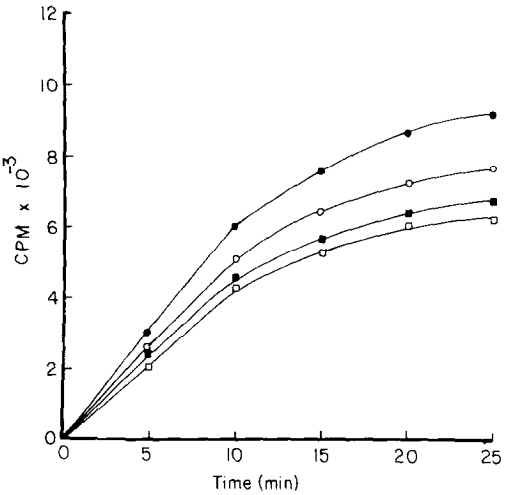

FIG. 3: Inhibition of Mg^{2+} dependent phosphorylation of erythrocyte ghosts by low concentrations of Ca^{2+}. Studies were done in the presence of 1.0 mM [γ -^{32}P]-ATP and 0 [Ca^{2+}] (●), 1.33 μM Ca^{2+} (○), 3.1 μM Ca^{2+} (■), and 5.0 μM Ca^{2+} (□).

added Ca^{2+} was unable to penetrate into all of the ghosts. This observation also indicates that Ca^{2+} increases the viscosity of preincubated ghosts by acting on the cytoplasmic surface of the membrane.

Ca^{2+} inhibition of Mg^{2+} dependent phosphorylation of ghosts. Figure 3 shows the time course of Mg^{2+} dependent phosphorylation in ghosts at 22°C. In the absence of Ca^{2+}, half-maximal phosphorylation was complete after approximately 8 minutes. It was found that 5 μM Ca^{2+}, which completely inhibits Mg-ATP dependent viscosity changes, inhibited Mg^{2+} dependent phosphorylation approximately 30% in the membranes. To determine if this inhibition could be due to Ca^{2+} phosphatase activity which dephosphorylates sites phosphorylated by Mg^{2+} dependent kinase activity, ghosts were preincubated for 30 minutes at 22°C in the presence of 1.0 mM [γ -^{32}P]-ATP. In some experiments 20 μM A23187 was included in the incubation medium to make the resealed ghosts leaky to Ca^{2+}. Addition of 10 μM Ca^{2+} to preincubated ghosts in the presence of A23187 resulted in a gradual decrease in total phosphorylation of approximately 25% after 20 minutes (Fig. 4). In ghosts preincubated without A23187, addition of Ca^{2+} produced only a 10% decrease in phosphorylation after 30 minutes incubation. In the absence of Ca^{2+}, phosphorylation increased slightly with time.

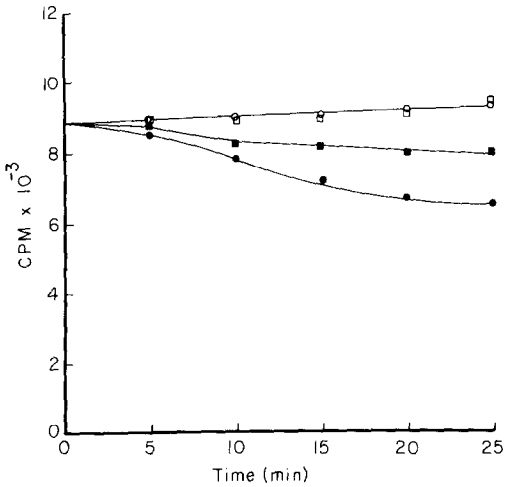

FIG. 4: Stimulation of dephosphorylation by Ca^{2+} of ghosts prephosphorylated with 5 mm $MgCl_2$ and 1 mM [γ -^{32}P]-ATP for 30 minutes at 22°C. After preincubation, 10 µM Ca^{2+} was added to tubes containing 0 A23187 (■) and 20 µM A23187 (●) at time 0 and the tubes were further incubated at 22°C. Ca^{2+} was not added to tubes containing 0 A23187 (□) and 20 µM A23187 (○).

DISCUSSION

Recently, Birchmeirer and Singer (6) hypothesized that erythrocyte membrane shape was maintained in a steady-state by opposing kinase and phosphatase activities which phosphorylate and dephosphorylate a membrane substrate, respectively. These workers (6) also suggested that Ca^{2+} may inhibit Mg-ATP dependent shape changes by inhibiting Mg^{2+} kinase activity. In this study, it was shown that low concentrations of Ca^{2+} not only inhibit shape or viscosity changes in ghosts but also can reverse Mg-ATP dependent viscosity changes. For instance, in ghosts preincubated with 1 mM Mg-ATP, Ca^{2+} increases the viscosity to exactly the same level in ghosts not preincubated with ATP (Fig. 2). Additionally, Ca^{2+} had no effect on the viscosity or shape in the absence of ATP. Therefore, it is clear that Ca^{2+} acts by modulating the effect of Mg-ATP on shape in this membrane preparation. If it is assumed that Mg-ATP induces shape changes via a Mg^{2+} kinase (6), then the effects of Ca^{2+} on viscosity or shape could readily be explained by the presence of a membrane bound Ca^{2+} phosphatase activity which dephosphorylates the sites phosphorylated by this Mg^{2+} kinase activity. In support of the existence of a Ca^{2+} phosphatase activity, it was shown that

Ca^{2+} stimulated dephosphorylation in ghosts approximately 25%. It is also conceivable that these coupled Mg^{2+} kinase and Ca^{2+} phosphatase activities could account for apparent Ca+Mg-ATPase activity in erythrocyte membranes not associated with Ca^{2+} transport (10,11). The Mg-ATP dependent viscosity changes in freeze-thawed membranes (5) and freshly prepared ghosts (Fig. 1) are inhibited by approximately 1 μM Ca^{2+} which could correspond to part of the Ca+Mg-ATPase activity in these membranes having a Kd for Ca^{2+} of 1 μM. Graham et al. (12) previously reported the presence of phosphoprotein phosphatase activity in the cytoplasm of erythrocytes, but this phosphatase activity is strongly inhibited by physiological ATP concentrations and therefore its role in erythrocyte membrane shape regulation is uncertain.

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