CATION CONTROL OF ERYTHROCYTE MEMBRANE SHAPE: Ca^{++} REVERSAL OF DISCOCYTE TO ECHINOCYTE TRANSITION CAUSED BY Mg^{++} AND OTHER CATIONS

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SUMMARY: White erythrocyte membranes, or ghosts, were monoconcave discocytes when incubated in 50mM N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid titrated to pH 7.4 with triethanolamine. If 3mM $MgCl_2$ was included in the incubation medium, the ghosts were predominantly echinocytes. The echinocytic form could also be induced by Co^{++}, Ni^{++}, Li^{+}, Na^{+}, K^{+}, NH_4^{+} and tetramethylammonium ion, all as chloride salts. The concentration of cation necessary for 50% of the ghosts to be echinocytes was correlated with the hydrated charge density of the cation with the most highly charged cations being the most effective. The cations Ca^{++}, Sr^{++}, Ba^{++} and La^{+++}, (also as chloride salts) did not induce the normal echinocytic form, but at high levels induced a few misshapen forms with some resemblance to echinocytes. Instead Ca^{++}, Sr^{++}, Ba^{++} and La^{+++} suppressed the formation of echinocytes in the presence of Mg^{++} and other ions. This suggests the presence of a specific Ca^{++} binding site important to shape control in the erythrocyte membrane.

INTRODUCTION: Normal intact human erythrocytes maintain the steady state configuration of a deformable biconcave disc or discocyte. Under various experimental conditions, erythrocytes can be induced to undergo a transformation to the spiculated form known as an echinocyte (1). In addition, it has been reported that inclusion of Ca^{++} in the lysis medium can cause echinocyte formation in resealed ghosts (2). Both intact erythrocytes and resealed ghosts contain intracellular constituents which could be involved in the transformation

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from discocyte to echinocyte. In order to determine whether these shapes can be caused solely through membrane constituents, it is necessary to induce their formation in a pure membrane system. The studies reported here demonstrate conditions under which echinocytes can be induced and inhibited in white erythrocyte ghosts.

EXPERIMENTAL METHODS: Whole blood from healthy donors was collected in heparin. Ghosts were prepared according to the method of Dodge et al. (3), with the following modifications: The lysis and all the hypotonic washes except the final one were done in the presence of 1mM EDTA. After the third hypotonic wash, the hypotonic phosphate wash buffer was replaced by a wash buffer of 28mM Tris-HCl, pH 7.4 at 4°C. After the third and final wash in Tris-HCl, the ghosts were resuspended in the final wash medium to a concentration of 5mg ghost protein /ml. The protein was determined by the method of Gornall et al. (4)

All of the salts used were reagent grade. All of the cations tested for shape changing effects were added as the chloride salts. All solutions were made using distilled deionized water that was essentially Ca^{++} - free (5). The incubation buffer consisted of* N-tris(hydroxy methyl) methyl -2- aminoethane sulfonic acid (TES)titrated to pH 7.4 at 30° with triethanolamine (TEA). The TES was passed over a chelex column to remove any cations present.

The incubation medium consisted of 50mM TES-TEA and any cations as indicated. The assay tubes containing the incubation medium were preincubated at 37°C for five minutes. Ghosts were then added to a final concentration of 1 mg protein/ml. The test tubes were then vortexed and incubated for an additional thirty minutes. Slides were prepared from the test tubes and observed under a Leitz phase contrast microscope using a 100x oil immersion objective. Several fields were observed for each assay tube. The number of echinocytes and discocytes per field was counted and the average percent of each type was recorded for each assay tube.

RESULTS: When the ghosts were incubated in a medium containing the 50mM TES-TEA buffer only, they were monoconcave discocytes. If Mg^{++} was included in the incubation medium the ghosts assumed an echinocytic form. Figure 1 shows that the percent of cells as echinocytes varied with the Mg^{++} concentration used and reached a maximum at 3-6 mM Mg^{++}.

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*Abbreviations used:
TES: N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid
TEA: Triethanolamine
TMA$^+$: Tetramethylammonium ion

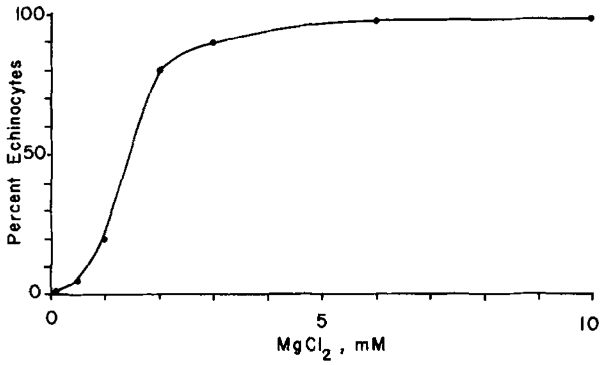

Figure 1: Effect of Concentration of Mg^{++} on Echinocyte Formation. Ghosts were incubated in 50mM TES-TEA buffer in the presence of various concentrations of $MgCl_2$. The ghosts were observed using phase contrast microscopy. The cells were counted as to the number of echinocytes and discocytes and an average value for percent echinocytes was recorded. This is the result of a typical experiment.

Other cations were also found to induce echinocyte formation when included in the incubation medium. These included Co^{++}, Ni^{++}, Li^{+}, Na^{+}, K^{+}, NH_4^{+}, and TMA^{+}. As with Mg^{++}, the percent of ghosts as echinocytes depended on the concentration of cation used. The order of effectiveness of these cations was such that the smaller and more highly charged ions had the stronger effect in causing echinocyte formation. Figure 2 demonstrates the correlation that was found to exist between the ionic concentration for 50% conversion to echinocytes and the hydrated charge density for each particular ion.

The cations Ca^{++}, Sr^{++}, Ba^{++} and La^{+++} did not induce an echinocyte form in these ghosts. Ghosts incubated in the presence of any of these ions at low concentrations were discocytes. Some misshapen cells were observed at concentrations above 5mM for Ca^{++}, Sr^{++}, and Ba^{++}, and above 10^{-2}mM La^{+++}.

However, these ions were found to inhibit the echinocyte formation caused by Mg^{++}. If 3mM Mg^{++} was present, Ca^{++} maximally inhibited echinocyte formation at 0.2mM, Sr^{++} at 0.5mM, Ba^{++} at 1.0mM, and

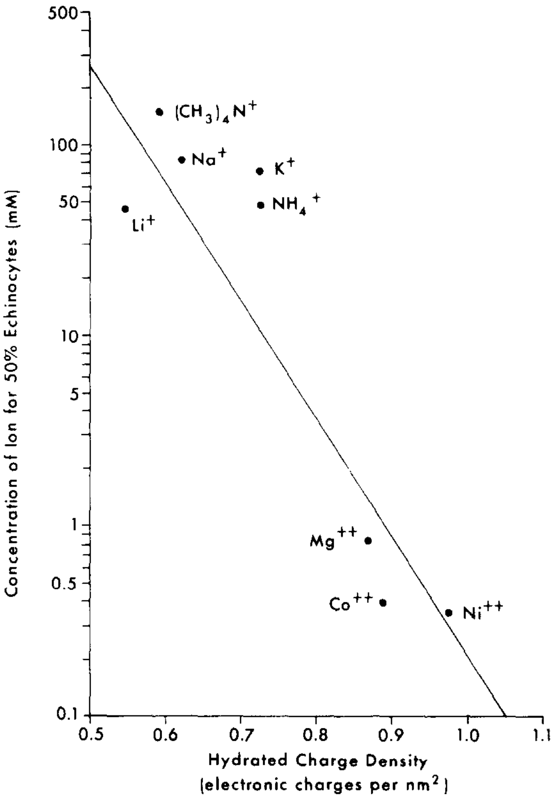

Figure 2: Correlation Between Hydrated Ionic Radius and Concentration of Ion Required to Cause 50% Echinocytes. Curves of percent echinocytes versus concentration of the chloride salt were observed as described in the Methods section. Hydrated charge densities were calculated from the hydrated radii of the ions (6).

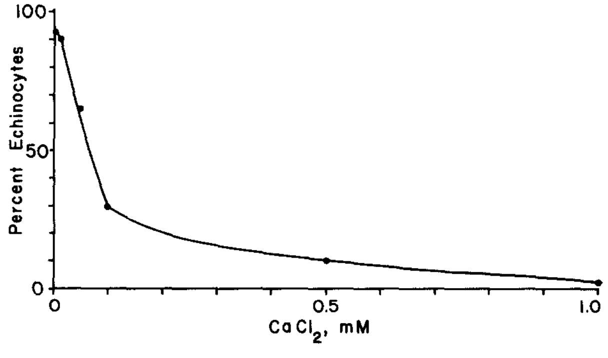

Figure 3: Effect of Ca^{++} on Mg^{++} Induced Echinocyte Formation. Ghosts were incubated with 50mM TES-TEA buffer and 3mM $MgCl_2$ in the presence of various concentrations of $CaCl_2$. The ghosts were observed as indicated in Figure 1. This is the result of a typical experiment.

La^{+++} at 0.005mM. Figure 3 illustrates the concentration-dependent nature of the Ca^{++} inhibition of echinocyte formation.

When ghosts were treated with $CaCl_2$ or $MgCl_2$ at 0^oC, both salts caused echinocyte formation at 4mM; the echinocytes which occurred in the presence of Ca^{++} reverted to discocytes upon incubation at 37^oC.

DISCUSSION: From the data presented here, it is apparent that certain cations can interact with the erythrocyte membrane to effect a shape change from discocyte to echinocyte. Presumably this shape change is due to binding of these cations to the membrane. This binding is nonspecific, since a number of monovalent and bivalent metal ions, as well as large cations, all can engender the echinocytic form. The affinity of the cation for the membrane depends on its size and charge; however, all ions tested, except for Ca^{++} and its relatives, were effective. This nonspecific effect of cations may be due to the collapse of the inner half of the bilayer upon binding of cations, as has been suggested by Johnson and Robinson (7).

On the basis of the correlation between concentration required to cause 50% echinocytes and the hydrated charge density, it would be expected that Ca^{++}, Sr^{++}, Ba^{++} and La^{+++} would all cause echinocyte formation at low concentrations. Their opposite effect in the presence of other cations suggests the existence of an ion-specific site associated with the reversal of echinocyte formation.

In addition to the observations reported here, the nonspecific effect of cations in producing echinocytes has been independently reported from two other laboratories (7,8). These reports were presented at nearly the same time as the original version of this manuscript, which emphasized the antagonistic effects of Mg^{++} and Ca^{++}. The present version has been slightly revised to reflect more accurately our current understanding of the relationship between their results and ours. Both of these laboratories report approximately equal effectiveness of Mg^{++} and Ca^{++} in producing echinocytes, apparently because they did not incubate ghosts at 37°C. Our results indicate that a brief incubation at 37°C causes a reproducible and specific effect of Ca^{++}, antagonistic to the echinocyte-causing effect of Mg^{++} and other cations. The high specificity of Ca^{++} and its analogs in causing this effect suggests that the change which occurs upon incubation is not a trivial degradative change, but rather the exposure of a Ca^{++} binding site important for shape control in the erythrocyte membrane.

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