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LOSS OF RESEALING ABILITY IN ERYTHROCYTE MEMBRANES

EFFECT OF DIVALENT CATIONS AND SPECTRIN RELEASE

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Summary

Washed human erythrocyte membranes can recover impermeability to macromolecules upon warming in solutions of sufficient ionic strength. This ability is rapidly lost from most ghost preparations in dilute salt solution at temperatures of 15°C or higher. Divalent cations both reseal ghosts in the absence of high ionic strength and prevent loss of resealing ability. The effective concentrations are 40 μM for Ca^{2+} and 200 μM for Mg^{2+}. The loss of resealing ability is associated with the release of spectrin polypeptides from the inner surface of the membrane. In ghost preparations that have not become irreversibly leaky, or in the presence of Ca^{2+}, loss of spectrin does not occur. These results suggest that an intact spectrin network is required for resealing to macromolecules, and divalent cations stabilize this network. In light of this information, the effect of temperature on resealing kinetics is described.

Introduction

After hypotonic hemolysis, red cell membranes are able to "reseal", that is, to recover impermeability to various solutes. Some membrane functions may be restored as well; for example, ATP-dependent ion transport has been reconstituted. It is known that the extent to which resealing occurs depends on the preparation of the ghosts (reviewed in ref. 1). White ghosts, i.e., those washed free of intracellular hemoglobin, can recover impermeability to macromolecules but not to smaller solutes [2,3], while less extensively washed membranes can reseal to small molecules and cations [4].

The ability to reseal is a transitory feature of the membrane. In the course of

Abbreviations: PMSF, phenylmethyl sulfonyl fluoride; SDS, sodium dodecyl sulfate.

determining divalent cation effects upon the resealing of white ghosts to macromolecules, we have found that red cell ghosts can rapidly lose the ability to reseal, while remaining morphologically intact, and that divalent cations act to prevent this loss. This loss of resealing ability can be correlated with the loss of certain membrane components. In this paper we describe the induction of permanent ghost permeability to macromolecules, the related protein loss from ghosts, and present evidence that Ca^{2+} stabilizes the protein structure of the erythrocyte membrane.

Materials and Methods

The ghost preparation in Tris buffer (Tris washed ghosts) has been described [2]. Erythrocytes are washed twice in 5 mM Tris · Cl, pH 7.4, 140 mM NaCl, 1 mM EDTA with removal of the buffy coat, and lysed in 20 volumes of 5 mM Tris, pH 7.4, 7 mM NaCl, 1 mM EDTA. The ghosts are collected by a 20 min spin at 10 000 rpm in a Sorvall SS34 rotor. After three washes in the same buffer, the ghosts are slightly pink. When necessary EDTA is removed by performing the final washes in 5 mM Tris, pH 7.4, 7 mM NaCl. All procedures are done at 4°C. Nearly all the experiments were performed using fresh blood from normal donors collected in heparin. Occasionally, these samples were stored overnight at 4°C. Some experiments used packed red cells obtained from the Southeastern Michigan Blood Center of the Red Cross. Hemoglobin for use as a macromolecular marker of impermeability was prepared by the method of Huntsman [5], and dialyzed against eight changes of the buffer used in the experiments. Other chemicals were reagent grade from Sigma and Mallinckrodt. Deionized, distilled water was used for all solutions.

The procedure for resealing measurements [2] has been slightly revised. Ghosts were mixed with an appropriate volume of a concentrated hemoglobin solution at 0°C in dilute salt for 10 min before beginning the experiment. This pre-mixing is necessary, since the internal volume of ghosts does not immediately equilibrate with added solutes. If resealing is begun without this equilibration step, less macromolecular marker will be trapped in the resealed ghost and the extent of resealing is underestimated. To begin resealing, an aliquot of the ghost-hemoglobin mixture is added to a large volume of buffer with the same concentration of hemoglobin, and the desired ionic composition. The temperature of the larger volume of solution is such that the final temperature after adding the cold ghost suspension is the desired incubation temperature (usually 30 or 37°C).

As before [2], resealing is quenched by diluting a sample of incubated ghosts in ice-cold isotonic buffer. The resealed ghosts are collected with a 20 min spin in a refrigerated clinical centrifuge. It can be shown that the hemoglobin containing resealed ghosts are pelleted by this centrifugation. The ghosts are washed once and lysed in a known volume of cyanide reagent with 0.1% Triton. Cyanmethemoglobin is assayed by absorbance at 415 or 540 nm. Resealed volume is calculated as the fraction of the hemoglobin in 1 ml of incubation mix found in the resealed ghost pellet divided by the number of ghosts per ml in the incubation, as determined by Coulter counting.

To determine the onset of irreversible permeability, ghosts are preequi-

librated with hemoglobin and incubated at various temperatures without added salt. Aliquots are removed at intervals, 80 mM NaCl is added, and the ghosts are allowed to reseal for an additional 60 min. Resealing is assayed as before. During preliminary incubations at 30°C, proteolysis of bands 1 and 2 was observed. Phenylmethyl sulfonyl fluoride (PMSF) (0.1 mM) was therefore included in all incubations.

Syringes are used throughout to minimize inaccuracies due to viscosity; all solutions contain 0.02% NaN_3 or 1 mM EDTA to prevent bacterial growth. All incubations were done in new Falcon plastic tubes, which are free from Ca^{2+} contamination by atomic absorbance.

SDS-gel electrophoresis was done by the modified [6] procedure of Fairbanks et al. [7]. For quantitation of the amount of bands 1 and 2 (nomenclature of Fairbanks et al. [7]) released during low salt incubation, the incubated ghosts were centrifuged at 30 000 $\times g$ for 15 min and a measured aliquot of the supernatant was lyophilized. The protein was redissolved in a small volume (usually 100 μl) of 10 mM Tris, pH 8, 1% SDS, 20 mM dithiothreitol, 1 mM EDTA with bromphenol blue as tracking dye. The samples were electrophoresed until all the polypeptides except 1 and 2 eluted from the bottom of the gel. This is necessary to resolve bands 1 and 2 for scanning, especially when large amounts are loaded on the gel. To construct a calibration curve, known amounts of ghost membrane were electrophoresed in the same run. A plot of peak height of Coomassie blue stained gels from the Gilford gel scanner vs. packed ghost volume was linear for bands 1 and 2. The amounts of bands 1 and 2 released from the membranes could then be determined.

Results

Effect of divalent cations on resealing

White erythrocyte ghosts are permeable to solutes at least as large as hemoglobin. Addition of 40 mM NaCl and warming reseals these ghosts to macromolecules, but not to small molecules [2]. Divalent cations are more effective than monovalent cations in resealing ghosts (Fig. 1, open circles). At 30°C, 2 mM Mg^{2+} or 0.2 mM Ca^{2+} is as effective as 80 mM NaCl in resealing ghosts to hemoglobin.

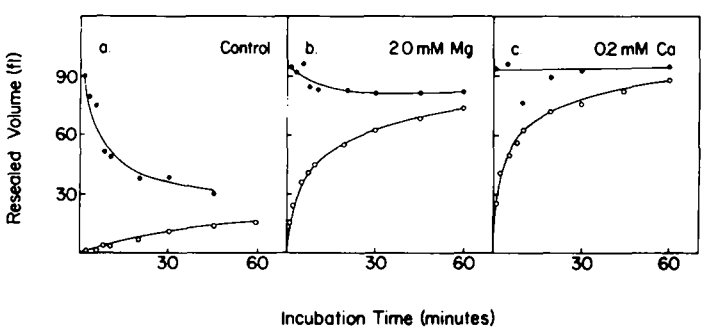

Fig. 1. Resealing or denaturation of washed ghosts. Tris-washed membranes were incubated in 5 mM Tris, 7 mM NaCl, pH 7.4 at 30°C, in the presence of hemoglobin as the marker for macromolecule resealing. At the times indicated, ghosts were assayed for resealing (○), or remaining ability to reseal after transfer to 80 mM NaCl (●). (a) control (b) 2.0 mM $MgCl_2$ in incubation (c) 0.2 mM $CaCl_2$ in incubation.

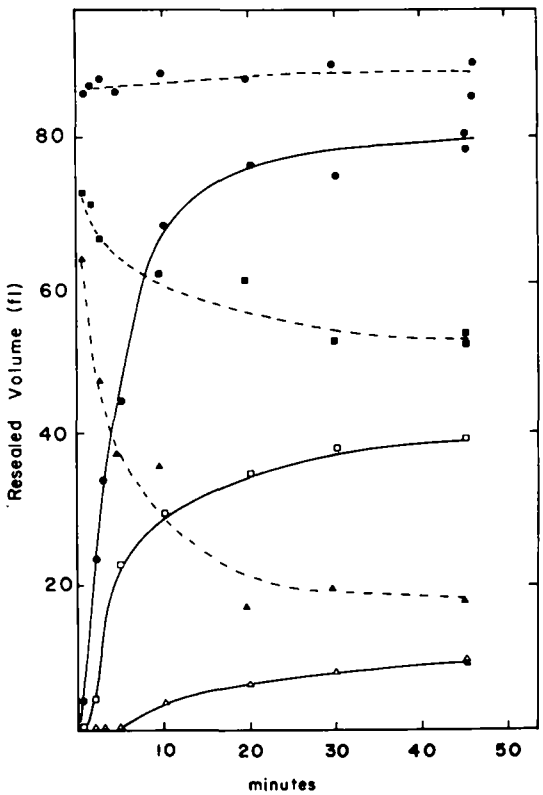

Fig. 2. Ionic strength dependence of resealing and denaturation. Tris-washed ghosts were incubated in 5 mM Tris, pH 7.4, with different amounts of NaCl: (△,▲) 10 mM; (⊏,■) 20 mM; (○,●) 40 mM. Resealing (open symbols) and loss of ability to reseal (closed symbols) were assayed as described in Methods.

Loss of resealing ability

Divalent cations have two distinguishable effects on ghost membrane resealing. They increase the rate of resealing and they prevent the onset of irreversible permeability. As noted earlier [2], ghosts do not reseal at 30°C in low ionic strength solutions. We have now observed that under these conditions, membranes also become irreversibly permeable (Fig. 1a, closed circles). As shown in Fig. 1b and c, Mg^{2+} and Ca^{2+} prevent this loss of resealing ability.

The temperature and ionic strength dependence of the onset of irreversible permeability was studied. Preliminary experiments showed that this loss does not occur in 80 mM NaCl, and the kinetics were determined by transferring incubated ghost aliquots into 80 mM NaCl at appropriate times. Fig. 2 shows the ionic strength dependence of both resealing and denaturation. It is of interest that the ionic strength dependence of the onset of irreversible permeability is the inverse of the ionic strength dependence of resealing. It appears that ghosts at 30°C necessarily either denature or reseal, depending on the ionic composition of the medium. We have been unable to find conditions in which warmed ghost membranes will neither reseal nor become irreversibly permeable.

The temperature dependence of the onset of irreversible leakiness (Fig. 3) was also similar to the previously determined temperature dependence of

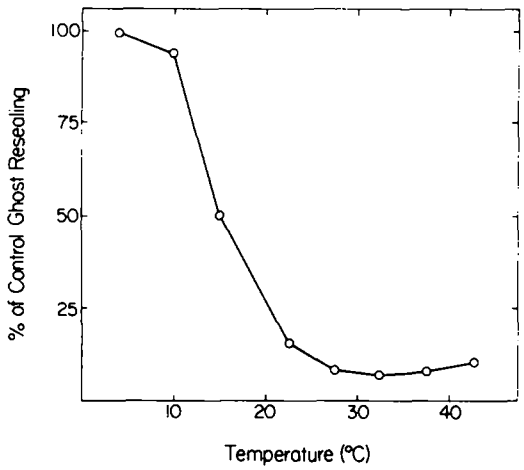

Fig. 3. Temperature dependence of the onset of irreversible permeability. Tris-washed ghosts were incubated in 5 mM Tris, 7 mM NaCl, 1 mM EDTA, pH 7.4 at 30°C. After 1 h of incubation, aliquots were transferred to 80 mM NaCl and the residual resealing ability was determined. The extent of resealing of ghosts kept in an ice bath was taken as 100%.

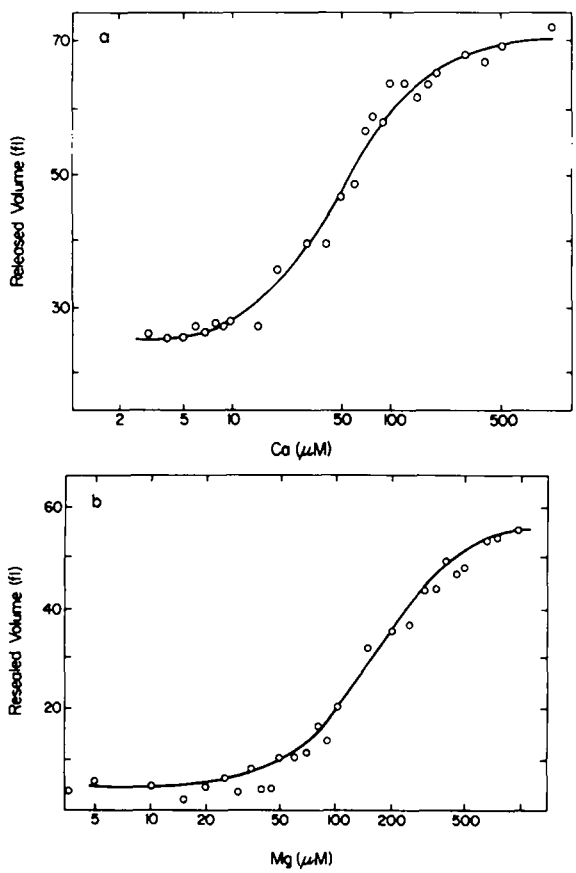

Fig. 4. Divalent cation concentration dependence of ghost resealing. Tris washed ghosts were incubated in 5 mM Tris, pH 7.4, 7 mM NaCl with the indicated amounts of Ca^{2+} or Mg^{2+}. Resealing after 6 min of incubation at 37°C was determined as described in Methods. (a) Ca^{2+} varied (b) Mg^{2+} varied.

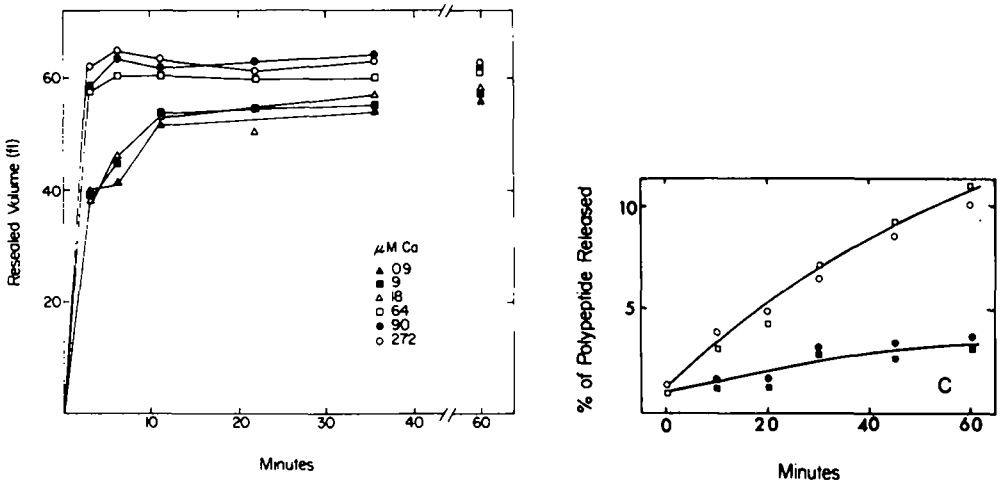

Fig. 5. Divalent cation concentration dependence of ghost resealing in high ionic strength. Tris-washed ghosts were incubated in 5 mM Tris, pH 7.4 80 mM NaCl, 37°C, with the indicated concentrations of Ca^{2+}. Resealing was assayed as described in Methods.

Fig. 6. Quantitation of polypeptides 1 and 2 released from incubated 5P8 ghosts. SDS gels were electrophoresed until all polypeptides except 1 and 2 ran off the gels, and the amounts of band 1 (○,●) and band 2 (□,■) was determined as described in Methods. The ghosts were incubated in 5P8 at 0°C (closed symbols) or at 30°C (open symbols).

resealing [2]. That is, ghosts at temperatures of 10°C or less do not reseal and do not become permanently leaky. At temperatures of 20°C or greater, however, ghosts lose the ability to reseal in 7 mM NaCl, but if the ionic strength is adequate [2] or divalent cations are present (Fig. 1), ghosts reseal. This results imply that at approximately 15°C, ghosts undergo a structural alteration such that they either recover their impermeability to macromolecules or become irreversibly leaky, depending upon the ionic composition of the medium.

Effective divalent ion concentrations

The concentration dependence of resealing on divalent cations was determined for Ca^{2+} and Mg^{2+} (Fig. 4). The half maximally effective concentrations were 40 μM Ca^{2+} and 200 μM Mg^{2+}. Since these experiments were performed in 7 mM NaCl, these concentrations represent the levels needed to prevent the onset of irreversible permeability. It is possible to show that Ca^{2+} and Mg^{2+} affect resealing rates even the presence of high NaCl levels. This is shown for Ca^{2+} in Fig. 5. Resealing rates increase between 18 and 64 mM Ca^{2+}, so that the effective Ca^{2+} concentrations are approximately the same in both high and low NaCl solutions.

Loss of spectrin in leaky membranes

It is important to point out that the observed loss of resealing ability is not due to simple fragmentation of the membranes. Ghosts in 5 mM Tris, 7 mM NaCl or in 5 mM phosphate, pH 8 (5P8) are stomatocytes [9], and their morphology is unchanged during the incubations. Vesiculation [7] or endo-

cytosis [10] is known to occur in erythrocyte membranes at 37° in low ionic strength solutions. This phenomenon is, however, very sensitive to ionic strength; e.g. membranes vesiculate in 0.5 mM sodium phosphate, but not in 5 mM sodium phosphate [7]. 7 mM NaCl affords sufficient ionic strength to prevent vesiculation.

Since membranes become irreversibly leaky at 30°C but not at 0°C in low salt, the proteins from ghosts incubated at these temperatures were compared. In the presence of 0.1 mM PMSF, no proteolysis occurs. A loss of spectrin (polypeptides 1 and 2) is observed, however (Fig. 6), in ghosts incubated at 30°C, which does not occur at 0°C. Many proteins are released from membranes by low ionic strength solutions at both 0 and 30°C (gesl not shown), but only spectrin release is increased at 30°C. In six experiments, the amount of spectrin lost in 60 min at 30°C varied from 5 to 15%. If Ca or Mg is present in the 30°C incubation, bands 1 and 2 are not released into the supernatant.

Temperature dependence of resealing

The observation of a temperature-dependent loss of resealing ability affects the interpretation of earlier results [2] on ghost resealing. It was reported that resealing rates increased with temperature; the extent of resealing, however, reached a plateau at 20°C and declined above 25°C. This was interpreted as a decrease in ghost volume above 25°C. However, the experimental protocol included a prewarming of the ghost preparation before mixing with salt and the impermeant marker; this permits heat induced loss of resealing ability. Redeterminations of the temperature dependence of resealing by a procedure which circumvents denaturation (Fig. 7) was therefore performed. The data for 20°C and below are the same as previously reported, but the total extent of resealing increases with temperatures above 20°C up to 40°C, where membranes undergo

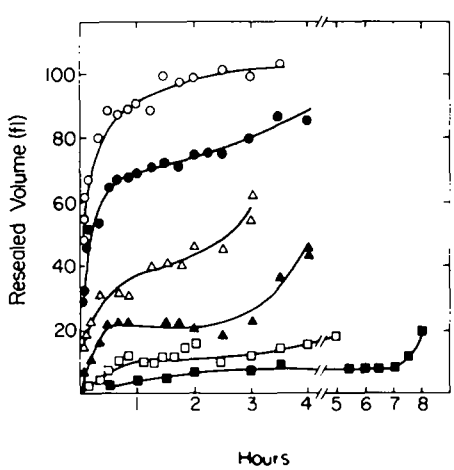

Fig. 7. Tris-washed ghost resealing as a function of temperature. Ghosts were resealed in 5 mM Tris, 140 mM NaCl, with hemoglobin as impermeant marker molecule. The procedure is described in Methods. Temperatures were (●) 15; (○) 20; (▲) 26; (△) 30; (■) 35; (□) 40°C. Kinetic runs were performed at 4 and 10°C, but are omitted since no resealing occurred.

fragmentation. The kinetics are, as noted before, biphasic, which is likely to be a consequence of ghost heterogeneity. The break in the Arrhenius plot for resealing [2] at 28°C is therefore attributable to the simultaneous occurrence of resealing and the onset of irreversible permeability.

Discussion

The principal observation here is that white erythrocyte ghosts can lose the ability to recover impermeability to macromolecules after relatively gentle treatments. This is not caused by vesiculation or proteolysis, but is accompanied by the specific loss of bands 1 and 2 from the membrane. Mono and divalent cations prevent protein loss and loss of resealing.

The influence of divalent cations, especially Ca^{2+}, on ghost resealing and permeability has been often studied. Usually, however, their effects on cation permeability have been observed, whereas this work describes resealing to macromolecules. There are differences in divalent cation effects on these two processes. For example, Bodemann and Passow [11] observed that the Ca^{2+} concentration dependence of cation resealing in unwashed ghosts was biphasic, reaching a maximum between 10 and 100 μM. Higher Ca^{2+} prevented resealing. By contrast, any Ca^{2+} concentration above 20 μM enhances macromolecule resealing. It seems possible that the biphasic effect on cation permeability reflects Ca^{2+} interaction with two distinct components of the membranes. At low levels (about 50 μM) Ca^{2+} interacts with a protein component, perhaps spectrin, bringing about membrane closure to both cations and macromolecules. Higher Ca^{2+} concentrations, however, affect K^+ permeability, inducing a specific K^+ leak [12,13], perhaps by interacting with the sodium transport system [14]. As observed, then, high Ca^{2+} would not diminish impermeability to macromolecules. Although Ca^{2+} and Mg^{2+} are antagonists in their effect on K^+ permeability [15], they have similar effects on macromolecule impermeability.

A recent paper by Bennett and Branton [16] describes some of the factors that control spectrin binding to the inner surface of the membrane. Many of the factors that promote spectrin binding and resealing to macromolecules are similar. For example, spectrin binding is promoted by an increase in KCl concentration from 0 to 20 mM; an ionic strength of about 35 mM is required for ghost resealing [2]. In the absence of KCl, Mg^{2+} or Ca^{2+} will permit spectrin binding; as shown here, macromolecular resealing in low salt solutions is brought about by low concentrations of Mg or Ca^{2+}. If spectrin is incubated at temperatures above 40°C, it will no longer bind to the ghost membrane; we have observed that ghosts heated above 40°C will not reseal. Taken together, these correlations suggest that spectrin must be bound to the cytoplasmic surface of the erythrocyte ghost membrane for resealing to macromolecules to occur.

It is unlikely that the loss of spectrin itself causes the onset of irreversible leakiness. Comparison of Fig. 1a and Fig. 6, for example, shows that resealing ability is lost more rapidly than spectrin. The more likely picture is that an alteration occurs in ghosts incubated in low salt, perhaps in spectrin or its binding site, which leads to both irreversible permeability and the loss of

spectrin. Moreover, the temperature dependences of resealing (Fig. 7) and of denaturation (Fig. 5) are similar; this suggests that at about 15°C, ghost membranes are converted to a state that will reseal if ionic conditions are appropriate, or will lose spectrin and become permanently leaky if ionic strength and divalent cation concentrations are low. There are some indications from spectroscopic evidence [17,18] that a structural change occurs in erythrocyte membranes around 15°C, and study of the structure of spectrin or its interactions with the membrane as a function of temperature in this range might be rewarding.

Acknowledgements

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