Shape transformation of erythrocyte ghosts 
depends on ion concentrations

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Resealed human erythrocyte ghosts undergo shape transformations similar to those of intact erythrocytes. The results indicate that the shape of these ghosts depends on the inner as well as on the outer NaCl concentration. A correlation between shape and calculated transmembrane potential was established similar to that for intact human erythrocytes.

Human erythrocyte ghosts undergo shape transformations similar to those of intact erythrocytes (1,2). The shape of unsealed erythrocyte ghosts is strongly affected by the ionic concentration of the medium (2,3). In the case of intact erythrocytes it was shown that the shape does not depend on the inside or on the outside cation concentration alone, but on the transmembrane potential (4,5). Increasing the transmembrane potential by changing the ionic concentration of the suspension medium or the cation content of the cytoplasm, a continuous echinocyte-discocyte-stomatocyte transformation was established. The molecular mechanism of this morphological alteration is unclear. There is some evidence that direct electric field effects on membrane constituents could be involved (6,7).

Keeping in mind the possible regulative importance of the transmembrane potential in development and maintenance of cellular function, this subject is of general interest. In the present paper the influence of extra- and intracellular ionic concentration, as well as of transmembrane potential, on the shape of resealed ghosts was investigated. Ghosts are a useful tool, since resealing of the membrane and restoration of ion permeability properties open the possibility of creating well-defined intracellular and extracellular ionic conditions.

Materials and Methods

Preparation of resealed ghosts

Human erythrocytes (type O Rh⁺) from different donors from a blood bank were used. The blood was stored in ACD medium at 4°C for not longer than 4 d after sampling. After centrifugation at 500 g and removal of the supernatant plasma and buffy coat, the cells were
washed twice (1500 g, 10 min) with unbuffered saline solution (156 mM NaCl; the pH was adjusted to 7.4 by adding NaOH). Resealed ghosts were prepared according to the procedure of Bodemann and Passow (8). All preparation steps were performed at 0°C. 1 vol. of red cell suspension (hematocrit 50%) was hemolyzed in 40 vol. of 4 mM MgSO₄ (by adding acetic acid in appropriate amounts the pH was adjusted to be 6.0 ± 0.1 in the final hemolysate. After 5 min incubation, buffered concentrated NaCl solution was added to establish the desired final intracellular NaCl concentration. For equilibration with the solute the suspension was held at 0°C for about 10 min. Ghosts with the following inside NaCl concentrations were prepared: 147 mM NaCl (hereafter called 'ghosts-147'), 99.7 mM NaCl ('ghosts-100') and 36.7 mM NaCl ('ghosts-37'). In this and all other cases in which solutions of reduced NaCl concentrations were used, an osmolarity of 290 mOsmol was maintained by adding sucrose in appropriate amounts. If not stated otherwise the pH was 7.4 (5.8 mM phosphate buffer). After resealing of the ghosts by incubation at 37°C for 45 min the suspension was washed three times in solutions of the same NaCl and sucrose concentrations as inside the ghosts. Enrichment in resealed ghosts was performed by sucrose density centrifugation (9).

Unsealed ghosts were prepared as described by Dodge et al. (10). Polyacrylamide gel electrophoresis of ghost membrane proteins was performed according to the method of Weber and Osborn (11) using 5% acrylamide gels (pH 7.2).

Evaluation of shape of erythrocyte ghosts

Shape investigations of ghosts were performed by dark field microscopy with a magnification of 640 x. Ghosts were suspended in solution of desired NaCl concentration (final hematocrit lower than 3%) at room temperature. After 5 min incubation the suspension was placed on a coverslip. After a further 3 min, the shape of the ghosts was evaluated within 1 min. Shapes were determined independently by different persons.

Preserving the shape by adding glutaraldehyde was avoided, because in that case irregular forms were observed. Because the forms of ghosts resemble those of normal erythrocytes, the shape index proposed by Glaser (4) was used for rapid quantification of the different shapes. The index estimation differentiates only between stomaticytes, echinocytes and discocytes (for details see Glaser (5)): shape index SI = 4 means 'exclusively stomaticytes', SI = 0 means 'exclusively discocytes' or a symmetric distribution around them and SI = -4 corresponds to 'exclusively echinocytes'.

Electrophoretic mobility measurements of ghosts

The electrophoretic mobility of intact erythrocytes and unsealed as well as resealed ghosts were measured by means of a cytosphrometer (Opton, FRG) at 20°C.

Calculation of transmembrane potential

The transmembrane potential of resealed ghosts was calculated according to the model already published in detail (5,12). This model, originally developed for intact human erythrocytes, can be applied to
resealed ghosts under certain conditions. The so called 'C-state' in this model, which is comparable to the situation of resealed ghosts due to their low rate constant of Na\(^+\)-efflux (13), assumes that the distribution of water, chloride, and the pH inside and outside are determined by the thermodynamic equilibrium which depends on the cation gradient across the cells as well as on the intracellular concentration of hemoglobin. The transmembrane potential was calculated by taking into account the conditions of electroneutrality, the pH dependence of the hemoglobin charge and the equilibration of osmotic pressure. Because of the dilution of cytoplasmic haemoglobin during hemolysis, a concentration in resealed ghosts of about 0.15 mM was assumed. As shown previously for erythrocytes there are only small deviations between calculated and measured values, indicating that the model accurately describes the real situation (14).

Results

Characterization of resealed ghosts

For temporarily maintaining a stable cation concentration gradient as well as a transmembrane potential across the human erythrocyte ghost membrane, a successful restoration of cation permeability with regard to Na\(^+\) is necessary. As shown in more detail in an accompanying paper (13), the measured rate constants of \(^{22}\text{Na}\(^+\)-efflux of ghosts at various extra- and intracellular ionic concentrations are similar to those of intact human erythrocytes (13,15; e.g. at 147 mM NaCl in the external medium the rate constants are 1.75 ± 0.71 · 10\(^{-3}\) min\(^{-1}\) for intact erythrocytes (ouabain insensitive) and 1.91 ± 0.24 · 10\(^{-3}\) min\(^{-1}\) for resealed ghosts at 147 mM NaCl intracellular concentration. Keeping in mind that the shape was determined within 9 min after transferring the ghosts to the desired medium, and that the rate constant of \(^{22}\text{Na}\(^+\)-efflux is of the same order as reported for intact erythrocytes, the model mentioned for calculating the transmembrane potential seems to be also applicable in the case of resealed ghosts.

The electrophoretic behaviour of unsealed ghosts corresponds qualitatively to that of intact erythrocytes (Fig. 1). A similar increase in electrophoretic mobility of all types of resealed ghosts was observed when the NaCl concentration outside was lowered; this suggests that the glycocalyx structure is not influenced by the resealing process in different media. The values are smaller than those for intact erythrocytes or for unsealed ghosts, perhaps because of an adsorption of Mg\(^{2+}\) ions in regions of the glycocalyx near the membrane surface similar to the adsorption of La\(^{3+}\) (16).

No differences in the pattern of membrane proteins (localization of bands and their relative intensity) between the different ghost types were observed by polyacrylamide gel electrophoresis (not shown).

Shape of resealed ghosts

The shape of resealed ghosts is similar to that of intact erythrocytes. In Fig. 2 the shape index SI of different ghost types is shown as a function of the outer NaCl concentration. Spherical echinocytes correspond to type III echinocytes according to the nomenclature of Bessis (17). Typical stomatocytes (type II) occurred at a shape index
Fig. 1. Dependence of electrophoretic mobility on extracellular NaCl concentration (pH 7.4): intact human erythrocytes (●), unsealed ghosts (▼) and resealed ghosts (○, ghosts-147; □, ghosts-100; △, ghosts-37). The measurements were performed at 20°C. In all cases the standard deviation was less than 3% of the average.

Fig. 2. The shape index, SI, of unsealed ghosts (▼) and resealed ghosts (○, ghosts-147; □, ghosts-100; △, ghosts-37) as a function of the extracellular NaCl concentration at 21°C. The standard deviation of the estimate of the shape index is presented for only one case, for reasons of clarity. At least 5 independent measurements were performed.
of about $SI = 0$. At higher shape indices the stomatocytes become more spherical (type I sphero-stomatocytes). The transition from stomatocytes to echinocytes occurs at a higher outside NaCl concentration when the salt content inside was increased. The shape behavior of unsealed ghosts was quite different from that of the resealed ghosts (Fig. 2). In this case only a slight dependence of the shape index on NaCl concentration was observed. When the shape index was plotted as a function of transmembrane potential, amazingly good agreement in the shape behavior of the different types of resealed ghosts was found (Fig. 3). At negative potentials the ghosts were preferentially echinocytes, whereas at positive potentials mainly stomatocytes were observed.

Performing linear regression analysis in the range from $\Delta\psi = -10$ mV to $\Delta\psi = 20$ mV gives a cross-over point ($SI = 0$) at 6 mV. These results are similar to those obtained in the case of intact erythrocytes (5) where the echinocyte-stomatocyte transition is somewhat more gradual (cross-over point at 25 mV).

Discussion

The first experiments on the effects of cation concentration on the shape of unsealed ghosts were carried out by Sheetz (3). In agreement with him, Lange et al. (2) suggested that the crenation of unsealed ghosts on increasing ionic concentration of the medium is caused by a stronger contraction of the more highly charged inner leaflet of the erythrocyte membrane than of the outer leaflet ('bilayer-couple' hypothesis). Furthermore, a redistribution of the lipids between the two leaflets resulting also in a net transfer of lipids was assumed to affect the membrane contour.

![Fig. 3. The shape index $SI$ of resealed ghost (O, ghosts-147; □, ghosts-100; Δ, ghosts-37) as a function of the transmembrane potential. The values of $SI$ presented were taken from Fig. 2.](image-url)
The dependence of the shape of resealed ghosts on the inner and outer NaCl concentration cannot be explained on the basis of this 'bilayer-couple' model. In that case the transition from stomatocytes to echinocytes should be shifted to a higher outside NaCl concentration when the ionic concentration inside the ghosts is lowered. However, our observations are in striking contrast to this. Assuming that the expansion as well as the contraction of the outer leaflet also plays an important role in shape transformation, the curves in Fig. 2 should also be reflected symmetrically on the abscissa. However, there are some doubts on this point. The shape of intact human erythrocytes as well as of white ghosts is maintained after treating the cells with neuraminidase (1,5). This suggests that the structure of the glycocalyx and the outer surface potential are not involved in shape transformation. Furthermore, the electrophoretic mobility measurements presented indicate no differences in the glycocalyx structure or the surface potential of the various resealed ghosts.

Johnson et al. (1) reported that with increasing ionic concentrations there was a reversible aggregation of the spectrin-actin lattice of unsealed ghosts, accompanied by crenation of the ghosts. This supports the rigidity pattern hypothesis proposed by us earlier (18). From these results, however, we conclude that the lateral organization of the network is not the dominant factor in echinocyte formation, because in that case the transformation from stomatocytes to echinocytes should be hindered by a low inside NaCl concentration.

Our data indicate clearly that the shape of resealed ghosts depends on the inner as well as on the extracellular NaCl concentration, as in intact human erythrocytes (5). The question of whether the similarity in shape behavior in response to the transmembrane potential is only an expression of a correlation between shape and membrane potential or is based on a causal relation remains open. This is related to the lack of a satisfactory theory explaining the mechanism of erythrocyte shape as well as to a poor understanding of the influence of the electric field on membrane structure and properties, especially at the molecular level. Also, we are aware that we have to distinguish carefully between transmembrane potential and the actual field in the membrane (20). As mentioned above there is some experimental evidence that the electric field influences the structure of membrane components (6,7). A simple dependence of the shape on the ratio of extra- to intracellular ionic concentration seems to be less probable because in this case the shape of unsealed ghosts should resemble those of resealed ghosts at a transmembrane potential of about 0 mV (Fig. 3).

It is necessary to point out that our results do not exclude the applicability of the 'bilayer-couple' mode to explain the shape of unresealed ghosts, as described by Lange et al. (2). The absence of divalent cations (2), which have a higher binding constant to phosphatidylserine than do monovalent cations, might enhance the sensitivity of the inner leaflet to cation concentration. This assumption is not supported by our experiments with unsealed ghosts. Aside from a slight shape dependence on salt concentration, only a small fraction of ghosts were found to be echinocytes at higher NaCl concentrations. The reasons for this contradiction are unknown. It could be caused by differences in the preparation of ghosts (3),
SHAPE OF ERYTHROCYTE GHOSTS

preserving the cytoskeleton more or less perfectly. Also the high phosphate buffer concentration, the higher pH (8.0) and the glutaraldehyde fixation used by Lange et al. (2), as well as temperature effects on cell shape (3,4), have to be taken into account. As pointed out above, we found irregular forms when glutaraldehyde was added.

Furthermore, there are structural differences between resealed and unsealed ghosts. A partial loss of asymmetric distribution of phospholipids (20) as well as of the heterogeneity of fluidity between the inner and outer leaflet (21) were reported for unsealed ghosts.

References