Influence of the intracellular and extracellular cation concentration on monovalent cation efflux of resealed human erythrocyte ghosts

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Tracer efflux measurements (\(^{86}\)Rb\(^+\) and \(^{22}\)Na\(^+\)) were performed on resealed human erythrocyte ghosts at different intra- and extracellular NaCl concentrations. Using a modified Goldman equation the observed alterations of the rate constants could be explained by taking into account the transmembrane and surface potentials, at constant permeability coefficient. These results emphasize the importance of membrane surface potentials in triggering ion transport across biological membranes.

The passive effluxes of K\(^+\) and Na\(^+\) from human erythrocytes are enhanced in isotonic solutions of low ionic strength (1-4). The increased K\(^+\) efflux was explained by Donlon and Rothstein (5) as a transmembrane potential-dependent change of the K\(^+\) permeability. The influence of surface potentials was not considered in that study. However, Wilbrandt (6), and Wilbrandt and Schatzmann (7), pointed out a possible role of membrane surface potentials in the increased K\(^+\) efflux at low ionic strength. As recently demonstrated by our group (8), the dependence of passive Rb\(^+\) efflux from human red blood cells on the extracellular ionic strength can be accounted for, at a constant permeability, by a modified Goldman equation that takes into account the surface potential of both leaflets:

\[
J_{i0} = \frac{P \cdot F \cdot (\Delta \psi_i - \psi_0)}{R \cdot T} \cdot \frac{c_i \cdot \exp(F \cdot (\Delta \psi_i)/R \cdot T)}{\exp(F \cdot (\Delta \psi_i)/R \cdot T - \exp(F \cdot \psi_0/R \cdot T)}
\]

where \(J_{i0}\) = efflux of an ionic species, \(P\) = permeability coefficient, \(c_i\) = intracellular concentration of the respective ion, \(\Delta \psi\) = transmembrane potential and \(\psi_i\) and \(\psi_0\) are the interior and exterior surface potentials, respectively. \(z, F, R, T\) are the usual charge number, Faraday constant, gas constant and temperature. It was argued that a relatively high negative interior surface potential is responsible for the increased rate constant observed at low ionic strength.

The aim of this paper is to extend these investigations using resealed human erythrocyte ghosts. Because of the restoration of cation permeability it is possible to modify the interior and exterior
surface potentials, as well as the transmembrane potential, by changing the ionic strength inside and outside. From the modified Goldman equation one would expect an increasing passive cation efflux from ghosts in response to a raised intracellular ionic strength (8), because in that case the interior surface potential becomes less negative and the transmembrane potential is enhanced (more positive).

**Materials and Methods**

Human erythrocytes of group O Rh+ from whole blood ACD preserves were used not later than 4 d after sampling (storage at 4°C). Resealed ghosts were prepared according to the procedure of Bodemann and Passow (9) as described in the accompanying paper (10). Ghosts with the following intracellular NaCl concentrations were prepared: 147 mM NaCl (hereafter called 'ghosts-147'), 99.7 mM NaCl ('ghosts-100') and 36.7 mM NaCl ('ghosts-37'). The NaCl concentrations given correspond to the concentration of the resealing media. Isotonicity was maintained by sucrose. The pH was 7.4 (5.8 mM phosphate buffer).

**22Na+- and 86Rb+-efflux measurements**

After a 2 h incubation in the presence of 86RbCl (1.8-2.2 MBq/ml) or 22NaCl (1.1-1.5 MBq/ml), the suspension of resealed ghosts (hematocrit 30%) was put into a diffusion chamber (vol. 0.1 ml) which was closed by a membrane filter (Sartorius, pore size 0.3 μm) and was continuously flushed with nonradioactive solution. The chamber was mounted perpendicular on a slowly rotating axis (12 r.p.m.) to prevent sedimentation. The time course of efflux was followed by continuously measuring the radioactivity of ghosts with a scintillation counter. All steps were performed at 37°C. The measuring procedure and equipment are described in detail elsewhere (8).

A typical experiment is shown in Fig. 1. To begin with, the resealing medium of the ghosts was used as inactive flushing solution; this was replaced after 100 min by a solution of a different NaCl concentration. In a number of experiments the initial suspending medium was finally used again for checking the reversibility of the observed rate constant changes. The fast compartment at the beginning of the experiment corresponds to the extracellular compartment in the chamber.

**Calculation of transmembrane potential**

The transmembrane potential of resealed ghosts was calculated according to the model already published in detail (11,12). This model, originally developed for intact human erythrocytes, can be applied also 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 (see Results), assumes that the distribution of water, chloride and intracellular and extracellular pH are determined by the thermodynamic equilibrium, which is set by the cation distribution across the membrane as well as by the concentration of hemoglobin inside. The
transmembrane potential could be 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 the cytoplasmic content during hemolysis, a concentration of hemoglobin in resealed ghosts of about 0.15 mM was assumed. As shown for erythrocytes previously, there are only small deviations between calculated and measured values, indicating that the model accurately describes the real situation (8,11,12).

Results

Characterization of resealed ghosts

Depending on the state of resealing, three types of ghost are distinguished (13): (1) type I ghosts, which reseal immediately after hemolysis; (2) type II ghosts, which can be loaded with the desired solutes and which reseal during the 37°C incubation; (3) type III
ghosts, which do not reseal. Because of the low percentage of type I ghosts (15) we distinguished only between leaky (type III) and resealed (type II) ghosts. An increase of the percentage of leaky ghosts was found by lowering the NaCl concentration inside, which is in agreement with the results of Lieber and Steck (14) (61.6 ± 4.4% resealed ghosts-147 and 49.7 ± 2.5% resealed ghosts-37). An enrichment in resealed ghosts could be achieved by centrifugation on a sucrose cushion (15). A significant effect of the sucrose cushion on the $^{22}\text{Na}^+$-efflux rate was not detected (e.g. for ghosts-147: $k = 1.82 \pm 0.01 \times 10^{-3}$ min$^{-1}$ without density centrifugation and $k = 1.92 \pm 0.06 \times 10^{-3}$ min$^{-1}$ after density centrifugation; t-test, error probability 1%). Storage of the ghosts for 4 d resulted in a slight but not significant increase of the $^{22}\text{Na}^+$-efflux rate constant, but in a significant increase of percentage of unsealed ghosts. The band pattern of membrane proteins obtained by SDS polyacrylamide gel electrophoresis shows no differences between ghosts-147, -100, and -37 (10). Furthermore, no differences of the electrophoretic mobility between the different ghosts were observed in similar external media (10), suggesting that the glycocalyx structure is not influenced by the resealing process in different media.

**Dependence of the $^{22}\text{Na}^+$- and $^{86}\text{Rb}^+$-efflux rate constant on the extracellular NaCl concentration**

The dependence of the rate constant of $\text{Na}^+$ efflux on extracellular NaCl concentration for ghosts with different intracellular ionic strength is presented in Fig. 2. For comparison the rate constant of

![Fig. 2. The Na$^+$ efflux rate constant of ghosts-147 (O), -100 (□), -37 (△) and erythrocytes + ouabain (8) (●) as a function of extracellular ionic strength. pH 7.4. T = 37°C. Osmolarity = 290 mOsM. The standard deviation of the rate constant is presented for ghosts-147 and -37 only for the sake of clarity.](image-url)
The passive, ouabain-insensitive Na\(^+\) efflux of intact human erythrocytes is shown (8). It can be seen that the rate constants of the resealed ghosts are of the same order as those measured on intact erythrocytes, indicating a successful resealing procedure. It is obvious that, similar to intact erythrocytes, the Na\(^+\) efflux of ghosts is enhanced by lowering the extracellular NaCl concentration. Furthermore, Na\(^+\) efflux is reduced by decreasing the intracellular NaCl concentration. The ouabain-insensitive Na efflux of intact erythrocytes consists of a furosemide-sensitive Na\(^+\)-K\(^+\) cotransport and a passive Na\(^+\) efflux (16). A furosemide-sensitive Na\(^+\)-K\(^+\) cotransport in resealed ghosts could not be detected (Table 1).

As with Na\(^+\) efflux, the Rb\(^+\) efflux rate constant was raised by decreasing the extracellular NaCl concentration or by increasing the intracellular concentration (Table 2). For comparison, the Rb\(^+\) efflux rate constants of intact erythrocytes at 147 mM and 5.7 mM extracellular (NaCl + KCl) concentration are \(0.81 \pm 0.09 \cdot 10^{-3}\) min\(^{-1}\) and \(6.36 \pm 0.42 \cdot 10^{-3}\) min\(^{-1}\), respectively (8). Although the rate constants of ghosts are higher, they are of the same order as those from intact erythrocytes.

### Table 2. Rb\(^+\) efflux rate constant (k) for ghosts-147 and -37, at 147 mM and 5.7 mM NaCl concentration outside

<table>
<thead>
<tr>
<th>Extracellular NaCl concn. (mM)</th>
<th>k \cdot 10^3 (min(^{-1})) for ghosts-147</th>
<th>k \cdot 10^3 (min(^{-1})) for ghosts-37</th>
</tr>
</thead>
<tbody>
<tr>
<td>147.0</td>
<td>(2.39 \pm 0.25) (5)</td>
<td>(1.50 \pm 0.12) (5)</td>
</tr>
<tr>
<td>5.7</td>
<td>(23.01 \pm 2.87) (6)</td>
<td>(5.75 \pm 0.60) (5)</td>
</tr>
</tbody>
</table>
Discussion

It is thought that the $^{22}$Na$^+$-efflux rate constant measured on resealed ghosts reflects a purely passive transport. This conclusion was drawn from the following points: (1) no Na$^+$-K$^+$ cotransport is likely because of the low inside concentration of K$^+$ and the absence of K$^+$ in the external medium; (2) due to the negligible ATP concentration an active Na$^+$ transport can be ruled out.

As with intact human erythrocytes, decreasing the extracellular NaCl concentration resulted in an increase of the $^{22}$Na$^+$-efflux rate constant for all ghost types. According to the modified Goldman equation, this could be caused by an increase of the transmembrane potential, as well as by a more negative exterior surface potential, without any alteration of the permeability constant. For instance in the case of ghosts-147, when the external NaCl concentration is lowered from 147 mM to 5.7 mM the trans-membrane potential increases from about -1 mV to about +85 mV ($T = 37^\circ$C), according to the model of Glaser (11,12). In the same concentration range the negative outer surface potential shifts from about -3 mV (147 mM NaCl) to about -21 mV (5.7 mM NaCl), calculated according to the model of Donath and Pastushenko (17) and assuming a surface area of 137 $\mu$m$^2$, 2 $\cdot$ 10$^7$ negative charges per ghost and a glycolcalyx thickness of 5.5 nm, similar to intact erythrocytes (18).

Using the relation $J = k \cdot c \cdot V/A$ ($V =$ ghost volume; $A =$ membrane surface area) we fitted the experimental data obtained with ghosts-147 to the modified Goldman equation by varying the permeability coefficient and the interior surface potential; a ghost volume of 55 $\mu$m$^3$ (19) and a cell surface of 137 $\mu$m$^2$ were assumed. Transmembrane potential and exterior surface potential were estimated as mentioned above. The exterior surface potential of the different ghost types are similar at various ionic strengths, as indicated by electrophoretic measurements (10). The best agreement with the modified Goldman equation was obtained with a permeability coefficient of about 7.2 $\cdot$ 10$^{-11}$ m/s and an interior surface potential of about -87 mV. A comparison of measured with estimated rate constants is given in Table 3. The agreement between these values is obvious. Using a similar approach, a permeability coefficient of 1.3 $\cdot$ 10$^{-10}$ m/s for Rb$^+$ efflux of intact human erythrocytes was determined (8). Furthermore, Bernhardt & Gröger estimated a permeability coefficient of 6.5 $\cdot$ 10$^{-11}$ m/s for the true passive Rb$^+$ efflux of intact erythrocytes after inhibition of the Na$^+$-K$^+$ cotransport by furosemide (unpublished results). This value is similar to our fitted value.

Comparing the rate constants of the different ghosts at the same extracellular NaCl concentration, a decrease of these values is observed by lowering the internal NaCl concentration. This is to be expected, since a reduced transmembrane potential and a more negative interior surface potential result in a lowered efflux according to the modified Goldman equation. For instance, the transmembrane potential of ghosts-37 is about -35 mV at 147 mM NaCl outside and +48 mV at 5.7 mM NaCl outside. These values are smaller than those of ghosts-147 under comparable situations (see above). Fitting the experimental values of the rate constant of ghosts-100 and ghosts-37 to the Goldman equation using the values given above, and a perme-
Table 3. Measured ($k_m$) and calculated ($k_c$) rate constants of Na⁺ efflux in solutions of different NaCl concentrations for ghosts-147

<table>
<thead>
<tr>
<th>Extracellular NaCl concn. (mM)</th>
<th>$k_m \cdot 10^3$ (min⁻¹)</th>
<th>$k_c \cdot 10^3$ (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>147.0</td>
<td>1.91 ± 0.24</td>
<td>1.52</td>
</tr>
<tr>
<td>99.7</td>
<td>2.02 ± 0.27</td>
<td>1.98</td>
</tr>
<tr>
<td>54.7</td>
<td>2.50 ± 0.62</td>
<td>3.25</td>
</tr>
<tr>
<td>36.7</td>
<td>4.77 ± 0.66</td>
<td>4.45</td>
</tr>
<tr>
<td>5.7</td>
<td>15.20 ± 1.96</td>
<td>15.25</td>
</tr>
</tbody>
</table>

ability coefficient of 7.2 $\cdot 10^{-11}$ m/s, interior surface potentials of about -91 mV (ghosts-100) and -103 mV (ghosts-37) were obtained. The shift of the negative surface potential inside corresponds qualitatively to the drop of the NaCl concentration inside. In view of the preferential location of phosphatidylserine at the inner leaflet (20) and the high negative charge density of spectrin (21), it is reasonable to assume a high interior surface potential.

To summarize, the dependence of the rate constants of $^{22}$Na⁺-efflux on extra- and intracellular NaCl concentration can be explained on the basis of a constant permeability coefficient by taking into account the surface, as well as the transmembrane, potential. This is in contrast to using the original Goldman equation, which takes into account only the transmembrane potential, in which case the permeability coefficient appears to increase at lower external ionic strength (not shown). These results emphasize the importance of membrane surface potentials in triggering ion transport across biological membranes.

As pointed out previously (8) the method allows only a rough approximation because of the strong correlation between the two parameters fitted. However, the difference between the theoretical and experimental values was in all cases less than 1.5%. This deviation was estimated using $D = (k_i - k_i')^2 / (k_i - k)^2$ (8) where $k_i$ is the measured rate constant, $k$ the average rate constant and $k_i'$ the calculated rate constant according to equation (1) using the best-fit values of the permeability coefficient and the interior surface potential.

The dependence of the passive Rb⁺ efflux rate constant on the intra- and extracellular NaCl concentrations is similar to that of $^{22}$Na⁺ and supports the conclusion drawn from the Na⁺ efflux experiments. We cannot exclude the possibility that structural membrane alterations are also involved in the dependence of the rate constant on the NaCl concentration. A membrane reorganization could be caused by the hemolyzing and resealing procedure itself as well as by the decreased ionic concentration of the resealing medium. However, as shown by Haest (22) and by Tanaka and Ohnishi (23), a loss of asymmetric distribution of phospholipids, as well as a loss of heterogeneity of membrane fluidity between the two leaflets, can be avoided if ghosts are resealed carefully.
References

2. LaCelle PL & Rothstein A (1966) J. Gen. Physiol. 50, 171-188.