Effect of Preparative Procedures on Ghostcells from Bovine Erythrocytes

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Several methods for the preparation of “white ghosts” or “resealed ghosts” were described in the recent literature. This article compares three methods to prepare white, resealed ghosts from bovine erythrocytes based on the principle of hypotonic lysis. The methods described differ by the removal of hemoglobin from the empty cells. The main difference between the standard centrifugation, the gelfiltration and the hollow-fibre diafiltration is the mechanical stress on the leaky membranes after swelling in hypotonic media. Mean cellular volumes, rates of potassium-efflux and the access of impermeable dyes to cytoplasmatic proteins are criteria to differentiate between ghostcell-populations.

Introduction

For a number of physical studies on membrane phenomena of erythrocytes the deeply coloured hemoglobin has to be removed. The resultant “empty” particles surrounded by the membrane envelope are called “ghosts”. The loading and subsequent separation of the loaded red blood cells from extracellularly administered substances is also important. In recent years efforts were made to prepare ghostcells from erythrocytes without damaging the membrane. Cells were obtained under conditions which guarantee a population of tightly resealed ghosts [1, 2]. The principal method described by several authors is based on hypotonic lysis followed by several washing-steps to remove the red blood cell dye in hypotonic media. Alternative methods for the preparation of ghosts in isotonic media [3, 4] or alternative possibilities for the removal of hemoglobin [5] are described. Recent investigations on membrane resealing and on properties of the ghost cells [6, 7] point out, that earlier assumptions on the resealing time of erythrocyte membranes in hypotonic media should be revised, i.e. the ghost-membranes begin to reseal even in hypotonic media. For flux- or permeation-experiments not only the intactness of the cell membranes is essential but also the volume to surface ratio should be known in order to determine permeability-coefficients. In addition to studies on erythrocyte membranes versatile methods should be available for the removal of extracellular material if erythrocytes are used as transport vehicles for encapsulated drugs.

Methods and Results

In our laboratory the water-exchange and the rate of pH-equilibration across membranes were measured on populations of ghostcells from human and bovine erythrocytes [8, 9]. The ghost cells were prepared by three methods:

i. standard centrifugation method (e.g. [10, 11]); lysis in hypotonic buffer (25 mosm) and several washing steps at low temperature with a maximal rca of 14,000 x g followed by the restoring of physiological ionic strength and incubation at 37 °C;

ii. a gelfiltration method; cell membranes and hemoglobin are separated by gelchromatography; the combined membrane fractions are treated as described above;

iii. a hollow-fibre diafiltration method where hemoglobin is removed with the filtrate while cells (cell membranes) circulate through bundles of hollow-fibres.

The following buffers were used for the preparation procedures: potassium phosphate 5 mM, pH 7.4 (hypotonic, buffer A), potassium phosphate 5 mM, 150 mM NaCl, pH 7.4 (isotonic, buffer B). Fresh bovine blood (anticoagulated with Na-citrate) was...
obtained from the local slaughter-house. After washing in isotonic buffer (buffer B) by low speed centrifugation periods the red blood cells were lysed in buffer A (final osmolarity 25 mosM). All preparation steps were performed in the cold-room at 4 °C. In the routine centrifugation method hemoglobin was removed by 5 successive centrifugation steps in hypotonic buffer at 14,000 x g. After restoring of physiological salt concentrations resealing was completed for all samples by an incubation period of 1 h at 37 °C.

The gel filtration method was performed using Sephacryl S-1000 columns (Pharmacia) with a total volume, $V_t$, of 150—300 ml. No lysis of intact erythrocytes was detectable when they were passed through the column in isotonic phosphate-buffered saline. Fig. 1A shows the separation of cell membranes from hemoglobin (preparative scale; low resolution). Flow rates of 0.2—1.0 ml/min were useful for a baseline-separation of the hemoglobin from the membrane-fractions. In Fig. 1B eluation-profiles of intact erythrocytes in isotonic saline and NaCl (for the determination of the entrapped solvent volume) are superimposed on a chromatogram for the fractionation of erythrocyte-ghost from hemoglobin. The void-volume $V_0$ at about 95—100 ml contains the membrane- and erythrocyte-peak, while $V_1$ (solvent volume) at 190—200 ml includes the hemoglobin- and NaCl-fractions. Ghost-cells were measured by turbidity, hemoglobin by absorption spectroscopy and NaCl by conductivity, respectively.

The diafiltration through hollow-fibre walls was performed in a closed circuit apparatus allowing to perform lysis, hemoglobin-removal and concentrating of the ghosts in the same system. Shell and tube type modules consisting of bundles of capillary membranes with surface areas of about 0.5 m² from Enka AG, Wuppertal, were used. The modules were equipped with microporous polypropylene hollow fibres (average pore size 0.2 μm) with inner diameters of 330 μm and a wall thickness of 150 μm (AC-CUREL®, Enka AG). Fig. 2A exhibits schematically the construction of a diafiltration apparatus. In Fig. 2B the time-profile of the hemoglobin-elution and of the saltconcentration in the filtrate are shown. Compared with the time to prepare white erythrocyte ghosts by gel filtration (about 4 h), a remarkable acceleration by a factor of at least 2 was reached with the diafiltration method. The possibility to increase the concentration of salts in the external medium before the hemoglobin is completely removed from the filtrate-cycle can lead to a further shortening of the time which the cells spend in the hypotonic medium. This is an advantage of the diafiltration over the centrifugation method.

After identical periods of resealing in media containing high K⁺-concentrations (1 h at 37 °C) physical measurements were performed to characterize
determination of the ratio of accessible protein in the band 1, 2-region to stained integral band 3-protein. EITC was added to the resealed ghost. In intact red blood cells EITC is non-penetrating and only extracellular amino-residues are labeled.

The results presented in Table I reveal that ghost cells prepared by the centrifugation method have a 7-fold higher permeability for \( K^+ \)-ions than those prepared by the gelfiltration-method. Diafiltration and gelfiltration lead to ghost-cells with 2- or 1-2-fold increased \( K^+ \)-permeability compared with intact erythrocytes, respectively. Potassium exchange-rates, \( k \), were determined from \( K^+ \)-efflux curves shown in Fig. 3 by an exponential fit of the experimental data. Permeability-coefficients, \( P_K \), were then calculated according to 

\[
P_K = k \cdot V/A,
\]

with the volume to surface ratio \( V/A \).

The mean cellular ghostvolumes \( V_m \), determined by the coulter-counter-technique in isotonic saline after restoring of physiological salt-concentrations and incubation at 37 °C, show an increase in the order hollow-fibre < centrifugation < gelfiltration with only a slight difference of about 2–3 \( \mu m^3 \) for the alternative methods. The striking decrease of the \( V_m \)-values from intact erythrocytes to ghostcells (bovine erythrocytes) is in accord with recently published data on human erythrocyte ghosts showing a reduction of \( V_m \) by the factor of about 2.5 after 2 h in hypotonic media [7].

The membrane proteins were stained by covalently binding the amino-specific eosin-derivative, EITC, to the accessible NH\(_2\)-residues. The fluorophore was

<table>
<thead>
<tr>
<th>Method</th>
<th>( P_K ) [cm/s](^a)</th>
<th>( V_m ) [( \mu m^3 )](^b)</th>
<th>( R(3/1, 2))^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>centrifugation</td>
<td>( 6.4 \times 10^{-9} )</td>
<td>14</td>
<td>0.5–1</td>
</tr>
<tr>
<td>hollow-fibre</td>
<td>( 2.0 \times 10^{-9} )</td>
<td>12</td>
<td>1–2</td>
</tr>
<tr>
<td>gelfiltration</td>
<td>( 1.0 \times 10^{-9} )</td>
<td>16</td>
<td>3–10</td>
</tr>
<tr>
<td>erythrocytes</td>
<td>( 0.9 \times 10^{-9} )</td>
<td>49</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

\(^a\) Permeability-coefficient for \( K^+ \) determined from \( K^+ \)-efflux measurements according to 

\[
P_K = k \cdot V/A
\]

with \( A = 90 \mu m^2 \) (bovine erythrocytes and \( V \)-values listed in the second column.

\(^b\) Mean ghostcell volume determined using the coulter-counter technique.

\(^c\) Ratio of EITC-labeled band 3-region to band 1, 2-region from quantitative scans of the fluorescence intensity of the SDS-electrophoresis gels.