CONCISE REPORT

Altered Response of Stored Red Cells to Ca\(^{2+}\) Stress

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Short-term Ca\(^{2+}\) loading of erythrocytes was used as a test for probing membrane protein susceptibilities toward intrinsic enzymes in cells from fresh and from stored blood. The proteolytic response, affecting mainly glycophorin and band 3, could be elicited only in fresh cells, whereas the transglutaminase-mediated cross-linking reaction was evident both in fresh and stored cells. Loss of the proteolytic response might be an important sign of erythrocyte damage from blood bank storage.

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ENRICHMENT of fresh human RBCs with Ca\(^{2+}\) (≥ 0.1 mmol/L) causes characteristic changes in the covalent structures of several membrane proteins. Work in our laboratory focused on two biochemical modifications of this type, each catalyzed by appropriate enzymes in the red cell. One is a transglutaminase-mediated reaction, resulting in the formation of polymeric clusters of membrane proteins through fusion by (γ-glutamyl)lysine cross-links. The other is a proteolytic event, readily recognizable by the degradation of glycophorin and band 3 following Ca\(^{2+}\) treatment of the cells. It is shown in the present report that the latter response is greatly reduced as a result of blood bank storage; following only seven days, the Ca\(^{2+}\)-evoked proteolytic phenomenon was barely demonstrable.

MATERIALS AND METHODS

Blood Collection and Storage: Isolation of RBCs

Blood was drawn in the ratio of 10 mL:1.4 mL of CPDA-1, which comprised 0.206 g citric acid, 1.66 g sodium citrate, 0.14 g sodium bisphosphate, 2.0 g dextrose, and 0.107 g adenosine per 63 mL. Collections from the same individual were divided into four aliquots: one for preparing washed RBCs immediately (day 0) and the rest to be kept at 4 °C for obtaining samples of washed RBCs on days 3, 7, and 14 of storage. In each instance, 2 mL of blood was centrifuged (Beckman J 21-B centrifuge JA-20 rotor, Palo Alto, Calif) at 3,000 rpm at 4 °C and the cells were washed four times with 30 mL of buffer K [100 mmol/L potassium chloride, 60 mmol/L sodium chloride, 10 mmol/L glucose, and 5 mmol/L Tris(hydroxymethyl)aminomethane or Tris hydrochloride, pH 7.4]. Each washing was followed by centrifugation as before; the cells were then resuspended in buffer K.

Ca\(^{2+}\) Stress

Response to Ca\(^{2+}\) treatment was tested by incubating 2.5 mL of the cells at 20% hematocrit and 37 °C with 2 mmol/L calcium chloride and 10 or 20 μmol/L A23187 (Calbiochem, San Diego). In some experiments, calcium loading was carried out in the presence of 1 or 2 mmol/L of pepstatin, a protease inhibitor. Controls with 2 mmol/L magnesium chloride as replacement for calcium chloride, were also tested. Final concentration of dimethylsulfoxide (DMSO), used as a solvent for ionophore A23187 as well as for pepstatin, was between 1.2% and 2.4% in the incubation mixtures. The three-hour incubation periods were terminated by addition of 30 mL of buffer K, in which 2.5 mmol/L EDTA was included (with readjustment of pH to 7.4). In order to exclude carry-over of membrane fragments, cells were centrifuged at 4 °C for 15 minutes at 3,000 rpm.

Isolation of Membrane

Packed cells were taken up in 30 mL of 5 mmol/L phosphate buffer of pH 8, containing 1 mmol/L iodoacetamide (Sigma, St Louis), 1 mmol/L benzamidine (Sigma), and 0.5 mmol/L/L phenylmethylsulfonylfluoride (Sigma). Membranes were removed by 30 minutes’ centrifugation at 4 °C and 18,000 rpm, and were washed four times in the above medium, each time followed by centrifugation and decanting of wash fluid.

Evaluation of the Proteolytic Response

Crossed immunoelectrophoresis was shown to be specially suitable for demonstrating the breakdown of glycophorin and band 3. Thus, combined sodium dodecyl sulfate (SDS) (Aldrich, Milwaukee) polyacrylamide gel (5%; [PAGE] Bio-Rad, Richmond, Calif) and agarose (1%, Marine Colloids, Rockland, Me, type LE) crossed immunoelectrophoreses were performed as described by Bjerrum and Bhakdi. Following solubilization in 2% SDS and 40 mmol/L dithiothreitol (15 minutes, 3 °C), 20-μg samples of solubilized membrane proteins were subjected to SDS-PAGE in 1.5-mm-thick slab gels using the procedure of Fairbanks et al with 0.1% SDS, and the runs were terminated when the pyronin-tracking dye (Sigma) migrated to a distance of 7.5 cm. The polyacrylamide support was trimmed to a width of 0.75 cm and was washed for 15 minutes at 22 °C in a buffer containing 38 mmol/L Tris, 100 mmol/L glycine (pH 8.7), and 1% Lubrol PX (Sigma). The slice was then placed 2 cm above the cathodic edge of the plate (7 × 10 cm) used for crossed immunoelectrophoresis, on top of a 2-mm-thick and 4-cm-wide agarose layer, which contained 3.5% Lubrol PX. The two layers were then sealed by application of a few drops of warm agarose (with 3.5% Lubrol PX) at the cathodic side. The polyspecific antibody-containing agarose with 2% Lubrol PX was cast as a 4-cm-wide and 1.5-mm-thick layer on the anodic side of the plate. On the top 2-cm portion of the plate, a 1.5-mm-thick 1% agarose layer was applied with monospecific rabbit antibody to human band 3 protein (a gift from Dr O.J. Bjerrum, Protein Laboratory, University of Copenha-
gen), in 2% Lubrol PX. Electrophoresis was performed at 2 vol/cm for about 16 hours, using the Tris-glycine buffer of pH 8.7 without Lubrol PX. The polyacrylamide band was removed prior to pressing, washing, and staining of the agarose.

Poly specific rabbit antibody against human erythrocyte ghost was obtained from Dako Corp., Santa Barbara, Calif (A 104 lot 099A). To 5 mL of antibody solution, 50 μL of human serum albumin (Pentex, Kankakee, III: 5 mg of lyophilized protein per milliliter of 100 mmol/L NaCl) were added. Following incubation at 4°C overnight, the precipitate was removed by centrifugation at 50,000 g for 15 minutes.

For analyzing SDS-PAGE protein profiles, 40-μg samples of membrane proteins were applied to each lane, and Coomassie Brilliant Blue R (Sigma) was used for staining.

RESULTS

In agreement with earlier observations, Ca²⁺ treatment of RBCs that were separated from fresh blood (ie, day 0) produced significant degradation of glycophorin. By crossed immunoelectrophoresis, only a trace of the normal slowest migrating glycophorin species (marked GP) was visible after three hours of incubation with Ca²⁺ and ionophore (Fig 1A). By contrast, glycophorin remained intact in the experiments in which the Ca²⁺ loading of cells was performed in the presence of pepstatin (Fig 1A'). In the absence of this protease inhibitor, also a considerable fragmentation of band 3 occurred, which gave rise to a an immunologically cross-reacting smaller unit, marked 3'.

Much less degradation of the transmembrane proteins was observed when blood stored for three days (Figs 1B and B') was used as the source of the RBCs. Following seven days of blood storage, the crossed immunoelectrophoretic patterns for glycophorin and band 3 were the same in the Ca²⁺ and ionophore-treated erythrocytes (Fig 1C) as in the control cells that were exposed to the combined treatment of Ca²⁺ and ionophore and pepstatin (Fig 1C'). The immunoprecipitation pattern of glycophorin and band 3 in the cells treated with Mg²⁺, ionophore, and pepstatin was indistinguishable from that shown in panel C'.

Analysis by SDS-PAGE showed formation of ε(γ-glutamyl)lysine cross-linked polymers¹ upon Ca²⁺ loading of RBCs, regardless of whether they were obtained from fresh (day 0, gel 1) or stored blood (day 14, gel 2); the polymer is denoted as X in Fig 2. It should be pointed out that previously we were able to dissociate the proteolytic event in Ca²⁺-treated erythrocytes from the transglutaminase-mediated cross-linking of membrane proteins only through the use of pepstatin as a differential inhibitor.² Examination of the cross-linking reaction without interference by proteolysis should henceforth be possible simply by studying RBCs from stored blood.

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Fig 1. Different susceptibilities to degradation of transmembrane proteins in fresh and stored erythrocytes during Ca²⁺-stress. RBCs were obtained either from fresh blood (day 0; A) or from three days' (B) and seven days' (C) stored blood. Ca²⁺ loading was performed with 20 μmol/L ionophore A23187 at 37°C for three hours. Panels A', B', and C' represent parallel experiments with 2 mmol/L pepstatin included in the incubation mixtures. On the crossed immunoelectrophoretic profiles of membrane proteins, vertical arrows indicate the position of the main glycophorin (GP) component; 3 and 3', respectively, denote band 3 protein and its proteolytic fragment.

Fig 2. SDS-PAGE of membrane proteins of fresh and stored erythrocytes. Erythrocytes were obtained from fresh blood (day 0, gel 1) and following 14 days of storage (gel 2), and were treated with Ca²⁺ and ionophore as in Fig 1, prior to isolating membranes. Gel 3 represents control cells from 14 days' stored blood, following incubation with Mg²⁺ and ionophore. Membrane protein cross-linking in the Ca²⁺-treated cells is denoted by X; positions for bands 1 to 4.5 are marked on the right.
As demonstrated by this work, intrinsic proteolysis of transmembrane proteins appears to be a characteristic response to Ca\textsuperscript{2+} stress only in RBCs from fresh blood. Loss of this property during storage could possibly be due to inactivation of proteases and might be an important sign of erythrocyte damage occurring in the blood bank. It is already known that old RBCs show greatly diminished protease activity.\textsuperscript{6}

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REFERENCES
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