Bioelectrochemistry 73 (2008) 151–154

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

Changes in the intracellular Ca^{2+} content in human red blood cells in the presence of glycerol

Olga A. Kofanova [a,b,*], Nina G. Zemlyanskikh [b], Lyubomira Ivanova [a], Ingolf Bernhardt [a]

[a] Laboratory of Biophysics, Faculty of Natural and Technical Sciences III, University of the Saarland, P.O. Box 151150, 66041 Saarbruecken, Germany
[b] Institute for Problems of Cryobiology and Cryomedicine of National Academy of Sciences of Ukraine, Pereyaslavskaya str.23, 61015 Kharkiv, Ukraine

ARTICLE INFO

Article history:
Received 10 March 2008
Received in revised form 21 April 2008
Accepted 24 April 2008
Available online 8 May 2008

Keywords:
Red blood cells
Intracellular Ca^{2+}
Fluo-4
Glycerol
Freeze–thawing

ABSTRACT

Changes of the intracellular Ca^{2+} content in human red blood cells (RBCs) in glycerol-containing solutions and after freeze–thawing the cells with glycerol and subsequent deglycerolization were investigated with the Ca^{2+}-sensitive fluorescent dye fluo-4 using fluorescence microscopy. In the glycerol-containing solutions the Ca^{2+} content increased when compared with a physiological medium (Hepes buffered saline solution (HBSS)). This effect was most likely a result of an inhibition of the Ca^{2+} pump. After inhibiting the Ca^{2+} pump using *o*-vanadate, the Ca^{2+} uptake was not significantly different in the cells in glycerol-containing and physiological medium. Freeze–thawing and deglycerolization of RBCs resulted in a more pronounced increase in the Ca^{2+} content. Also in this case, the Ca^{2+} pump seemed to play a major role.

1. Introduction

The elevation in the intracellular free Ca^{2+} content and modification of the Ca^{2+} pump activity can play an important role in the cellular metabolic transformations underlying adaptation against stress conditions. Such conditions arise, for example, during cooling down to ultralow temperature (−196 °C) and long-term storage of red blood cells (RBCs) in blood banks. Glycerol is one of the most effective compounds used for cell protection against extreme physical and chemical insults during the freeze–thawing processes [1,2]. Incubation of RBCs in hypertonic solutions of glycerol results in changes of cell shape and volume that can induce alterations in the cell membrane permeability for cations including Ca^{2+} [3] and alteration of enzyme activity of the Ca^{2+} pump [4].

Human RBCs do not have intracellular Ca^{2+} stores and also lack a cell membrane Na^+/Ca^{2+}-exchanger. Hence, for human RBCs, the intracellular Ca^{2+} concentration depends on the balance of the Ca^{2+} influx and efflux across the cell membrane. The Ca^{2+} efflux is realized by the Ca^{2+} pump, the only high affinity Ca^{2+} transporting system by which Ca^{2+} can be removed from cells [5]. Under osmotic stress and cooling an increase of the intracellular Ca^{2+} content in RBCs can be caused by several ways. One possibility would be the activation of specific or nonspecific cation channels. This could lead to an increase of the intracellular Ca^{2+} concentration under *in vitro* conditions even when Ca^{2+} is not included as a component in the extracellular medium. Trace amounts of Ca^{2+}, i.e. up to 10^{-6}–10^{-5} M are always present in salt solutions as an admixture [6]. Another possibility relates to modifications of the Ca^{2+} pump activity.

During freeze–thawing RBCs a variety of factors influence the cells in spite of glycerol protection and therefore, these factors could influence the cell membrane structure and properties. As a consequence ion transport pathways and barrier properties of the membrane could be affected.

The aim of the investigation was to study how the incubation of RBCs with the cryoprotectant glycerol and the process of cell deglycerolization after freeze–thawing influence the intracellular Ca^{2+} content. To monitor the Ca^{2+} content, the Ca^{2+}-sensitive fluorescence dye fluo-4 was used. For measuring the Ca^{2+} uptake, *o*-vanadate has been applied to inhibit the Ca^{2+} pump.

2. Materials and methods

2.1. Blood and solutions

Blood was used from healthy human donors and obtained from the blood bank of the University Hospital of the Saarland University (Homburg). Blood was drawn in CPDA medium and RBCs were separated by centrifugation (1200 g, 5 min) at room temperature. The cells were washed 3–4 times with a sodium chloride-based physiological solution containing (mM): NaCl 150, glucose 10, Tris-(hydroxymethyl)aminomethane (Tris) 10 titrated with HCl to pH 7.4 at room temperature. Plasma and buffy coat were aspirated.

* Corresponding author. Institute for Problems of Cryobiology and Cryomedicine of National Academy of Sciences of Ukraine, Pereyaslavskaya str.23, 61015 Kharkiv, Ukraine. Tel.: +38 050 969 3673; fax: +38 057 775 3693.
E-mail address: o.kofanova@gmail.com (O.A. Kofanova).

1567-5394/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bioelechem.2008.04.025

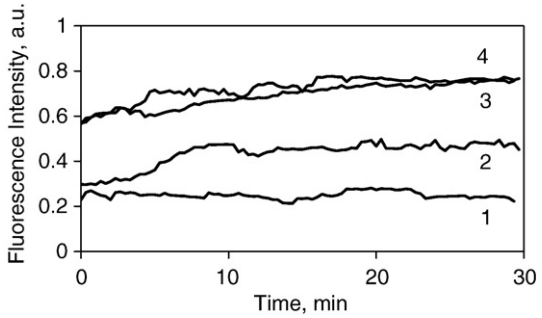

Fig. 1. Fluorescence intensity of RBCs labeled with fluo-4. Curves 1 and 3: cells in HBSS in the absence (1) and presence (3) of *o*-vanadate, curves 2 and 4: cells in glycerol-containing solution in the absence (2) and presence (4) of *o*-vanadate. Ortho-vanadate (10^{-3} M) was present to inhibit the Ca^{2+}-pump. Each curve presents the average fluorescence intensity of 240 RBCs from 6 donors.

2.2. Freezing and deglycerolization

The freezing was performed as described in [7] with some modifications. The cryoprotective solution contained 3.25 M glycerol, 220 mM mannitol, and 120 mM NaCl. The glycerol-containing medium was added to RBCs in a 1:1 volume ratio. The incubation time was 20 min. Then the RBC suspension was frozen by immersion into liquid nitrogen (temperature −196 °C). The thawing was carried out in water bath at 42 °C with permanent shaking. Frozen–thawed RBCs were centrifuged 3 times at 1200 g to separate the cells from glycerol. For the first centrifugation glycerol was replaced by 600 mM NaCl, for the second and third centrifugation the physiological solution containing 150 mM NaCl has been used.

2.3. Measurement of the intracellular free Ca^{2+} concentration

Intracellular free Ca^{2+} levels of single cells were monitored using an inverted fluorescence microscope (Nikon) and the Ca^{2+}-sensitive fluorescent dye fluo-4 AM (4 μM). This indicator has a high affinity binding for Ca^{2+} (K_d=345 nM) and a very large fluorescence intensity increase in response to Ca^{2+} binding (>100 fold). The application of fluo-4 for Ca^{2+} measurements in intact RBCs has been reported by our group elsewhere [8]. Aliquots of fluo-4 AM were mixed with Pluronic F-127 (20% DMSO in H_2O) and prepared to give a final concentration of 1 M (stock solution). The non-ionic detergent was used to assist in dispersion of the non-polar AM ester in aqueous media. Washed RBCs (1% haematocrit) were incubated with the fluo-4 AM for 45 min at 37 °C in the dark. Cells were then washed in HEPES/NaOH-buffered saline solution (HBSS) containing (mM) NaCl 145, KCl 7.5, 10 HEPES, pH 7.4 to remove any dye and were re-suspended (0.015% haematocrit) in the same medium. In some experiments when RBCs were incubated in the presence of glycerol or pretreated with ortho-vanadate (*o*-vanadate, final concentration 1 mM) the corresponding substances were present in the loading media. Finally, an aliquot of stock solution was added to give a final $CaCl_2$ concentration of 2 mM.

2.4. Reagents

All chemicals used (except the chemicals mentioned below) were purchased from Sigma-Aldrich (Munich, Germany). Tris and EGTA were obtained from Fluka (Buchs, Switzerland), fluo-4 AM was obtained from Molecular Probes (USA).

2.5. Statistical treatment of results

Each curve presented in the figures represents the mean value of at least 240 single RBCs of 6 different donors. The significance of differences was tested by ANOVA according to Student's *t*-test. Differences were considered significant if $p<0.05$.

3. Results

To see whether the presence of glycerol (and mannitol) in the medium has any influence on the Ca^{2+} content in the RBCs, experiments have been performed in the HBSS as well as in the glycerol-containing medium. In both solutions the change of the Ca^{2+} content was measured in the absence or presence of 1 mM *o*-vanadate, an inhibitor of the Ca^{2+} pump, over a time interval of 30 min. Fluo-4 has been used for qualitative determinations of the Ca^{2+} content. It should be noticed that a time period of about 2–5 min was necessary to let the cells settle down to the cover slip surface and to start the experiment. As shown in Fig. 1 in the HBSS in the absence of *o*-vanadate the fluorescence intensity of fluo-4, i.e. the Ca^{2+} content, was very low but constant over 30 min (curve 1). In the presence of *o*-vanadate in the HBSS a continuous increase in the Ca^{2+} content was observed. The Ca^{2+} content was significantly higher in the presence of *o*-vanadate in comparison to the situation where the substance was absent (curve 3). The fluorescence intensity of fluo-4 in the presence of glycerol was represented in curves 2 and 4. In the glycerol-containing solution in the absence of *o*-vanadate an increase of the Ca^{2+} content could be seen during the first 10 min of the experiment (curve 2). The Ca^{2+} content was higher than in the control (cp. curves 1 and 2). The presence of *o*-vanadate in the glycerol-containing solutions resulted in continuous increase of fluorescence intensity of fluo-4 as well as the control in HBSS when Ca^{2+} pump was inhibited (cp. curves 3 and 4).

After freeze–thawing of RBCs in the glycerol-containing solution and the following removal of the cryoprotectant, the fluo-4 fluorescence intensities in the presence or absence of *o*-vanadate were significantly higher in comparison with the corresponding fluorescence intensities in the HBSS and in the glycerol-containing solutions without freezing the cells (cp. Figs. 2 and 3). After cryopreservation the Ca^{2+} content was more or less constant over 30 min in the absence of *o*-vanadate (Fig. 2, curve 1) but increased continuously in the presence of *o*-vanadate (Fig. 2, curve 2) similarly as for unfrozen cells in HBSS and glycerol-containing solution.

In Fig. 3 fluorescence microscopy images of RBCs incubated under different conditions, as shown in Figs. 1 and 2, are presented. The images were taken after the 30 min fluorescence intensity recordings.

4. Discussion

During freeze–thawing any cells are exposed to a number of stress factors [9,10]. The transition from the liquid phase to the solid state is coupled to processes of crystal formation [11] leading to an increase in salt concentration in the supercooled liquid [12,13], an increase in osmotic pressure [14], phase transitions and the lateral separation of membrane lipids as well as dehydration of macromolecules [15,16]. The application of organic compounds for cryopreservation is required to protect cells against the extreme events that accompany the freeze–

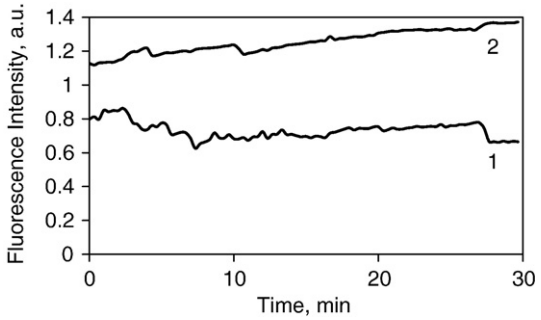

Fig. 2. Fluorescence intensity of RBCs labeled with fluo-4. Cells in HBSS in the absence (curve 1) or presence (curve 2) of *o*-vanadate (10^{-3} M) after cryopreservation and deglycerolization. Each curve presents the average fluorescence intensity of 240 RBCs from 6 donors.

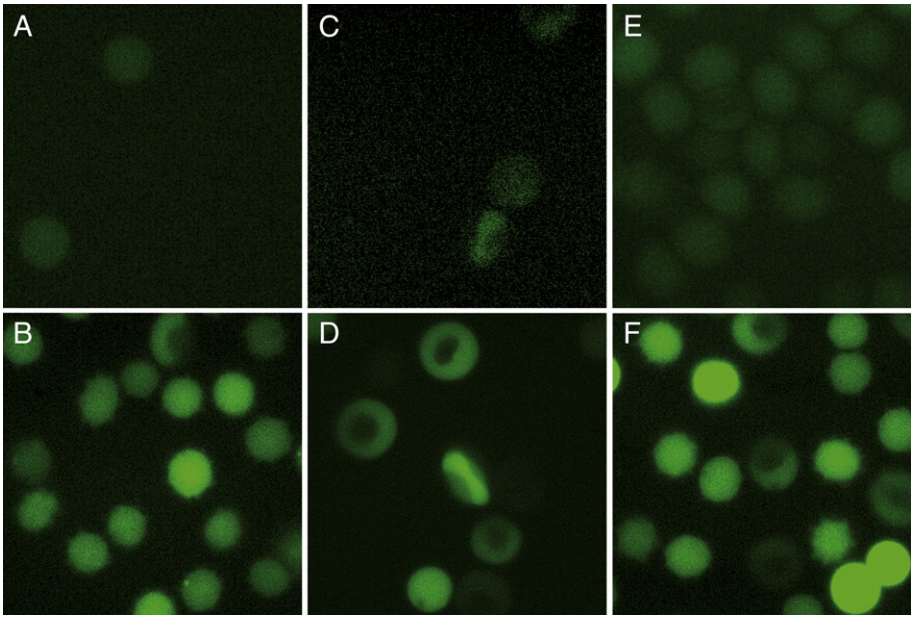

Fig. 3. Fluorescence microscopy images of RBCs in *o*-vanadate-containing and *o*-vanadate-free media. A and B: cells in HBSS in the absence (A) or presence (B) of *o*-vanadate, C and D: cells in glycerol-containing medium in the absence (C) or presence (D) of *o*-vanadate, E and F: cells in HBSS after cryopreservation and deglycerolization in the absence (E) or presence (F) of *o*-vanadate. The concentration of *o*-vanadate was 10^{-3} M.

thawing processes. Determining changes of the intracellular Ca^{2+} content in response to added cryoprotectants and physical and chemical factors affected by freeze–thawing can be useful to understand how cell stabilization is achieved.

The control experiment (HBSS without *o*-vanadate) clearly showed that the intracellular Ca^{2+} content was constant over 30 min, i.e. the Ca^{2+} pump is intact (Fig. 1, curve 1). The slight increase of the Ca^{2+} content in the glycerol-containing medium in the absence of *o*-vanadate, especially at the beginning of the experiment (Fig. 1, curve 2), can be explained in the following ways: (i) the Ca^{2+} pump was affected in this solution and could not fully operate and/or (ii) an enhanced Ca^{2+} uptake was induced which could not be compensated by the pump. In any case, the curves are significantly different. Since the addition of *o*-vanadate did not show a significant difference of the data obtained in HBSS and glycerol-containing solutions (Fig. 1, curves 3 and 4), it seems more likely that glycerol in the absence of *o*-vanadate affected the Ca^{2+} pump (see above).

Freeze–thawing RBCs with glycerol and subsequent deglycerolization induced an increase in the fluorescence intensity (Fig. 2) in comparison with control cells (Fig. 1) indicating a rise in intracellular Ca^{2+} levels. This increase of the Ca^{2+} levels in the cells could be again a result of (i) a reduction of the Ca^{2+} pump activity and/or (ii) an enhancement of the Ca^{2+} influx into the cells. However, it can be seen from Fig. 2 (curve 1) that the Ca^{2+} pump is still active and not completely blocked after cryopreservation. In addition, enhanced phosphatase activity of the Ca^{2+}-ATPase has been shown in saponin-permeabilized RBCs after freeze–thawing and deglycerolization [4]. After freeze–thawing RBCs with glycerol and under the condition that the Ca^{2+} pump was inhibited using *o*-vanadate, an increase of the intracellular Ca^{2+} content was observed, which was comparable to the increase under control conditions. Therefore, it is reasonable to assume that during storage of the cells in the cold the membrane function was compromised, which led to an uptake of Ca^{2+} that could not be compensated by the Ca^{2+} pump. Since trace amounts of Ca^{2+} are present during freeze–thawing (even when Ca^{2+} was not added to the extracellular solution, see Introduction), the Ca^{2+} uptake might occur during this process. During cryopreservation, i.e. at low temperature, the Ca^{2+} pump activity is close to zero. At the time the fluorescence intensity was measured it can be assumed that the Ca^{2+} pump was again fully operating. The changes in the Ca^{2+} content in RBCs in the glycerol-containing solution and after freeze–thawing were, however, smaller than those reported to be necessary to cause cell damage. This assumption is supported by the successful application of cryopreserved RBCs for transfusion [17].

Summarizing the results, we demonstrated that glycerol had only a small effect on the Ca^{2+} content of RBCs. Freeze–thawing RBCs with glycerol and subsequent deglycerolization resulted in an increase of the intracellular Ca^{2+} content that could be related to compromising permeability properties of the plasma membrane and/or change in the Ca^{2+} pump activity under these conditions.

Acknowledgement

This work was supported by a DAAD fellowship granted to Olga Kofanova.

References

[1] C.R. Valeri, G. Ragno, L.E. Pivacek, G.P. Cassidy, R. Srey, M. Hansson-Wicher, M.E. Leavy, An experiment with glycerol-frozen red blood cells stored at −80 degrees C for up to 37 years, Vox. Sang. 79 (2000) 168–174.
[2] K. Gekko, S.N. Timasheff, Mechanism of protein stabilization by glycerol: Preferential hydration in glycerol–water mixtures, Biochem. 20 (1981) 4667–4676.
[3] I.B. Bakaltcheva, C.O. Odeyale, B.J. Spargo, Effects of alkanols, alkanediols and glycerol on red blood cell shape and hemolysis, Biochim. Biophys. Acta 1280 (1996) 73–80.
[4] N.G. Zemlyanskikh, O.A. Kofanova, L.A. Babiychuk, Modification of erythrocytes' Ca^{2+}-ATPase activity under glycerol and freezing effect and in media with different ionic strength, Probl. Cryobiol. 15 (2005) 173–183.
[5] P.J. Romero, E.A. Romero, The role of calcium metabolism in human red blood cell ageing: a proposal, Blood Cell Mol. Diseases 25 (1999) 9–19.
[6] C. Patton, S. Thompson, D. Epel, Some precautions in using chelators to buffer metals in biological solutions, Cell Calcium 35 (2004) 427–431.
[7] A.W. Rowe, E. Eyster, A. Kellner, Liquid nitrogen preservation of red blood cells for transfusion; a lowglycerol-rapid freeze procedure, Cryobiol. 5 (1968) 119–128.
[8] L. Kaestner, W. Tabellion, E. Weiss, I. Bernhardt, P. Lipp, Calcium imaging of individual erythrocytes: problems and approaches, Cell Calcium 39 (2006) 13–19.
[9] P. Mazur, The role of intracellular freezing in the death of cells cooled at supraoptimal rates, Cryobiol. 14 (1977) 251–272.
[10] P. Mazur, S.P. Leibo, E.H.Y. Chu, A two-factor hypothesis of freezing injury, Exp. Cell Res. 71 (1972) 345–355.
[11] K. Muldrew, L.E. McGann, Mechanisms of intracellular ice formation, Biophys. J. 57 (1990) 525–532.

[12] P. Mazur, N. Rigopoulos, Contribution of unfrozen fraction and of salt concentration to the survival of slowly frozen human erythrocytes: influence of warming rate, Cryobiol. 20 (1983) 274–289.
[13] H.T. Meryman, R.J. Williams, M. Dougas, Freezing injury from "solution effects" and its prevention by natural or artificial cryoprotection, Cryobiol. 14 (1977) 287–302.
[14] K. Muldrew, L.E. McGann, The osmotic rupture hypothesis of intracellular freezing, Cryobiol. 30 (1993) 620.
[15] J.H. Crowe, L.M. Crowe, Effects of dehydration on membranes and membrane stabilization at low water activity, in: D. Chaptman (Ed.), Biological Membranes, Academic Press, London, 1984, pp. 57–103.
[16] L.M. Crowe, R. Mouradian, J.H. Crowe, S.A. Jackson, C. Womersley, Effects of carbohydrates on membrane stabilization at low water activity, Biochim. Biophys. Acta 779 (1984) 141–150.
[17] C.R. Valeri, G. Ragno, The survival and function of baboon red blood cells, platelets, and plasma proteins: a review of the experience from 1972 to 2002 at the Naval Blood Research Laboratory, Boston, Massachusetts, Transfusion 46 (2006) 1–42.