ISSN 0006-2979, Biochemistry (Moscow), 2006, Vol. 71, No. 8, pp. 900-905. © Pleiades Publishing, Inc., 2006.
Original Russian Text © N. G. Zemlyanskikh, O. A. Kofanova, 2006, published in Biokhimiya, 2006, Vol. 71, No. 8, pp. 1112-1118.

Modulation of Human Erythrocyte Ca^{2+}-ATPase Activity by Glycerol: The Role of Calmodulin

N. G. Zemlyanskikh* and O. A. Kofanova

Institute for Problems of Cryobiology and Cryomedicine, National Academy of Sciences of Ukraine, Pereyaslavskaya str. 23, 61015 Kharkov, Ukraine; fax: (805) 737-33084; E-mail: cryo@online.kharkov.ua

Received September 15, 2005
Revision received March 23, 2006

Abstract—The effect of an intracellular cryoprotectant glycerol on human erythrocyte Ca^{2+}-ATPase activity and possible involvement of calmodulin in the regulation of Ca^{2+}-pump under these conditions were investigated. The experiments were carried out using saponin-permeabilized cells and isolated erythrocyte membrane fractions (white ghosts). Addition of rather low concentrations of glycerol to the medium increased Ca^{2+}-ATPase activity in the saponin-permeabilized cells; the maximal effect was observed at 10% glycerol. Subsequent increase in glycerol concentrations above 20% was accompanied by inhibition of Ca^{2+}-ATPase activity. Lack of stimulating effect of glycerol on white ghost Ca^{2+}-ATPase may be attributed to removal of endogenous compounds regulating activity of this ion transport system. Inhibitory analysis using R24571 revealed that activation of Ca^{2+}-ATPase by 10% glycerol was observed only in the case of inhibitor administration after modification of cells with glycerol; in the case of inhibitor addition before erythrocyte contact with glycerol, this phenomenon disappeared. These data suggest the possibility of regulation of human erythrocyte Ca^{2+}-ATPase by glycerol; this regulatory effect may be attributed to both glycerol-induced structural changes in the membrane and also involvement of calmodulin in modulation of catalytic activity of the Ca^{2+}-pump.

DOI: 10.1134/S0006297906080128

Key words: erythrocyte, Ca^{2+}-ATPase, calmodulin, glycerol

During cryopreservation cells are subjected to complex effects of various stress factors. Transition of liquid phase into a solid state is coupled to processes of crystal formation, increase in salt concentration in the supercooled liquid, the increase in osmotic pressure, phase transitions and lateral separation of membrane lipids, dehydration of macromolecules, etc. [1]. Use of cryoprotectants may significantly alter the development of these processes at ultralow temperatures [2]. The diversity of structural and functional properties of biological objects determines the diversity of their cryopreservation modes and especially cryoprotectant agents employed for optimization of conditions required for maintenance of cell integrity during the freeze-thawing cycle.

Glycerol is widely employed in cryobanks of various countries for long-term storage of blood [3-5]. For erythrocytes, the terminally differentiated cells, properties of membranes and a special mode of functioning of transport and signaling systems [6] during various stages of cryopreservation may play a decisive role in stabilization of these cells. Changes in intracellular calcium under stress conditions represent one of the most important trigger mechanisms of metabolic regulation finally underlying death or adaptation of cells to the extreme treatments. In human erythrocytes, plasma membrane Ca^{2+}-ATPase (PMCA) represents the only mechanism responsible for active transport of Ca^{2+} ions against the gradient of calcium ion concentration [7]. Changes of catalytic properties of the Ca^{2+}-pump and its Ca^{2+} translocation capacity at various stages of cryopreservation may significantly influence stability of erythrocytes.

The effects of organic solvents on Ca^{2+}-ATPase activity from various biological sources have been investigated in many studies [8-12]. However, results on the effect of the same compound on the activity of Ca^{2+}-ATPase are often contradictory. The latter may be attributed to differences of approaches and model systems employed. The stimulating effect of certain organic solvents including glycerol was demonstrated using the

Abbreviations: CaM) calmodulin; PMCA) plasma membrane Ca^{2+}-ATPase; DMSO) dimethylsulfoxide; SR) sarcoplasmic reticulum.

* To whom correspondence should be addressed.

enzyme isolated from erythrocyte plasma membrane [13]. However, it is possible that this effect is observed in the model system, which does not reflect a particular effect of the compound on Ca^{2+}-ATPase in intact cells. In fact, the isolated enzyme lacks its natural lipid bilayer environment and addition of amphiphilic organic solvents just mimics the activation effect due to partial restoration of hydrophobic contacts. Dimethylsulfoxide (DMSO) and glycerol also stabilized sarcoplasmic reticulum (SR) Ca^{2+}-ATPase [14]. These compounds influenced partial reactions of the catalytic cycle. Particularly, it was shown that ATP hydrolysis/synthesis ratio which was 10 times higher for solubilized enzyme than for its membrane-bound form could be restored by cosolvents up to parameters of the catalytic activity of this enzyme in SR vesicles.

Organic solvents (cryoprotectants) added into medium may not only influence the rates of certain stages of the enzymatic reaction but also change regulatory properties of Ca^{2+}-ATPase. Studying the effect of glycerol on Ca^{2+}-ATPase, it was found [15] that glycerol activated hydrolysis of pseudosubstrate, *p*-nitrophenylphosphate; the activation of phosphatase activity occurred in a dose-dependent manner. It is possible that synergistic action of glycerol and Ca^{2+} are attributed to dehydration of substrate binding and Ca^{2+}-binding domains of Ca^{2+}-ATPase.

However, the effect of an organic solvent on Ca^{2+}-ATPase was not consistent in all models. For example, opposite results were obtained using DMSO. The latter stimulated Ca^{2+}-ATPase activity of isolated purified enzyme [13]. However, experiments employing inverted closed membrane vesicles obtained after hypotonic shock of erythrocytes did not reveal any influence of DMSO on the activity of this Ca^{2+}-pump [16]. Detailed analysis revealed that DMSO did not influence basal Ca^{2+}-ATPase activity, but it did inhibit calmodulin (CaM)-stimulated activity of this enzyme [17].

Thus, effects of various organic solvents (including glycerol) on erythrocyte Ca^{2+}-ATPase are complex and may involve various aspects of its functioning.

In this study, we have investigated the effect of glycerol on the activity of human erythrocyte Ca^{2+}-ATPase and possible involvement of CaM in the regulation of Ca^{2+}-pump functions under these conditions.

MATERIALS AND METHODS

The following chemicals were used in this study: ATP sodium salt, Tris, compound R24571 (inhibitor of CaM-regulated reactions) (Sigma, USA); Hepes, EGTA (Serva, Germany); KCl, $MgCl_2$, $CaCl_2$, and other chemicals of chemically pure and specially pure grades from local suppliers.

Erythrocytes of II(A) male donor blood taken with Glugicir (Biopharm, Ukraine) and stored at 2-4°C for 2-4 days were used as the research object.

Before experiment, erythrocytes were sedimented at 3000 rpm using an OPN-3 centrifuge (Dastan, Kyrgystan). After plasma and leukocyte layer were removed, erythrocytes were washed three times with 3-4 volumes of medium containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.4. The final wash was carried out in medium A (135 mM KCl, 10 mM Tris, 10 mM Hepes (pH 7.4), 0.025 mM $MgCl_2$).

Erythrocyte Ca^{2+}-ATPase activity was assayed as described earlier [18]. Aliquots of washed erythrocytes were added to medium B containing 0.04% saponin, 135 mM KCl, 10 mM Tris, 10 mM Hepes (pH 7.4), 0.025 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA, 1.1 mM $CaCl_2$. The composition of this medium was also supplemented with various concentrations of glycerol (5-60%). Final concentration of cells in the medium corresponded to 10% hematocrit value (about 10^6 cells per 1 μl). For analysis of ATP hydrolysis, cells were incubated at 37°C for 20 min. The reaction was stopped by adding cold TCA solution up to final concentration of 5%, and protein was sedimented by centrifugation at 1000 rpm. Glycerol-induced changes of Ca^{2+}-ATPase activity were evaluated by difference in P_i accumulation in the Ca^{2+}-containing and Ca^{2+}-free media (medium B did not contain $CaCl_2$).

Cells in samples were counted in a Goryaev chamber.

Isolation of erythrocyte membranes (white ghosts). Erythrocytes were treated with 30 volumes of hypotonic medium (5 mM KCl, 10 mM Tris-HCl, pH 7.6) in an ice bath for 10 min. White erythrocyte ghosts were sedimented by centrifugation using a refrigerated K-24 Janetzki centrifuge (VEB MLW Zentrifugenbau, Germany) at 20,000*g*. After decanting the supernatant, the membrane pellet was washed 3-4 times using the same mode.

For evaluation of white erythrocyte ghost Ca^{2+}-ATPase activity, aliquots of the isolated membrane fractions were added to medium B and the enzyme activity was determined as described above (for Ca^{2+}-ATPase of saponin-permeabilized erythrocytes).

Possible involvement of CaM in erythrocyte Ca^{2+}-ATPase activation in the presence of 10% glycerol was evaluated using calmidazolium R24571, an inhibitor of CaM-regulated reactions [19]. In the first series of experiments 0.04% saponin, 20 μM R24571, and medium A were added to cells. After cell lysis glycerol and components of the reaction mixture dissolved in the medium A were added up to the following final concentrations: 1 mM ATP, 1 mM EGTA, 1.1 mM $CaCl_2$, and 10% glycerol. In the second series of experiments, medium A containing 0.04% saponin and 10% glycerol was added to cells. After cell lysis and putative modification of Ca^{2+}-ATPase and intracellular modulator of this enzyme by glycerol, we added the reaction components (up to standard final concentrations), 20 μM R24571 and 10% glycerol (the cryoprotectant concentration was the same during enzyme modification and the beginning of the enzymatic reaction). After incubation at 37°C for 20 min, the

reaction was stopped by TCA addition. Changes in Ca^{2+}-ATPase activity were evaluated by difference of P_i accumulation as described above.

The amount of P_i was determined as described in [20]. Briefly, aliquots of deproteinized supernatants (100 μl) were mixed with 2 ml of acetate buffer (1.5 M CH_3COOH-CH_3COONa, pH 4.3) containing 3.7% formaldehyde and 10% ethanol. After that 0.1 ml of 2% ammonium molybdate and then immediately 0.2 ml of 6.75 mM $SnCl_2$ were added to each sample. Samples were read at λ = 660 nm using a Lomo SF-46 spectrophotometer (LOMO, Russia). The calibration curve was linear up to 10 μg of P_i per sample.

Protein content was determined by the method of Bradford [21].

Statistical analysis of results (n = 10 in each group) was performed using StatGraphics plus 2.1 for Windows. The Kolmogorov–Smirnov test revealed that all experimental data followed normal distribution. Statistical significance of changes observed was evaluated using Student's t-test. All results represent mean ± SEM.

RESULTS

Erythrocyte incubation with glycerol is accompanied by cell saturation with the cryoprotectant and its concentration in cytoplasm gradually reaches steady-state (equilibrium) level. Although glycerol easily passes through the plasma membrane of human erythrocytes, equilibration of its extra- and intracellular concentrations requires several minutes [2, 22]. This is rather significant time interval compared with the turnover rate of the enzyme in the catalytic cycle, which is usually realized within micro-/millisecond range [23]. So plasma membrane transport systems may exhibit different behavior in response to changes of parameters of the medium at the initial period of glycerol addition and after reaching its equilibrium. The time course of saturation of cells with glycerol and changes Ca^{2+}-ATPase activity during this process may be modeled by studying the enzymatic reaction rate in media with different cryoprotectant concentrations.

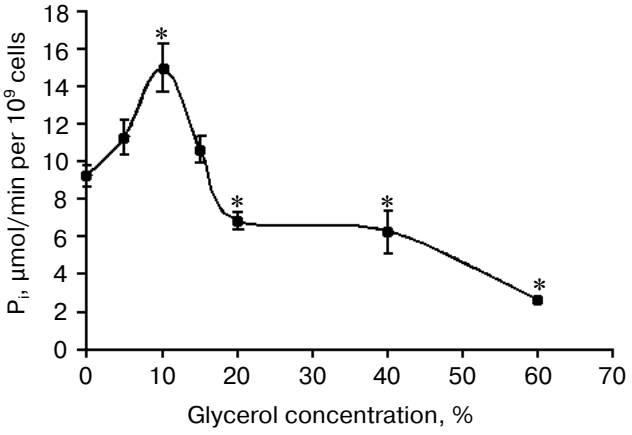

Fig. 1. Effect of glycerol on Ca^{2+}-ATPase activity in saponin-permeabilized erythrocytes. Results represent mean ± SEM of 10 independent experiments. Asterisk shows statistical significance compared with control: *$p < 0.05$.

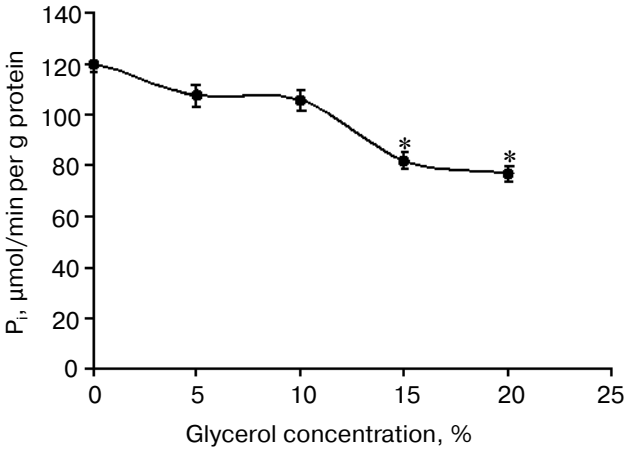

Fig. 2. Effect of glycerol on erythrocyte white ghost Ca^{2+}-ATPase. Data represent mean ± SEM of 10 independent experiments; *$p < 0.05$ compared with control.

Figure 1 shows the dependence of Ca^{2+}-ATPase activity of saponin-permeabilized erythrocytes on the change of glycerol concentrations in the reaction medium. It is characterized by biphasic behavior. At low glycerol concentrations Ca^{2+}-ATPase activity increases. Maximal stimulation was in the presence of 10% glycerol in the medium. Subsequent increase in glycerol concentrations caused a decrease in Ca^{2+}-ATPase activity, and at 20% glycerol the enzyme activity decreased below control values. Further increase in glycerol concentration resulted in gradual progressive decrease in Ca^{2+}-ATPase activity.

For evaluation of possible contribution of endogenous intracellular regulators in the glycerol-induced changes in Ca^{2+}-ATPase activity, we used erythrocyte white ghosts. Preparation of this membrane fraction is accompanied by removing all intracellular components and gives a possibility to evaluate the glycerol effect on activity of membrane bound enzyme just within its natural membrane environment. Figure 2 shows that the increase in glycerol concentration causes a monotonous decrease in Ca^{2+}-ATPase activity. This decrease in the enzyme activity became statistically significant at 15% glycerol in the medium. The qualitative difference between results obtained using these two models consists in the absence of stimulating effect of 10% glycerol in erythrocyte white ghosts. These data suggest possible involvement of endogenous modulators (removed during white ghost preparation) in the regulation of Ca^{2+}-ATPase by glycerol.

During recent decades, various modes of PMCA regulation have been recognized [24-26]. Calmodulin (CaM) is one of the most effective and the best-studied regulator [24, 27]. Stimulation of PMCA activity after CaM binding to the CaM-binding site of Ca^{2+}-ATPase occurs due to conformational changes in the autoinhibitory domain of this enzyme [24, 27, 28]. The involvement of CaM in regulation of Ca^{2+}-ATPase in saponin-permeabilized cells was demonstrated under various pathological conditions [29, 30]. Certain evidence exists that CaM may not only activate but also inhibit erythrocyte PMCA activity during hypothermic blood storage [31].

For investigation of possible involvement of CaM in the regulation of Ca^{2+}-ATPase by glycerol, we used R24571, an inhibitor of CaM-regulated processes [19]. These experiments were designed for evaluation whether CaM influences activity of this enzyme after its activation by glycerol, or manifestation of glycerol effect requires the presence of CaM and its inactivation on subsequent stages is ineffective for the development of the subsequent process. Figure 3 shows the results of these experiments.

We found that addition of R24571 to intact saponin-permeabilized human erythrocytes was accompanied by stimulation of Ca^{2+}-ATPase (Fig. 3b). This suggests that CaM in such cells subjected to short-term storage at 2-4°C (see "Material and Methods" section) causes inhibition of the enzyme activity. Similar results were obtained in another laboratory [31] using W-7 as the CaM inhibitor. When R24571 was added to the saponin-permeabilized cells before 10% glycerol (Fig. 3c), Ca^{2+}-ATPase activity slightly increased ($p < 0.05$) compared with control (Fig. 3a), but it was significantly lower than that assayed in the presence of glycerol alone, when this inhibitor was not employed (Fig. 3e). In the case of R24571 addition after 10% glycerol (Fig. 3d), the enzyme stimulation reached the same level as in the presence of glycerol alone (Fig. 3e). This suggests that maximal activation of Ca^{2+}-ATPase in the presence of glycerol involves CaM.

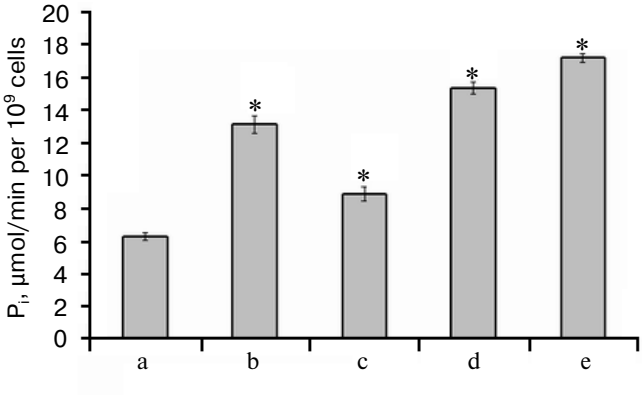

Fig. 3. Effect of 10% glycerol and R24571, an inhibitor of CaM-regulated reactions, on Ca^{2+}-ATPase activity of saponin-permeabilized human erythrocytes. Ca^{2+}-ATPase activity assayed in: a) medium B; b) medium B containing 20 μM R24571; c) medium B containing 20 μM R24571 with subsequent addition of 10% glycerol (final concentration); d) medium B containing 10% glycerol with subsequent addition of 20 μM R24571; e) medium B with 10% glycerol. Data represent mean ± SEM of 10 independent experiments; *$p < 0.05$ compared with control.

DISCUSSION

Use of organic compounds in cryobiology is technologically required for protection of biological objects against extreme factors accompanying the freeze–thawing processes. Study of Ca^{2+}-ATPase modification by various cryoprotectants and understanding of the role of this alteration in mechanisms underlying cell stabilization may represent an important step for directed regulation of metabolism and improvement of cell cryopreservation modes.

Our experiments revealed that the effect of glycerol on human erythrocyte Ca^{2+}-ATPase depends on structural organization of the model systems used. In saponin-permeabilized erythrocytes, glycerol caused biphasic changes in Ca^{2+}-ATPase with maximal stimulation at 10% glycerol in the medium. Removal of cytoplasmic components (including soluble endogenous regulators of PMCA) and use of erythrocyte white ghosts as the model changed Ca^{2+}-ATPase catalytic behavior in the presence of glycerol. Comparison of glycerol effects on Ca^{2+}-ATPase activity in various model systems suggests possible involvement of endogenous regulators in modification of this enzyme in the presence of the cryoprotectant.

Use of R24571, an inhibitor of CaM-regulated reactions, revealed that reaching the maximal stimulating effect of glycerol on Ca^{2+}-ATPase may involve CaM. The presence of 10% glycerol may abolish inhibitory effect of CaM found in cells subjected to hypothermic storage. A putative mechanism explaining these processes may consist in appearance of oxidized forms of CaM under hypothermic conditions characterized by imbalance between anti- and prooxidant systems [32]. Oxidation of CaM methionine residues is considered as one of the posttranslational protein modifications [33], influencing both CaM affinity to Ca^{2+} and its high affinity binding to protein targets [34, 35]. In the oxidized states, CaM may form new contact interactions with CaM-binding sequence of Ca^{2+}-ATPase and stabilizes it in the inhibitory conformation. Native (intact) CaM cannot displace oxidized molecules of this protein from Ca^{2+}-ATPase and activate it [34, 35]. Changes in physicochemical properties of the solution in response to glycerol administration into the medium may influence both structural properties of CaM and the autoinhibitory domain of PMCA. In such conditions interaction between CaM and its protein tar-

get may be either more effective or less effective; it also might represent a precondition for displacement of the oxidized CaM by native molecules of this regulator followed by formation of adequate response of PMCA on the regulatory signal. Possibility of such changes in protein–protein interactions in the presence of amphiphilic compounds may be attributed to induction of thermodynamically unfavorable states developed in surface zones of macromolecules during their contact with solvent. The latter promotes increase in protein–protein interactions, i.e., leads to decrease in contact area between protein and solvent [36, 37].

However, inhibitory analysis of the putative role of CaM in the regulation of human erythrocyte Ca^{2+}-ATPase in the presence of 10% glycerol revealed that the latter might exert the stimulatory effect in a CaM-independent manner (Fig. 3c). Although this effect was not detected in experiments with erythrocyte white ghosts, it is reasonable to suggest that certain structural modification may also be considered as one of the reasons underlying increase in Ca^{2+}-ATPase activity. Increased concentrations of glycerol may influence mobility of membrane proteins and their aggregate state; this may facilitate conformational transitions of the enzyme [38].

In conclusion, we should stress that Ca^{2+}-ATPase behavior in media with various glycerol contents may play an important role in adaptation of cells to unfavorable environmental factors. For example, stimulation of catalytic activity of the Ca^{2+}-pump by cryoprotectant concentrations corresponding to the initial stage of cell saturation found in saponin-permeabilized erythrocytes may promote their stabilization. Under these conditions, such stimulation may compensate increased rate of Ca^{2+} entry into cells induced by structural changes of the plasma membrane in response to osmotic pressure of the glycerol medium. Inhibition of the catalytic activity by ~20-25% after reaching equilibration at the stage preceding freezing may lead to increase in cytosolic Ca^{2+}. Nevertheless, this may positively influence cell stability provided that this increase would not exceed physiological oscillations. For example, moderate increase in intracellular Ca^{2+} is accompanied by the increase of cytoskeleton rigidity and may result in mechanical resistance of erythrocytes [39]. It is relevant to suggest that glycerol-induced changes in metabolism together with changes in physicochemical properties of the medium determining parameters of solution crystallization promote erythrocyte stabilization in the process of a freeze–thawing cycle.

Thus, results of our study suggest that the intracellular cryoprotectant glycerol may influence various properties of erythrocyte Ca^{2+}-ATPase stored under hypothermic conditions (2-4°C) for 2-4 days. These include regulatory sensitivity to CaM. This raises the possibility of metabolic changes of cells by regulation of Ca^{2+} level and erythrocyte stabilization during cryopreservation under glycerol protection.

REFERENCES

1. Gulevsky, A. K., Bondarenko, V. A., and Belous, A. M. (1988) *Barrier Properties of Biomembranes at Low Temperatures* [in Russian], Naukova Dumka, Kiev.
2. Pushkar, N. S., Shrago, M. I., Belous, A. M., and Kalugin, Yu. V. (1978) *Cryoprotectants* [in Russian], Naukova Dumka, Kiev.
3. Valeri, C. R., Ragno, G., Pivacek, L. E., Cassidy, G. P., Srey, R., Hansson-Wicher, M., and Leavy, M. E. (2000) *Vox. Sang.*, **79**, 168-174.
4. Meryman, H. T., and Hornblower, M. (1977) *Transfusion*, **17**, 438-442.
5. Lelkens, C. C., Noorman, F., Koning, J. G., Truijens-de Lange, R., Stekkinger, P. S., Bakker, J. C., Lagerberg, J. W., Brand, A., and Verhoeven, A. J. (2003) *Transfusion*, **43**, 157-164.
6. Konev, S. V. (1987) *Structural Lability of Biological Membranes and Regulatory Processes* [in Russian], Nauka i Tekhnika, Minsk.
7. Lew, V. L., Daw, N., Perdomo, D., Etzion, Z., Bookchin, R. M., and Tiffert, T. (2003) *Blood*, **102**, 4206-4213.
8. Barata, H., and de Meis, L. (2002) *J. Biol. Chem.*, **277**, 16868-16872.
9. Rocha, G. B., Landeira-Fernandez, A. M., and de Meis, L. (1998) *FEBS Lett.*, **231**, 73-77.
10. De Meis, L., and Inesi, G. (1988) *J. Biol. Chem.*, **263**, 157-161.
11. Galina, A., and de Meis, L. (1991) *J. Biol. Chem.*, **266**, 17978-17982.
12. Gladilin, A. K., and Levashov, A. V. (1998) *Biochemistry (Moscow)*, **63**, 345-356.
13. Benaim, G., and de Meis, L. (1989) *FEBS Lett.*, **244**, 484-486.
14. Martins, O. B., and de Meis, L. (1985) *J. Biol. Chem.*, **260**, 6776-6781.
15. Alves, G. G., Lima, L. M., Favero-Retto, M. P., Lemos, A. P., Peres-Sampaio, C. E., Meyer-Fernandes, J. R., Vieyra, A., and Sola-Penna, M. (2001) *Biosci. Rep.*, **21**, 25-32.
16. Rubinacci, A., Fuller, B., Waytack, F., and de Loecker, W. (1986) *Cryobiology*, **23**, 134-140.
17. McConnell, E. J., Wagoner, M. J., Keenon, C. E., and Raess, B. U. (1999) *Biochem. Pharmacol.*, **57**, 39-44.
18. Pokudin, N. I., Petrunyaka, V. V., and Orlov, S. N. (1988) *Biokhimiya*, **5**, 753-758.
19. Grietzen, K., Sadorf, J., and Bader, H. (1982) *Biochem. J.*, **207**, 541-548.
20. Rathbum, W., and Betlach, V. (1969) *Analyt. Biochem.*, **28**, 436-445.
21. Bradford, M. M. (1976) *Analyt. Biochem.*, **72**, 248-254.
22. Belous, A. M., Gordienko, E. A., and Rosanov, L. F. (1987) *Freezing and Cryoprotection. Membrane Biochemistry* (Boldyrev, A. A., ed.) [in Russian], Vysshaya Shkola, Moscow.
23. Filomatori, C. V., and Rega, A. F. (2003) *J. Biol. Chem.*, **278**, 22265-22271.
24. Strehler, E. E., and Zacharias, D. A. (2001) *Physiol. Rev.*, **81**, 21-50.
25. Padanyi, R., Raszty, K., Penheiter, A. R., Filoteo, A. G., Penniston, J. T., and Enyedi, A. (2003) *J. Biol. Chem.*, **278**, 35798-35804.

26. Enyedi, A., Verma, A. K., Filoteo, A. G., and Penniston, J. T. (1996) *J. Biol. Chem.*, **271**, 32461-32467.
27. Carafoli, E. (1991) *Physiol. Rev.*, **71**, 129-153.
28. Penheiter, A. R., Bajzer, Z., Filoteo, A. G., Thorogate, R., Torok, K., and Caride, A. J. (2003) *Biochemistry*, **42**, 12115-12124.
29. Malaya, L. T., Petrunyaka, V. V., and Rudik, Yu. S. (1991) *Vestnik AMN SSSR*, **7**, 54-58.
30. Mavrov, I. I., Goncharenko, M. S., Petrunyaka, V. V., Tereschenko, E. A., Kondakova, A. K., and Stepanyuk, L. V. (1990) *Ukr. Biokhim. Zh.*, **3**, 72-76.
31. Rybina, V. V., Elenskaya, I. A., and Kaimachnikov, N. P. (2001) *Biol. Membr. (Moscow)*, **18**, 287-293.
32. Kravchenko, L. P., Belous, A. M., and Shanina, I. V. (1994) *Cryo-Lett.*, **3**, 135-145.
33. Bigelow, D. J., and Squier, T. C. (2005) *Biochim. Biophys. Acta*, **1703**, 121-134.
34. Yao, Y., Yin, D., Jas, G. S., Kuczer, K., Williams, T. D., Schoneich, C., and Squier, T. C. (1996) *Biochemistry*, **35**, 2767-2787.
35. Chen, B., Mayer, M. U., and Squier, T. C. (2005) *Biochemistry*, **44**, 4737-4747.
36. Douzou, P. (1986) *Cryobiology*, **23**, 38-47.
37. Carpenter, J. F., and Crowe, J. H. (1988) *Cryobiology*, **25**, 244-255.
38. Rubtsov, A. M., Boldyrev, A. A., Liqun Yang, McStay, D., and Quinn, P. J. (1994) *Biochemistry (Moscow)*, **59**, 1263-1268.
39. Liu, F., Mizukami, H., Sarnaik, S., and Ostafin, A. J. (2005) *Struct. Biol.*, **150**, 200-210.