Changes in the Structure and Functions of Membranes in Erythrocytes and Ehrlich Ascites Carcinoma Cells under the Influence of a New Generation Hybrid Antioxidant IKhFAN-10

O. M. Vekshina, L. D. Fatkullina, Yu. A. Kim*, and E. B. Burlakova

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 143, No. 4, pp. 402-406, April, 2007
Original article submitted August 30, 2006

We studied the effect of a new generation hybrid antioxidant IKhFAN-10 on the structure and function of cell membranes (organization of the lipid bilayer and proteins and activity of ion channels). The test preparation proposed for the therapy of neurodegenerative diseases modified properties of membranes in erythrocytes and Ehrlich ascites carcinoma cells and affected functional activity of cells. We determined the doses of this antioxidant, which did not cause side effects.

Key Words: *antioxidants; heat denaturation; Ca^{2+}-dependent K^+ channels; light scattering; microviscosity*

Alzheimer's disease is a membrane disorder characterized by oxidative stress and significant changes in the system for regulation of lipid peroxidation and structure, lipid composition, and function of membranes [6]. One of the approaches to the search for new drugs that can be used in correction of Alzheimer's disease is the development of combined antioxidant preparations with several pathological targets. New generation hybrid antioxidants (AO) were synthesized at the Institute of Biochemical Physics. IKhFANs, derivatives of phenosan [β-4-hydroxy-(3,5-ditert-butyl-4-hydroxyphenyl) potassium propionate], consist of the AO part, choline residue responsible for anticholinesterase activity, and fatty acid fragment modulating membrane viscosity and promoting to permeation through the blood-brain barrier. IKhFANs have high AO activity and serve as cholinesterase inhibitors in human erythrocytes [5]. Here we studied the effects of IKhFAN-10 on membranes.

N. M. Emanuel Institute of Biochemical Physics, Russian Academy of Sciences, Moscow; *Institute of Cell Biophysics, Russian Academy of Sciences, Pushchino. ***Address for correspondence:*** olgavek@yandex.ru. O. M. Vekshina

MATERIALS AND METHODS

Erythrocytes served as an object of the study. Erythrocytes are one of the first targets for circulating drugs. Moreover, structural elements in the erythrocyte membrane are similar to those in various cells of the organism. However, erythrocytes lack complete signal transduction system typical of less specialized cells. Therefore, a model system of Ehrlich ascites carcinoma (EAC) cells served as the second object of the study. It is characterized by the presence of a complete signal transduction system from the outer surface to the internal structures and typical cell response to the signal.

Erythrocytes and erythrocyte ghosts were isolated from outbred albino rats. Cell integrity was estimated by the degree of erythrocyte hemolysis. The hemoglobin absorption maximum was measured spectrophotometrically (575 nm) in the supernatant after erythrocyte sedimentation [1]. EAC

0007-4888/07/1434-0426 © 2007 Springer Science + Business Media, Inc.

cells, erythrocytes, and erythrocyte ghosts were preincubated in the presence or absence of AO at 37°C for 45 min, precipitated, and resuspended before the study.

We studied the following parameters of membrane structure and functions: microviscosity of the lipid bilayer, thermostability of membrane protein domains, and conductance of Ca^{2+}-dependent K^+ channels in the presence of IKhFAN-10 in concentrations of 10^{-4}-10^{-7} M. These parameters were selected taking into account the fact that modification of membrane lipids and proteins during Alzheimer's disease leads to significant changes in membrane fluidity. Microviscosity determines function of membrane-bound proteins, *e.g.* Ca-K channels in the plasmalemma, which are activated in response to high intracellular Ca^{2+} concentration typical for various diseases. Activity of Ca^{2+}-dependent K^+ channels was measured potentiometrically with a K^+-selective electrode [3]. Ca^{2+}-induced K^+ release was initiated by addition of calcium ionophore A23187 (2-4 μM) under conditions of low K^+ content (less than 1 μM). Thermograms of erythrocyte ghost membranes were recorded using a DASM-4 differential adiabatic scanning microcalorimeter.

The isolation of EAC cells and recording of perpendicular light scatter were performed on a Perkin-Elmer-44B spectrofluorometer at 510 nm [2]. Microviscosity of membranes in erythrocytes and EAC cells was measured on an ER-200D SRC electron paramagnetic resonance (EPR) spectrometer (Bruker) at room temperature using 2 paramagnetic spin probes: 2,2,6,6-tetramethyl-4-capryloyl-hydroxypiperidine-1-oxyl primarily located in the surface layer of the membrane lipid bilayer and 5,6-benzo-2,2,6,6-tetramethyl-1,2,3,4-tetrahydro-γ-carbolin-3-oxyl permeating deep regions of annular lipids. The rotational correlation time for a probe was calculated from EPR spectra.

RESULTS

IKhFAN-10 in a concentration of 10^{-4} M induced complete erythrocyte hemolysis and maximum release of K^+ into the incubation medium. The curves for the dependence of K^+ release and degree of hemolysis under the influence of IKhFAN-10 in lower concentrations were different (Fig. 1). The erythrocyte hemolysis curve had a maximum at high concentration of AO, but did not differ from the control in the presence of test preparation in lower concentrations (10^{-17}-10^{-6} M). The dependence of K^+ release was described by an S-shaped curve. IKhFAN-10 in concentrations of 10^{-10}-10^{-6} M induced the release of 20% K^+. K^+ release decreased in the presence of 10^{-15}-10^{-11} M IKhFAN-10, but slightly increased under the influence of IKhFAN-10 in concentrations of 10^{-17}-10^{-16} M (Fig. 1). As distinct from IKhFAN-10 in low concentrations (10^{-17}-10^{-6} M), this AO in high concentrations (10^{-5}-10^{-4} M) probably impairs the integrity of erythrocyte membrane. It should be emphasized that in intact erythrocytes we observed two maxima for activation of Ca^{2+}-dependent K^+ channels at low AO concentrations.

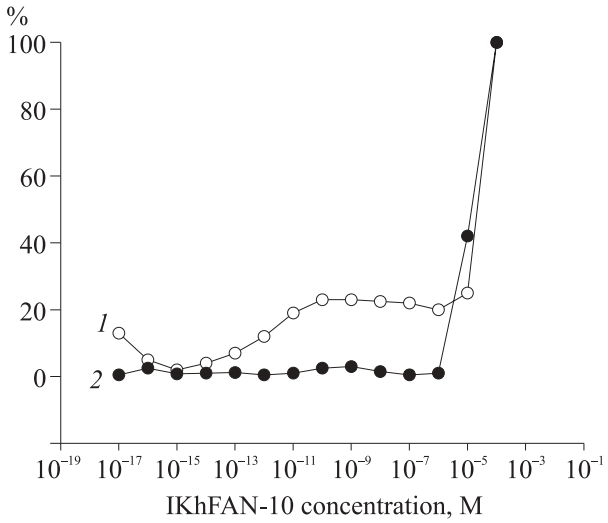

Fig. 1. Effect of IKhFAN-10 on K^+ release from erythrocytes (*1*) and their hemolysis (*2*).

The effect of IKhFAN-10 on the ATP-activated intracellular Ca^{2+} signaling system [2] was tested on EAC cells. The interaction of ATP with purinoceptors on the cell surface was followed by Ca^{2+} signal generation followed by activation of Ca^{2+}-dependent K^+ and Cl^- channels, which resulted in changes in cell volume and light scatter [2]. IKhFAN-10 in a concentration of 10^{-5} M significantly modulates light scatter, which reflects changes in cell shape and channel function (Fig. 2). Application of IKhFAN-10 in a concentration of 10^{-5} M inhibited Ca^{2+} signal transduction induced by the influence of ATP on purinoceptors of the plasmalemma. IKhFAN-10 in a concentration of 10^{-8} M or lower was ineffective. Therefore, IKhFAN-10 in a concentration $\leq 10^{-8}$ M produces no side effects on EAC cells.

Five heat-induced transitions were identified in thermograms of rat erythrocyte ghosts (A, B_1, B_2, C, and D; Fig. 3, *a*). Preincubation of erythrocyte ghosts with IKhFAN-10 and phenosan for 45 min was followed by a slight shift in transition temperature. A-transition reflecting denaturation of the domain in the membrane skeleton (complex of α-spectrin, β-spectrin, and actin) was shifted towards a high temperature region. B_1-transition caused by denaturation of the membrane domain (an-

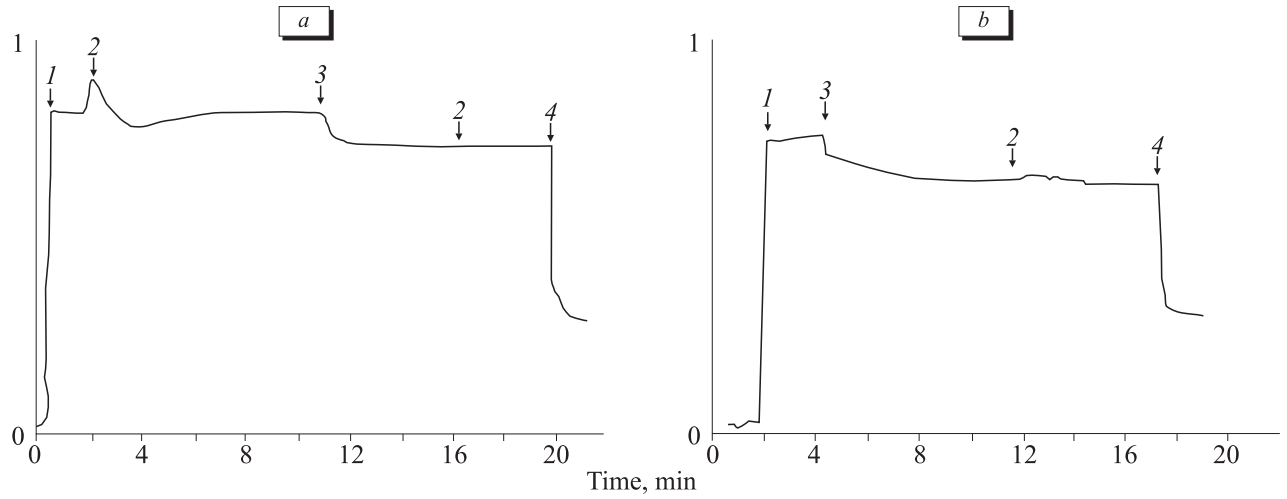

Fig. 2. Effect of IKhFAN-10 on ATP-dependent Ca^{2+} signaling system in EAC. Kinetic curves of light scatter of diluted cell suspension. EAC (*1*), ATP (*2*), IKhFAN-10 (10^{-5} M, *3*), and Triton X-100 (*4*).

kyrin, band 4.1 and 4.2 proteins, and dematin) remained unchanged. The following transitions were shifted toward the low temperature region: B_2-transition (denaturation of the cytoplasmic fragment in band 3 protein), C-transition (denaturation of a 55-kDa membrane fragment in band 3 protein, ion channels), and D-transition (effect of unidentified proteins and temperature-dependent membrane vesiculation). Ethanol distorted the peaks of B_1-, B_2-, and D-transitions in thermograms, while treatment with AO returned the thermograms to normal (except D-transition; Fig. 3, *b*, *c*). Hence, IKhFAN-10 (10^{-6} M) had a stabilizing effect on the structure of isolated membranes.

The effects of a pharmacologically active substance on the isolated membrane and whole cell membrane can significantly differ in the concentration dependence and quality characteristics. Therefore, erythrocyte ghosts were isolated after pretreatment of whole erythrocytes with IKhFAN-10. Preincubation of erythrocytes with IKhFAN-10 in concentrations of 10^{-6} and 10^{-5} M changed thermograms of erythrocyte ghosts (Fig. 3, *c*). Erythrocyte ghosts preincubated with ethanol served as the control. A-transition peak in thermograms of these ghosts decreased compared to untreated ghosts. Published data show that disappearance of A-transition is accompanied by the loss of deformability in erythrocytes and ghost membranes [1,4]. Treatment of erythrocytes with IKhFAN-10 restored A-transition peak. These changes were probably accompanied by recovery of the cytoskeleton. Treatment of erythrocytes with AO was followed by a shift in the temperature maxima toward the low temperature range. Their amplitude also changed under these conditions. Increasing the concentration of IKhFAN-10 to 10^{-5} M led to fusion of B_1 and B_2 transitions peaks into one B-transition peak. Under normal conditions, these changes accompany a decrease in the ionic strength. It can be hypothesized that IKhFAN-10 in high concentrations (10^{-6} and 10^{-5} M) induce significant structural changes in the whole erythrocyte membrane.

EPR data showed that IKhFAN-10 in a wide concentration range modulates membrane microviscosity in erythrocytes and EAC cells (Table 1).

AO increased viscosity of the lipid bilayer and annular lipid domains (probes 1 and 2) in erythro-

TABLE 1. Relative Changes in Microviscosity of the Membrane Lipid Bilayer under the Influence of IKhFAN-10 in Various Concentrations

Object of study	Probe	IKhFAN-10 concentration, M						
		10^{-16}	10^{-14}	10^{-12}	10^{-10}	10^{-8}	10^{-6}	10^{-4}
Erythrocytes	1	1.12	1.11	1.09	1	1.02	1.12	1.15
	2	1.09	1.02	0.96	1.01	1	1.01	1.18
EAC cells	1	1.09	0.91	0.78	0.8	0.91	0.69	1.1
	2	1.05	0.66	0.84	0.97	1.12	0.93	1.15

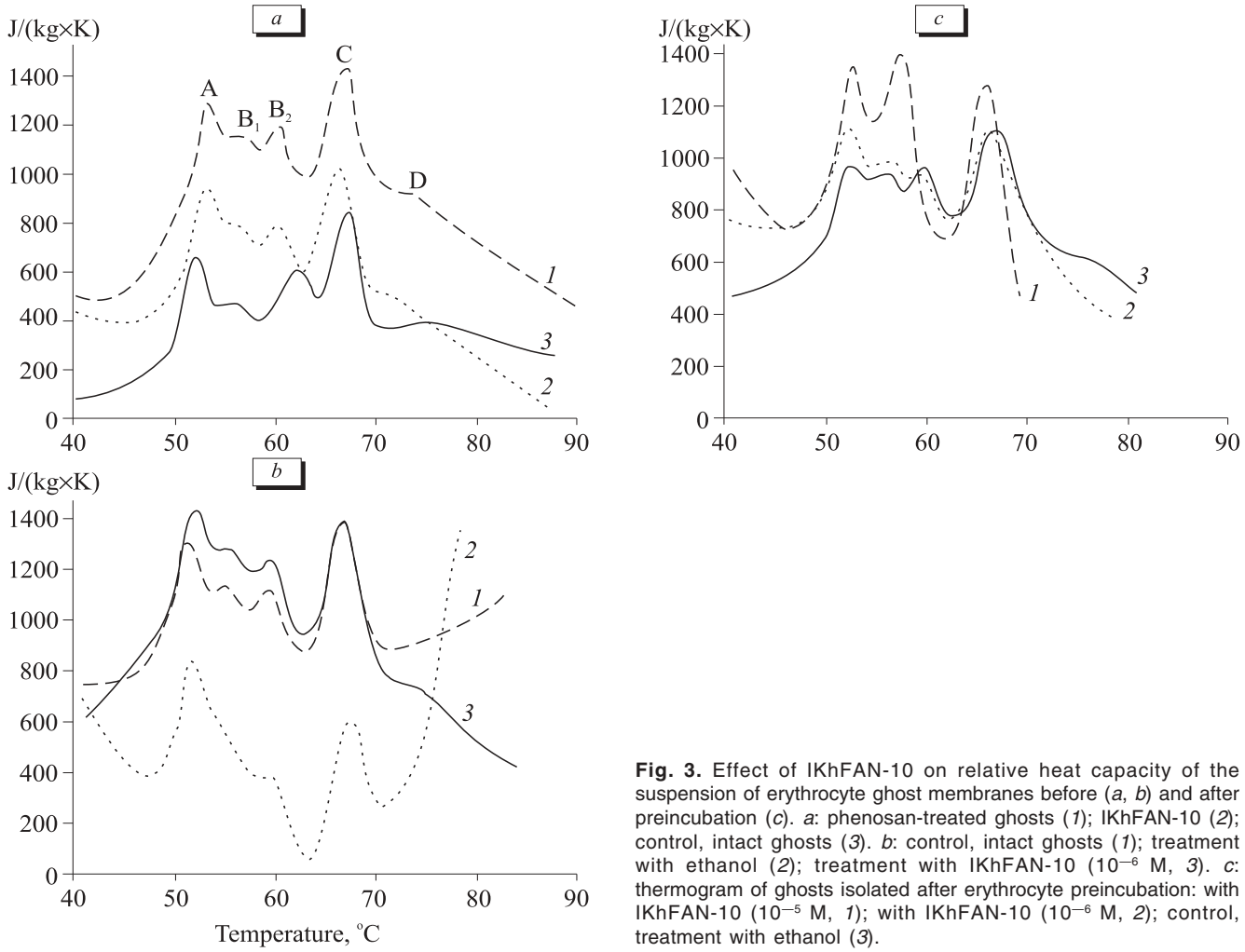

Fig. 3. Effect of IKhFAN-10 on relative heat capacity of the suspension of erythrocyte ghost membranes before (*a*, *b*) and after preincubation (*c*). *a*: phenosan-treated ghosts (*1*); IKhFAN-10 (*2*); control, intact ghosts (*3*). *b*: control, intact ghosts (*1*); treatment with ethanol (*2*); treatment with IKhFAN-10 (10^{-6} M, *3*). *c*: thermogram of ghosts isolated after erythrocyte preincubation: with IKhFAN-10 (10^{-5} M, *1*); with IKhFAN-10 (10^{-6} M, *2*); control, treatment with ethanol (*3*).

cyte membranes compared to untreated membrane samples. These changes were most significant under the influence of AO in concentrations of 10^{-16}-10^{-14} and 10^{-6}-10^{-4} M, which correlates with 2 peaks of the increased K^+ release from erythrocytes and is consistent with the dependence of channel function on microviscosity. Thermostability of cytoskeleton protein domains remained unchanged in the presence of AO in high concentrations. These data indicate that treatment with the test preparation in high concentration induces structural modification, including changes in microviscosity of the lipid phase and annular lipids in the erythrocyte membrane and variations in the structure of cytoskeleton proteins. Direct potentiometry showed that these changes affect function of Ca^{2+}-dependent K^+ channels in erythrocytes. IKhFAN-10 in low concentrations increased microviscosity and activated K^+ channels, but had little effect on membrane structure. AO in very high concentration (10^{-4} M) impairs the integrity of erythrocyte membrane. In EAC cells, IKhFAN-10 in various concentrations significantly decreased microviscosity of surface lipids and annular lipids, but in the presence of IKhFAN-10 in concentrations of 10^{-16} and 10^{-4} M microviscosity in these cells increased. These changes correlated with light scatter data and potent effect of AO (10^{-5} M) on the Ca^{2+} signaling system in EAC cells (Ca^{2+}-dependent channels for regulation of cell volume). AO in lower concentrations (10^{-8} M) decreased microviscosity and, therefore, had no effect on variations in cell volume. Probably, the Ca^{2+} signaling system is practically unaffected by IKhFAN-10 in this concentration.

Thus, studying the influence of IKhFAN-10 on cell model system (EAC cells) allowed us to estimate the limits of concentrations, at which this preparation caused no side effects. After exogenous application, IKhFAN-10 in pharmacological concentrations (10^{-8} M) had no effect on the Ca^{2+} signal transduction system. Application of IKhFAN-10 in lower concentrations (10^{-5} M) is accompanied by a significant change in Ca^{2+} signaling system. We conclude that IKhFAN-10 should be

used in concentrations of no more than 10^{-8} M. IKhFAN-10 in the specified concentrations causes no side effects on this system. Experiments on erythrocytes showed that preincubation with IKhFAN-10 in concentrations of 10^{-5}-10^{-6} M induced structural changes in ghost membranes, which correlates with the degree of hemolysis and activity of Ca^{2+}-dependent K^+ channels. A correlation between changes in the shape of erythrocytes and degree of hemolysis was demonstrated previously [4]. IKhFAN-10 in lower concentrations is less effective. The effect of IKhFAN-10 disappears with a further decrease in its concentration. Probably, the test preparation in a concentration of no more than 10^{-8} M has little effect on the structure of cytoskeleton and function of cell ion channels. IKhFAN-10 in ultralow concentrations (10^{-15}-10^{-17} M) induces little changes in activity of K^+ channels. Treatment with AO in these concentrations does not impair cell integrity, significantly increases viscosity of the upper layer of the lipid bilayer, and slightly increases viscosity of its deep layers containing integral proteins. The proposed hybrid AO in ultralow concentrations can have a targeted effect, *e.g.* activate ion channels. IKhFAN-10 in these concentrations does not cause side effects impairing cell structure and function.

We are grateful to Yu. S. Tarakhovskii for his help in preparing the manuscript for publication.

REFERENCES

1. A. K. Gulevskii, V. V. Ryazantsev, and A. M. Belous, *Nauchnye Doklady Vysshei Shkoly. Biol. Nauki*, 29-36 (1990).
2. V. P. Zinchenko, V. A. Kasymov, V. V. Li, and N. P. Kaimachnikov, *Biofizika*, **50**, No. 5, 1055-1069 (2005).
3. N. V. Maksimova, S. Yu. Chizhevskaya, Yu. A. Karpov, and Yu. V. Postnov, *Kardiologiya*, **5**, 45-49 (1999).
4. E. Yu. Parshina, L. Ya. Gendel', and A. B. Rubin, *Biofizika*, **49**, No. 6, 1094-1098 (2004).
5. E. M. Molochkina, I. B. Ozerova, and E. B. Burlakova, *Free Radic. Biol. Med*., **33**, Iss. 2S1, No. 610, 229-230 (2002).
6. M. R. Prasad, M. A. Lovell, M. Yatin, *et al*., *Neurochem. Res*., **23**, No. 1, 81-88 (1998).