Obtaining «ghosts» of red blood cells with a set ion concentration as models for studying Na+, K+ - ATPase

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Abstract. The article considers the production of red blood cell «ghosts» and their application as a model for studying Na+, K+ - ATPase. Various variants of liposomes are used as test objects for studying ion transport, but the best model of biological membranes for studying ion transport and ATPase pumps are the «ghosts» of red blood cells. The activity of blood Na+/K+ - ATPase is determined mainly in the suspension of red blood cell «ghosts». This is due to the need to remove the interfering compound – hemoglobin - and obtain "«ghosts»" that are necessary for accurate measurement of the activity of the enzyme. In this scientific work, studies were conducted to determine the concentrations of Na+ and K+ ions in the blood and red blood cells of cows and calves of various ages by atomic absorption and atomic emission spectroscopy. The values of these concentrations of Na+ and K+ ions were used to obtain red blood cell «ghosts». «Ghosts» of red blood cells with a set concentration of sodium and potassium ions were obtained by the method of J. T. Dodge, in the modification of Zhumadilov Zh. Sh. and Gening T. P. and in our modification. Studies of the concentration of Na+ and K+ ions in the obtained «ghosts» of red blood cells showed their full compliance with the calculated values.

1 Introduction

The ionic composition of the intracellular environment differs from the ionic composition of the environment. This is most typical of monovalent sodium and potassium cations.

Ionic asymmetry is used for the transport of sugars, amino acids, organic acids and other compounds into the cell and the removal of metabolic products from it, the generation of excitation in nerve and muscle cells, the transmission of a hormonal signal, and many other processes.

The erythrocyte membrane is composed by 60% of phospholipids, essentially phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and phosphatidylserine. It has also some phospholipidic minor components such as phosphatidylinositol, phosphatidylethanolamine, phosphatidic acid, lysophosphatidylcholine and lysophosphatidylethanolamine. Non-sterified cholesterol represents about 30% of the lipidic erythrocyte membrane composition, and the last 10% are glycolipids [16]. At the physiologic pH, the majority of phospholipid content is electrically neutral, although phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine are negatively charged. With the exception of sphingomyelin and lysophosphatidylcholine, the bulk of phospholipids have two fatty acid chains attached to a glycerol backbone.

The erythrocyte cytoskeleton consists of several proteins that form a filamentous network under the lipid bilayer. The network is composed of spectrin, ankyrin, actin, and protein 4.1. Protein 4.1 of red blood cells (Figure 2) is a multifunctional protein essential for maintaining erythrocyte shape and membrane mechanical properties, such as deformability and stability [12]. Cytoskeletal proteins interact with integral proteins and lipids of the bilayer to maintain membrane integrity. The cytoskeleton has an important role in erythrocyte shape, flexibility, and lipid organization [13].

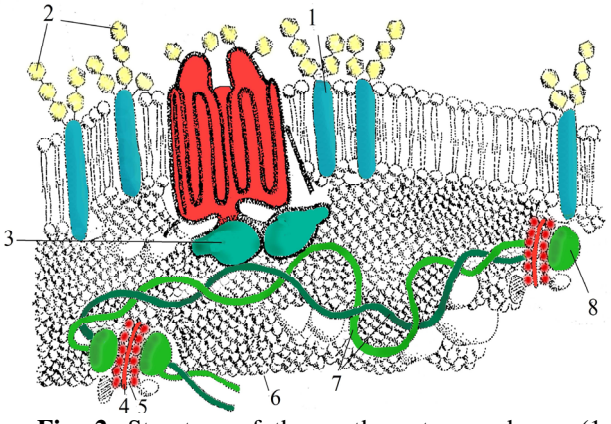

Fig. 2. Structure of the erythrocyte membrane (1-Glycophorin, 2-Carbohydrate, 3-Ankirin, 4-Tropomyosin, 5-Actin, 6-Lipid bilayer, 7-spectrin dimmer, 8- Protein 4.1)

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The active transport of ions against their concentration gradient is carried out by ion pumps, in particular by Na, K-ATPase, which transports sodium and potassium ions through the biological membrane.

During recent years biophysical experiments using a variety of techniques have been focusing on the ion move ments through the Na,K-ATPase [35, 36, 37].

The study of Na/K-ATPase began in 1957 by J. Skou. Na/K-ATPase is an integral enzyme of the membranes of all cells involved in the regulation of their ion homeostasis (Figure 1). Na/K-ATPase is the largest protein complex in the family of P-type cation pumps. The minimum functional unit is a heterodimer of the α- and β-subunits [26].

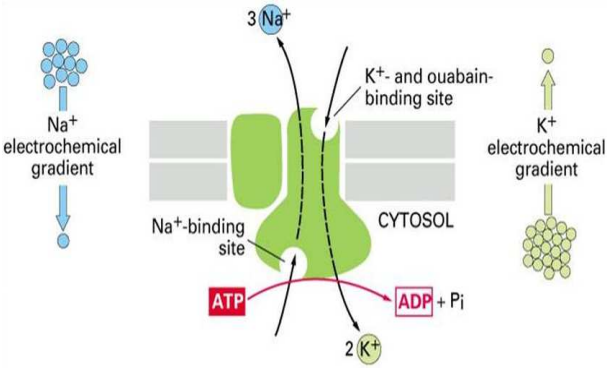

Fig. 1. Schematic structure of Na/K-ATPase and it function

The Na/K-ATPase consists of two subunits, α and β.

Some information on structural features of Na/K-ATPase has been gained from electron microscopic images [32, 33, 34].

Na,K-ATPase possesses two charged sites for two Na^+ or two K^+ ions and a third neutral site specific for a Na^+ ion (Figure 3).

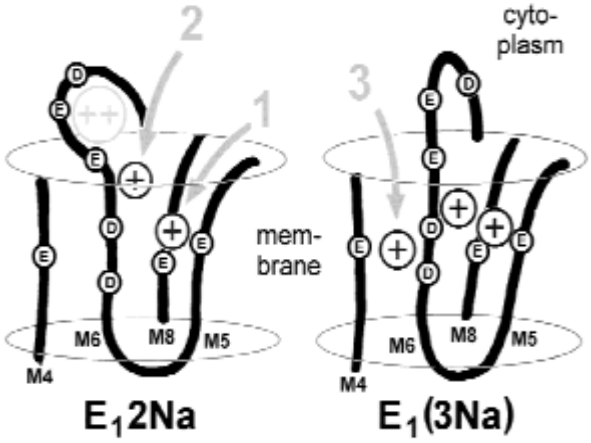

Fig. 3. Hypothetical structure-function relation of the cytoplasmic ion binding sites of the Na,K-ATPase. Binding of three Na^+ ions [39]. The negatively charged amino acids D (aspartate) and E (glutamate) are placed according to a proposal of Vilsen et al. [38].

An effective way to investigate ion transport is the detection of charge movements in the course of the transport process [37, 40, 41, 42].

The classic object of study was the giant squid axon. Subsequently, it was found that the most convenient objects for studying the mammalian enzyme are the «ghosts» of red blood cells, which are shells consisting of the cytoplasmic membrane of red blood cells, with various protein structures integrated into it, for example, Na, K-ATPase. It has long been known that the cell residues after hypotonic hemolysis are by no means disrupted or disintegrated cells; on the contrary, the «ghosts» seem to retain many of the properties of intact blood corpuscles.

Various variants of liposomes are used as test objects for studying ion transport, but the best model of biological membranes for studying ion transport and ATPase pumps are the «ghosts» of red blood cells.

In recent decades, red blood cell «ghosts» have been widely used in biological research. This is due to several reasons. First, due to the fact that dead red blood cells («ghosts» of red blood cells) may appear in the bloodstream as a result of many pathological influences. At the same time, it was found that the «ghosts» of red blood cells, charged less negatively than normal red blood cells [6], could cause the development of renal failure [14]. In this regard, it is necessary to remove them from the bloodstream [9].

Among the various biological carriers, an erythrocyte-derived vector possesses great potential. Red blood cells («ghosts») are excellent carrier candidates owing to the following advantages [11–14]: 1) «ghosts» are easily available because they are the most abundant cells in the body. 2) «Ghosts» can provide considerable intracellular space for reagents as «ghosts» lack a nucleus and other organelles. 3) «Ghosts»-derived carriers do not produce toxic side effects because autologous «ghosts» are biocompatible, and «ghosts» possess osmotic fragility, which allows them to physically load cargos without chemical additives. 4) «Ghosts» have plastic deformability owing to their biconcave-disk shape, which enables «ghosts» to deform through pulmonary capillary and splenic sinus blood vessel walls [27-30].

Second, they can be used as simple models to study the functioning of ion pumps in cell membranes. Providing oxygen transport, red blood cells are highly specialized cells of the body, consisting of 25% of hemoglobin [2]. The activity of blood Na^+/K^+ - ATPase is determined mainly in the suspension of red blood cell «ghosts». This is due to the need to remove the interfering compound - hemoglobin - and obtain "«ghosts»", which are necessary for accurate measurement of the activity of the enzyme [3, 5, 13].

Third, there are a number of publications that consider red blood cells as a system for directed drug transport [7]. For the first time, the introduction of chemical compounds into red blood cells was carried out in 1953 during an attempt to load ATP into red blood cell "«ghosts»" [31]. In 1973, the successful sealing of therapeutic drugs in erythrocyte "«ghosts»" for direct delivery to target organs was described [16, 21, 22].

Since the 70s of the XX century, the possibility of creating drug delivery systems directly to the pathological focus by binding drug molecules and

certain other molecules (vectors) that are tropic to certain cells (the "tagging" method), as well as by enclosing drug molecules in "bioactive" capsules based on semi-permeable artificial or natural membranes (the "packaging " method") [1, 11, 9]. A separate direction formed as a result of studying the possibility of transporting drugs to the pathological focus is the development of delivery systems that use natural containers as carriers - shaped elements of human or animal blood that are not covered with the corresponding antibodies to target cells [10, 1].

A well-known method for obtaining «ghosts» of red blood cells is the method of Dodge et al. (1963) [18], in addition, there are other methods for obtaining «ghosts», also based on hypoosmotic hemolysis of red blood cells [18, 20].

2 Materials and methods

The object of the study was cows and calves of Simmental and black-and-white breeds. The animals were selected according to the principle of analogues, taking into account their origin, body weight, age and development. All the animals were kept in stereotypical conditions of feeding and keeping, with constant observance of the daily routine and provision of the necessary conditions.

Blood for research in cattle was taken from the jugular vein. As an anticoagulant, heparin was used, which was added to the samples at the rate of 4-6 units per 1 ml of blood. The heparinized blood was immediately placed in a thermos with ice and after 15-20 minutes was delivered to the laboratory, where tests were performed.

The separation of red blood cells from plasma was carried out by centrifugation in a refrigerated centrifuge for 30 minutes at 3000 rpm. After separation, the red blood cells were washed several times with a sterile isotonic solution.

«Ghosts» of red blood cells with a set concentration of sodium and potassium ions were obtained by the method of J. T. Dodge [19], Zhumadilov Zh. Sh. and Gening T. P. [4] in our modification.

To do this, venous blood with heparin was delivered to the laboratory, where red blood cells were washed twice from the plasma with isotonic sodium chloride solution by centrifugation at 3000 rpm for 5 minutes at 4°C. A sevenfold volume of distilled water cooled to 0°C was added to the erythrocyte sediment and centrifuged at 8000 rpm for 25 minutes at 4°C. The attachment fluid, which contains hemoglobin, was sucked off, and a sevenfold volume of solutions was added to the resulting red blood cell «ghosts»:

a) cow red blood cell «ghosts»-Na-3.74, K-0.57 mmol×l^{-1};

b) red blood cell «ghosts» of calves (6 days) - Na-1.99 mmol×l^{-1}, K-1.54 mmol×l^{-1};

c) «ghosts» of red blood cells of calves (3-4 months) - Na-3.42 mmol×l^{-1}, K-0.66 mmol×l^{-1}.

The suspension was incubated for 15-20 minutes at 4°C. Then 1/9 of the volume of 9% sodium chloride was added to restore the integrity of the erythrocyte membrane and incubated for 30 minutes at 37°C.

The content of Na^+ and K^+ in erythrocytes and blood plasma was determined by atomic absorption and atomic emission spectroscopy [8, 23].

The advantages of atomic spectroscopic methods – atomic emission and atomic absorption - are their versatility and expressiveness, which is especially important when analyzing a large number of samples. The method of flame photometry is widely used in the practice of clinical laboratories for the determination of alkali metals in biological fluids due to the ease of implementation, fast execution and availability of equipment [24, 25]. The widespread use of inductively coupled plasma atomic emission spectroscopy, despite the attractiveness of the method as a multi-element method, is constrained by the high cost of equipment [26]. Atomic absorption spectrometry for the determination of potassium and sodium in biological objects, including in clinical studies, is used quite rarely. As in flame photometry, atomic absorption shows the effect of the mutual influence of alkali metals [26]. On the other hand, modern atomic absorption spectrophotometers can record both the absorption and emission of atoms.

3 Results and Discussion

Figure 4 shows the results of studies on the content of sodium and potassium in red blood cells and blood plasma of cattle of various ages.

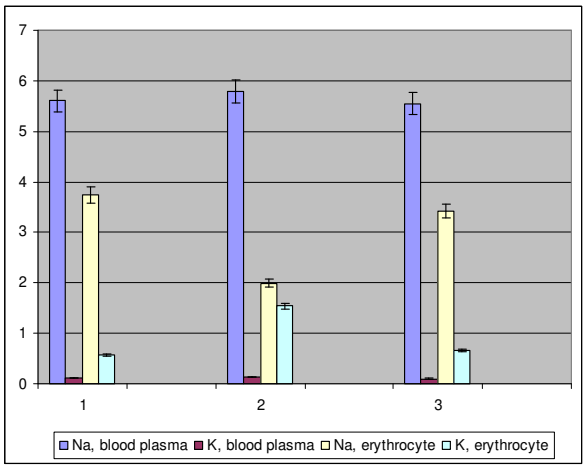

Fig. 4. The content of Na^+ and K^+ in blood plasma and red blood cells of cattle: 1-mother cows, 2-calves at the age of 6 days, 3-calves at the age of 3-4 months.

The analysis of this figure shows that in erythrocytes cows mothers Na^+-3.74±0.03 mmol×l^{-1}, K^+ 0.57 ±0.03, in erythrocyte calves 6 days of age Na^+ - 1.99 ±0.02 mmol×l^{-1}, K^+ 1.54±0.12 mmol×l^{-1}, and the calves at the age of 3-4 months Na^+ was 3.42±0.06 mmol×l^{-1}, K^+ 0.66±0.08 mmol×l^{-1}.

Thus, the concentrations of sodium and potassium were determined to obtain red blood cell «ghosts» with a set ion concentration: cows - Na-3.74, K-0.57 mmol×l^{-1};

calves (6 days) - Na-1.99 mmol×l^{-1}, K-1.54 mmol×l^{-1}; calves (3-4 months) - Na – 3.40 mmol×l^{-1}, K – 0.66 mmol×l^{-1}.

Figure 5 shows the results of studies of the Na and K content in the obtained «ghosts» of red blood cells.

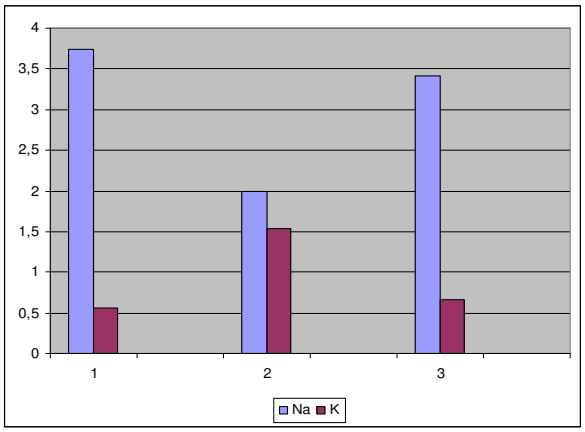

Fig. 5. The content of Na^+ and K^+ in the «ghosts» of red blood cells of cattle: 1-mother cows, 2-calves at the age of 6 days, 3-calves at the age of 3-4 months.

Analysis of this figure shows that the highest content of Na^+ and K^+ in the «ghosts» of red blood cells of cows of mothers, less is in the «ghosts» of red blood cells of calves at the age of 3-4 months, and the lowest their content is in calves at the age of 6 days.

4 Conclusion

The ionic composition of cells differs from the ionic composition of the environment. This is most typical for monovalent sodium and potassium ions. A difference in the concentrations of monovalent cations is created on the plasma membrane.

Ionic asymmetry is used to transport sugars, amino acids, organic acids and other compounds into the cell and remove metabolites from it, generate excitation in nerve and muscle cells, transmit a hormonal signal, and many other processes.

The active transport of ions against their concentration gradient is carried out by ion pumps, in particular by Na, K-ATPase, which transports sodium and potassium ions through the biological membrane.

The study of Na, K-ATPase began in 1957 by J. Skou. Na, K-ATPase is an integral enzyme of erythrocyte membranes involved in the regulation of ion homeostasis of cells. The classic object of study was the giant squid axon.

Subsequently, it was found that the most convenient objects for studying the mammalian enzyme are the «ghosts» of red blood cells.

«Ghosts» of red blood cells with a set concentration of sodium and potassium ions were obtained.

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