Recording Maximal Ca-ATPase Activity in Intact Human Erythrocytes by Means of a Ca-Selective Electrode

N. V. Maksimova and V. V. Petrov

UDC 616.151.8

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol.116, № 11, pp. 493-497, November, 1993
Original article submitted May 20, 1993

Key Words: *human erythrocytes; Ca-ATPase; methodology; Ca-selective electrode*

The accumulated data concerning the key role of calcium as a biological regulator of vital cell processes have prompted the development of appropriate methods for the determination of the level of free calcium in the cytoplasm and studies of the mechanisms of its maintenance [1, 3, 5, 6, 20]. Transmembrane Ca^{2+} transport mediated by channels, carriers, or pumps can be assessed by the methods of isotope exchange, fluorescent analysis, atom-adsorption spectroscopy, or by means of Ca-selective electrodes in either intact cells or cell organelle fractions [2, 7, 8, 13, 15, 17].

In mammalian erythrocytes the activity of Ca-ATPase and the rate of ATP-dependent Ca^{2+} accumulation are most commonly measured by inorganic phosphorus accumulation using enclosed inverted vesicles, because the active center of ATPase is located on the inner surface of the plasma membrane [4, 21-23]. In measurements of the active Ca^{2+} transport in intact cells the intracellular concentration of Ca^{2+} ions should be preliminarily increased with Ca-ionophores ionomycin or A23187, or by long-term incubation in the cold in the presence of a high Ca^{2+} concentration in the incubation medium [14].

Such investigations are usually based on the isotope method of ^{45}Ca transport or atom-adsorption spectroscopy, since the use of fluorescent probes is complicated by the presence of hemoglobin [4,6,14].

Cardiological Research Center, Moscow. (Presented by E. I. Chazov, Member of the Russian Academy of Medical Sciences)

The search for simple and less laborious methods for the investigation of Ca^{2+} transport was the aim of this study.

MATERIALS AND METHODS

The study was performed on an erythrocyte suspension, obtained from whole blood of healthy donors. The blood was drawn from the ulnar vein and collected in tubes with 50 IU heparin per ml blood. The whole blood was kept in the cold at the temperature of melting ice for no more than 3 hours, and then centrifuged at 4000 rpm, at 2-4°C for 5 min with a K-23 centrifuge (Germany). After removal of the plasma and buffy coat, the erythrocytes were washed 4 times with 5 volumes of the following medium: 75 mM NaCl, 75 mM KCl, 0.2 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES-OH, pH 7.4, t=4°C. The medium for the first two washes also contained EGTA (100 μM). Packed erythrocytes adjusted to 70-80% hematocrit were stored in the cold. All experiments were conducted in a thermostatically controlled cuvette with 2 ml of the above medium at 37°C with constant stirring. The measurements were performed with a Ca-selective electrode (Orion Research, USA), and readings were taken with a pH-meter (Radelkis, Hungary) hooked up to an automatic writing device. The circuit included a compensator. The reagents used were: $MgCl_2$, $CaCl_2$, A23187 from Merck, glucose, EGTA, HEPES, bovine serum albumin from Serva, and chemically pure reagents from Soyuzreakhim.

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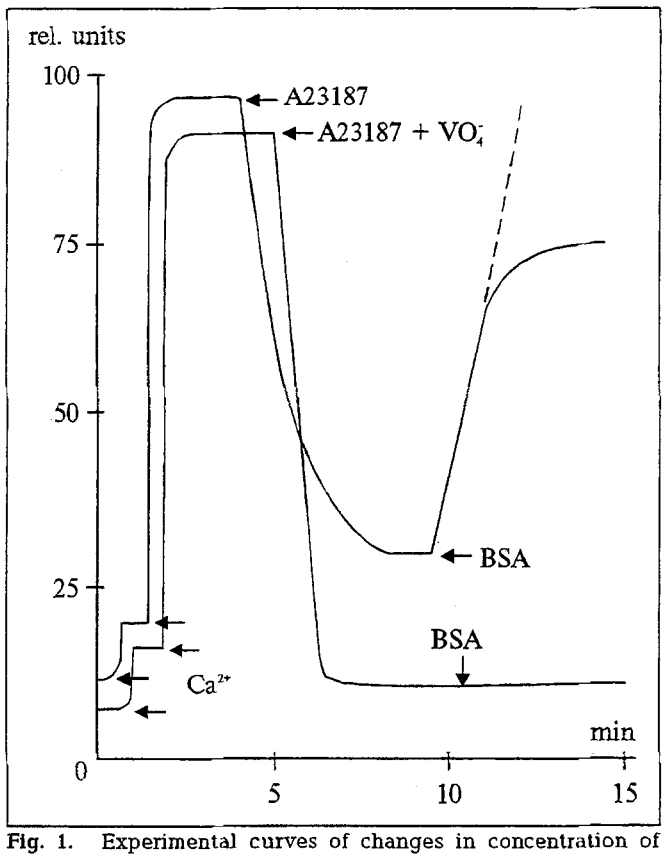

Fig. 1. Experimental curves of changes in concentration of Ca^{2+} ions in Na^+/K^+ medium in the presence of erythrocyte suspension. Arrows indicate the addition of: E – erythrocytes (200 µl); Ca^{2+} – $CaCl_2$ (50 µM); A23187 (8 µM); VO_4 – sodium orthovanadate (500 µM); BSA – bovine serum albumin (0.35%).

RESULTS

In cells with a large number of potential- and receptor-dependent channels compensation for the enhanced cation influx is accomplished in parallel with Ca-ATPase by the Na^+/H^+ exchange system as well [9,10,18,19]. In mammalian erythrocytes Ca-APTase is the only mechanism of antigradient Ca transport. Under normal physiological conditions this transport system produces Ca release as fast as 0.2-4 µmol Ca^{2+}/liter/cell/minute. This value is in conformity with the rate of $^{45}Ca^{2+}$ entry into quin-2 loaded cells with the external Ca^{2+} concentration being 1 mM (0.3-1 µmol/liter/cell/min) [7]. The activation of Ca-ATPase occurs with the intracellular Ca^{2+} rise and results in rapid restoration of the basal state [14].

To enhance the outward calcium flow and to obtain the maximal rate we increased the concentration of free Ca^{2+} in the erythrocytes with Ca-ionophore A23187 in the presence of 25-100 µM Ca^{2+} in the medium. The changes of the calcium ion concentration in the medium in the course of the experiment are presented in Fig. 1. The measurements were performed in calcium-free Na^+/K^+ medium. The minor increase of the Ca^{2+} concentration after the erythrocytes were added is due to admixtures. The subsequent increase is due to the introduction a 10-100 µM standard. Addition of calcium ionophore A23187 to the erythrocyte suspension caused a rapid decrease of the Ca^{2+} concentration in the medium due to its transfer into the cells along the concentration gradient. The "loading" process in the erythrocytes culminated after 3 minutes in an equilibrium state, the inward flow being compensated by the outward Ca-ATP mediated transport. For complete inhibition of ionophore-induced Ca^{2+} transport Co^{2+} or bovine serum albumin (BSA) are commonly used. BSA forms complexes with the ionophore and promotes its removal from the membrane [14]. Figure 1 (curve *1*) shows the increase of the cation concentration in the medium after the protein is added, which corresponds to the calcium release from the erythrocytes.

Calcium release may be attributed either to passive efflux along the concentration gradient or to functioning of the Ca-pump. To separate the active and passive flow we used orthovanadate, an inhibitor of Ca-ATPase [12].

The calcium efflux was decreased by 80-90% for concentrations of orthovanadate of 0.5 to 2.5×10^{-4} M and completely inhibited at 5×10^{-4} M (Fig.1, *2*). Under these conditions both the entry rate and the amount of calcium accumulated in the cells were increased due to the abscence of antigradient efflux. Thus, from the results obtained calcium release in response to BSA may be assumed to be connected with Ca-ATPase function.

In subsequent experiments we tried to choose the conditions for maximal efficiency of the Ca-pump and optimal conditions for its recording. Figure 2, *a* shows the dependence of the initial rate of calcium release upon BSA addition on the Ca^{2+} concentration in the Na^+/K^+ medium. As is seen from the figure, the initial rate increases with the calcium concentration up to 30 µM. In the concentration range from 30 to 65 µM the rate of efflux is maximal. A further increase of calcium in the incubation medium leads to a reduction of the calcium efflux, the rate being 50% of the maximal value at a concentration of 100 µM.

Figure 2, *b* shows the dependence of calcium uptake by the cells (degree of loading) on the calcium concentration in the Na^+/K^+ medium. Increasing Ca^{2+} to 30 µM is attended by a linear rise of the calcium uptake in the erythrocytes. At Ca^{2+} concentrations higher than 30 µM the calcium uptake reaches a plateau and remains unchanged

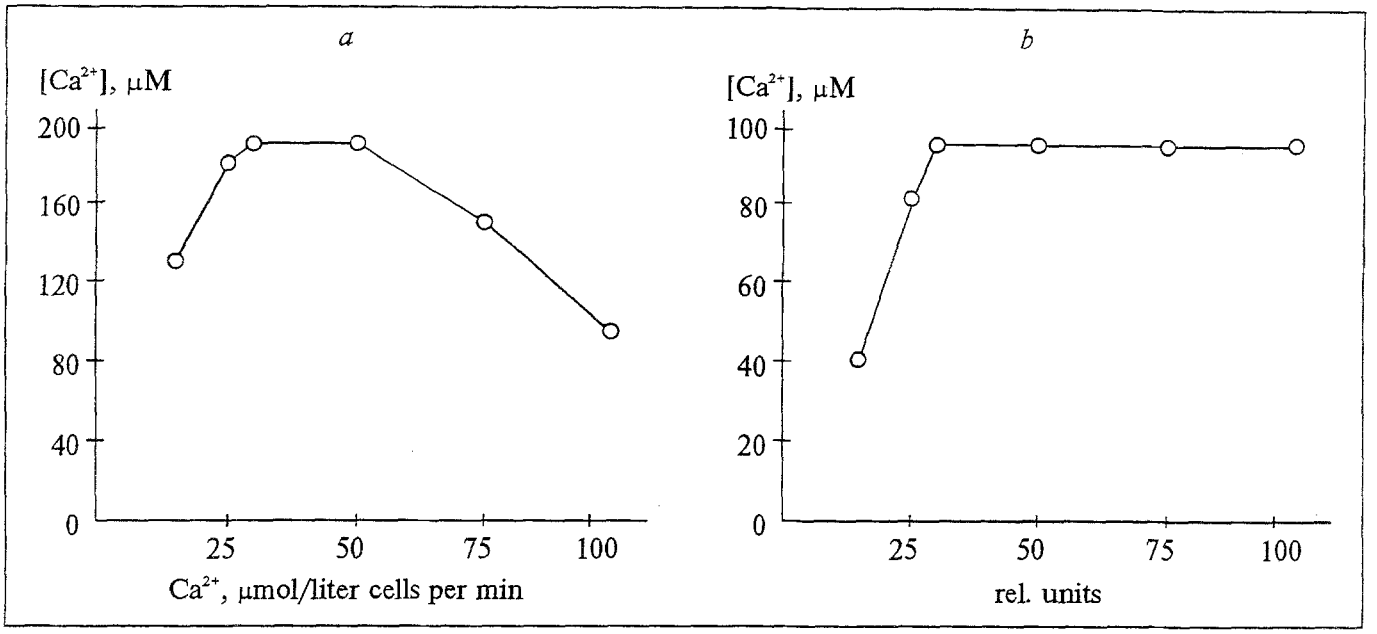

Fig. 2. Rate of Ca–ATPase mediated Ca^{2+} efflux from erythrocytes (*a*) and degree of loading of erythrocytes with calcium (*b*) as a function of calcium concentration in incubation medium.

for a cation increase to 100 μM. In comparing Figs. 2, *a* and 2, *b*, we see that the initial rate of Ca^{2+} efflux from the erythrocytes depends on the amount of calcium accumulated. The Ca-pump acts with maximal efficiency in maximally loaded cells up to a Ca^{2+} concentration in the medium of as much as 65 μM. At higher concentrations the calcium uptake remains at the maximal level, the Ca^{2+} release being reduced.

Thus, the Ca^{2+} concentration in the medium should be restricted to the range from 30 to 65 μM.

The activity of Ca-ATPase depends on the level of free calcium in the cell, which is determined both by the concentration of cation in the incubation medium and by the amount of ionophore added.

The dependence of the initial rate of calcium efflux on the ionophore concentration in Na^+/K^+ medium is presented in Fig. 3, *a*. The rate of Ca^{2+} efflux rises for an increase of the concentration of A23187 to 1 μM. In the concentration range from 1 to 2.5 μM the initial rate of calcium efflux is a constant value. At a concentration of ionophore of 4 μM a slight (10%) reduction of Ca^{2+} release occurs, followed by complete inhibition of the Ca-ATPase-mediated efflux at a concentration of 6 μM. The dependence of the amount of calcium accumulated in the erythrocytes on the ionophore concentration in the medium is depicted in Fig. 3, *c*. The dependence is seen to be direct up to a concentration of ionophore as high as 2 μM and disappears at higher concentrations, the amount of calcium accumulated being constant and maximal. When we compare Figs. 3, *a* and 3, *b*, it becomes evident that the rate of Ca-ATPase-mediated efflux depends directly on the degree of loading of the cells with calcium only for low ionophore concentrations. Under these conditions maximal activation of the enzyme cannot be achieved, probably due to the fact that the removal of calcium from the cell is compensated by its influx induced by ionophore, whose distribution on the plasma membrane surface depends on its concentration in the suspension. In the range of ionophore concentrations from 1 to 2.5 μM the rate of calcium efflux is maximal, since the amount of carrier added is enough to produce maximal saturation of the cells with calcium, the latter being a prerequisite for maximal activation of the Ca-pump. The amount of BSA (0.25%) is adjusted so that the ionophore is completely removed from the membrane. The addition of 0.25% BSA with A23187 concentrations of 4 μM or more was not enough for complete removal of the ionophore from the membrane, which resulted in a decrease of the net efflux due to ionophore-produced influx. The maximal rate was restored with a twofold protein concentration (0.5%). It should be noted, however, that this effect does not appear at a concentration of ionophore of 6 μM, in spite of an increase of BSA to 0.75%, upon which complete inhibition of Ca-ATPase was observed. At this concentration of ionophore irreversible changes probably take place in the erythrocyte membrane, affecting the structure of the cytoskeleton. Treat-

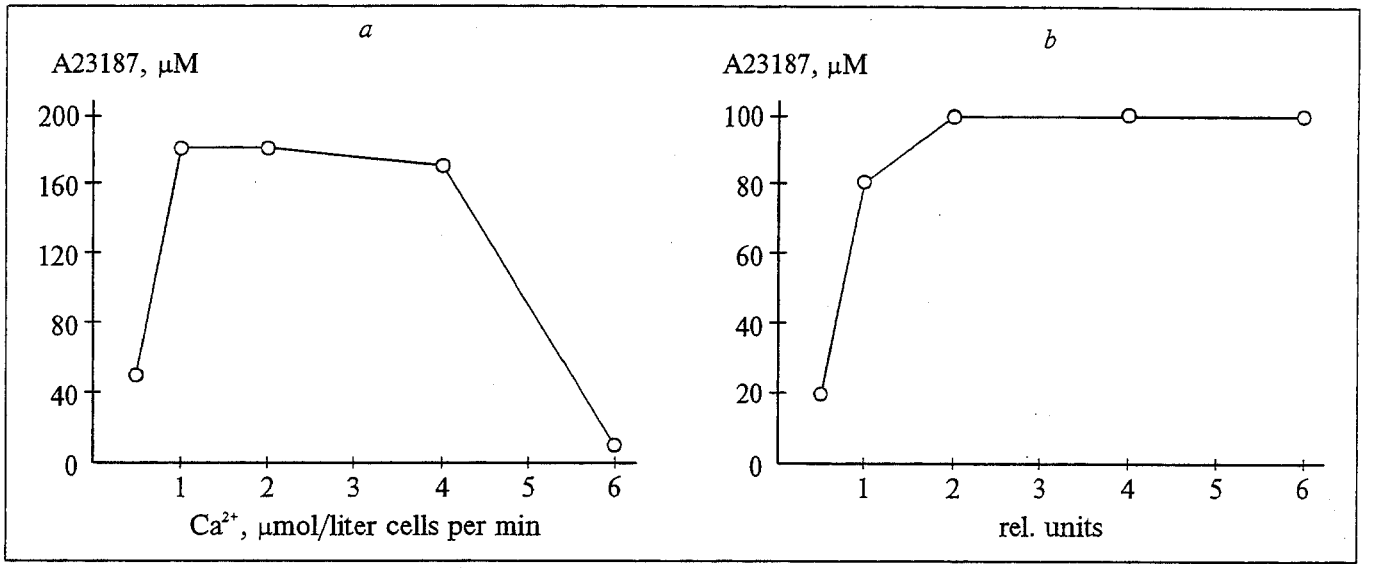

Fig. 3. Rate of Ca–ATPase mediated Ca^{2+} efflux from erythrocytes (*a*) and degree of loading of erythrocytes with calcium (b) as a function of A23187 concentration in incubation medium.

ment with 10^{-5} M ionophore is known to alter the viscosity of the erythrocyte membrane. Inner membrane proteins may form cross-links or aggregates as the level of intracellular Ca^{2+} rises, this affecting the protein-lipid interactions in the erythrocyte membrane [16].

On the basis of these results we chose a 1-2 μM concentration of ionophore to obtain the maximal rate of Ca^{2+} efflux.

Since the formation of the ionophore-protein complex is a limiting factor in the recording of the Ca^{2+} efflux, we tried to determine the appropriate BSA concentration for producing the maximal efflux rate. With low protein concentrations the rate of calcium release into the medium is low due to the presence of ionophore in the membrane. The calcium efflux increases with the protein concentration, the maximal rate being observed with 0.25-0.5% BSA in the incubation medium. Increasing the protein content in the suspension results in a reduction of the efflux recorded, probably due to the ability of BSA to complexate not only with ionophore but also with Ca^{2+} ions. In subsequent experiments we used 0.35% BSA.

Thus, we determined the necessary conditions for recording the maximal rate of Ca^{2+} efflux mediated by Ca-ATPase. Under our experimental conditions (human erythrocyte suspension, 10% Ht, in Na^+/K^+ medium containing 35 to 50 μM free Ca^{2+} and 2 to 5 μM A23187) 0.35% BSA is enough to record the maximal rate of Ca^{2+} efflux of the order of 185-200 μmol Ca^{2+}/liter/cell/min with relative error of less than 5%.

In the course of the experiment the suggestion arose that the presence of an ionophore-protein complex in the measurements may considerably affect the value of the Ca^{2+} efflux recorded. To clarify this, we changed the course of procedure of the experiment by transferring the erythrocyte suspension to the cold after the standard loading procedure to inhibit Ca-ATPase. Ten minutes later BSA was added, and the suspension was incubated at 4°C for 10 min. The incubation mixture was then pelleted in the cold, the supernatant was removed, the pellet was transferred to 2 ml of the measurement medium (t=37°C), and the Ca^{2+} efflux was recorded immediately. The rate of Ca^{2+} efflux under these conditions did not differ from that obtained from the original experiments.

The data allow us to conclude that in intact erythrocytes loaded previously with calcium by adding ionophore, the initial rate of Ca^{2+} efflux measured under standard conditions immediately after the addition of protein (Fig. 1) is determined by the maximal activity of Ca-ATPase.

At present, $^{45}Ca^{2+}$ or the membrane fractions technique is used for recording the active Ca^{2+} transport in erythrocytes, which greatly prolongs the experiments. At the same time, it is known that long-term incubation of erythrocytes in the presence of Ca-ionophore A23187 may cause irreversible structural alterations in the membrane and transformation of the cells into echinocytes and spheroechinocytes [16]. Nor is it advantageous from the energy point of view. Erythrocytes have no oxidative phosphorylation system. The ionophore-induced decrease of the intracellular Ca^{2+} concentration to 2-5 μM leads to a 10-20-fold drop of the ATP content as soon as the first 10-15 minutes of incubation even in the presence of glucose [11].

In conclusion it should be noted that the method proposed here using the Ca-selective electrode makes it possible to evaluate precisely and simply the function of Ca-ATPase in intact erythrocytes without either $^{45}Ca^{2+}$ or a long washing procedure being required. This method may be applied as a routine test for differential diagnostics in patients with essential hypertension and other disorders.

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