

Influence of divalent cations Ca^{2+} and Zn^{2+} on the activation of posthypertonic hemolysis of human erythrocytes

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Human erythrocytes (RBC) were incubated for various time at 37 °C in hypertonic solutions of NaCl (1.5 M) or sucrose (1.2 M) and then rehydrated in isotonic NaCl or sucrose media in the presence or absence of divalent cations Ca^{2+} and Zn^{2+} . After equilibration in hypertonic sucrose both cations significantly increased the extent of hemolysis at concentration dependent manner, but show more complex response after equilibration in hypertonic NaCl media. In this case both Ca^{2+} and Zn^{2+} ions lose their activation ability during rehydration in isotonic NaCl media, and Ca^{2+} did so in isotonic sucrose. Dehydration of cells in hypertonic sucrose solutions in the presence of 100 mM of cations Na^+ , K^+ and Mg^{2+} leads to decrease the extent of lysis after rehydration but does not influence activation ability of Ca^{2+} and Zn^{2+} , whereas addition of Ca^{2+} to hypertonic sucrose solution completely abolishes activation effect of both ions. The model of posthypertonic lysis is presented according to which action of cations Ca^{2+} and Zn^{2+} may be explained by their specific binding to activatory and inhibitory membrane sites, regulating membrane permeability during reswelling from hypertonic salines.

Introduction. It is well known that an attempt to transfer red blood cells from hypertonic media to isotonic one bring about RBC membrane damage and hemolysis which is called posthypertonic hemolysis [1]. Data, obtained by several authors [1—4] suggest a significant role of composition of hypertonic solution, time of exposure, tonicity of the solution after dilution, cell volume and initial tonicity after which RBC were subjected to hypertonic treatment by dialysis [5] or freezing and subsequent rehydration or thawing [2]. It is still remain unclear why RBC can not restore their initial volume after dehydration without membrane damage.

Divalent cation such as Ca^{2+} and Zn^{2+} are effective protectors against membrane damage inflicted by variety of hemolytic agents, including viruses, bacterial and animal toxins, and detergents [3, 6—9] as well as hypotonic conditions [10]. We have shown that this is also true for posthypertonic hemolysis [11]. These common features suggest an universality of the mechanism of pore formation in RBC mem-

brane, which is partially independent on hemolytic stimuli. In this study we show that divalent cations Ca^{2+} and Zn^{2+} together with their inhibiting effect [11] exhibit additional property to activate posthypertonic hemolysis under conditions where cells were rehydrated in the cation contained media. This property demonstrates distinction between posthypertonic membrane pores and those initiated by action of other lysins thus suggesting differences in the mechanism of their formation and molecular structure.

Materials and Methods. Human RBC were obtained from a clinical blood bank. They were washed 3 times in a 10-fold excess of tris-buffered saline (TBS = 150 mM NaCl, 20 mM tris-HCl, pH 7.4) using 2.000 g for 3 min on each occasion. 0.1 ml of RBC were incubated in 0.90 ml of 1.2 M sucrose or 1.5 M NaCl both with 5 mM phosphate buffer at pH 7.4 at 37 °C and then 10 μl of erythrocyte suspension were transferred into 1 ml of isotonic media of various composition at room temperature. After 5 min incubation the cell suspension was centrifuged (2.000 g, 3 min) and hemoglobin content in the supernatant

was determined by absorption at 415 nm. The percentage hemolysis was calculated from a comparison with the optical density of a fully lysed sample.

The distribution of cell volumes in the samples was measured using a Coulter-type apparatus with an orifice $50 \mu\text{m}$ long \times $5 \mu\text{m}$ in diameter [12, 13] using a current $< 0,3 \text{ mA}$. $2 \cdot 10^{15}$ cells were sampled in each measurement and the mean linear velocity through the orifice was $< 1 \text{ m/sec}$: the cells were not significantly deformed [12].

Results. Fig. 1 shows the time dependence of posthypertonic hemolysis of RBC, dehydrated in hypertonic NaCl and sucrose media, and rehydrated in isotonic electrolyte (NaCl) and non-electrolyte (sucrose) media in the presence or absence of divalent cations. Prolonged cell incubation under hypertonic conditions results in a gradual increase in the extent of hemolysis.

The effect of cations depends on the type of both dehydrative and rehydrative media. Zn^{2+} and Ca^{2+} cations increased lysis by 2- and 3-fold, respectively, relative to control value after dehydration in sucrose hypertonic medium, independently of the type of rehydrative medium. However after dehydration in NaCl hypertonic medium Zn^{2+} cations fail to produce activation of hemolysis during rehydration in electrolyte medium, whereas Ca^{2+} ions inhibit it (Fig. 1,

A). There was no appreciable effect of Ca^{2+} in non-electrolyte rehydrative medium though it slightly activated hemolysis at short-term exposure, and Zn^{2+} ions stimulated lysis (Fig. 1, C). Stimulation of posthypertonic lysis by divalent cations was a function of cation concentration.

Fig. 2 and 3 show the dependence of posthypertonic hemolysis on the concentration of Ca^{2+} and Zn^{2+} ions during rehydration in NaCl and sucrose isotonic media. In both type of rehydrative medium Ca^{2+} ions activated posthypertonic hemolysis of cells, dehydrated in hypertonic sucrose, while poorly affected hemolysis of RBC, dehydrated in hypertonic NaCl and rehydrated in isotonic sucrose. The extent of posthypertonic hemolysis of these RBCs was considerably reduced during rehydration in sucrose-containing media (Fig. 2, B). In contrast to Ca^{2+} , Zn^{2+} ions lose their stimulatory ability only subsequent rehydration in NaCl medium (Fig. 3, A). Moreover, the concentration of Zn^{2+} ions which is required for activating lysis, has shown to be three order of magnitude lower than the corresponding concentration of Ca^{2+} ions (compare Fig. 2 and 3). These data show that treatment of cells in hypertonic NaCl solution followed by rehydration in isotonic NaCl solution eliminates the effect of posthypertonic hemolysis activation caused by both Ca^{2+} and Zn^{2+} ions. Hence,

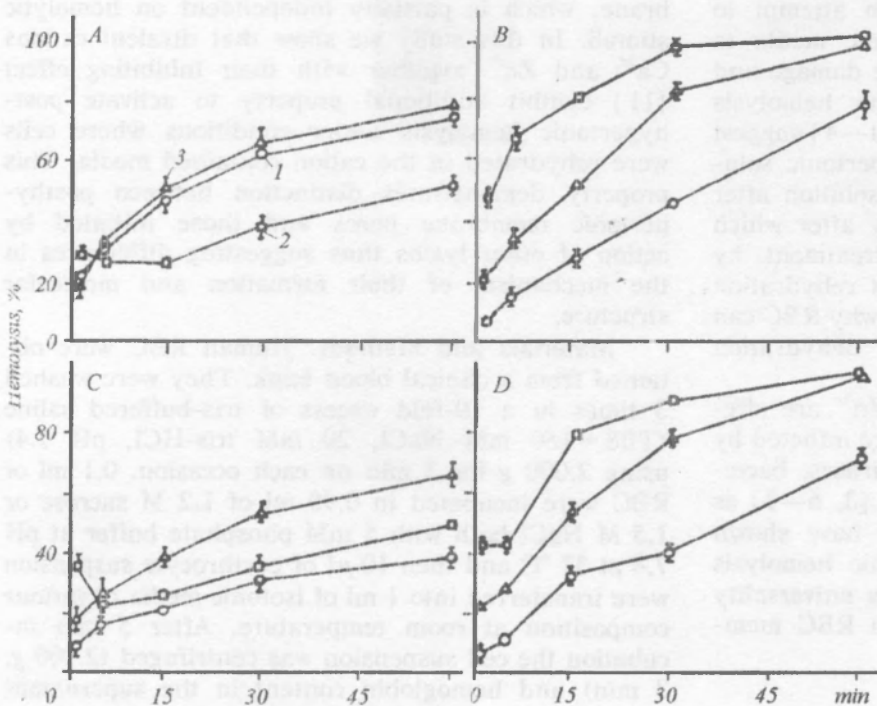


Fig. 1. Effect of divalent cations on posthypertonic hemolysis. RBC were incubated selected time in hypertonic media of 1.5 M NaCl (A, C) or 1.2 M sucrose (B, D) at 37°C following by dehydration in isotonic NaCl (A, B) or sucrose (C, D) solutions in the absence (1) or presence (2) of 100 mM Ca^{2+} or 0,1 mM Zn^{2+} (3)

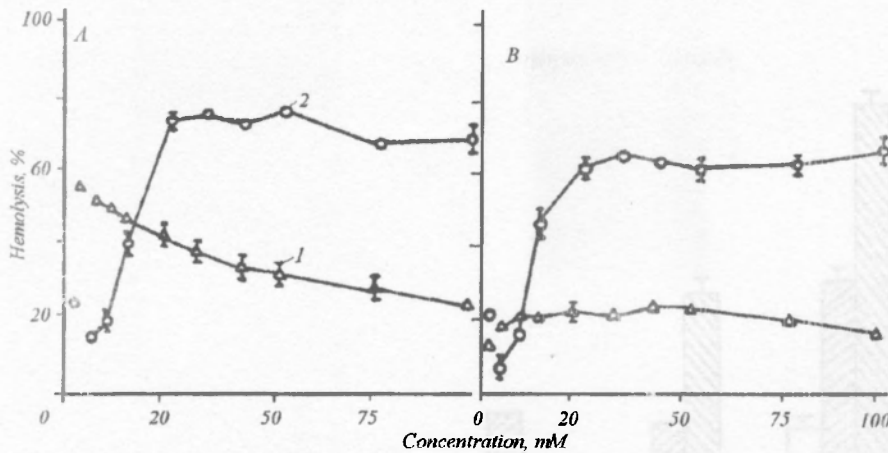


Fig. 2. Effects of Ca^{2+} ions on posthypertonic hemolysis. RBC were incubated 15 min in hypertonic solutions of 1.5 M NaCl (1) and 1.2 M sucrose (2) and then transferred to isotonic NaCl (A) and sucrose (B) media contained various amount of Ca^{2+}

the alteration occurring in the membrane and cytoplasm of cells, dehydrated in saline medium, differ from those in non-electrolyte medium (sucrose). This may be due to different changes in transmembrane potential and effluxes of cations and anions, resulting in various alterations in the intracellular content and pH.

Hypertonic non-electrolyte medium causes a decrease in the content of K^+ and Cl^- ions in cytoplasm, accompanied by an increase in pH [12]. In hypertonic electrolyte medium leakage of K^+ cations will not result in the corresponding loss of Cl^- anions due to inward Cl^- gradient. Hence, hypertonic treatment leads to significantly altered Cl^- contents in cells treated by non-electrolyte solutions. To assess the role of electrolytes in the mechanism of posthypertonic hemolysis activation by Ca^{2+} and Zn^{2+} cations, RBC were dehydrated in 1.2 M sucrose in the presence of 100 mM Na^+ , K^+ , Mg^{2+} and Ca^{2+} salts. The data represented in Fig. 4 show that dehydration

in sucrose medium in the presence of Na^+ , K^+ and Mg^{2+} reduces the level of posthypertonic hemolysis by 2–3-fold, and does not influence the ability of Ca^{2+} and Zn^{2+} ions to activate posthypertonic hemolysis as they do in the case when dehydration was performed in pure non-electrolyte medium. In contrast, Ca^{2+} ions when present in dehydrative medium, completely eliminate the posthypertonic hemolysis activation, effect is not due to simple rise in ionic strength. This is also supported by the analysis of volume distribution of rehydrated RBC, obtained after dehydration in electrolyte and non-electrolyte media (Fig. 5).

Activation of posthypertonic hemolysis by Zn^{2+} ions after cell dehydration in hypertonic sucrose is accompanied by an increased in amount of cells and ghosts in the swollen subpopulation indicating that under these conditions a larger number of cells undergo hemolysis. This finding differs from corresponding volume distributions, obtained for Ca^{2+} ions, where reduced modal volume of swollen subpopulation

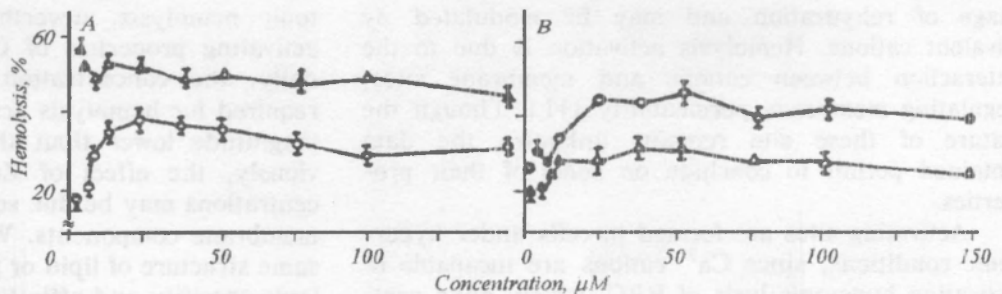


Fig. 3. Effects of Zn^{2+} ions on posthypertonic hemolysis. Conditions and definitions of symbols are the same as for Fig. 2

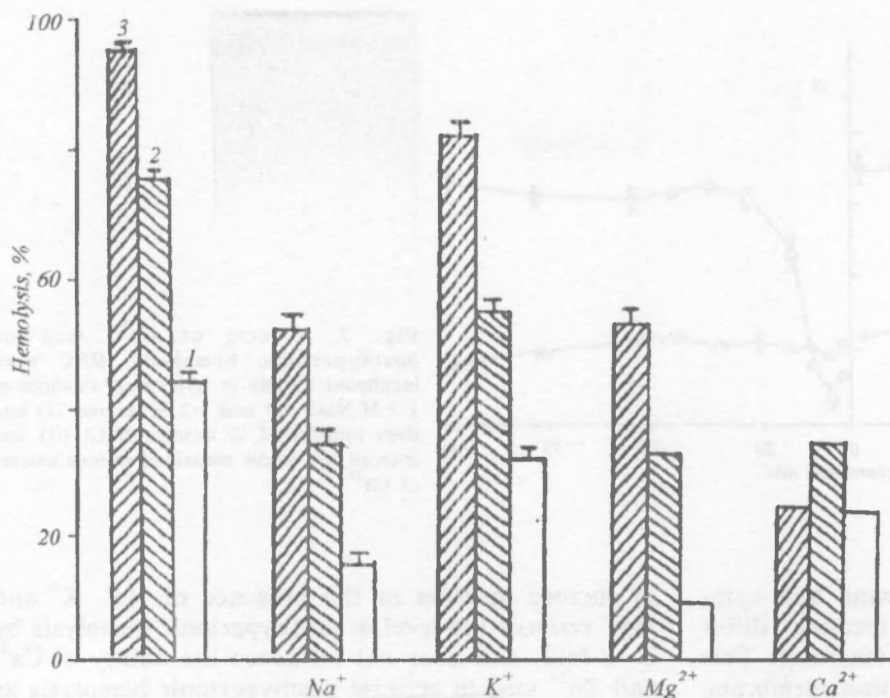


Fig. 4. Effects of preincubation of RBC in hypertonic sucrose media in the presence of mono and divalent cations in the concentration 100 mM on the extent of posthypertonic hemolysis. RBC were incubated for 30 min at 37 °C and then rehydrated in isotonic sucrose solutions which contain no cations (1) or 0.1 mM Zn²⁺ (2), or 100 mM Ca²⁺ (3)

was found. The later supports the view that Ca²⁺ ions facilitate formation of large membrane pores which restrict colloidal-osmotic swelling of cells, resulting in the formation of ghost without cells acquiring critical hemolytic volume. Zn²⁺ ions do not affect volume distributions (Fig. 5, A) as well as hemolysis of rehydrated RBC after dehydration in NaCl medium (Fig. 3). On the other hand, the inhibiting action of Ca²⁺ ions in this case resulted in a reduced swelling of the cells which also correlated with their influence on hemolysis. The revealed peculiarities in the Ca²⁺ and Zn²⁺ ions action on posthypertonic hemolysis activation and volume distributions after cell rehydration demonstrate critical role of shrinking in the development of cell injury during rehydration. At this stage a prerequisites for irreversible changes in cells are formed. They are subsequently manifested at the stage of rehydration and may be modulated by divalent cations. Hemolysis activation is due to the interaction between cations and membrane sites, regulating membrane permeability [11]. Though the nature of these site remains unknown the data obtained permit to conclude on some of their properties.

Activating sites are formed in cells under hypertonic conditions, since Ca²⁺ cations are incapable of activating hypotonic lysis of RBC, which, alike post-

hypertonic hemolysis, results in the swelling of cells. Ca²⁺ is known to inhibit hypotonic hemolysis [10]. Sites are cation specific. This is supported by the fact that posthypertonic hemolysis is activated by only Ca²⁺ and Zn²⁺ cations. Other cations such as Mg²⁺, Na⁺, K⁺, Ch⁺ are capable of inhibiting but not activating posthypertonic hemolysis [11]. This may be due to electrostatic screening of charges in the mouth of a hemolytic pore [8]. Despite the concentration of Ca²⁺ ions which is required for activation is high enough (> 20 mM), their action is not accounted for by the screening effect, because only incubation of RBC in hypertonic medium in the presence of 100 mM Ca²⁺ completely eliminates the activating effect of Ca²⁺ and Zn²⁺ on posthypertonic hemolysis. A similar incubation in the presence of the other ions, though reducing the level of posthypertonic hemolysis, nevertheless, does not affect the activating properties of Ca²⁺ and Zn²⁺ (Fig. 4). Finally, the concentration of Zn²⁺ ions (~ 30 μM) required for hemolysis activation is by three order of magnitude lower than that of Ca²⁺ (50 mM). Obviously, the effect of Zn²⁺ ions in such low concentrations may be due solely to specific binding with membrane components. Whether or not one and the same structure of lipid or protein nature with differing ionic specificity and affinity to Ca²⁺ and Zn²⁺ cations is

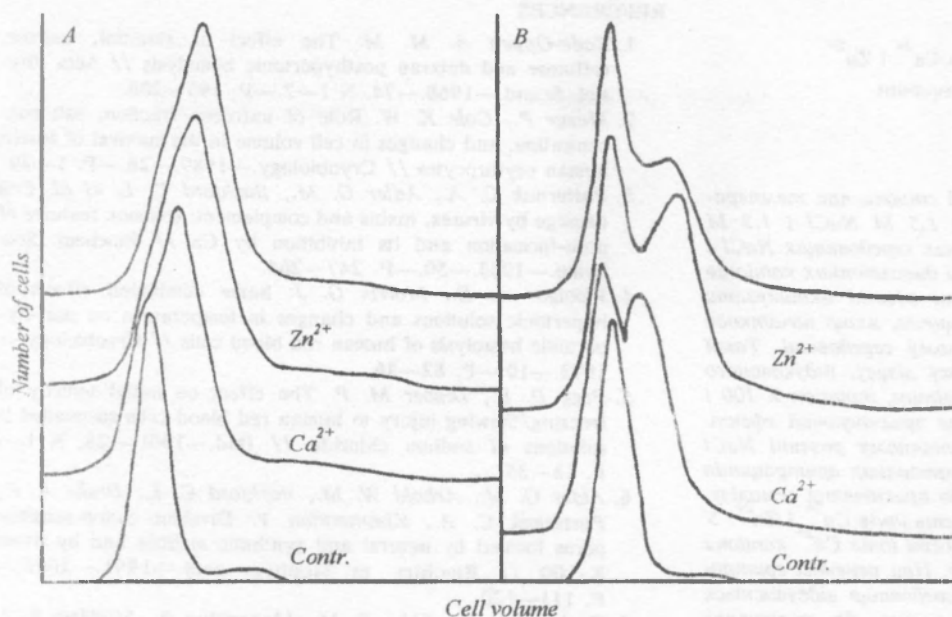


Fig. 5. Volume distributions of RBC after 15 min dehydration in hypertonic NaCl (A) and sucrose (B) media followed by rehydration in isotonic NaCl solution in the absence and presence of 0.1 mM Zn^{2+} and 100 mM Ca^{2+} . Contr.-volume distribution of control erythrocytes in isotonic NaCl medium

responsible for posthypertonic hemolysis activating effect of both Ca^{2+} and Zn^{2+} . Such an ability demonstrates the specific action of Ca^{2+} on the membrane and/or cytoplasmic structures which are responsible for activation remains unclear. Parallelism was shown in the activating action of Ca^{2+} and Zn^{2+} for cell incubated in hypertonic sucrose (Fig 1, B, D). A failure to detect a corresponding parallelism following RBC incubation in hypertonic NaCl, after which posthypertonic hemolysis is not longer affected by Ca^{2+} ions during rehydration in both isotonic solutions (Fig. 1, A, C), as well as the maintenance of Zn^{2+} activating properties following rehydration in non-electrolyte isotonic medium (Fig. 3, B), suggest that sites for Ca^{2+} and Zn^{2+} ions may be different.

The notion that modes of Ca^{2+} and Zn^{2+} activation action are different is additionally supported by the data on the volume distributions of the mixed population of cells and ghosts, obtained after rehydration in media in the presence of these ions (Fig. 5). The volumes of ghosts in the presence of Ca^{2+} (right pick of distribution) are considerably lower than those in the presence of Zn^{2+} , which testifies to larger dimensions of pores. Earlier we have shown that both monovalent and divalent ions, as well as EDTA and sucrose, inhibit posthypertonic hemolysis after addition into the medium a short while after onset of hemolysis [11]. Since such an influence was also ion-specific and could not be accounted for by the screening effect, it has been suppose that cations

specifically bind to blocking sites on the external membrane surface, and close hemolytic pores in membranes. Here we observe the activating effect of the same Zn^{2+} and Ca^{2+} cations (but not Mg^{2+}) when the ions are originally present in the rehydrative medium. The data reported elsewhere [3, 6—9] demonstrate that divalent and monovalent cations inhibit membrane defects, produced by the action of hemolytic agents of various origin. Their activating influence has also been shown for RBC lysis induced by melittin [14].

However, phenomenon of lysis activation by divalent cations were not observed on model lipid systems (liposomes), with composition close to RBC membranes, [14]. Hence, activation seems to require some protein components of RBC membrane. The latter is also supported by the fact that inhibition of lysis in lipid vesicles systems requires Zn^{2+} concentrations to be by 10—100-fold higher than those for RBC [14, 15]. Hence, one may conclude that reswelling of RBC from hypertonic media leads to formation of composite pores in their membranes. The structure of the pores involves at least two activating ion-specific and one blocking site for divalent cations. It is probably that membrane proteins participate in pore formation, while the exact structure of the pore and the ability of sites to interact with divalent cations depends on electrolyte and non-electrolyte composition of dehydrative and rehydrative media.

С. В. Пателарос

Активуючий вплив дивалентних катіонів Ca^{2+} і Zn^{2+} на постгіпертонічний лізис еритроцитів людини

Резюме

Еритроцити людини інкубували в різні строки при температурі 37°C у гіпертонічних розчинах $1,5\text{ M NaCl}$ і $1,2\text{ M}$ сахарози та регідратували в ізотонічних середовищах NaCl і сахарози в присутності та відсутності дивалентних катіонів Ca^{2+} і Zn^{2+} . Встановлено, що останні здатні активувати постгіпертонічний лізис (ПЛ) еритроцитів, якщо початкова дегідратація здійснюється в сахарозному середовищі. Такої активації не спостерігається у випадку лізису, індукованого різними гемолітичними агентами (мелітин, тритон X-100 і т. і.), де ці катіони проявляють лише пригнічуючий ефект. Попередня інкубація клітин у гіпертонічному розчині NaCl згасаний ефект усуває. Обробка дегідратованих еритроцитів катіонами Na^+ , K^+ , Mg^{2+} призводить до пригнічення гемолізу, однак не впливає на активуючу здатність іонів Ca^{2+} і Zn^{2+} . У випадку дегідратації клітин у присутності іонів Ca^{2+} катіони втрачають здатність активувати ПЛ. При переносі еритроцитів із гіпертонічних в ізотонічні середовища відбувається формування мембранних гемолітичних пор, до структури яких входять два активуючих іоноспецифічних і один блокуючий сайти для дивалентних катіонів. Вказані сайти здатні регулювати формування мембранних пошкоджень при взаємодії з катіонами Ca^{2+} і Zn^{2+} .

С. В. Пателарос

Активирующее влияние дивалентных катионов Ca^{2+} и Zn^{2+} на постгипертонический лизис эритроцитов человека

Резюме

Эритроциты человека инкубировали в разное время при температуре 37°C в гипертонических растворах $1,5\text{ M NaCl}$ и $1,2\text{ M}$ сахарозы и регидратировали в изотонических средах NaCl и сахарозы в присутствии или отсутствии дивалентных катионов Ca^{2+} и Zn^{2+} . Установлено, что последние способны активировать постгипертонический лизис (ПЛ) эритроцитов, если начальная дегидратация происходит в сахарозной среде. Данной активации не наблюдается в случае лизиса, индуцированного разными гемолитическими агентами (мелиттин, тритон X-100 и т. д.), где эти катионы оказывают только ингибирующий эффект. Предварительная инкубация клеток в гипертоническом растворе NaCl указанный эффект устраняет. Обработка дегидратированных эритроцитов катионами Na^+ , K^+ , Mg^{2+} приводит к ингибированию гемоліза, однако не влияет на активирующую способность ионов Ca^{2+} и Zn^{2+} . В случае дегидратации клеток в присутствии ионов Ca^{2+} катионы утрачивают способность активировать ПЛ. При переносе эритроцитов из гипертонических в изотонические среды происходит формирование мембранных гемолитических пор, в структуру которых входят два активующих ионоспецифических и один блокирующий сайты для дивалентных катионов. Указанные сайты способны регулировать формирование мембранных повреждений при взаимодействии с катионами Ca^{2+} и Zn^{2+} .

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