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Cation-sensitive pore formation in rehydrated erythrocytes

Sergey V. Rudenko *, Stamatios V. Patelaros

Institute for Problems of Cryobiology and Cryomedicine of the Ukrainian Academy of Sciences, 23 Pereyaslavskaya Str., 310015 Kharkov, Ukraine

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Abstract

Rehydration of red blood cells (RBC) in isotonic media after dehydration in hypertonic electrolyte or nonelectrolyte saline leads to their posthypertonic hemolysis (PH). Ca^{2+} ions at a concentration of more than 5 mM stimulated hemolysis of RBC treated by hypertonic sucrose but not NaCl if rehydration was carried out in the presence of cations. Zn^{2+} produced a more complex response of stimulation followed by inhibition as a concentration is increased. Mg^{2+}, Ca^{2+}, Zn^{2+}, EDTA and sucrose exhibited only inhibition when added to isotonic NaCl media immediately after onset of rehydration or later on. At low ionic strength inhibition produced by divalent cations was markedly reduced and sucrose was ineffective. An equimolar concentration of EDTA abolished the inhibition of PH by Zn^{2+} ions if they were introduced into the isotonic media after the cells, but activated hemolysis when rehydration was carried out in the presence of ions. The same divalent cations prevented shape transformation and hemolysis induced by melittin if they interacted with the plasma membrane prior to the addition of melittin. Subsequent chelation of cations by EDTA triggers the full sequence of events characteristic to the action of melittin alone and resulted in cell spherulation followed by hemolysis. Inhibition of melittin-induced hemolysis produced by all cations was reversible because EDTA abolished the action of divalent cations and even stimulated hemolysis in isotonic sucrose. Similarities in the mode of action of divalent cations and EDTA on posthypertonic hemolysis which is attributed to endogenous stimuli and melittin-induced hemolysis as far as the exogenous agent is concerned imply that in both cases common intrinsic mechanisms are involved in the process of cation-sensitive pore formation in erythrocyte membranes, while differences indicate that more complex pores are formed during posthypertonic injury.

Keywords: Dehydration; Rehydration; Hemolysis; Melittin; Divalent cation; Membrane lesion

1. Introduction

It has been shown that the agents as diverse as the Sendai virus, melittin, α-toxin, activated complement and detergents induce leakage of ions and low molecular weight metabolites through membrane lesions in various types of cells. Most types of lysis are known to be inhibited by Ca^{2+} and other divalent cations such as Zn^{2+} [1–5] and protons [2]. Circumstantial evidence has been presented that divalent cations and protons protect the cells by binding to the outside of cells [2]. On the other hand, a stimulatory action of divalent cations on peptide-induced hemolysis and lysis of phospholipid vesicles has also been reported [4]. The inhibitory and activating action of divalent cations with respect to formation of membrane lesions, induced by pore-forming substances, has been found to strongly depend on the experimental conditions such as the ionic strength, phospholipid composition of vesicles, type of cells, etc. It has been suggested that the ability of divalent cations to inhibit hemolysis of RBC induced by a variety of agents possessing distinct molecular structure and the mode of action are due to some common intrinsic membrane components, comprising the pore structure itself [1,6]. Because pore-forming substances are, mainly, exogenous agents, it seems important to confirm or refute this view in the case when any exogenous contributions to the membrane damage are certainly excluded. Cell rehydration from hypertonic electrolyte or nonelectrolyte solutions is a useful example of this type of damage which is called posthypertonic hemolysis [7–9]. When the cells loose the intracellular water below the critical threshold they will be damaged after returning them to the isotonic or nearly isotonic conditions [7–9]. Although posthypertonic injury has been detected in cells of various types [10], the molecular mechanism of hemolysis during rehydration is still poorly understood. It is reasonable to suppose that endogenous processes, taking place in dehydrated cells are re-

* Corresponding author. Fax: +7 572 720084.

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sponsible for this damage. The purpose of the present paper is to study the process of pore formation in the erythrocyte membrane during cell reswelling from hypertonic salines and to investigate the interaction of divalent cations with binding sites which may be involved in the regulation of pore structure as well as their possible localization on the membrane surface. To better understand the role of endogenous components in the process of pore formation the action of divalent cations on the posthypertonic pore will be compared with the corresponding action on the lesions, produced by the exogenous agent melittin.

2. Materials and methods

Ionophore A23187, Tris-HCl and EDTA were supplied by Calbiochem. $CaCl_2$, $MgSO_4$, $ZnSO_4$ were of the highest grade available. Melittin free from phospholipase A_2 was the generous gift of Dr. C. Dempsey (Bristol University).

2.1. Hemolysis assay

Human erythrocytes were obtained from bank-collected blood by washing three times in a 10-fold excessive Tris-buffered saline (TBS) (150 mM NaCl, 10 mM Tris-HCl, pH 7.4). The buffy coat and top of pellet were removed after each centrifugation ($2000 \times g$, 3 min). 0.1 ml of packed cells were transferred into 0.9 ml of prewarmed at 37° C concentrated solutions of sucrose (1.2 M, 5 mM phosphate buffer, pH 7.4) or NaCl (1.5 M, 5 mM phosphate buffer, pH 7.4), which may contain ions or A28187, and incubated on water bath at 37° C for 15 or 30 min. 10 μl of the cell suspension were then added to 1 ml of isoosmotic (310 mosM) TBS or sucrose saline (0.27 M sucrose, 10 mM Tris buffer, pH 7.4) at room temperature (19–21° C) in the presence or absence of $CaCl_2$, $MgSO_4$ or $ZnSO_4$ for 5 min, and spun down for 2 min ($2000 \times g$). The supernatant was removed and its absorbance at 415 nm was determined relative to a suitable control. The percentage of hemolysis was calculated by comparing with the absorbance of a fully lysed sample. Results presented are the mean ± S.E. for three to eight independent determinations using various blood samples. The dynamics of erythrocyte hemolysis in isotonic saline of different composition were measured spectrophotometrically [11–13]. The RBC suspension was constantly stirred and its absorbance at 720 nm was recorded continuously. Normally a concentration of cells, injected into a cuvette, was (4–6) $\cdot 10^5$ cells/ml. To investigate the influence of divalent cations on the time-course of hemolysis aliquots of cations (2–100 μl) were added directly into the cuvette from concentrated stock solutions (1 M) prior to or after injection of cells. As reported elsewhere [11,12] a decrease in absorbance reflects a decrease in the amount of intact cells in the cuvette during hemolysis, absorbance, therefore, is inversely proportional to the extent of hemolysis.

2.2. Determination of the distributions in the volumes of cells and ghosts

Volume distributions of cells and ghosts, which were produced as a result of posthypertonic hemolysis were measured using a Coulter-Counter type apparatus [14–16]. In the present study a cylindrical, 50 μm long, and 50 μm diameter orifice was used in a system of transducers provided for a practically complete hydrodynamic focusing of cells. The flow rate in the experiments was below 1 m/s of the mean linear velocity of a cell suspension flowing through the orifice. Each measurement cycle ana-

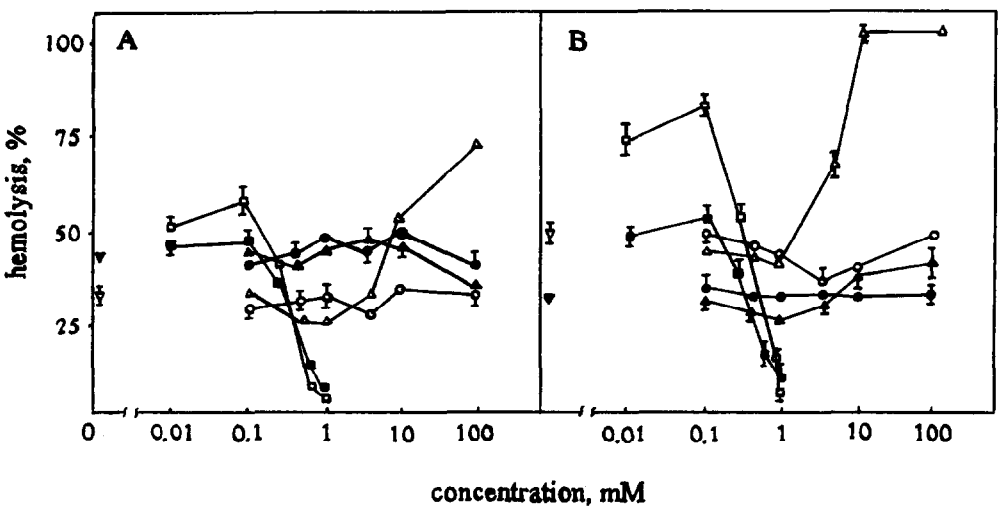

Fig. 1. Influence of divalent cations Mg^{2+} (○,●), Ca^{2+} (△,▲) and Zn^{2+} (□,■) on hemolysis of RBC rehydrated in isoosmotic NaCl (A) or sucrose (B) media in the presence or absence (▽,▼) of ions after 15 min incubation at 37° C in hypertonic electrolyte (1.5 M NaCl, close symbols) or nonelectrolyte (1.2 M sucrose, open symbols) salines.

lyzed 2^{15} cells and the current across the orifice did not exceed 0.2 mA, so that no electrical breakdown [14] and no significant deformation of cell membrane [15,16] occurred, the method provided a measure for the true volume of both RBC and their ghosts (particles) in suspension.

3. Results

3.1. Influence of divalent cations on posthypertonic hemolysis

Fig. 1 shows the influence of divalent cations Mg^{2+}, Ca^{2+} and Zn^{2+} on posthypertonic hemolysis of the erythrocytes, preincubated in hypertonic NaCl and sucrose media, and then transferred into the isotonic media with high and low ionic strength. The concentration of NaCl and sucrose in hypertonic solutions was chosen to produce a comparable value of hemolysis after rehydration. Zn^{2+} at both low and high ionic strength initially stimulates hemolysis but subsequently inhibits when the concentration is increased. The activating and inhibitory action of Zn^{2+} ions is obvious for cells treated with hypertonic sucrose. In the case of hypertonic NaCl medium the inhibitory effect of Zn^{2+} almost completely coincided with that for sucrose. Ca^{2+} ions activate hemolysis of cells dehydrated in sucrose during rehydration in both isotonic media up to the maximum concentration tested but have no effect on cells dehydrated in NaCl. In contrast to other cations Mg^{2+} is ineffective in producing changes in PH in the whole range of concentrations (0.1–100 mM). The main features of PH and the mode of action of divalent cations are similar in the electrolyte and nonelectrolyte rehydrative media, however, in all cases lysis is higher when dehydration and rehydration are performed in the same type of medium (NaCl or sucrose). It should be noted that after dehydration of cells in NaCl Zn^{2+} fails to activate PH during rehydration in NaCl but not in sucrose solutions. Thus, the presence of divalent cations in rehydrative medium could produce either an activation or inhibition of posthypertonic hemolysis depending on the concentration used.

3.2. Inhibition of posthypertonic hemolysis

It is known that divalent cations Ca^{2+}, Mg^{2+} and Zn^{2+} inhibit membrane defects induced by a variety of perturbants [1–6]. However, in the case of PH (Fig. 1) inhibition was observed only at a relatively high concentration of Zn^{2+} (> 0.5 mM) and was not detected for other cations. At these concentrations Zn^{2+} may precipitate Hb [4] giving an artifactual decrease in the measured values of hemolysis. To exclude this possibility we carried out control experiments where Zn^{2+} at the maximal final concentration (1 mM) was added into the samples referring 100% blank of hemolysis. No changes in absorbance were detected after addition of Zn^{2+} ions, indicating an absence of Hb precipitation. Hence, the possible role of Hb precipitation may be ruled out. Time-courses of posthypertonic hemolysis during cell rehydration in isotonic media in the presence and absence of divalent cations are shown in Fig. 2. The dynamics of hemolysis for control cells and cells rehydrated in the presence of an activating concentration of Zn^{2+} (0.1 mM) were close to the kinetic curves of the first order (Fig. 2a). Reduced absorbance changes and a biphasic response were observed at higher Zn^{2+} concentration. These data indicate that inhibition is progressive in time. Inhibition was also observed after addition of the same amounts of divalent cations 12 s after the onset of hemolysis, the time at which approx. 50% reduction in the whole absorbance change was reached. It is interesting that Zn^{2+} at a concentration of 0.1 mM fails to accelerate

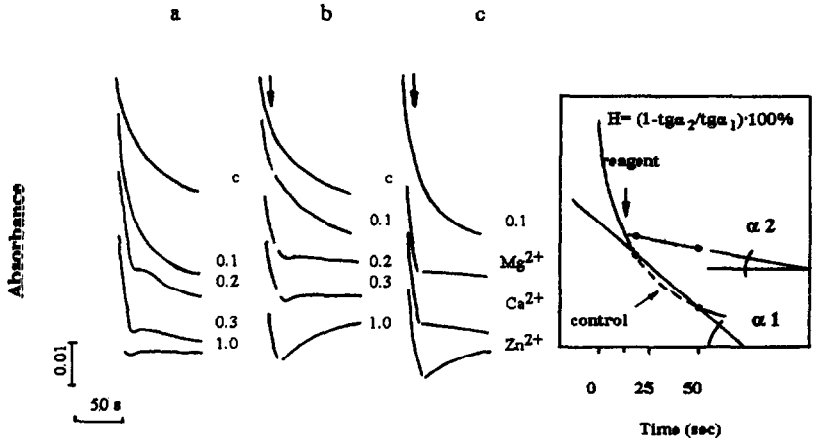

Fig. 2. Effect of Zn^{2+} on time-courses of hemolysis during RBC rehydration in isoosmotic NaCl media, containing Zn^{2+} ions (panel a), or when ions were added at time indicated by arrows to give a final concentration in mM (panel b); panel c shows the influence of Mg^{2+}, Ca^{2+} and Zn^{2+} ions added from a concentrated stock solution to obtain final concentration 50 mM or 1 mM for Zn^{2+}, on Zn^{2+}-stimulated hemolysis, i.e., rehydration in the presence of 0.1 mM Zn^{2+} in the isotonic media. RBC were incubated for 30 min in 1.2 M sucrose solutions at 37° C. The inset gives a definition of inhibition parameter H used to quantitatively assess the inhibition produced by addition of reagents.

Table 1
Inhibition of posthypertonic hemolysis (%) produced by divalent cations, EDTA and sucrose

Type of hemolysis	Medium	Inhibitors (50 mM)				
		Ca	Mg	EDTA	sucrose	Zn (1 mM)
Control	NaCl	72 ± 4	95 ± 5	81 ± 7	90 ± 5	100
Zn-activated (0.1 mM)	NaCl	72 ± 6	83 ± 4	100	58 ± 3	100
Ca-activated (10 mM)	NaCl	44 ± 16	76 ± 16	94 ± 5	88 ± 6	100
Control	sucrose	39 ± 4	42 ± 3	60 ± 3	0	100
Zn-activated (0.1 mM)	sucrose	40 ± 8	40 ± 8	62 ± 5	0	100
Ca-activated (5 mM)	sucrose	15 ± 12	32 ± 10	80 ± 10	33 ± 8	100

RBC were incubated at 37° C in 1.2 M sucrose for 30 min and then transferred into isotonic media. 12 s later aliquots of inhibitors were added and the extent of inhibition was calculated from hemolysis traces as indicated in the inset to Fig. 2. In the case of cation-activated PH, RBC were rehydrated into the media which contain the indicated amount of divalent cations.

hemolysis, while a larger concentration acts in a manner as if the ions were originally presented in the rehydrative medium. Additional experiments have shown that Zn^{2+} in any concentration in the range from 0.01 to 1 mM fails to activate hemolysis when introduced after onset of hemolysis (not shown). Zn^{2+} at a concentration of 1 mM causes an increase in absorbance, an effect which was never observed using other cations (Fig. 2b, c). This may be probably due to Zn^{2+}-induced Hb precipitation in the vicinity of the plasma membrane, but not to precipitation of Hb in solution (see above) indicating a specificity of Zn^{2+} action compared to other divalent cations. We leave this question outside the scope of the present paper. To assess inhibition quantitatively we defined an inhibition parameter as indicated in Fig. 2. The extent of inhibition produced by divalent cations, sucrose and EDTA is summarized in Table 1. Inhibition parameters of Zn^{2+}-activated PH (i.e., rehydration in isotonic media in the presence of 0.1 mM Zn^{2+}) and Ca^{2+}-activated PH (i.e., rehydration in the presence of 10 mM Ca^{2+} in isotonic NaCl, and 5 mM Ca^{2+} in isotonic sucrose solutions, respectively) are shown in comparison with control values. Zn^{2+} was found to be the most potent inhibitor for either type of PH in both electrolyte and nonelectrolyte media, producing 100% inhibition in all cases. Sucrose exhibited a strong inhibitory effect in isotonic NaCl, except for Zn^{2+}-activated PH, but it was ineffective in isotonic sucrose solutions. In all cases the inhibitory effect of Ca^{2+}, Mg^{2+} and EDTA was less in isotonic sucrose compared to corresponding effects in isotonic NaCl. The same discrimination between electrolyte and nonelectrolyte isotonic media was observed for the monovalent cations Na^+ and K^+ [17]. These data imply that a different structure or size of the pore – presumably a larger pore size in sucrose media – may be formed during posthypertonic hemolysis.

3.3. Combined action of divalent cations and EDTA on PH

We have shown that the divalent cations Ca^{2+} and Zn^{2+} in appropriate concentrations stimulated PH in cation-contained media and inhibited it when were added later. Such dual influence implies that the effect may be due to the interaction of divalent cations with lipid and/or protein components of the plasma membrane (sites) which might regulate the dimension and/or the number of pores arising at the rehydration stage. The differences in cation concentration required to produce activation and inhibition may be explained by variations in ion specificity and affinity of sites toward divalent cations. In order to evaluate the ion specificity and affinity of sites as well as the reversibility of cation binding PH was investigated in the presence and absence of the cation chelator EDTA. As follows from Table 2, PH was the same during rehydration of cells in the isotonic media in the presence of an equimolar mixture of Zn^{2+} and EDTA at the whole range of concentrations used. Principally, other results were obtained after addition of EDTA into the media which previously contained Zn^{2+}. In this situation EDTA not only the canceled blocking effect of Zn^{2+}, but also stimulated hemolysis above control value. Fig. 3 illustrates the role of order in addition of cations and EDTA. Zn^{2+} and EDTA produce no changes in time-courses of hemolysis in the following cases: (i) in the presence of EDTA alone, (ii) in the presence of an equimolar mixture of Zn^{2+} and EDTA in the whole concentration range used in the present study (0.01–1 mM) (for example Fig. 3, track 2), (iii) when EDTA was added after onset of Zn^{2+}-activated hemolysis.

Table 2
Effect of Zn^{2+} and EDTA on posthypertonic hemolysis (%)

	Media	Concentration of Zn (mM)				
		0.01	0.1	0.3	0.7	1
Zn	NaCl	54 ± 6	60 ± 5	44 ± 8	7 ± 0.4	5 ± 7
Zn + EDTA	NaCl	44 ± 4	40 ± 12	40 ± 9	38 ± 8	38 ± 8
Zn + addition of EDTA	NaCl	48 ± 5	67 ± 11	76 ± 2	82 ± 9	88 ± 3
Zn	sucrose	56 ± 17	71 ± 17	35 ± 7	10 ± 11	5 ± 6
Zn + EDTA	sucrose	24 ± 16	31 ± 8	23 ± 2	26 ± 4	28 ± 16
Zn + addition of EDTA	sucrose	51 ± 8	64 ± 13	53 ± 11	53 ± 10	56 ± 3

RBC were incubated 30 min in a 1.2 M sucrose solution at 37° C and rehydrated into isotonic NaCl or sucrose media in the presence of Zn^{2+}, an equimolar amount of Zn^{2+} and EDTA or when equimolar concentration of EDTA was restored 1 min after the onset of hemolysis.

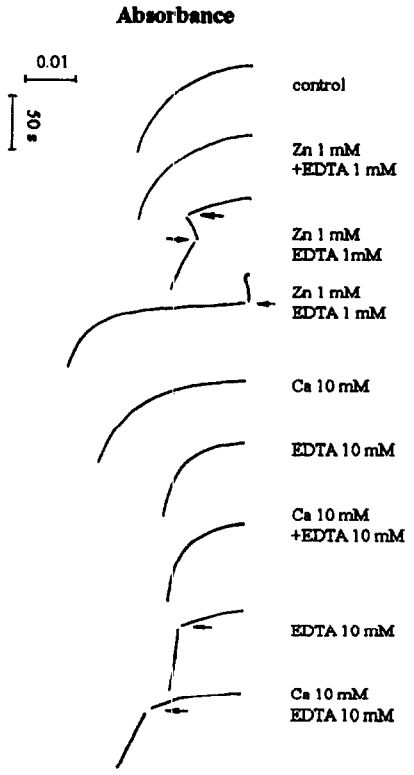

Fig. 3. Effect of Zn^{2+}, Ca^{2+} and EDTA on time-courses of hemolysis during rehydration in isoosmotic NaCl media. Arrows indicate the addition of cations and EDTA to the final concentration shown. Captures without arrows denote that rehydration was performed in the media in the presence of indicated amounts of these reagents.

Inhibition of PH by Zn^{2+} ions was reversible (tracks 3,4). Moreover, EDTA strongly increased the rate and extent of changes in absorbance for cells which were initially reexpanded in the presence of 1 mM Zn^{2+} (track 4). This correlated well with the hemolysis data presented in Table 2. Equivalent results with Zn^{2+} ions were obtained in the isotonic sucrose media (not shown). Slightly enhanced concentrations of EDTA (5 and 10 mM) brought about a week inhibition of PH either in the presence or absence of Ca^{2+} (Fig. 3, tracks 5,6). In general these data indicate that the mechanism of interaction of divalent cations with the plasma membrane during PH is complex and depends on the free cation concentration, the time of cell exposure in isotonic media and the presence of a cation chelator. The main conclusion is that if a significant amount of divalent cations is bound in the complex Me^{2+}-EDTA, the ions do not affect PH. However, the action of EDTA after the addition of ions would depend on the type of ion (Ca^{2+} or Zn^{2+}) and its concentration (Fig. 3). To define a possible localization of activating and inhibitory sites on the internal or external leaflet of the membrane the cells were treated by hypertonic sucrose solutions in the presence of divalent cations and ionophore A23187 which is known to facilitate the transport of cations inside the cells [2]. The data in Table 3 show that various treatments including treatment with A23187 and EDTA, 0.1 mM Zn^{2+}, and 1 mM Ca^{2+} in the presence and absence of A23187 do not change the extent of posthypertonic hemolysis. Treatment by 0.1 mM Ca^{2+} and 1 mM Zn^{2+} enhanced hemolysis which was further stimulated by A23187. Additional experiments have shown that Zn^{2+}-induced stimulation was due to Zn^{2+} action at the rehydration step of the procedure (see Materials and methods) where the residual concentration of Zn^{2+} (0.01 mM) became activating. EDTA (0.01 mM) fully prevented this stimulation, so Zn^{2+} by itself also does not cause changes in PH. Hence, PH is affected only in the case of hypertonic treatment of the cells in the presence of 0.1 mM Ca^{2+} and 0.1 mM Ca^{2+} plus A23187. The same results were obtained after rehydration of the cells in an isoosmotic sucrose solution (not shown).

3.4. Effect of divalent cations on volume distribution of cells and ghosts after rehydration

Fig. 4 shows that rehydration in isotonic NaCl solution significantly affected the volume distribution of erythrocytes generating ghosts which become visible in the right shoulder of the distribution or in the additional peak with a larger volume relative to the initial volume of the cells. The presence of a blocking concentration of Zn^{2+} (1 mM) in rehydrative media reduced the number of cells in the swollen subpopulation (curve 4). Subsequent addition of EDTA additionally reduced the volume of this subpopulation (curve 5), whereas rehydration in the media with both Zn^{2+} and EDTA did not significantly change a distribution relative to control (curve 6). Despite the large differences in the extent of control and Ca^{2+}-activated PH the cells had a similar volume distributions (curves 2,7). There is, therefore, an absence of correlation between ion-modulated posthypertonic hemolysis and cell swelling taking place

Table 3
Influence of Ca^{2+}, Zn^{2+}, EDTA and ionophore A23187 on hemolysis (%) after cells rehydration

Media	Control	A23187	A23187 + EDTA	0.1 mM Zn	0.1 mM Zn +A23187	1 mM Zn	1 mM Zn +A23187	0.1 mM Ca	0.1 mM Ca +A23187	1 mM Ca	1 mM Ca +A23187
NaCl	51 ± 2	49 ± 4	56 ±2	56 ± 4	55.7 ± 1.4	66 ± 3	69.8 ± 1.3	73 ± 2	77 ± 6	51 ± 2	61 ± 3
Sucrose	55 ± 1	53 ± 4	57.3 ± 1.6	53 ± 3	53 ±2	68 ± 2	73 ±3	69 ± 5	85 ± 5	51 ± 2	59 ± 2

RBC were treated 15 min in hypertonic sucrose solution (1.2 M) at 37° C in the presence or absence of divalent cations, 1 mM EDTA or 5 μM A23187 and then transferred into isotonic NaCl or sucrose solutions.

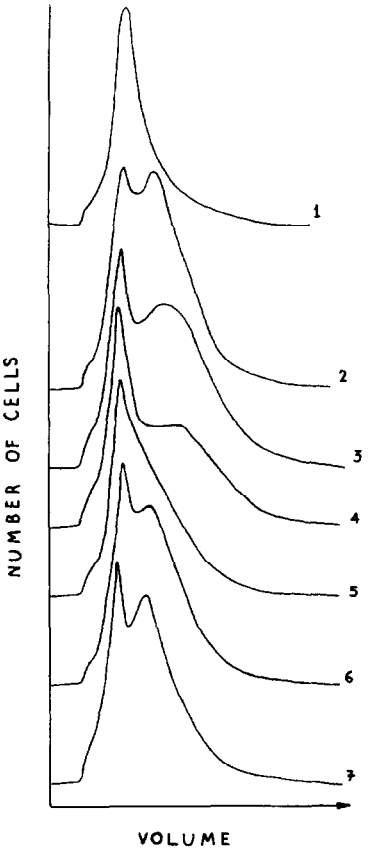

Fig. 4. Volume distributions of mixed population of rehydrated cells and ghosts obtained after RBC rehydration in isoosmotic NaCl media in the presence of an activating or inhibitory concentration of cations and EDTA. Curve 1 represents control distribution of cells in isotonic saline. 2, rehydrated erythrocytes. 3 and 4, RBC, rehydrated in the presence of Zn^{2+} at a concentration of 0.1 mM and 1 mM, respectively. 6, RBC rehydrated in the presence of 1 mM Zn^{2+} and 1 mM EDTA. 5, the same as 6 except that EDTA was added to the suspension of rehydrated cells 1 min after the onset of rehydration. 7, RBC rehydrated in the presence of 50 mM Ca^{2+}.

for example in hypotonic or lysolecithin-induced lysis [18,19]. This is apparent for Zn^{2+}-activated and Zn^{2+}-inhibited PH (curves 3,4), where swollen subpopulation takes place at both high and low extent of hemolysis. The similarities in volume distributions in the absence as well as in the presence of both Zn^{2+} and EDTA (curves 2,6) and their significant differences in the media without EDTA provide additional support to the view that the affinity of Zn^{2+} ions in the Zn^{2+}-EDTA complex exceeds those of both activating and inhibitory sites. It is interesting to note that the addition of a small amount of EDTA (1 mM) to the cells after interacting them with blocking concentrations of Zn^{2+} results in a decrease in the volume of ghosts, despite the increase of hemolysis (curve 5). A similar decrease in ghosts volume was also detected after applying an activating concentration of Zn^{2+} (not shown).

3.5. Influence of divalent cations on inhibition of melittin-induced hemolysis.

Fig. 5 shows the effect of divalent cations on time-courses of melittin-induced hemolysis in isotonic electrolyte and nonelectrolyte media. Melittin was 5-fold more effective in sucrose media. An additional difference was found in melittin-induced alteration of cell shape. In nonelectrolyte saline melittin induces an abrupt increase in absorbance concomitantly with the disappearance of absorbance noise, which corresponds to the rapid transformation of cells into smooth spheres (for details, see Ref. [13]) followed by hemolysis. A similar but not identical effect was observed after the addition of EDTA into media which contained both melittin and divalent cations. Divalent cations added after completing a stage of melittin-induced shape transformation and at the onset of hemolysis inhibited this in both media. This inhibition was reversible

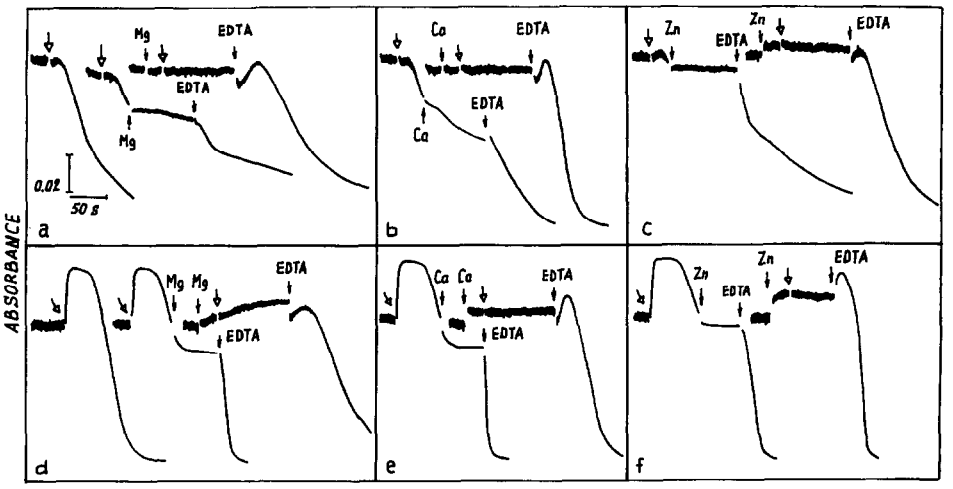

Fig. 5. Effects of divalent cations and EDTA on melittin-induced hemolysis in 150 mM NaCl (a, b, c) or 270 mM sucrose (d, e, f) media. Open arrows indicate the addition of melittin in final concentration 0.5 μg/ml (a, b, c) or 0.1 μg/ml (d, e, f). Close arrows show an addition of Mg^{2+} and Ca^{2+} at a final concentration of 25 mM or EDTA 50 mM for (a, b, d, e); and Zn^{2+} 1 mM (c) or 0.2 mM (f) and EDTA 2 mM for (c,f).

inasmuch as subsequent addition of EDTA prevented inhibition in NaCl and even stimulated hemolysis in sucrose. However, the presence of divalent cations in the cell suspension prior to the addition of melittin completely prevented the melittin-induced shape transformation as well as hemolysis in both media except Mg^{2+} in sucrose saline where a slight cell crenation was observed. It seems that divalent cations occupy the same membrane sites, which normally interact with melittin permitting its insertion into membrane. This is supported by the fact that chelation of divalent cations by EDTA initiates the full sequence of events resulting in shape transformation followed by hemolysis similar to those induced by melittin in cation free media. These data show that divalent cations are much more effective in prevention of melittin-induced pore formation than in inhibition of the preexisting pore. Although the rate and extent of shape transformation induced by melittin in nonelectrolyte media greatly exceeds those in electrolyte media the main features of the action of divalent cations are similar in both cases.

4. Discussion

Dehydration of erythrocytes in hypertonic media leads to changes in cell shape [20], decrease in cell volume [21], deformability [22] permeability for cations [23–26], stimulates endogenous transglutaminase [27], and others enzymes [28]. Osmotically stressed cells in contrast to fresh cells become highly sensitive to chilling [29], centrifugation [30], hypertonic stress or freezing [31,32] and rehydration [7–9,33]. The fact that hypertonic treatment per se does not cause a significant damage of cells while leaving them sensitive to changes in temperature or ionic environment implies that a variety of membrane defects arises mainly during changes in environment conditions. The present data support the view that posthypertonic injury is not due to solute 'loading' of cells in hypertonic conditions [9,33]. Moreover, dissociation between the extent of hemolysis and volume changes during Ca^{2+} and Zn^{2+} modulated PH and action of EDTA (Fig. 4) suggest that the overall mechanism of PH is not a colloidal-osmotic one in the sense that hemolysis is a result of cell swelling up to a maximal critical volume as in the case of hypotonic lysis [18] or lysis induced by toxins or other pore-forming substances [19,34]. PH, hence, may be attributed to the formation of pore permeable for hemoglobin while swelling and ghost formation occur secondary as a result of an increase in the membrane permeability. Besides, data show that Ca^{2+} and Zn^{2+} in contrast to Mg^{2+} possess the ability to stimulate PH when initially present in rehydrative media. On the other hand, all ions as well as EDTA and sucrose are able to inhibit it when introduced later (Fig. 2). The blocking effect of these substances was stronger in electrolyte compared to nonelectrolyte media, whereas the activating effect was dependent on the composition of dehydrative media. These complex data, nevertheless, permit to propose a simple and clear interpretation. We suggest that pore formation during reswelling may be regulated by at least two classes of cation binding sites on the erythrocyte membrane.

One class of sites is activating, their specific binding to cations Ca^{2+} and Zn^{2+} but not to Mg^{2+} as well as Na^{+} and K^{+} (unpublished data) leads to the stimulation of hemolysis (Fig. 1). They possess a three order of magnitude higher affinity for Zn^{2+} ions than for Ca^{2+} as judged from the concentration dependence of the effect. Failure to find activation by Zn^{2+} and Ca^{2+} in the presence of equimolar EDTA, as well as the inability of EDTA to block Ca^{2+} and Zn^{2+}-activated lysis, suggest that the affinity of activating sites to divalent cations is close to the affinity of these ions for EDTA. Results may be nicely explained in terms of competition for divalent cation between activating sites and EDTA. The data that the activation effect of divalent cations could not be restored by adding cations as quickly as possible after the onset of rehydration may be explained on the basis that activating sites live relatively short, comprising cell reswelling toward isotonic volume followed by inactivation or disappearing. The fact that the mode of activation and inhibition is the same during rehydration at both high and low ionic strength indicates that the regulatory binding sites do not contain charged groups with a high protonated ability. This is also confirmed by a weak pH dependence of PH (unpublished data) in contrast to the corresponding properties of hemolysis induced by some toxins known to be inhibited at low ionic strength and low pH [6,35,36].

The second class of sites is inhibitory. These sites are less specific to cations because hemolysis was inhibited in a similar manner by all divalent as well as monovalent cations, EDTA and sucrose, Zn^{2+} had the highest affinity among cations tested. Inhibition of PH by a variety of di- and monovalent cations, EDTA and sucrose was accompanied by an increase in either ionic strength or osmolarity of media suggesting a role of cell volume. Indeed, a decrease in volume of rehydrated cells facilitates blocking primarily at high ionic strength (Table 1). Several findings support the conclusion that blocking sites are located on the external leaflet of the rehydrated erythrocyte membrane: (i) blocking occurs virtually after addition of blocking agents, (ii) reversibility of blocking as judged by releasing action of EDTA or washing divalent cations (data not shown), (iii) blocking does not depend on the intracellular concentration of Zn^{2+} or Ca^{2+} after loading cells using A23187. This is in agreement with the mode of action of cations on membrane lesions produced by melittin, α-toxin or Triton X-100 [1,5] in RBC or lipid vesicles [4,37], it is a common feature among pores induced by exogenous and endogenous stimuli. Crespo et al. [23] found that RBC dehydration produced a subpopulation of RBC with enhanced permeability for monovalent cations, which was not blocked by the known transport inhibitors and did not

depend on intracellular Ca^{2+} concentration and ATP content. Up to now there is no additional information regarding the mechanism of this alteration in permeability. Our data confirm the existence of a subpopulation of prolytic susceptible cells (Fig. 4) which swell during rehydration suggesting the involvement of activating and inhibitory sites in these new transport pathways. Failure to find either a significant activation or inhibition of PH after cell exposure at hypertonic solutions in the presence of Ca^{2+} or Zn^{2+} (Table 3) indicates that neither activating nor inhibitory sites are exposed to the external leaflet of the membrane in hypertonic media. The simplest assumption is that they appear on the cell surface during the rehydration process itself. Indeed, the fact that an elevated intracellular Ca^{2+} concentration increases posthypertonic hemolysis supports the view that activating sites are initially located inside the cells. The mechanisms causing their exposure and the molecular nature of the ligands to which divalent cations bind are unclear. The questions must await elucidation of the molecular structure of pores. Nevertheless, it is obvious that triggering of both classes of sites correlate with a volume expansion of the shrunken cell as a result of inflicted osmotic gradient and water flow toward the cell interior. A causal role of each of these variables remains to be identified. Despite the clear differences between the mechanisms of posthypertonic hemolysis and hemolysis induced by melittin they reveal surprising similarities in many respects. Both have the same dependence on ionic strength of media showing larger extent of hemolysis at low ionic strength [4], and both are inhibited when the ionic strength is increased [4]. Both types of lysis may be reversibly blocked by divalent cations such as Ca^{2+} and Zn^{2+} which protect cells by an action at the extracellular side of the plasma membrane [1–5]. EDTA stimulates both cation-inhibited PH and cation-inhibited melittin-induced hemolysis in nonelectrolyte media (Figs. 3 and 5). There are also some important differences: (i) divalent cations when initially presented into rehydrative media stimulate PH, but significantly inhibit melittin-induced hemolysis, (ii) EDTA and sucrose are able to inhibit PH in electrolyte media (Table 1), but stimulate melittin-induced hemolysis (Fig. 5). The fact that PH may be inhibited by both cationic (divalent cations), anionic (EDTA) and nonelectrolyte (sucrose) compounds and stimulated by Ca^{2+} and Zn^{2+} at an early stage of damage strongly suggests that the structure of the posthypertonic pore is much more complex and different from that of the pore induced in the RBC membrane by melittin. In the present experiments we have not detected even a weak stimulation of melittin-induced hemolysis by Ca^{2+} and Zn^{2+} in nonelectrolyte media as reported by Portlock et al. [4]. In both media these ions exhibited inhibitory action in the whole range of concentrations used (1–200 μM) for Zn^{2+} and (1–50 mM) for Ca^{2+}. Moreover, it appears that inhibition was even more severe in sucrose media. The reason for these discrepancies is not clear, though it may be due to the differences in procedure of preparation of the cells and the concentration. To explain the activation ability of divalent cations Portlock et al. [4] proposed that the lytic activity of melittin is associated with its binding to at least two types of anionic sites at the RBC membrane, only one of which is involved in the hemolytic mechanism. Our results regarding the actions of divalent cations on melittin-induced hemolysis may be interpreted assuming the existence of only one lytic site. Divalent cations, when initially present in media occupy lytic membrane sites thus preventing the mechanism of pore formation, though, possibly, do not prohibit melittin insertion into the membrane. Pasternak et al. [38] and Alder et al. [3] have shown that the presence of divalent cations does not affect melittin binding to Lettre cells. EDTA deleting divalent cations from lytic sites permits melittin to create a pore, which is accompanied by a redistribution of membrane components [39] resulting in cell crenation. As shown in Fig. 5 crenation is a prerequisite of melittin-induced hemolysis especially at low ionic strength and may reflect the initial stage of pore formation. This is in contrast with observation of Katsu et al. who has reported onset of hemolysis from discocytes [40]. Thus, divalent cations will interfere with pore formation when initially present in media as well as block preexisting pore. All this supports the view that membrane damage caused by endogenous (posthypertonic hemolysis) and exogenous (melittin-induced hemolysis) factors affects some common intrinsic components of the membrane responsible for common properties of two types of pores. Since membrane proteins (band 3-glycophorin complex) are probably involved in pore formation induced by melittin [39,41] one may expect that they are also implicated in the regulation of pore formation during posthypertonic hemolysis.

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