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Effect of Zinc Ions on Melittin-Induced Hemolysis of Erythrocytes

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Divalent cations (Ca^{2+} and Zn^{2+}) were shown to inhibit melittin-induced hemolysis of erythrocytes in the hemolysis phase, the degree of inhibition increasing with increasing cation concentration. In addition, Zn^{2+} caused a concentration-dependent change in the initial stages of the interaction between melittin and the red cell membrane, slowing melittin insertion and hemolysis rate. Comparison of the dependencies between hemolysis rate and melittin and cell concentrations in the medium, as well as features of the inhibitory activity of cations under various conditions with corresponding data concerning melittin-induced lysis of lipid vesicles, indicates that melittin-induced hemolysis and melittin-induced lysis of lipid vesicles has little in common; this suggests an insignificant role of melittin–lipid interactions in the mechanism of cell hemolysis. The data show that the erythrocyte membrane contains an intrinsic blocking mechanism, whose efficacy can be synergistically enhanced by Zn^{2+} ions, protecting the cells against the lytic action of melittin.

KEY WORDS: melittin, erythrocytes, hemolysis, divalent cation.

Melittin, the main component of bee venom, consists of 26 amino acid residues and is widely used to study mechanism of translocation as well as incorporation of peptides and proteins into model biological membranes [1]. Melittin has been shown to form potential-dependent ion channels at submicromolar concentration [2-4] and to increase the permeability and leakage of lipid vesicles [5-7]. At micromolar concentration melittin causes lysis of erythrocytes [5, 8-11], but the molecular mechanism of this phenomena remains obscure. The idea that hemolysis is the result of melittin–lipid interaction is based on the ability of melittin to disrupt the membrane of lipid vesicles. De Grado et al. [8] found melittin binding to erythrocyte membrane, creating transient pores through which Hb leaks. This property was sufficient to explain experimental biphasic kinetics of hemolysis. Others have found an all-or-none response in erythrocyte hemolysis and proposed a colloid-osmotic mechanism where hemolysis is due to the formation of relatively small pores followed by colloid-osmotic swelling of the cells [11]. The dimension of the pores depended on the melittin concentration in the media [9]. Comparing the lysis of lipid vesicles of varying lipid composition (including compositions similar to that of erythrocyte membranes) with melittin-induced hemolysis, Portlock et al. [5] concluded that protein–melittin interaction may be important in the hemolytic mechanism. Melittin has been shown to interact with proteins such as calmodulin [12, 13], Ca- [14], Na,K-, and H,K-ATPase [15, 16], and band 3 protein [17, 18]. Divalent cations protect cells against the lytic action of viruses, toxins, and other agents [2, 5, 19, 20], including melittin. The specific action of divalent cations can therefore be used as a tool to study details of the lysis induced by various agents [5]. In this study we investigated the ability of Zn^{2+} to inhibit melittin-induced hemolysis. Comparison of the present data with data on melittin-induced lysis of lipid vesicles show that the two types of lysis have little in common; this contradicts suggestions of the importance of melittin–lipid interaction and supports a role of melittin–protein interactions. Furthermore, the data indicate the existence of an intrinsic blocking mechanism (IBM) in the erythrocyte membrane which protects cells against the lytic action of melittin in synergism with Zn^{2+}.

MATERIALS AND METHODS

In the present experiments only fresh blood was used. Some drops of blood from the donor's finger were mixed with 10 ml of physiological solution (150 mM NaCl,

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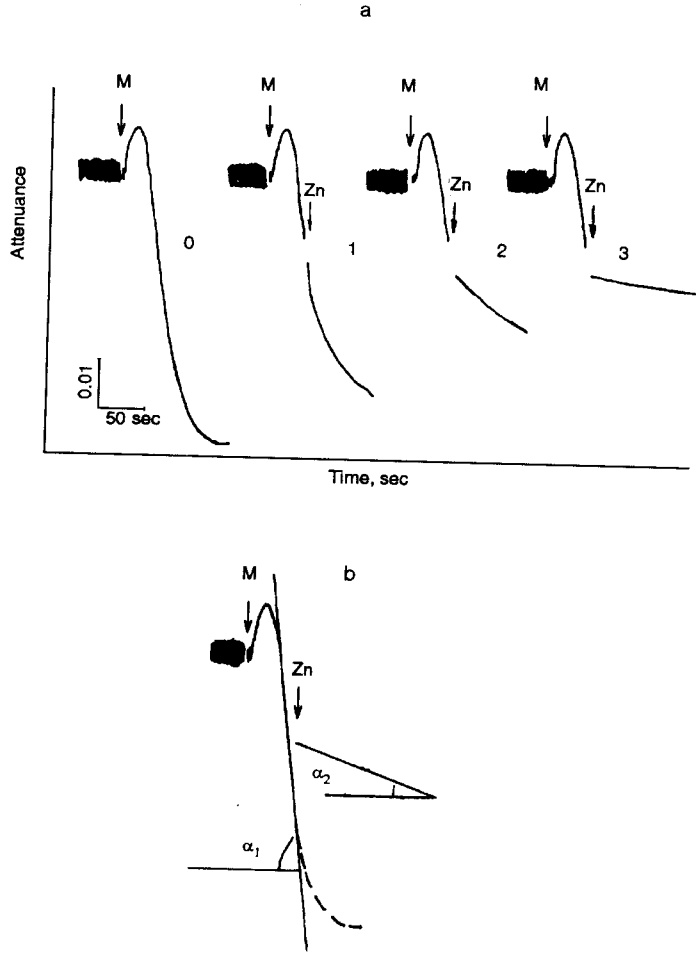

Fig. 1. a) Inhibitory effect of Zn^{2+} on melittin-induced hemolysis of erythrocytes during the hemolysis phase. Arrows marked M indicate additions of melittin to final concentration 0.25 μg/ml. Curve (0) is without Zn^{2+}. Arrows marked Zn indicate additions of Zn^{2+} to final concentrations of 0.25 mM (1), 0.5 mM (2), and 1 mM (3). b) Illustration of the method for determination of the coefficient of inhibition $k = (1 - \frac{\tan \alpha_2}{\tan \alpha_1}) \cdot 100\%$ used for estimating the inhibitory activity of cations.

10 mM Tris-HCl, pH 7.4) and washed twice by centrifugation (2000*g*, 3 min). Part of the erythrocyte pellet (30 μl) was suspended in 0.5 ml of physiological solution and used within a few hours as the stock suspension. The dynamics of erythrocyte hemolysis was measured spectrophotometrically at 720 nm [21-22]. An aliquot (6-7 μl) of the erythrocyte suspension was placed into a spectrophotometer cuvette (2 ml) with magnetic stirrer; the initial attenuance was 0.12-0.13. This corresponds to $1 \cdot 10^6$ cells per ml as detected by a Coulter counter. Attenuance changes in cell suspensions after addition of melittin or Zn^{2+} were continuously recorded. The rate of hemolysis was calculated from kinetic curves as tanα, where α is the maximal slope of the attenuance curve versus time. When the rate of melittin-induced hemolysis was measured as a function of cell concentration, the measured rate of hemolysis was normalized to the initial attenuance. Because the attenuance is proportional to cell concentration, the measured rate of decrease in attenuance is proportional to the rate of hemolysis [22]. The attenuance of a lysed suspension of cells at the given wavelength is zero. Phospholipase A_2-free melittin was a generous gift of Dr. C. Dempsey (Bristol University, UK). Other reagents were of the highest grade available. The experiments were carried out at room temperature (22-24°C).

RESULTS

Figure 1 shows typical time courses of attenuance changes after addition of melittin to cell suspensions. Adding various concentration of Zn^{2+} to the media after

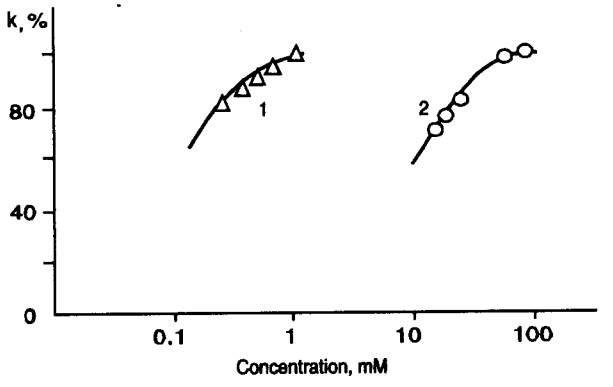

Fig. 2. Effect of Zn^{2+} (1) and Ca^{2+} (2) concentration on the inhibition coefficient of melittin-induced hemolysis $k = (1 - \frac{\tan \alpha_2}{\tan \alpha_1}) \cdot 100\%$.

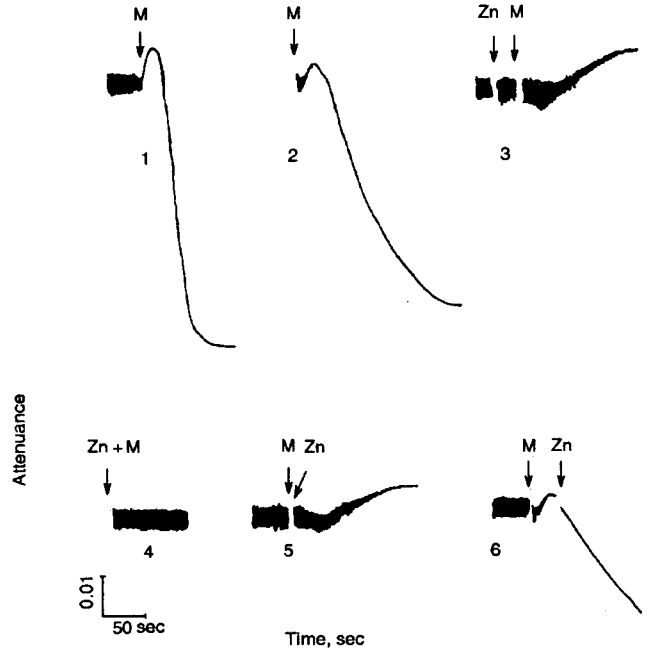

Fig. 3. Effect of order of addition of Zn^{2+} and melittin into erythrocyte suspensions on kinetics of melittin-induced hemolysis. Concentrations: Zn^{2+}, 75 μM; melittin, 1 μg/ml; erythrocytes, $1 \cdot 10^6$ cells per ml.

the onset of hemolysis inhibits hemolysis. The extent of inhibition was calculated as shown in Fig. 1b (100% inhibition corresponds the full arrest of hemolysis after the addition of cations). Figure 2 shows the dependence of the inhibition coefficient k on the concentration of Zn^{2+} and Ca^{2+}. As seen, both cations inhibit melittin-induced hemolysis, Zn^{2+} being more effective that Ca^{2+}. At 0.1 mM Zn^{2+} produced 50% inhibition, whereas 100-fold more Ca^{2+} (10 mM) is required to produce the same effect. The cation-induced inhibition is reversible because an equimolar amount of EDTA prevents inhibition (data not shown). These data suggest that the divalent cations block preexisting melittin-induced pores in erythrocytes, which open again after chelating of the cations. Thus, the affinity of the cations for binding to the sites responsible for blocking is less than to EDTA. This result is in agreement with those of others obtained using other lytic agents, cell types, and experimental procedures [2, 5, 19, 20, 23]. It might be expected that Zn^{2+} ions have an effect on pore formation as well as melittin insertion into the membrane. Thus, in some experiments we varied the order of addition of cells, melittin, and Zn^{2+} to the medium.

Typical attenuance traces are shown in Fig. 3. The order of addition of the reagents significantly affects the

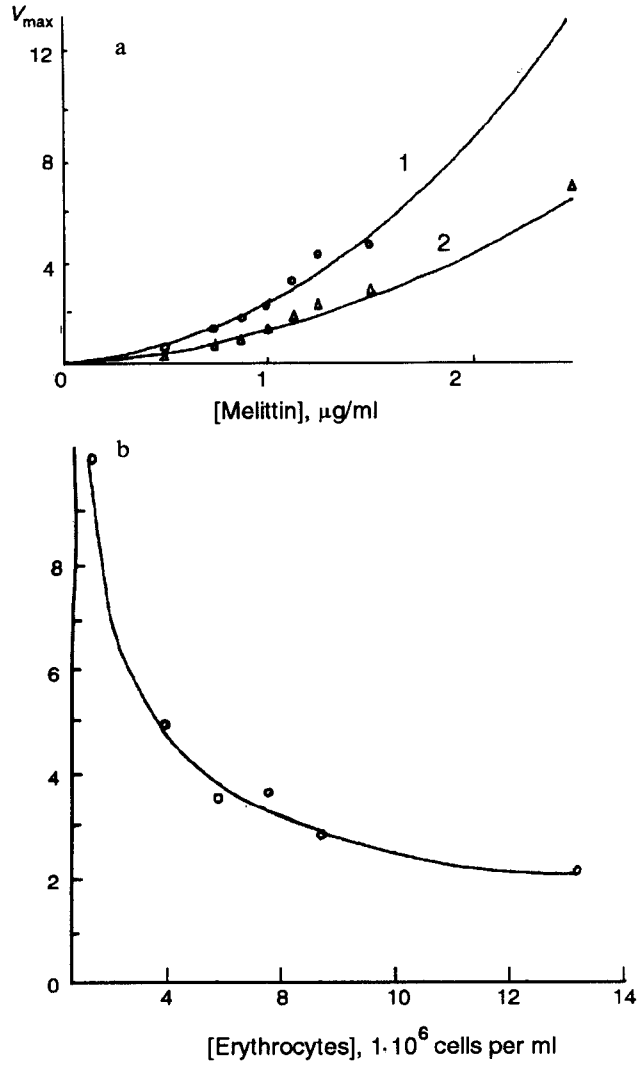

Fig. 4. Influence of melittin (a) and cell (b) concentration on maximal rate of melittin-induced hemolysis. a: 1) melittin was added to erythrocyte suspension 50 sec after the addition of cells to the medium; 2) erythrocytes (final concentration $1 \cdot 10^6$ cells/ml) were added to medium containing the indicated amounts of melittin. The solid lines are the theoretical curves obtained by least-squares fitting to the expression $V = Ac^n$, where c is concentration of melittin, n the power coefficient (1.96 and 1.8 for curves (1) and (2), respectively), and A a constant coefficient (2.2 and 1.2 for curves (1) and (2), respectively). b) Melittin (final concentration 2 μg/ml) was added to the medium 50 sec after the cells. The solid line represents the dependence $V = A/\sqrt{c}$.

kinetics of the melittin-induced hemolysis. For instance, adding the cells into medium containing melittin (curve 2) leads to a twofold decrease in the rate of hemolysis compared to the control, where melittin is added 50 sec after the cells (curve 1). The effect of Zn^{2+} also depended on the order of addition. Addition of Zn^{2+} before the addition of melittin decreases the rate of hemolysis and increases the lag period before hemolysis begins (curve 3). A similar result was obtained when Zn^{2+} was added immediately after melittin (curve 5). However, when Zn^{2+} was added a little longer after melittin, when the attenuance was at its maximum, the effect was significantly less (curve 6). The most significant effect of Zn^{2+} was obtained when cells were introduced into medium containing Zn^{2+} and melittin. No attenuance changes were detected within the time range comparable to that of other similar experiments. This indicated that no hemolysis or shape transformation occurred (curve 4). Because the Zn^{2+} concentration used here was relatively low for the inhibition of hemolysis (Fig. 2; as seen also from curve 6), this suggests that Zn^{2+} has an effect on the initial stages of melittin-induced pore formation and/or melittin insertion into membrane. To investigate this in more detail the rate of melittin-induced hemolysis was measured at

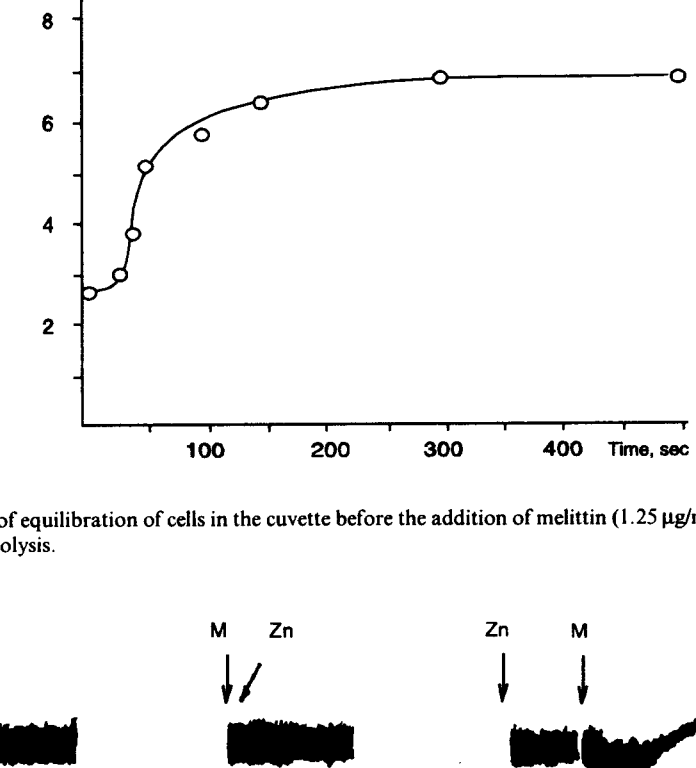

Fig. 5. Effect of time of equilibration of cells in the cuvette before the addition of melittin (1.25 μg/ml) on maximal rate of melittin-induced hemolysis.

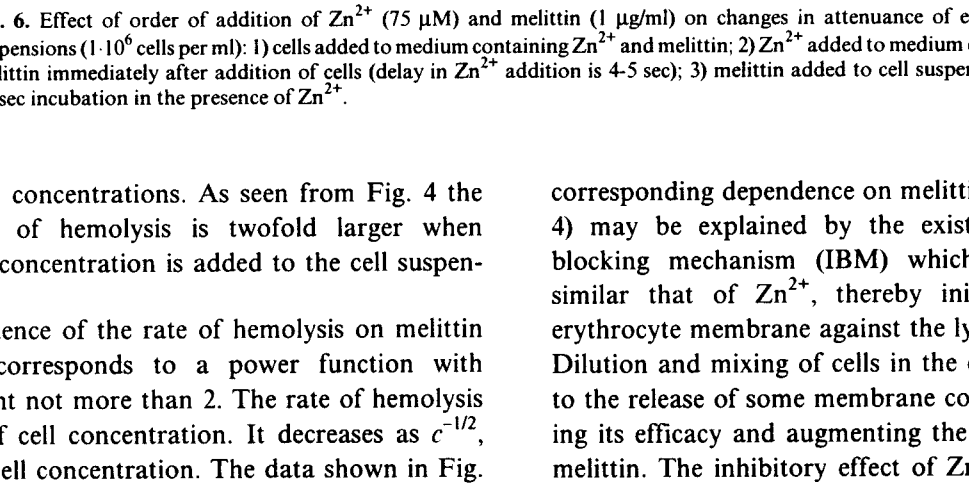

Fig. 6. Effect of order of addition of Zn^{2+} (75 μM) and melittin (1 μg/ml) on changes in attenuance of erythrocyte suspensions ($1 \cdot 10^6$ cells per ml): 1) cells added to medium containing Zn^{2+} and melittin; 2) Zn^{2+} added to medium containing melittin immediately after addition of cells (delay in Zn^{2+} addition is 4-5 sec); 3) melittin added to cell suspension after 50-sec incubation in the presence of Zn^{2+}.

various melittin concentrations. As seen from Fig. 4 the maximum rate of hemolysis is twofold larger when melittin at any concentration is added to the cell suspension.

The dependence of the rate of hemolysis on melittin concentration corresponds to a power function with power coefficient not more than 2. The rate of hemolysis is a function of cell concentration. It decreases as $c^{-1/2}$, where c is the cell concentration. The data shown in Fig. 5 indicate that the rate of hemolysis depends on the time between addition of cells and melittin to the cuvette. In other words, after dilution erythrocytes became more sensitive to the lytic action of the same amount of melittin. This result suggests that the aforementioned data can be explained within the scope of one suggestion; we believe that the time dependence of the melittin effect and the corresponding dependence on melittin concentration (Fig. 4) may be explained by the existence of an intrinsic blocking mechanism (IBM) which acts in a manner similar that of Zn^{2+}, thereby initially protecting the erythrocyte membrane against the lytic action of melittin. Dilution and mixing of cells in the cuvette probably lead to the release of some membrane component, thus reducing its efficacy and augmenting the sensitivity of cells to melittin. The inhibitory effect of Zn^{2+} may act in synergism with the IBM, nonadditively enhancing its power. If so, a natural test of this suggestion could be experiments in which Zn^{2+} is added to the cell suspension together with melittin immediately after the addition of the cells as in Fig. 3, curve 4. Another test consists of incubation of the erythrocytes for a fixed time period (50 sec) in the presence of Zn^{2+}, to preserve the efficacy

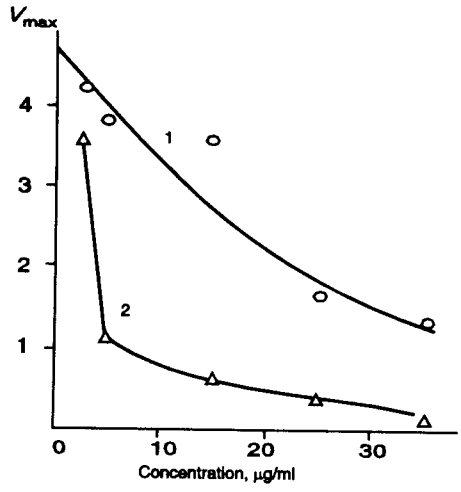

Fig. 7. Effect of Zn^{2+} on maximal rate of melittin-induced hemolysis: 1) melittin (final concentration 0.9 μg/ml) was added to the cell suspension ($1 \cdot 10^6$ cells per ml) 50 sec after the addition of the cells into the cuvette. Zn^{2+} was added to the same suspension 25 sec after addition of the cells; 2) cells were added into medium containing Zn^{2+} and melittin.

of the IBM, followed by addition of melittin. The results of these experiments are shown in Fig. 6.

Indeed, adding Zn^{2+} immediately after cells leads to the same result as when cells were introduced into medium containing Zn^{2+} and melittin, i.e., full prevention of the melittin-induced changes in erythrocyte state (curves 1 and 2). Incubation of cells in the Zn^{2+}-containing medium, while retarding the melittin-induced changes, does not fully prevent them (curve 3). This suggests that Zn^{2+} is not able to prevent desorption of the IBM components from the erythrocyte membrane. Thus, we conclude that Zn^{2+} acts in synergism with the IBM at an earlier stage of melittin–membrane interaction. The actual mechanism may be due to decreasing insertion of melittin into the membrane with corresponding inhibition of the ability of melittin to form hemolytic holes. Indirect evidence for this suggestion came from the kinetic curves shown in Figs. 3 and 6 obtained in the presence of Zn^{2+}. As shown in [24], the shape transformation of erythrocytes is accompanied by an increase in attenuance and decrease in attenuance fluctuations, which is due to the mixing of cells in suspension. As seen in Figs. 1 and 3, decrease in attenuance fluctuations reflecting the transformation of the erythrocytes to smooth spheres precedes hemolysis. The disk–sphere transformation is confirmed by direct microscopic analysis. Erythrocytes become spherical when melittin is inserted into the outer leaflet of the membrane. A similar phenomena is detected during the insertion of other lytic and nonlytic membrane-active agents [10, 24-26]. Hence, the extent of shape transformation detected by attenuance changes may be used as qualitative parameter reflecting the process of melittin insertion in the presence of Zn^{2+} because Zn^{2+} *per se* does not change the attenuance. From Fig. 3 (curves 3 and 5) it is obvious that Zn^{2+} increases the time of for the insertion of melittin into the erythrocyte. When cells are introduced into the medium in the presence of Zn^{2+} and melittin (Fig. 3, curve 4) or when the ions are added immediately after the cells (Fig 6, curve 2), melittin is apparently not inserted. Therefore, Zn^{2+} competes with melittin for common binding sites on the erythrocyte membrane, so that an ability of the ions to prevent melittin insertion depends on the state of the IBM. Although the elements comprising the IBM remain unidentified, our data, as well as data known from the literature, permit us to conclude that membrane-bound cations like Ca^{2+} and weakly associated peripheral membrane proteins may be included. Increasing concentrations of the cation chelator EDTA (1-5 mM) stimulate melittin-induced hemolysis. Intensive washing of cells in isotonic saline has the same result. On the other hand, a small amount of albumin (1-5 μg/ml) or plasma proteins inhibit hemolysis. Decreasing ionic strength by placing cells into isotonic sucrose solution or increasing pH increase erythrocyte sensitivity to melittin [5], whereas raising ionic strength decreases it, which suggests a significant role of electrostatic interactions in the IBM. The wide range of sensitivity of erythrocytes obtained from various donors or even the same donor on different days to melittin treatment under the same experimental conditions indicates variability of the IBM. Considering this variability, during the above experiments we tested a control sample of blood to be sure that rate of hemolysis did not change by more than 20% from the beginning to the end of an experiment. Non-additive synergism between Zn^{2+} and IBM is clearly seen from the dependencies between hemolysis rate and Zn^{2+} concentration obtained under various conditions of addition. As seen in Fig. 7, five-to-six-fold less Zn^{2+} is required to reduce twofold the rate of melittin-induced hemolysis when the efficacy of the IBM is maximal, that is when the cells are added to medium containing the mixture of Zn^{2+} and melittin, as compared with the case when the corresponding effectiveness is reduced due to initial washing of erythrocytes in the cuvette. Therefore, the differences in the blocking ability of Zn^{2+} may be satisfactory explained on the basis of its action in synergism with the intrinsic blocking mechanism.

DISCUSSION

It is widely accepted that lytic activity of melittin can by explained on the basis of melittin–lipid interactions. This view is based on the ability of melittin to cause leakage of lipid vesicles [2, 3, 5-11] and to form ion-selective channels in planar lipid membranes [3, 4]. It was

expected that features of melittin-induced lysis of lipid vesicles and erythrocyte hemolysis should have much in common. However, the detailed comparison revealed significant differences in the mode of action of divalent cations and ionic strength on melittin-induced lysis of lipid vesicles and on the hemolysis of human erythrocytes [5]. It turned out that the divalent cations Ca^{2+} and Zn^{2+} were able either to activate or inhibit melittin-induced hemolysis depending on concentration and ionic strength, effects not observed with lipid vesicles. Phosphate ions inhibited hemolysis but were unable to prevent the lysis of vesicles [5]. The concentration of cations required to inhibit or activate melittin-induced lysis of vesicles has been shown to be 10-fold larger compared to erythrocyte [5, 6]. These and other data suggest that the mechanism of melittin-induced hemolysis includes the interaction of melittin with at least two types of membrane sites, only one of which directly participates in hemolysis and may be connected with membrane proteins [5, 18]. This is confirmed by the fact that the lytic ability of melittin derivatives correlates well with their corresponding ability to aggregate intramembrane particles, i.e., band 3–glycophorin complexes [17, 18]. The present data also permit comparison between features of melittin-induced hemolysis and data concerning melittin-induced lysis of lipid vesicles. The dependence of rate of hemolysis on melittin concentration (Fig. 4) is a weak power function with coefficient less than two, whereas a third-power law was obtained in the case of lipid vesicles [7]. Our result is in agreement with data obtained earlier [8] where proportional dependence of a fast phase of hemolysis on melittin concentration has been shown. The rate of hemolysis depends on the concentration of erythrocytes c as $\sqrt{1/c}$, whereas this value for lipid vesicles depends on vesicle concentration as $\sqrt{1/c^3}$ [7]. The partition coefficient of melittin between synthetic lipids and solution in of the order of 10^4 M^{-1} [7]. Our estimate of the partition coefficient in the present experiments gives a value of the order of 10^6 M^{-1}, close to a value obtained by others [8]. This value is close to the melittin–protein binding constant, for instance for calmodulin, $4 \cdot 10^8$ M^{-1} [27]. Significant differences were observed also in the mode of action of Zn^{2+} relative to these two systems. Whereas Zn^{2+} was shown to be able to either activate or inhibit melittin-induced lysis of lipid vesicles depending on their phospholipid composition [5], primarily inhibitory action of cations was detected for erythrocytes in isotonic saline depending on cell state. As seen in Fig. 5, the lytic effect of melittin depends on preincubation condition. The same is true for the inhibitory effect of Zn^{2+}, which can be enhanced 10-fold depending on experimental conditions (Fig. 7). In the latter case Zn^{2+} inhibits at very low concentration, suggesting binding to a membrane site with high affinity. Because such effects have not been observed using phospholipid vesicles of various composition, including zwitterionic as well charged lipids, it is possible that this site is associated with membrane proteins. It is known that melittin binding to lipid vesicle membrane occurs in a time interval less that 1 sec [1, 28].

The present results show that the time for melittin incorporation into the external leaflet of the membrane significantly increases in the presence of Zn^{2+} and exceeds that for lipid vesicles. This comparison indicates that there is little if anything in common between the features of melittin-induced hemolysis and the lysis of lipid vesicles except for the fact that the kinetics of both processes could be described by the same function with exponential and linear terms [7, 8]. It follows that lipid vesicles, which are a widely used model to study melittin–lipid interactions, are inappropriate to study the mechanism of melittin-induced lysis of erythrocyte membranes, where lipids play a minor role. On the other hand, these data show that membrane proteins are the main target for melittin-induced lysis. The action of Zn^{2+} either alone or in synergism with the IBM decrease the lytic effect of melittin. Our data show that analysis of the mechanism of melittin-induced hemolysis is complicated by the existence of an IBM which may act in synergism with divalent cations. Because the nature of this mechanism is obscure and there is no means to control it status, it is not surprising that data obtained by some authors are different [5, 8, 18]. In any case, to better understand the mechanism of melittin-induced hemolysis it is important to transfer the main attention from melittin–lipid to melittin–protein interactions. This is especially important because a main contribution of proteins to the stabilization of the membrane pore during hypotonic hemolysis of erythrocyte has been recently found [29].

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