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Modulation of Melittin-Induced Hemolysis of Red Blood Cells

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Various chemical agents with respect to their action on melittin-induced hemolysis can be subdivided into three main groups: neutral substances, inhibitors, and activators of hemolysis. Inhibitors—which include the divalent cations Ca^{2+} and Zn^{2+}, albumin, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), and some other agents—inhibit melittin-induced hemolysis of erythrocytes; their blocking ability is significantly increased when the agents act on the early stages of peptide–membrane interaction. Melittin causes hemolysis at a rate that depends on time of preliminary incubation of the cells in physiological saline, irrespective of the presence or absence of inhibitors and activators. The rate of hemolysis increases with increasing preincubation time. These effects may be due to the existence of melittin-specific membrane inhibitory components (MICs) which initially protect the cell against the lytic action of peptides, but are probably desorbed from the membrane surface during cell dilution and incubation in physiological saline. A model of melittin-induced hemolysis is proposed according to which the features of hemolysis are determined by consecutive stages of peptide–membrane interactions and depend on whether or not an anti-lytic triple complex, including a membrane inhibitory component, an inhibitor, and the peptide is formed.

KEY WORDS: melittin, erythrocytes, hemolysis, divalent cations, albumin, DIDS, membrane defects.

Despite much literature on various aspects of the action of melittin on biological and model membranes (for review see [1]), the mechanism of its lytic action remain unresolved. As far as mechanism of action of melittin and other lytic agents is concerned, it is generally accepted that if an agent is able to increase permeability of lipid vesicles or to form ion channels in a planar lipid bilayer, there is no need to implicate the idea of a specific target receptor to explain the mode of action of the agent [2-6]. On the other hand, recent data have shown that melittin produces a lytic effect on erythrocytes interacting mainly with protein rather than lipid components of membranes [7-10]. Thus, this does not exclude completely models according to which melittin-induced hemolysis is due to interaction of the peptide with a receptor regulating cell lysis. Alternatively, membrane proteins could modulate the interaction of melittin with membrane patches where lytic pores are finally formed. It is known that divalent cations, chlorpromazine, and some other substances inhibit melittin-induced hemolysis, an effect which is believed to be due to their ability to close preexisting melittin-induced pores [11, 12]. If the interaction of melittin with some membrane receptors is involved in the actual hemolytic mechanism, one should expect that inhibitors would produce modulation especially at early stages, namely at the stages of birth and formation of pores. Therefore, investigation of the modulation of melittin-induced hemolysis could provide important information regarding the mechanism of the lytic action of melittin.

In this work the influence of various chemical substances on the modulation of peptide–membrane interactions has been studied. We have studied the effect of various inhibitors of melittin-induced hemolysis, such as divalent cations Zn^{2+} and Ca^{2+}, chlorpromazine [9, 10], DIDS[3], albumin, serum, and other agents when they acted prior to or simultaneously with melittin.

The results are consistent with the view that the significant increase in the blocking ability of inhibitors when they act in concert with melittin is due to the presence of membrane inhibitory components (MICs) that

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[3]Abbreviations: DIDS) 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; CTAB) cetyltrimethylammonium bromide; CCCP) carbonyl cyanide *m*-chlorophenylhydrazone; SDS) sodium dodecyl sulfate; LS) lytic site; NLS) non-lytic site; MIC) membrane inhibitory component.

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play a protective role and represent a special class of non-lytic sites for melittin.

MATERIALS AND METHODS

Human erythrocytes were obtained adding some blood drops to 10 ml of physiological solution (150 mM NaCl, 10 mM Tris-HCl, pH 7.4), mixed, and washed twice by centrifugation (2000*g*, 3 min). The leukocyte coat was discarded and 30 μl of cell pellet was suspended in 0.5 ml of physiological solution and used within a few hours as the stock suspension. Plasma and serum were obtained by pelleting whole blood samples or samples diluted in physiological solution. In the present experiments only fresh autologous plasma and serum were used. Concentrations of plasma proteins were determined using the photometric method described in [13]. Phospholipase A_2-free melittin was a generous gift of Dr. C. Dempsey (Bristol University, UK). Chlorpromazine-HCl, EDTA, trifluoperazine, human serum albumin, iodoacetamide, valinomycin, CCCP, and DIDS were from Serva (USA); A23187, CTAB, N-ethylmaleimide, and SDS from Sigma (USA); dithiothreitol from Reanal (Bulgaria); mannitol from Chemapol (Czechoslovakia); colchicine from Fluka (Switzerland); Triton X-100 from Merck (Germany); L-α-lysolecithin from Calbiochem (USA); 2-mercaptoethanol from Ferak (Germany); adrenalin hydrochloride and noradrenalin hydrochloride from Russian sources. Other reagents of "chemically pure" grade were from domestic sources. The experiments were carried out at room temperature (22-24°C).

Determination of Hemolysis. The dynamics of erythrocyte hemolysis during the interaction of the cells with lytic peptides was measured spectrophotometrically at 720 nm as described earlier [9, 10, 14]. The rate of hemolysis v was calculated from kinetic curves as tanα, where α is the angle between the linear part of the kinetic curve and the time axis. Figure 1 shows typical time courses of attenuance changes after addition of cells into the medium containing peptide (1) or medium containing both peptide and divalent cations (2). The interaction of peptide with erythrocyte leads initially to an increase in attenuance, which corresponds to a phase of shape transformation, followed by attenuance decrease attributed to a phase of swelling and hemolysis [9, 10, 14, 15]. As seen, the presence of divalent cations increases the period (delay time) between introduction of the cells and the maximum of attenuance and decreases hemolysis rate. Defined in such a manner the delay time T_d is an accurate and objective quantitative parameter to assess the blocking ability of cations and others inhibitors at early stages of the interaction of the peptide with the cell. Fluorescence studies were carried out on a Hitachi F-4010 spectrofluorimeter.

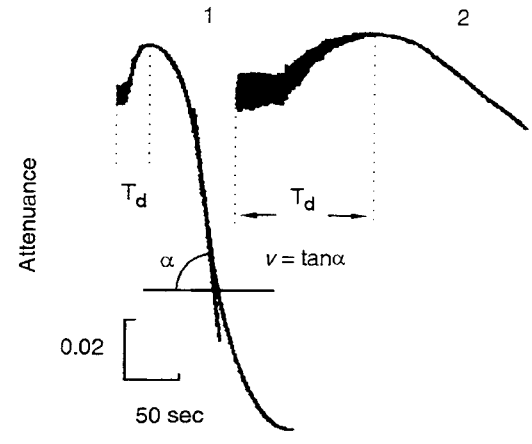


Fig. 1. Typical time courses of attenuance of erythrocyte suspensions after addition of cells into medium containing peptides (1) or medium containing both peptides and divalent cations Zn^{2+} or Ca^{2+} (2). T_d is the delay time from the onset of hemolysis measured from the moment of cell introduction up to the moment of achievement of the maximal value of attenuance.

RESULTS

Data on ability of the divalent cations Zn^{2+} and Ca^{2+} to retard melittin-induced hemolysis are shown in Fig. 2. As seen, the maximal inhibitory effect is achieved when the cells were introduced into the medium containing both peptides and cations (curves 1). Inhibition was lower when the peptides were added to the cell suspension 50 sec after addition of the cells to the solution (curves 2).

Figure 3 illustrates the dependence of hemolysis rate on time of equilibration of the cells in the cuvette preceding addition of peptide and the effect of cations Zn^{2+} and Ca^{2+}. It is evident that the presence of cations reduces several-fold the rate of hemolysis compared with the control. Nevertheless, the rate is increased with characteristic time 50-100 sec after incubation of the cells in physiological saline both in the presence as well as in the absence of cations. This effect varies several-fold in various blood samples.

The data in Table 1 regarding the action of other substances on melittin-induced hemolysis confirms the view that the effect of inhibitors depends significantly on the order of mixing reagents in the experimental hemolysis assay. Inhibitors produce the maximal effect when the cells are introduced into medium already containing peptides and the indicated amount of inhibitor. The protective action of inhibitors is reduced on incubating the cells in physiological saline in the presence of the inhibitor for 100-200 sec prior to addition of the same

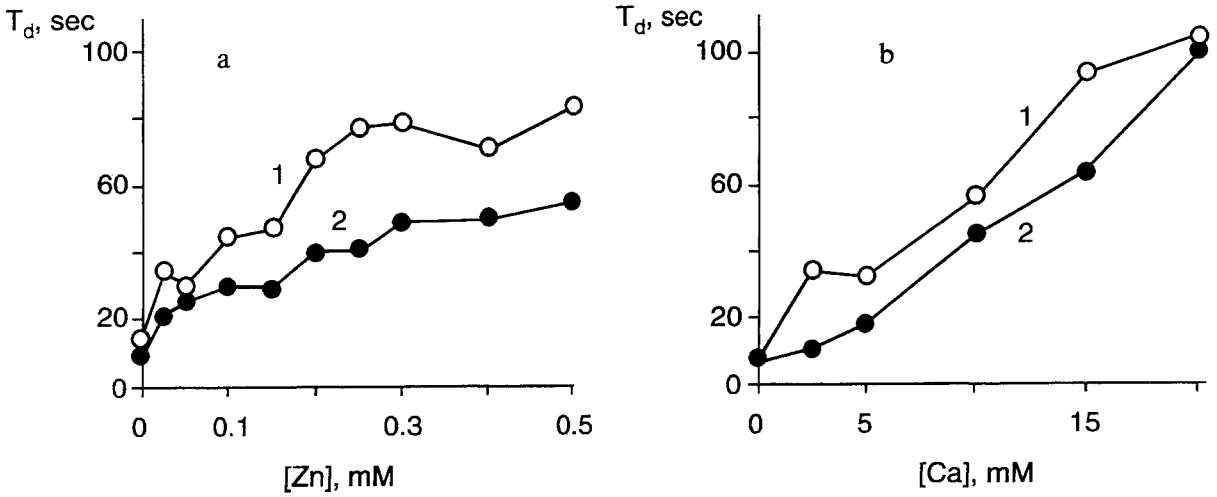


Fig. 2. Effect of Zn^{2+} (a) or Ca^{2+} (b) on delay time of hemolysis induced by melittin: 1) cells were introduced into medium containing peptides and cations; 2) cells were introduced into medium containing cations followed by addition of the peptide 50 sec after the cells.

amount of peptide. So, to characterize quantitatively the effect of these substances toward hemolysis, we have used both parameters: v_0, rate of hemolysis when cells are introduced into medium containing peptide and the investigated reagent, and v_{200}, the rate of hemolysis after addition of the same amount of peptide to cells which were initially equilibrated for 200 sec with the corresponding reagent. Both of these parameters were normalized to the control rate of hemolysis defined by cells added to inhibitor-free medium containing only melittin.

As seen from Table 1, the substances can be subdivided into three groups according to their mode of action: neutral agents, activators, and inhibitors of melittin-induced hemolysis. Interestingly, some agents are able to change their mode of action with respect to the type of hemolysis, that is whether the rate of hemolysis was measured introducing the cells directly into the medium containing peptide and agents (v_0) or introducing peptide after cell incubation during 200 sec in the presence of the agent (v_{200}). For example, lysolecithin was recognized as an inhibitor with respect to v_0, but it became an activator relative to v_{200}. Similar changes in the mode of action were found for A23187, valinomycin, and mercaptoethanol. This indicates that during incubation of cells in physiological saline some changes occur to the cell membrane, which induces changes in cell susceptibility not only to the action of the peptide, but also sometimes in opposite directions, to the action of additional modifiers, such as activators or inhibitors of hemolysis. Among inhibitors, albumin and DIDS possessed maximal inhibitory ability, so peculiarities of their action were studied in more detail.

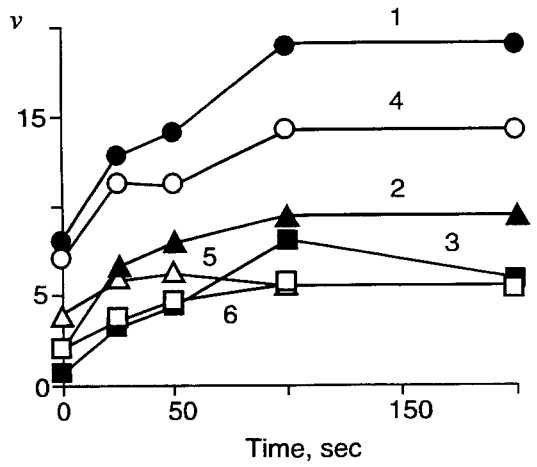


Fig. 3. Effect of time of cell equilibration in the cuvette preceding addition of melittin (1.5 μg/ml) in the absence of cations (1, 4) or in the presence of Zn^{2+} (75 μM) (2, 5) or Ca^{2+} (7.5 mM) (3, 6) on the rate of melittin-induced hemolysis; 1-3) donor 1; 4-6) donor 2. Definition of rate of hemolysis is given in Fig. 1.

Figure 4a shows the ability of albumin to retard hemolysis under different regimes of adding the cells and peptide into medium containing albumin. In both cases, an effective retarding of hemolysis is observed and at some concentration of albumin $v_0 = v_{200}$, which is not characteristic for Zn^{2+} and Ca^{2+} and many other substances (Table 1). Figure 4 (b and c) shows Dixon plots of hemolysis rate versus concentration of inhibitors. This dependence for albumin is linear for v_{200}, but non-linear for v_0. In the latter case negative cooperativity in inhibition is observed, that is, increasing the concentration of the inhibitor decreases its efficacy. Both dependences had

TABLE 1. Effect of Agents on Rate of Hemolysis Induced by Melittin at 1.5 or 4 μg/ml Concentrations with Albumin, Serum, and Plasma[a]

Agent	v_0/v_c	v_{200}/v_c	Characterization
Control	1	2.59 ± 0.67	
Iodoacetamide (1 mM)	0.81	3.81	N
N-ethylmaleimide (1 mM)	0.44	3.3	N
Dithiothreitol (6.5 mM)	0.39	3.8	N
Sucrose (50 mM)	0.89	3.9	N
Mannitol (50 mM)	1.64	3.8	N
Adrenalin (1 mg/ml)	0.77	1.55	N
Noradrenalin (2 mg/ml)	0.68	1.55	N
Colchicine (0.3 μM)	0.8	3.4	N
CCCP (20 μM)	0.85	4.0	N
Triton X-100 (4 μg/ml)	3.48	8.9	A
SDS (0.5 μM)	2.38	6.2	A
L-α-lysolecithin (0.84 μM)	0.17	6.2	I, A
EDTA (5 mM)	4.8	8.85	A
A23187 (5 μM)	0.8	5.7	N, A
Zinc (75 μM)	0.28	1.2	I
Calcium (7.5 mM)	0.08	0.73	I
Human serum albumin (20 μg/ml)	0.3	0.36	I
Human serum (20 μg/ml)	0.3	0.3	I
Human plasma (20 μg/ml)	0.19	0.35	I
Valinomycin (1 μM)	0.32	3.2	I, N
Chlorpromazine-HCl (20 μM)	0.32	1.32	I
Trifluoperazine (10 μM)	0.37	1.19	I
2-Mercaptoethanol (0.2%)	0.02	2.95	I, N
CTAB (10 μM)	0	0	SI
DIDS (10 μM)	0	0	SI

[a] v_0, rate of hemolysis after introduction of cells to final concentration 10^6 cells per ml into medium containing peptide and agent; v_{200}, rate of hemolysis after melittin addition to cells incubated for 200 sec in the presence of agent; v_c, rate of hemolysis of control cells after introducing them into medium containing only melittin. The agent was characterized as neutral (N) if it does not change the rate of hemolysis more than twofold in either direction, as an activator (A) or an inhibitor (I) if the rate of hemolysis was increased or decreased more than twofold relative to control, respectively, and as a strong inhibitor (SI) for as much as tenfold inhibition.

similar inhibition constants. Similar result was obtained for DIDS; however, in this case the inhibition constants were different.

It should be explained why the blocking effect depends on the time of cell equilibration in the physiological saline. Separate experiments revealed that this phenomenon is not associated with deformation of the cells during their mixing into the cuvette. Deformation, therefore, can be excluded as a potential source of cell susceptibility to the action of the peptide.

One might suggest that increased hemolysis rate is due to increased ability of melittin to bind to the cell membrane. However, calculation of partition coefficient of melittin between erythrocyte and medium, which had an order of $3 \cdot 10^6$ M^{-1} [16] for three donors, revealed not increase but decrease in corresponding values for v_{200} relative to v_0 at 25, 34, and 49%, respectively. This means that despite variations which exist among different donors, in all cases the cells bind less of the peptide after a 200 sec period compared with the zero-time moment, although they lyse with enhanced rate (Fig. 3, Table 1). Thus, we see an absence of direct correlation between hemolytic potency of the peptide and its ability to bind to the membrane. In this respect, erythrocytes differ from liposomes, where increasing concentrations of peptides in the membrane results in proportional enhancement of lysis rate [17]. This suggests that changes in susceptibility of cells are due to corresponding changes in the structure of the membrane occurring in the initial stages of peptide–membrane interactions.

The significance of these stages in determining subsequent steps of hemolysis is illustrated by data shown in Fig. 5. It is seen that EDTA and equimolar mixture of Zn^{2+} and EDTA practically have no effect on the dynamics of hemolysis relative to control (curves 1-3). At concentrations above 1 mM, EDTA became a stimulator

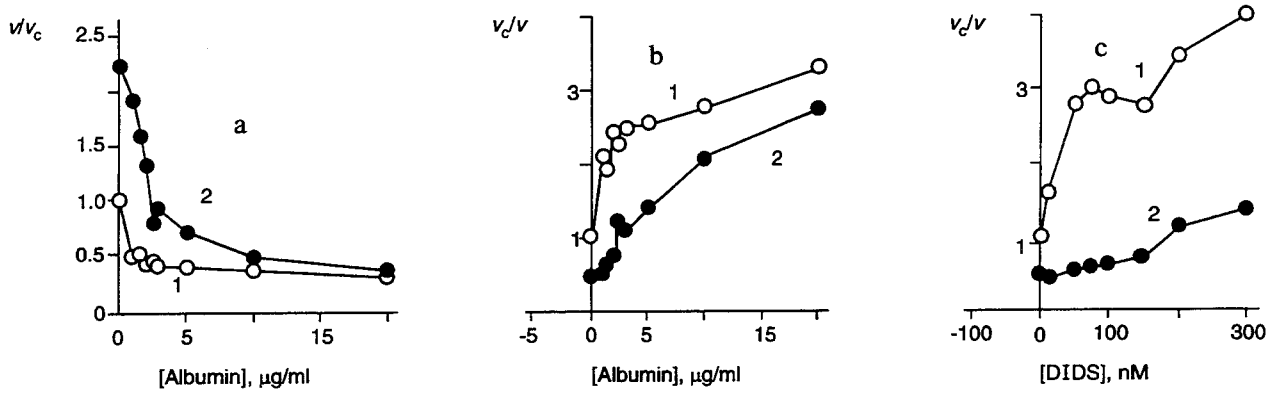


Fig. 4. Dependence of rate of hemolysis normalized to the rate of a control sample v_c which was determined after introducing cells into medium containing melittin (4 μg/ml) on the concentration of albumin (a) and dependence of reciprocal normalized rate of hemolysis on concentration of albumin (b) and DIDS (c): 1) cells added to medium containing melittin and inhibitor; 2) melittin added to cells 200 sec after introduction of cells in medium containing the inhibitor.

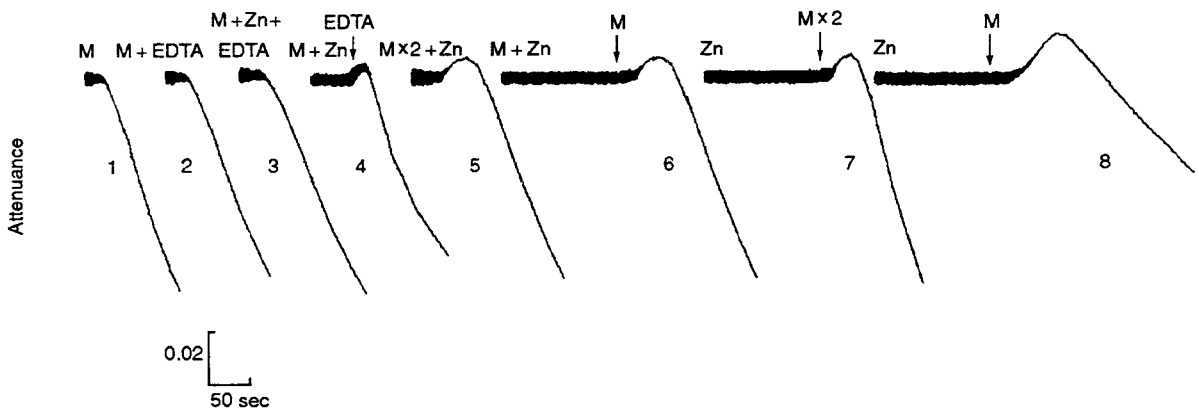


Fig. 5. Typical spectrophotometer traces obtained on placing cells in medium containing melittin (1 μg/ml) and additionally EDTA (75 μM) or Zn^{2+} (75 μM). Arrows indicate addition of reagents. Labels without arrows denote that the reagents were added to the incubation medium prior to the cells. M, melittin.

of hemolysis. The first result may be explained on the basis that Zn^{2+} and EDTA creating a Zn^{2+}–EDTA complex have lost the ability to inhibit or stimulate hemolysis because the affinity of components in the complex exceeds that of the corresponding ability to interact with membrane sites. Introducing EDTA into the medium after interaction of the cells with both peptide and Zn^{2+} causes a hemolysis rate of which is equal to the control (curve 4). This indicates that in chelating blocking ions of Zn^{2+} from membrane sites, EDTA permits the peptide to produce the lytic effect. For the interpretation of the data it is significant to note that an equal rate of hemolysis in these variants of the experiments shows that the susceptibility of the cells did not change during incubation in the presence of both peptide and Zn^{2+}; this usually takes place during incubation in the presence of only Zn^{2+} or other inhibitors (Fig. 3, Table 1). The same is evident from curves 5 and 6, derived for doubled concentration of melittin. In this case melittin produces hemolysis with the same rate without dependence on whether the cells were placed into the medium containing the final concentration of melittin and Zn^{2+} or these final concentration were produced after incubation of the cells with Zn^{2+} and half the amount of melittin for 200 sec. This indicates that the presence of melittin in the early stage of interaction is necessary to increase the blocking ability of Zn^{2+}. When the cells are incubated in the media in the presence of Zn^{2+}, melittin taken at the same concentration will lyse them with higher rate (compare curves 5 and 7; the beginning of curve 6 and curve 8).

In order to establish to what extent inhibitors are able to modify melittin itself, the fluorescence technique has been employed to study the interaction of melittin with some inhibitors. Figure 6 shows emission spectra o

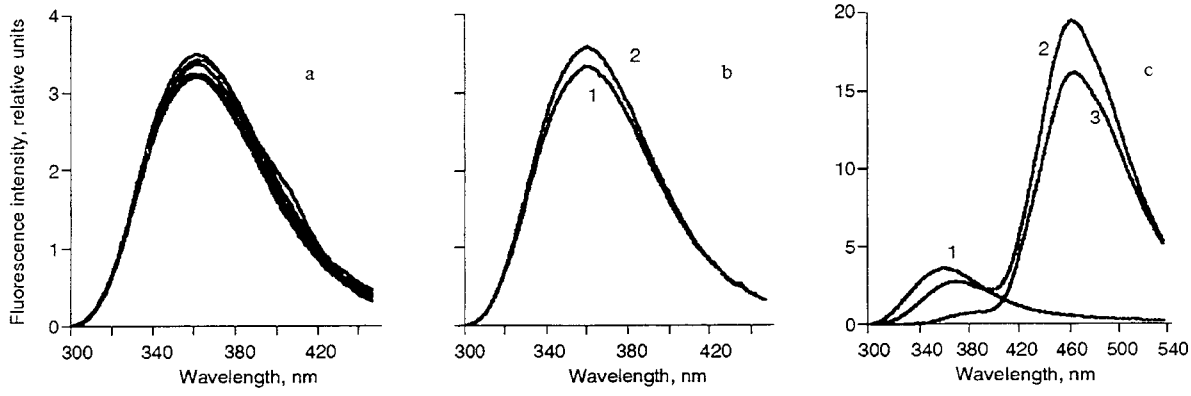


Fig. 6. Fluorescence emission spectra of melittin (1.5 μg/ml) in physiological saline of NaCl in the absence and presence of Ca^{2+} (a), DIDS (b), and chlorpromazine (c): a) curves for melittin and melittin in the presence of Ca^{2+} at concentrations 5, 10, 15, and 20 mM; b) melittin (1) and melittin plus 200 nM DIDS (2); c) melittin (1), melittin plus chlorpromazine (60 μg/ml) (2), and chlorpromazine (60 μg/ml) (3). The excitation wavelength was 290 nm.

tryptophan fluorescence (melittin contains a single tryptophan in position 19) in the presence of Ca^{2+}, DIDS, and chlorpromazine at concentrations which produce appreciable blocking effects. As seen, neither cations nor DIDS, as well as lanthanum and CTAB (data not shown) change the intensity or position of the fluorescence maximum, indicating an absence of alterations in the structure of the melittin molecule and, therefore, the absence of direct interactions between them. Determination of interaction of melittin and Zn^{2+} failed due to intensive emission of samples containing Zn^{2+} in the wavelength range which is characteristic for melittin emission. However, interaction between Zn^{2+} and melittin was not detected by circular dichroism in the absence as well as in the presence of lipids [11]. Chlorpromazine substantially decreased the fluorescence intensity of melittin and caused a red shift of the maximum. At the same time, the presence of melittin in the mixture with chlorpromazine increased by 22% the intensity of chlorpromazine emission. Identical spectra were recorded at excitation wavelength of 270 nm (data not shown). These alterations occurring simultaneously in the spectra of both molecules points to interaction between the molecules, which presumably results in formation of a complex structure where tryptophan is located in a more polar environment [18]. Thus, it seems that melittin does not interact directly with inhibitors such as cations, DIDS, and CTAB. This excludes the interaction of melittin with inhibitors as a possible explanation of the increased ability of the inhibitors to inhibit hemolysis in the early stages of peptide–membrane interaction.

On the other hand, no changes in shape or volume of cells was detected during incubation in the presence of inhibitory concentrations of the inhibitors. As a rule, such types of changes are detected for agents possessing a strong membranotropic effect [19]. This makes unlikely that the effects observed ensue from a direct interaction between cells and the inhibitors.

DISCUSSION

The present data can be explained using the following model. It is believed that hemolysis is the result of peptide binding to a lytic site (LS) that activates formation of a lytic pore. In addition, the peptide is able to bind to a non-lytic site (NLS)—this association does not lead to hemolysis. Data reported here and obtained elsewhere [7-9, 20] strongly suggest the existence of NLS on the erythrocyte membrane, which has presumably protein nature. Divalent cations and other inhibitors produce inhibition at the expense of competition with melittin for the lytic site. This competition significantly distinguishes this mechanism from the known ability of inhibitors to block preexisting lytic pores, where larger concentrations of inhibitors must be used [4, 11, 12]. A characteristic of the proposed model is the existence of membrane inhibitory components (MICs), which represent a special class of non-lytic sites. These components, which are normally under dynamic sorption equilibrium, nevertheless are weakly associated with the membrane. In the framework of this model, a significant increase in blocking ability of divalent cations and other inhibitors at early stages of interaction between peptide and membrane can be explained by formation of a triple complex including peptide, inhibitor, and MIC. The main property of this complex allowing the present data to be explained, particularly why v_0 in all cases is less than v_{200}, is its strong membrane association and inability for release from the membrane surface. This is based on the follow-

ing observations: 1) an absence of time-dependent changes in cell susceptibility to the lytic action of melittin (Fig. 5, curves 5 and 6) that is characteristic for formation of double complexes between melittin and inhibitors (Fig. 3); 2) cells have reduced ability to respond to additional increase in peptide concentration (Fig. 5, curves 6 and 7); 3) there are no changes in erythrocyte morphology (Fig. 5, compare the beginning of curve 6 and curve 8). Furthermore, the peptide is not directly associated with the membrane, although it is included in the triple complex, because there is no strong peptide association under the present conditions as has been established elsewhere [16]. We explain this fact on the basis that the peptide does not directly interact with the membrane but is connected with the membrane via a transitory element—MIC. So, on one hand, an inhibitor (for example Zn^{2+}) increases the blocking ability of MIC, interfering with the interaction of the peptide and the LS; on the other hand, the peptide itself should be present to increase association of both MIC and Zn^{2+} with the membrane. As seen, MICs are good for explaining all the experimental results.

Our view about a MIC corresponds well to a recently discovered homologous restriction factor (HRF) [21] or membrane protein CD59 [22] which plays a role of natural blocker of hemolysis induced by homologous complement. It cannot be excluded that MICs and these natural blockers have a close molecular basis, representing a cell defense system directed against external (but not all) virulent agents. In our case albumin was the only agent among all other inhibitors which removed the dependence of cell susceptibility to lytic action of peptide versus time of cell equilibration in physiological saline (Fig. 4a). This suggests that albumin can play the role of MIC, in contrast to other inhibitors such as divalent cations and chlorpromazine.

The action of other chemical agents is also consistent with the proposed model. Since nonelectrolytes, sulfhydryl reagents, reducing agents, and catecholamines have no substantial effect on hemolysis, it is suggested that modification of sulfhydryl groups of membrane proteins, at least during the experimental time, plays a minor role in the hemolytic mechanism. Similarly, the neutral effect of catecholamines indicates that membrane adrenoreceptors are not included in the hemolytic mechanism. It should be noted that treatment of cells by reagents causing activation of endogenous lipases (valinomycin, A23187) which finally leads to supplementation of the membrane by free fatty acids and lysolipids results in activation of hemolysis. Probably this is a basis for the observed property of these substances to change in a time-dependent manner the mode of their action from inhibitory to activating (Table 1). This is consistent with the fact that treatment of cells by phospholipase A_2 leads to activation of hemolysis [10]. Activation of hemolysis can be explained by selective interaction of activators which are mainly related to the class of detergents (Table 1), with another class of strongly membrane associated NLS, displacing melittin and thus increasing its lytic power [8]. The proposal of the existence of such a class of NLS is confirmed by the data that incorporation of band 3 into lipid vesicles reduces lytic activity of melittin [20]. This is in agreement with the fact that DIDS, which interacts covalently mainly with band 3 [23], is a strong inhibitor of melittin-induced hemolysis.

The observed effects are difficult to interpret as being a result of direct interaction between melittin and inhibitors which lead to formation of a double complex having reduced lytic activity because it seems unlikely that all substances interact with melittin in the same manner (Table 1). The fluorescence data also show that in most cases there is no direct interaction between melittin and inhibitors of hemolysis. The same is confirmed by circular dichroism in the case of Zn^{2+}, irrespective of the presence or absence of lipids [11].

Thus, it becomes clear that the erythrocyte membrane contains two classes of NLS—strongly and weakly associated with the membrane. The latter class of sites represents the membrane inhibitory components.

In conclusion, it should be noted that the existence of melittin-specific MICs which play a significant role in melittin-induced hemolysis could be a significant factor during the interaction of red blood cells or other cells with virulent factors such as viruses, toxins, or pore-forming agents. This point, however, should be confirmed in each case separately. For example, equinatoxin-II, an agent possessing a lytic activity exceeding that of melittin [3, 5, 6] and used here for comparison, did not show the features characteristic for melittin. For this toxin we did not find any differences between v_{200} and v_0 in the presence and absence of divalent cations, among them only Zn^{2+} having conserved its inhibitory properties (data not shown).

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