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MODULATORY EFFECT OF PEPTIDE STRUCTURE AND EQUILIBRATION CONDITIONS OF CELLS ON PEPTIDE-INDUCED HEMOLYSIS

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It is shown that Red Blood Cells (RBC) hemolysis induced by lytic peptides melittin (M), and melittin analog [Ala-14]melittin (P14A) depends on peptide structure, an order of interaction between cells and peptides, and the presence of inhibitors such as divalent cations, chlorpromazine, albumin and other agents. Maximal protective effect of inhibitors was observed when peptides and inhibitors acted simultaneously after introducing the cells into the media containing both peptides and inhibitors. Protection was reduced after equilibration of cells in the saline in the presence of inhibitors. This effect is proposed to originate from synergistic interaction between blocking agents and putative weakly bound membrane inhibitory components (MICs) playing protective role. The present data suggest that albumin may be referred to such membrane inhibitory component in contrast to other inhibitors such as divalent cations and chlorpromazine, because incubation of cells in the presence of these agents, in contrast to albumin, did not prevent enhancement of RBC susceptibility to lytic action of the same amount of peptide. P14A had a larger hemolytic activity compared to melittin demonstrated 3-fold larger value of binding to RBC and ghost membranes and was recognized as a peptide having minimal sensitivity to action of blocking agents. Chlorpromazine and albumin specifically inhibited hemolysis induced by melittin but were significantly less effective in inhibition of P14A-induced hemolysis.

Key words: *melittin, [Ala-14]melittin, erythrocyte, hemolysis, divalent cations, chlorpromazine, albumin, plasma.*

Introduction

Melittin, (M) lytic peptide from bee venom, consisting of 26 amino acid residues is widely used to study mechanism of translocation as well as incorporation of peptides and proteins into model biological membranes (Dempsey, 1990; Habermann, 1972). Melittin has been shown to produce potential-dependent ion channels acting in submicromolar concentration (Pawlak, 1991) and causes permeability increase and leakage of lipid vesicles (Kaszuba, Hunt, 1990; Alder et al., 1991; Ohki et al., 1994; Portlock et al., 1990). At micromolar concentration melittin causes cell crenation, release of membrane fragments (Katsu et al., 1988, 1989) and lysis of erythrocytes (Katsu et al., 1988, 1989; De Grado et al., 1982; Tosteson et al., 1985), acting via unknown mechanism. The relative contribution and interrelations between melittin-lipid and melittin-protein interactions in hemolytic mechanism also remain obscure. Particularly it is not clear whether melittin interacts with specific membrane sites or its lytic effect is solely due to nonspecific disruption of the integrity of membrane structure. It is known that variety of factors such as presence of divalent cations (Alder et al., 1991; Bashford et al., 1988) or other inhibitors (Portlock et al., 1990) in the media, size and preparative procedure of lipid vesicles (Kaszuba, Hunt, 1990; Portlock et al., 1990; Van Veen, Cherry, 1992), their phospholipid content (Portlock et al., 1990; Monette, Van Calsteren, Lafleur, 1993; Subbarao, MacDonald, 1994) surface charge (Monette, Lafleur, 1995) and presence of incorporated proteins (Van Veen, Cherry, 1992), temperature (De Grado et al., 1982) as well as peptide structure (Dempsey, Sternberg, 1991; Cornut et al., 1994) can modulate lytic power of peptide. Furthermore, kinetic of hemolysis has been shown to depend on the order in addition of peptides and cells in the reaction medium (Rudenko, Nipot, Pavlyuk, 1995).

In this paper, the role of the mode of peptide-membrane interactions in the mechanism of hemolysis induced by melittin and melittin analog [Ala-14] melittin (P14A) in which the Pro residue at position 14 has been replaced by Ala (Dempsey, Sternberg, 1991) has been examined. It was found that an order of interaction between peptide, inhibitor and membrane can modulate time course of hemolysis, peptide structure being a predominant factor underlying mode of peptide interaction with membrane surface in producing hemolytic pores.

Materials and methods

In the present experiments only fresh blood was used. Some blood drops from donor finger were mixed with 10 ml of isotonic Tris buffered saline (TBS) (150 mM NaCl, 10 mM Tris-HCl, pH 7.4) and washed

twice by centrifugation (2000×g, 3 min). 30μl of erythrocyte pellet was suspended into 0.5 ml of TBS and used during some hours as stock-suspension. Melittin free of phospholipase A_2 and [Ala-14]melittin (P14A) were a generous gift of Dr. Dempsey C. (Bristol University, UK). Chlorpromazine-HCl and human serum albumin (HSA) were from Sigma. Concentration of plasma proteins was determined according to the method of Bradford using HSA as a standard (Scopes, 1982). Other reagents were the highest grade available.

Preparation of hypotonic and hypertonic ghosts. Ghost membranes were prepared from blood obtained from blood bank. To prepare hypotonic ghosts, 0.05 ml of washed packed cells were incubated into 0.5 ml of hypotonic solution of NaCl (50 mM NaCl, 10 mM Tris-HCl, pH 7.4) for 1 min at 0°C followed by restoration of isotonicity adding 10 ml of cold TBS and collected by centrifugation (4000× g, 10 min). Two type of hypertonic ghosts were obtained as a result of posthypertonic hemolysis (Rudenko, Patelaros, 1995). 0.05 ml of packed cells were incubated in 0.5 ml of prewarmed at 37°C hypertonic solutions of 1.5 M NaCl or 1.4 M sucrose for 15 min. After that, 10 ml of dilute NaCl solution with appropriate concentration was added to restore isotonic conditions followed by incubation for next 10 min at room temperature. Remnant cells in these later solutions were pelleted by centrifugation (2000×g, 3 min), and ghosts remaining in the supernatant were concentrated by subsequent centrifugation (4000×g, 10 min). Ghost pellets were diluted by TBS to obtain final concentration appr. 10^8 ghosts per ml and used in binding experiments within 2 h.

Hemolysis assay. The dynamics of erythrocyte hemolysis and alteration of their shape during interaction with melittin and other peptides were measured spectrophotometrically (Rudenko, Patelaros, 1995; Rudenko, Crowe, Tablin, 1998). Erythrocyte suspensions were constantly stirred and their absorbance at 720 nm was recorded continuously. 6-7 μl of stock erythrocyte suspension was placed into the spectrophotometer cuvette (2 ml), so that the initial value of absorbance was 0.12-0.13. This value corresponds to a concentration of cells into the cuvette ~$0.8 \cdot 10^6$ cells/ml as detected by Coulter-Counter. Aliquots of peptides from concentrated stock solutions were added directly into a cuvette with or without the erythrocyte suspension. Time of mixing was approximately 2 sec. Because absorbance is proportional to cells concentration, the measured rate of absorbance changes is proportional to the rate of hemolysis (Rudenko, Patelaros, 1995; Rudenko, Crowe, Tablin, 1998). The rate of hemolysis was calculated from kinetic curves as tangent of α (tgα), where α is the angle between linear part of the absorbance curve and time axis. Absorbance of lysed suspension of cells at given wavelength equals zero. All experiments were carried out at room temperature (20-22°C).

Determination of peptide binding to cell and ghost membranes. Associated monomer peptide to lipid ratio (mol per mol) r, can be related to the free (aqueous) concentration of monomeric peptide, c, by equation $r=(\Gamma/\gamma)\cdot c$ with a partition coefficient Γ and pertinent thermodynamic activity coefficient γ taking into account the possible interaction between associated peptide molecules (Schwarz, Zong, Popescu, 1992). The above expression has been used to define partition coefficient Γ for melittin and P14A. The free concentration of peptides in the media remaining after peptide interaction with defined number of RBC or ghosts membranes (unbound peptides) was calculated on the basis of hemolysis of the second equal portion of cells in the same media using appropriate calibration as dependence of hemolysis rate versus free peptide concentration in the media obtained in the separate experiment using the same cell preparation and the same sequence of addition of cells and peptides into the cuvette (Rudenko, Nipot, 1996). Usually, binding was determined allowing the cells or ghosts to interact with peptides for 150 s. This time is sufficient for complete hemolysis of 10^6 cells per ml to occur at present experimental conditions.

Results

Influence of divalent cations on the onset of peptide-induced hemolysis. Fig. 1 shows the typical spectrophotometer traces obtained after addition of RBC into solution containing peptides without or with divalent cations. Initial increase in absorbance with corresponding decrease in absorbance noise reflects isovolumetric shape transformation toward symmetric spherical form (Rudenko, Crowe, Tablin, 1998), whereas subsequent decrease in absorbance reflects RBC swelling and hemolysis (Rudenko, Patelaros, 1995). As seen from Fig. 1, divalent cations increase a delay (lag-time) between addition of cells and maximal value of absorbance which reflects onset of hemolysis. This value was used to assess quantitatively blocking ability of agents.

Fig. 2 shows the effect of Zn^{2+} on delay time of RBC hemolysis induced by melittin and P14A, respectively. Divalent cations retarded onset of peptide-induced hemolysis as concentration is increased. However, the blocking ability of cations was approx. twice less when the same amounts of peptides were introduced into the cuvette 50 s after the cells. This effect was reduced as far as action of P14A is concerned. The same features were observed for protective action of Ca^{2+} and La^{3+} ions with the exception that Ca^{2+} produced the same effect acting at 10-50-fold larger and La^{3+} at lower concentration as compared with Zn^{2+} (data not shown). Nevertheless, the amount of divalent cations that increased delay time two-fold were relatively low to reduce significantly the rate of peptide-induced hemolysis when peptide-induced pores have been already formed. These amount of cations only slightly reduced hemolysis rate being introduced

after peptides (data not shown), therefore this strong inhibition reveals an additional inhibitory property of ions at the initial stage of membrane-peptide interaction, primary at the stage of peptide incorporation into membrane and/or at the stage of pore formation distinct from known ability to close preexisting peptide-induced pore (Alder, Arnold, Bashford et al., 1991; Bashford, Alder, Graham et al., 1988; Rudenko, Nipot, Pavlyuk, 1995).

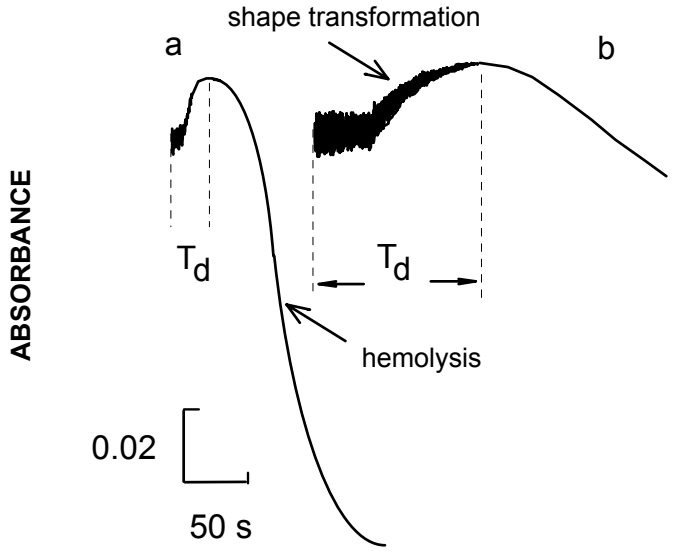

Fig. 1. Typical time-course of peptide-induced absorbance changes after injection of the cells into cuvette in the absence (a) and presence (b) of divalent cations
T_d - delay time of the onset of hemolysis

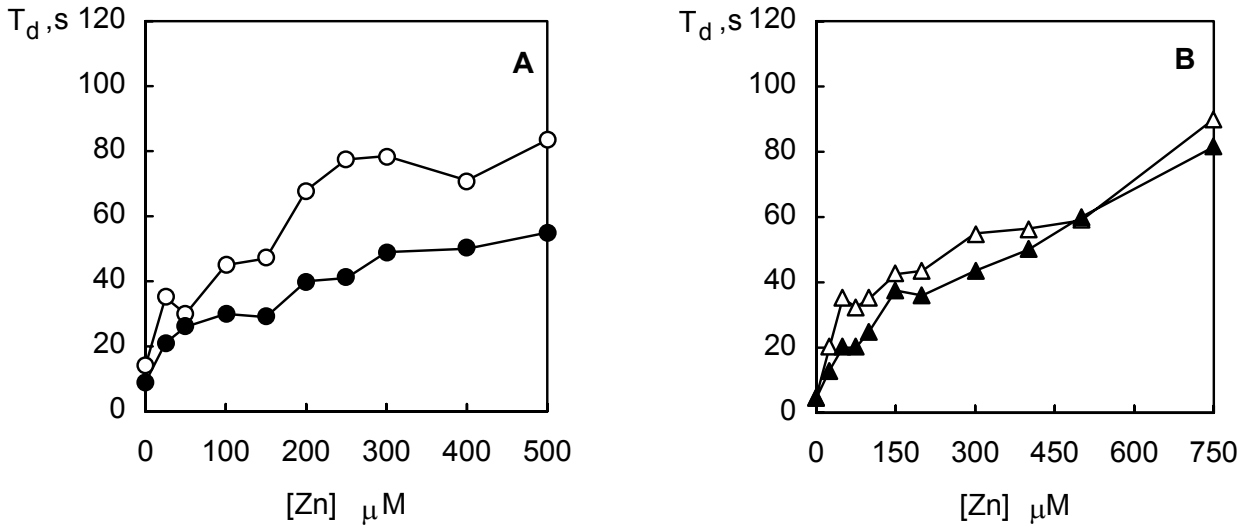

Fig. 2. Effect of Zn^{2+} on delay-time of beginning of hemolysis induced by melittin (2.5 μg/ml) (A) and P14A (0.3 μg/ml) (B)
Open symbols: RBC were introduced into the media containing peptides and indicated amount of Zn^{2+}
Closed symbols: RBC were introduced into the media containing Zn^{2+}
Peptides were added 50 s after the cells. T_d -was measured as indicated in Fig. 1

Influence of divalent cations, chlorpromazine, albumin and plasma on time-dependent changes in RBC susceptibility to lytic action of peptides. Existence of time-dependent changes in RBC susceptibility to lytic action of the same amount of melittin established earlier (Rudenko, Nipot, Pavlyuk, 1995; Rudenko, Nipot, 1996). Although exact molecular mechanism of this phenomenon was not described,

it has been suggested that these changes are attributed to the existence of membrane inhibitory components (MICs). The possibility exist that divalent cations themselves might be included in MICs. To verify this, time-dependent changes in RBC susceptibility to lytic action of peptides occurring in the presence of divalent cations Zn^{2+} or Ca^{2+} and other inhibitors were assessed.

Fig. 3 illustrates that both cations were unable to prevent a raising in cell susceptibility to hemolysis induced by peptides. Moreover, susceptibility increased even more in the presence than in the absence of Ca^{2+}. Zn^{2+} ions slightly enhanced cell susceptibility relative to P14A-induced hemolysis but reduced it relative to hemolysis induced by melittin. These data show that divalent cations can produce quite different inhibitory effect against hemolysis induced by the same amount of peptide depending on time of cell incubation in physiological saline in the presence of cations and type of peptide. Fig. 4 shows typical examples of M-induced hemolysis in the absence or presence of Ca^{2+} and chlorpromazine. Both Ca^{2+} and chlorpromazine as well as many other inhibitors (for example DIDS, (Rudenko, Nipot, 1996)) decrease rate of hemolysis υ_0 compared with control value υ_c obtained in the absence of inhibitor. However, if cells were incubated for 200 s in the presence as well as in the absence of inhibitor rate of hemolysis υ_{200} induced by the same amount of melittin increased relative to υ_0. Therefore, a ratio $\upsilon_{200}/\upsilon_0$ can be used to assess quantitatively a raising in cell susceptibility to lytic action of peptides.

The effects of other inhibitors on increase in RBC susceptibility are shown in Fig. 5. Similar to divalent cations, chlorpromazine exhibited inhibitory effect on peptide-induced hemolysis (Fig. 4), but was incapable to prevent time-dependent enhancement in RBC susceptibility to lysis induced by melittin. Action of P14A was significantly different. In this case chlorpromazine as well as albumin did not affect cell susceptibility up to maximum concentration tested. As seen from Fig. 6 these particular effects are due to poor ability of chlorpromazine and albumin to inhibit hemolysis induced by P14A in contrast to hemolysis induced by melittin. In the case of melittin albumin showed complex influence initially giving rise to an increase followed by a decrease in time-dependent susceptibility as a concentration of albumin is increased. In the presence of large amounts of albumin up to 15 μg/ml rate of peptide-induced hemolysis became time-independent because $\upsilon_0 = \upsilon_{200}$.

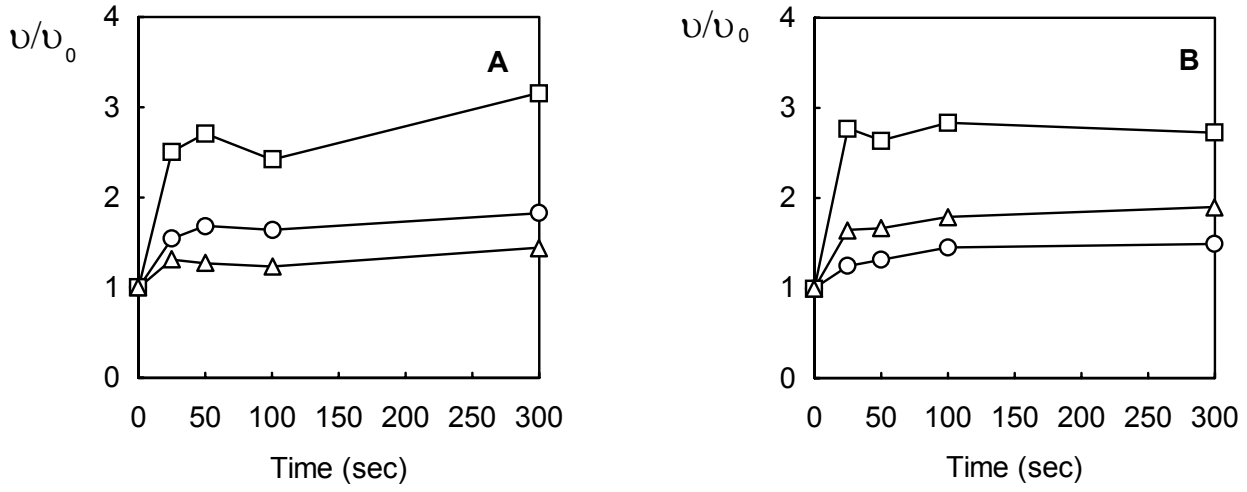

Fig. 3. Dependence of relative rate of hemolysis induced by melittin (1 μg/ml) (A) and P14A (0.1 μg/ml) (B) on duration of RBC equilibration in the cuvette in the absence (○) or presence of 7.5 mM Ca^{2+} (□) or 75 μM Zn^{2+} (Δ) prior to addition of peptides

υ_0 - the rate of hemolysis when RBC were added to the medium containing peptides

Binding of peptides to RBC and ghost membranes. Data in Table 1 show that partition coefficient for P14A is about 3-fold larger compared to melittin. This provides a good explanation for enhanced hemolytic power of this peptide. The fact that membrane of various type of ghosts prepared by using different procedures which included incubation at high and low ionic strength binds peptides at the same extent as erythrocyte suggest that ghost membrane contain the same amount of binding sites as native cell.

Table 1.

Partitioning coefficients (Г/γ), M^{-1} of melittin and P14A for RBC and ghost membranes (mean±SD)

object	Melittin	P14A
native cells	$(4.4\pm1.6)\cdot10^6$ (n=9)	$(10.1\pm3.7)\cdot 10^6$ (n=12)
hypotonic ghosts	$(4.5\pm2.4)\cdot10^6$ (n=6)	$(12.6\pm3.4)\cdot 10^6$ (n=7)
hypertonic ghosts (NaCl)	$(4.5\pm2.0)\cdot10^6$ (n=6)	$(9.0\pm2.3)\cdot 10^6$ (n=7)
hypertonic ghosts (sucrose)	$(4.2\pm2.2)\cdot 10^6$ (n=6)	$(8.0\pm2.2)\cdot 10^6$ (n=7)

Discussion

Data obtained so far show that lysis of cells or model lipid vesicles induced by lytic peptides like melittin is a highly variable process depending strongly on peptide structure, environment and type of cells or vesicles. Changes in each of these parameters may alter actual sequence of peptide interaction with lipid bilayer such as peptide incorporation, self-association and final pore formation. Molecular structure of peptide is important, because melittin, for example, can adopt different conformations in solution, micelles and lipid bilayer (Dempsey, 1990; Okada, Wakamatsu, Miyazawa et al., 1994) and a minor modification in the amino acid sequence of melittin results in dramatic changes in its biological activity (Perez-Paya, Houghten, Blondelle, 1994). This is true for melittin analog P14A (Dempsey, Sternberg, 1991) and related straight α-helix peptides (Cornut, Buttner, Dasseux et al., 1994; John, Jähnig, 1992), possessing higher hemolytic activity, but reduced ability to form channels in planar bilayer.

It is known that lytic power of melittin depends strongly on phospholipid composition of bilayer (Portlock, Clague, Cherry, 1990; Subbarao, MacDonald, 1994), presence of cholesterol (Monette, Lafleur, 1995) and especially of charged species (Ohki, Marcus, Sukumaran et al., 1994; Portlock, Clague, Cherry, 1990; Monette, Lafleur, 1995). Environmental condition like value of pH, ionic strength, the presence of alcohol or divalent cations (Kaszuba, Hunt, 1990; Katsu, Ninomiya, Kuroko, 1988; Bashford, Alder, Graham, et al., 1988; Monette, Lafleur, 1995) also modulate, often in a diverse manner, an ability of peptide to lyse cells and lipid vesicles. In the case of RBC along with uncertantities in the true conformation which melittin adopts in RBC membrane, it is though to interact with both lipid and protein membrane components (Portlock, Clague, Cherry, 1990; Van Veen, Cherry, 1992; Cuppoletti, Abbott, 1990). The presence of proteins in the membrane is an additional source of modulation in lytic action of peptide because proteins can either affect lipid component of the membrane or directly interact with melittin. It is known that melittin tightly interacts with some proteins with association constant two to tree order of magnitude as much as lipids (Hui, Stewart, Cherry, 1990; Iio, 1993). On the other hand, membrane proteins modulate the mode of melittin interaction with annular and bulk lipids.

In this paper, we analyze a type of modulation of M-induced hemolysis of human erythrocytes, which take place presumably at an early stage of peptide-membrane interactions. The present data reveal a general rule in the lytic action of the peptides and action of inhibitors of peptide-induced hemolysis. Rate of hemolysis is less and inhibitory efficacy of inhibitors is higher when peptides interact with RBC membrane at the stage of cell dilution in our hemolysis assay. During subsequent incubation in physiological saline, the cells become more susceptible to the same amount of peptides irrespective of the presence or absence of inhibitors in the media and less susceptible to the same amount of inhibitors. This observation, which is valid also for many other type of inhibitors different in chemical structure (Rudenko, Nipot, 1996) permits to rule out the possibility that this effect is due to direct interaction between peptides and inhibitors.

Since these changes are accompanied by an increase in susceptibility and corresponding decrease in inhibitor potency, it is reasonable to suggest that native RBC membrane contains membrane inhibitory components (MICs) which could change their properties during cell incubation in physiological saline. One possibility is that time-dependent increase in RBC susceptibility is due to desorption of MICs from membrane surface, whereas increased ability of divalent cations and other inhibitors to protect cells at an early stage of peptide-membrane interaction is due to synergistic interaction of inhibitor and MICs that prevents their desorption in the presence of peptides thus increasing protective efficacy. Indeed, data from Figs. 3, 4 and 5 show that such inhibitors as divalent cations and chlorpromazine do not prevent time-dependent changes in RBC susceptibility. In all cases the rate of hemolysis was larger after incubation of the cells for 200 s in the physiological saline both in the presence or absence of inhibitors. This result may be rationalized as inability of these inhibitors per se to prevent desorption of MICs and suggest further that cations and chlorpromazine are hardly involved in the structure of MICs. In contrast, albumin and plasma (Fig. 5) (in the case of melittin

and P14A but not bee venom) fully prevented time-dependent susceptibility changes as concentration is increased up to 15-20 μg/ml, suggesting albumin and possibly other plasma proteins to be either an intrinsic elements of MIC or elements capable to interact strongly with putative MICs anchoring them to membrane surface. In any case, a weak association makes unlikely these MICs which in fact, represent a non-lytic site, to be a membrane lipid. Alternatively, time-dependent changes in susceptibility may be due not to desorption of inhibitory components but to time-dependent changes in conformation of labile membrane elements responsible for inhibition that results in partial loss of their inhibitory properties. Unfortunately, the data available can not make a choice between these two possibilities at present. In any case the present results show that actual mechanism of peptide-induced hemolysis is complicated by interaction of peptides with non-lytic class of sites which can modulate hemolysis especially at an early stage of peptide-membrane interactions.

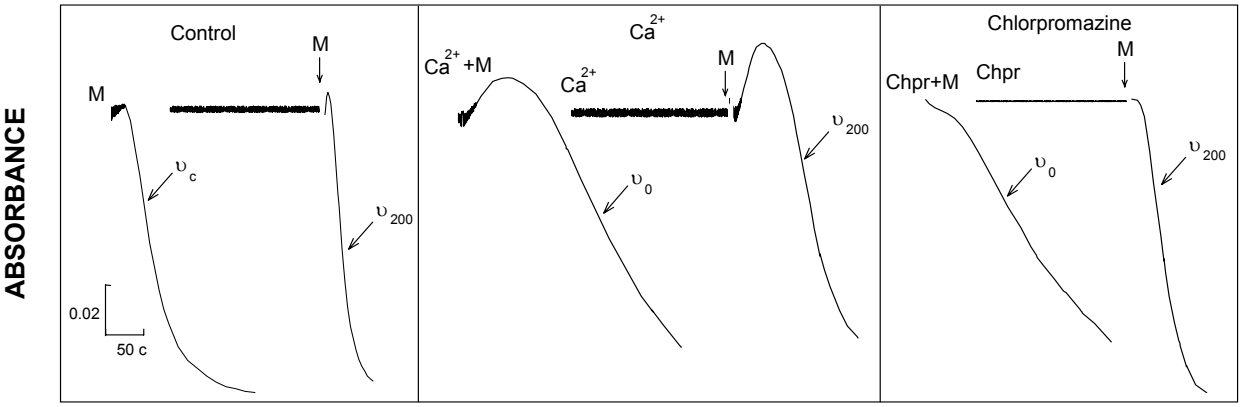

Fig. 4. Changes in absorbance of RBC suspension during hemolysis induced by melittin (4 μg/ml), in the media without inhibitors (control) and in the media containing Ca^{2+} (7.5 mM) or chlorpromazine (25 μg/ml)

Arrows indicate addition of melittin

Captures without arrows indicate that peptides and inhibitors are initially present in the media

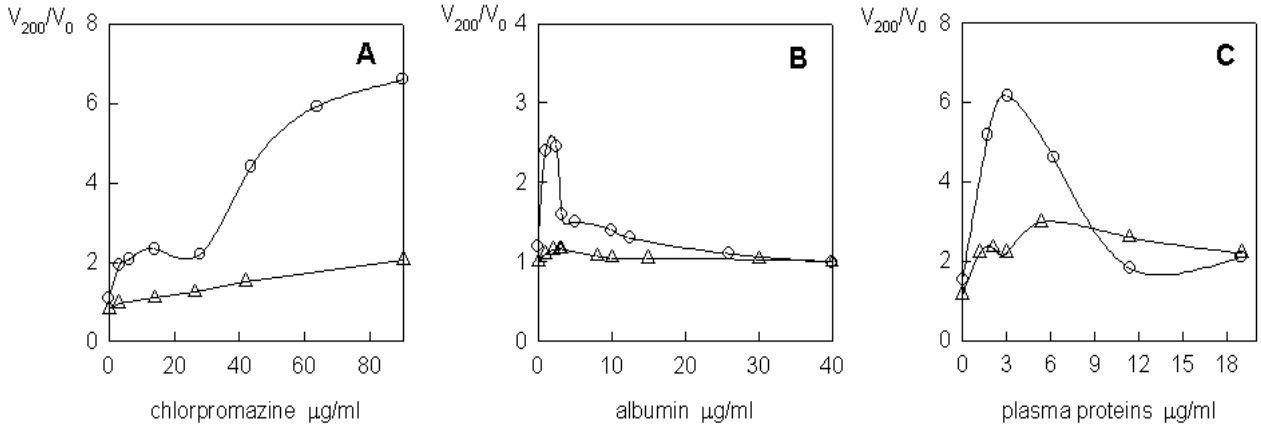

Fig. 5. Effect of chlorpromazine (A), albumin (B) and plasma (C) on relative rate of hemolysis induced by melittin (4 μg/ml) (○) and P14A (0.7 μg/ml) (Δ)

υ_0 - rate of hemolysis when RBC were introduced into the media containing peptides and inhibitors

υ_{200} - the rate of hemolysis when peptides were added to the cuvette 200 s after placing the cells into the media containing inhibitors

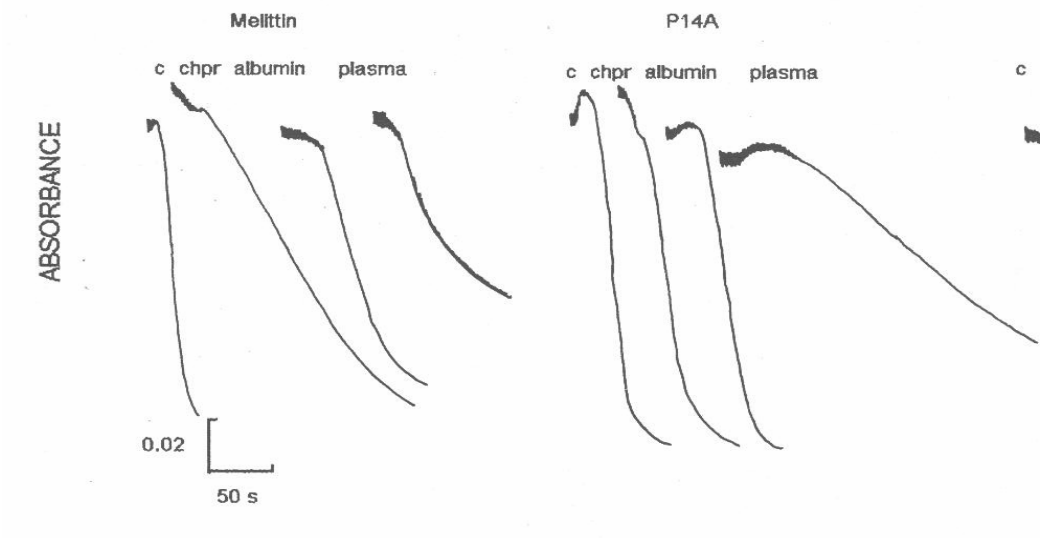

Fig. 6. Changes in absorbance of RBC suspension during hemolysis induced by melittin (4 μg/ml) and P14A (0.35 μg/ml) in the media containing chlorpromazine (25 μg/ml), albumin (10 μg/ml), and plasma (12 μg/ml)

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МОДУЛИРУЮЩИЙ ЭФФЕКТ СТРУКТУРЫ ПЕПТИДА И УСЛОВИЙ ЭКВИЛИБРАЦИИ КЛЕТОК НА ПЕПТИД-ИНДУЦИРУЕМЫЙ ГЕМОЛИЗ

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Показано, что гемолиз эритроцитов, индуцируемый литическим пептидом мелиттином и его аналогом Ala-мелиттином, зависит от структуры пептида, порядка взаимодействия между клетками и пептидами и присутствия ингибиторов, таких как двухвалентные катионы, хлорпромазин, альбумин и другие. Максимальный протектирующий эффект ингибиторов наблюдался, когда пептиды и ингибиторы действовали одновременно после помещения клеток в среду, содержащую смесь пептидов и ингибиторов. Протекция уменьшалась после эквилибрации клеток в растворе в присутствии ингибиторов. Предполагается, что этот эффект обусловлен синергичным взаимодействием между блокаторами и слабо связанными с мембраной мембранными ингибирующими компонентами (МИК), играющими протектирующую роль. Полученные данные показывают, что альбумин может быть отнесен к таким ингибирующим компонентам, в отличие от других ингибиторов, таких как катионы и хлорпромазин, поскольку инкубация клеток в присутствии этих агентов, в отличие от альбумина, не предотвращала увеличения чувствительности клеток к литическому действию тех же количеств пептида. P14A обладал большей литической активностью по сравнению с мелиттином, в 3 раза большей величиной константы связывания с мембранами нативных клеток и их теней, и минимальной чувствительностью к действию ингибиторов. Хлорпромазин и альбумин специфически ингибировали гемолиз, индуцированный мелиттином, но были слабо эффективны в отношении гемолиза, индуцированного P14A.

Ключевые слова: *мелиттин, [Ala-14] мелиттин, эритроциты, гемолиз, двухвалентные катионы, хлорпромазин, альбумин, плазма.*

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