УДК 577.1:612.111

PECULIARITIES OF LYTIC ACTION OF MELITTIN AND ITS ANALOG [ALA-14]MELITTIN

S.V. Rudenko[1], Wajdi Khalaf Jamil Madanat[2]

[1]*Institute for Problems of Cryobiology and Cryomedicine, National Academy of Sciences of the Ukraine, 23 Pereyaslavskaya Str., Kharkov, 61015 Ukraine, e-mail: rsv@rsv.concom.kharkov.ua*

[2]*V.N. Karazin Kharkov National University, 4 Svobody Sq., Kharkov, 61077 Ukraine*

Received December 12, 2005

It is shown that lytic peptides melittin (M), melittin analog [Ala-14]melittin (P14A) and whole bee venom act in a different manner relative to human red blood cells (RBC). The normalized rate of hemolysis depended linearly on the relative amount of P14A in the mixture with both melittin and bee venom. Dose-response curve for P14A showed saturation only when lytic effect of membrane bound melittin was inhibited by chlorpromazine. This indicates that melittin and P14A produce hemolysis acting independently on each other. In contrast to melittin, but similar to bee venom, P14A also reduced volume of lysed cells. Non-linear effects of cells shrinkage induced by mixtures of P14A with melittin and bee venom suggest that synergistic action of peptide analogs underlies the mechanism of this phenomena. In addition, data suggest that melittin and P14A produce lytic effect through binding to different classes of sites on RBC membrane. We conclude that the structure of lytic peptide is a predominant factor which in concert with mode of peptide-membrane interactions modulates its lytic power.

KEY WORDS: melittin, [Ala-14]melittin, erythrocyte, hemolysis, divalent cations, chlorpromazine.

Melittin, a naturally occurring antimicrobial peptide, exhibits strong lytic activity against both eukaryotic and prokaryotic cells. Mechanism of melittin action is complex and depends on type of cells [1-3], phospholipid and protein content of the membrane [4-8], environment conditions such as presence of divalent cations [2, 9] other inhibitors [6,10,11], temperature [12] and the order in addition of peptides and cells in the reaction medium [10,11]. Many works show that hemolytic effect of melittin depends on peptide structure [13- 22], however it still remains unclear whether modifications in structure change melittin-lipid or melittin protein interactions. In this paper, we compared lytic ability of melittin, melittin analog [Ala-14]melittin (P14A) in which the Pro residue at position 14 has been replaced by Ala [13,14], and whole bee venom where melittin acts in synergism with phospholipase A_2 [23]. It was found that peptide structure is a predominant factor underlying mode of peptide interaction with membrane surface in producing hemolytic pores. The important conclusion made out if these results is that two peptide analogs, melittin and P14A cause hemolysis interacting with different classes of binding sites on human erythrocyte membrane.

MATERIALS AND METHODS

In the present experiments only fresh blood was used. A few 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 (2000g, 3 min). 30μl of erythrocyte pellet was suspended into 0.5 ml of TBS and used during several 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). Whole bee venom (Sigma) was dissolved in distilled water and centrifuged subsequently to remove the non-dissolved compounds. Concentration of the bee venom solution was determined by its dry weight after evaporation. Chlorpromazine-HCl was from "Sigma".

The dynamics of erythrocyte hemolysis and alteration of their shape during interaction with melittin and other peptides were measured spectrophotometrically [10] 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 in 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 [10]. 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. All experiments were carried out at room temperature (20-22°C).

The specific RPS technology, used for measuring volume distribution of erythrocytes and ghosts, has been reported elsewhere [10, 24, 25]. Coulter-type sizing, in RPS produces resistive pulses as particles pass through a flow-limiting orifice with a constant flow maintained across it. The magnitudes of these resistive pulses are displayed in the form of spectra (256-channel histograms), the modal peaks and other characteristics of which are analyzed by computer. In the present study 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 analyzed 2^{15} cells and current across the orifice did not exceed 0.2 mA, so that no electrical breakdown [25] and no significant deformation of cell membrane [24] did occur. The method provided a measure for the true volume of both RBC and their ghosts (particles) in suspension.

RESULTS AND DISCUSSION

As was stated earlier the true kinetic of M-induced hemolysis depends on the order of addition of cells, peptide and inhibitor in reaction medium [10,11]. Here we tested this effect for P14A in comparison with melittin. Fig. 1 shows that adding P14A 150 s after incubating the cells in the presence of Zn^{2+} induces shape transformation followed by hemolysis (curve 4). This behavior is in contrast to the simultaneous action of P14A and of Zn^{2+} where only limited shape transformation and no hemolysis were detected within similar time-scale (curve 5). It seems that P14A becomes less effective when Zn^{2+} is present at early (not at later) stages of interactions. Synergistic interaction of P14A and Zn^{2+} with RBC membrane at this early stage makes cells less prone to lysis induced by addition of a second equal portion of P14A as compared with the case when a double amount of P14A was directly added 150 s after incubation of cells in the presence of Zn^{2+} (compare curves 5 and 6). In the other words, same final amount of P14A can evoke quite different cell response depending on the order of addition of Zn^{2+} and peptide in the RBC suspension. To compare lytic properties of melittin and P14A concentrations of peptides in the present experiments were chosen to produce similar rates of hemolysis. The features of action of others peptides melittin and bee venom in dependence on the order of addition of Zn^{2+} and EDTA were identical to those of P14A (not shown).

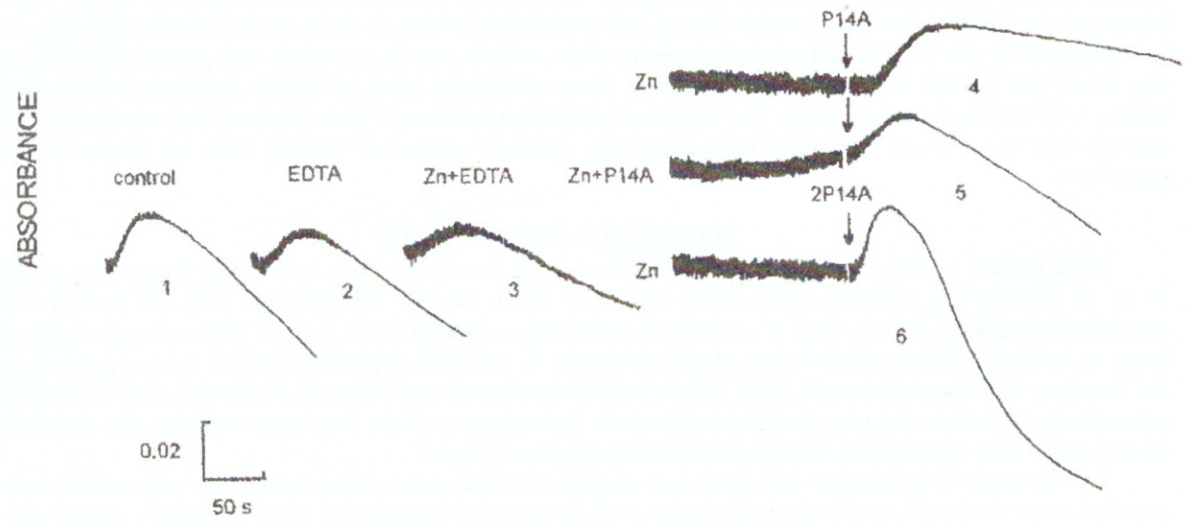

Figure 1. Time-courses of absorbance changes induced in RBC suspension by P14A. Arrows indicate addition of P14A (0.075 μg/ml) or double amount of peptide indicated as 2P14A. Captures without arrows indicate that peptides, EDTA (75 μM) or Zn^{2+} (75 μM) are initially present in the media.

It is generally believed that M-induced hemolysis has a colloid-osmotic nature [12,26]. Fig. 2 compares time-courses of changes in volume of cells during hemolysis. RPS technique provides simultaneous measurements of volume of both intact cells and ghosts (particles) formed as a result of hemolysis [25]. There were significant differences in volume changes induced by melittin, P14A and bee venom. Melittin caused swelling of the cells although final ghost volume did not reach maximal volume (~1.5 relative to normal isotonic volume). This indicates that hemolysis induced by melittin does not solely correspond to colloid-osmotic mechanism, as for instance in the case of lysolecithin-induced hemolysis [27] and may be attributed to formation of small amount of large pores permeable for hemoglobin [12]. In contrast to melittin, P14A induced only transient swelling at the beginning of hemolysis followed by shrinking toward to value close to normal physiological volume. Bee venom produced a large time-dependent shrinking of RBC particles during hemolysis. Significant differences in volume alterations induced by both peptides imply that exact mechanism of peptide-membrane interactions leading to pore formation and hemolysis is not the same in all cases. This implies that peptides interact with RBC membrane in a different way depending on peptide structure and presence of additional enzymes.

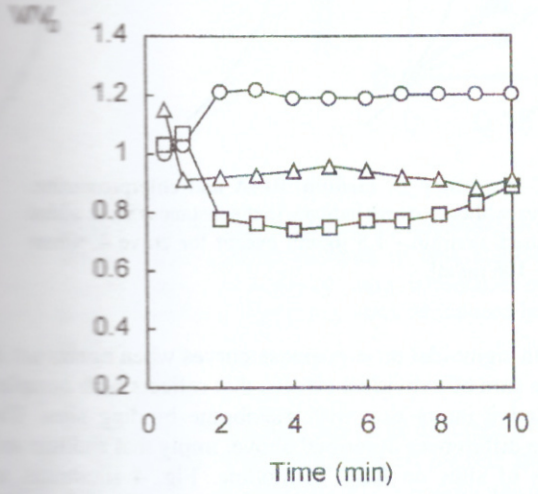

Figure 2. Time-courses of changes in mean relative volume of particles (cells plus ghosts) during hemolysis induced by melittin (1 μg/ml) (○), P14A (0.45 μg/ml) (△) and bee venom (1.8 μg/ml) (□). RBC at final concentration 10^6 cells/ml were added to the media containing peptides and changes in particle volume was measured using RPS technique. The concentration of peptides were chosen to obtain similar rate of hemolysis which was completed within 4 minutes. Volume distribution histograms were measured 5 min after interaction of RBC with peptides. V_0-volume of control cells in isotonic saline.

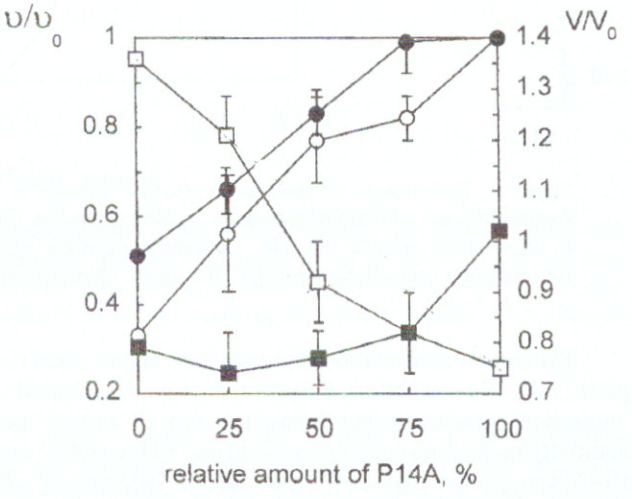

Figure 3. Dependence of normalized rate of hemolysis (○,●) and relative mean volume (□,■) of erythrocytes on relative amount of P14A in the mixtures of P14A with melittin (open symbols) and bee venom (closed symbols). RBC were added to the media, containing peptides with concentrations producing similar rates of hemolysis. Rate of hemolysis υ was normalized to the rate of hemolysis υ_0 induced by 100% P14A, and cell volume V measured 3 min after interaction of RBC with peptides was normalized to the initial volume of the cells V_0 in isotonic saline. (mean±S.E.)

In order to assess interrelations between melittin and P14A in producing hemolysis and volume changes of RBC, these parameters were measured after combined action of peptides in various proportions. Equivalence of mode of action of both peptides should result in effects to be linearly dependent on the amount of each peptide in the mixture. Indeed, Fig. 3 shows that the normalized rate of hemolysis, in fact, linearly depends on relative amount of P14A in the mixture of both melittin and bee venom. This indicates that melittin and P14A produce hemolysis acting independently on each other. Interrelations between changes in volume of RBC and content of peptide mixture are more complex. In this case, increasing the relative amount of P14A increases a capability of bee venom to shrink RBC membrane. For example, at 75% of P14A in the mixture with bee venom there was a decrease in the volume of RBC up to 0.8 relative to isotonic volume, whereas neither P14A nor bee venom themselves produced any shrinkage at those concentrations. The same tendency was observed for mixture of P14A and melittin. In this case melittin potentiated shrinking action of P14A with maximal effect at 50%. Non linear interrelations between RBC shrinkage

and relative amount of P14A in the mixture of melittin and bee venom strongly suggest cooperative interactions between peptides, especially in the presence of PLA_2 (bee venom).

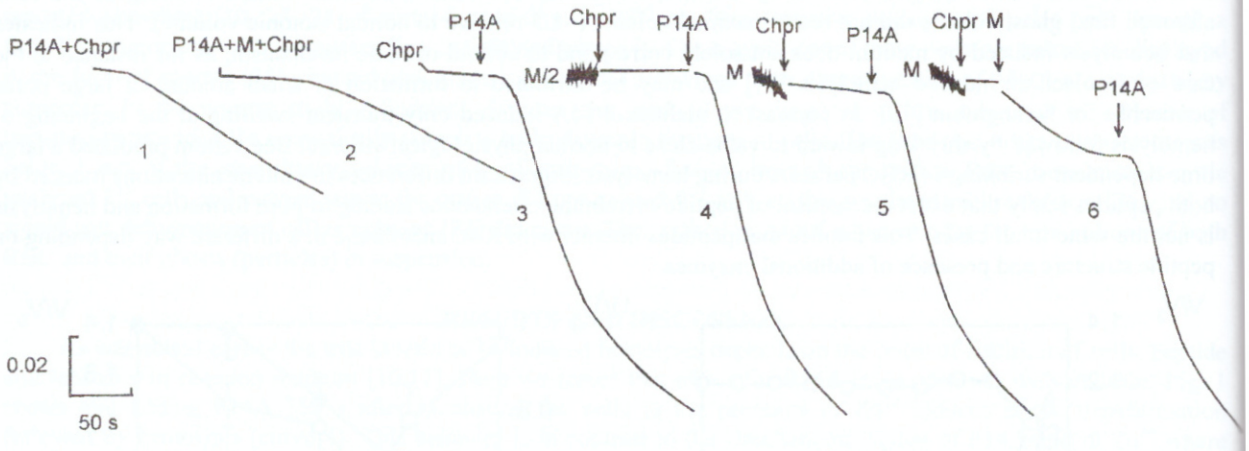

Figure 4. Time-course of absorbance changes induced in RBC suspension by melittin, P14A and chlorpromazine. Arrows indicate addition of substances in the suspension. Captures without arrows indicate that substances were added to the solution prior to the cells. Concentration of the agents used: melittin - 1.5 μg/ml, except for curve 4, where concentration was twice less; P14A - 0.3 μg/ml; chlorpromazine - 106 μg/ml.

Synergistic interrelations between lytic agents often result in sigmoidal dose-response curves when agents act in pairs [1,2]. The total effect, therefore, does not correspond to the sum of individual effects, that reflects both complex interaction between agents themselves as well as their cooperative interaction with membrane binding sites. The additivity in the lytic action of melittin and P14A as well as some differences described above, imply that melittin and P14A can produce lytic effect acting via independent classes of sites on RBC membrane. Fig. 4 illustrates an additional experiment confirming this suggestion. Here the property of chlorpromazine to inhibit significantly M-induced hemolysis and only at moderate extent P14A- induced hemolysis has been exploited [10]. Supposing an existence of only one class of binding sites common for melittin and P14A occupation of a part of these sites by melittin should result in reduced hemolysis subsequently induced by P14A as occurs with combined action of active and non-active forms of some toxins [28]. However, as shown in Fig. 4 the rates of P14A- induced hemolysis, in fact, do not depend on the presence or absence of melittin in reaction mixture. In the presence of chlorpromazine which completely inhibits M-induced pore (as seen from curves 4-6) the rate of hemolysis closely corresponds to the rate of hemolysis induced by P14A alone. This means that melittin does not interfere with P14A in producing lysis suggesting that P14A interacts with lytic site which chemical nature is different from that of melittin-binding site. The fact that at least two strong inhibitors of M-induced hemolysis - chlorpromazine and albumin, are non-effective with respect to P14A-induced hemolysis also lies in line with this conclusion [10]. This shows that peptide homologs can have a specific inhibitors which inhibit lytic action of only one peptide being ineffective relative to the other one. Alternative explanation of present results is that P14A being a more active peptide possessing higher partitioning coefficient [10] could expel chlorpromazine from the melittin-binding lytic site. This possibility, however, seems unlikely because at this situation one should expect increasing in a total rate of hemolysis as a result of abolishing the protection exerted by chlorpromazine on M-induced pore. Fig. 4 shows that this is not a case. Dose-response curve for P14A shown in Fig. 5 obtained under experimental conditions depicted in curves 4-5 (Fig. 4) indicate saturation only when lytic effect of membrane bound melittin was inhibited by chlorpromazine. In the absence of melittin, dose-response dependences were linear irrespective of the presence or absence of chlorpromazine. This clearly demonstrates that P14A can not interact with sites occupied by melittin and blocked by chlorpromazine. Consequently, failure to find any positive contribution of melittin to the lytic effect of P14A in the presence of chlorpromazine -a more specific inhibitor of M-induced hemolysis, and saturation in dose-response curve for P14A, obtained in the presence of melittin but not in its absence (Fig. 5), strongly suggest that these peptide analogs interact with different classes of sites on RBC membrane.

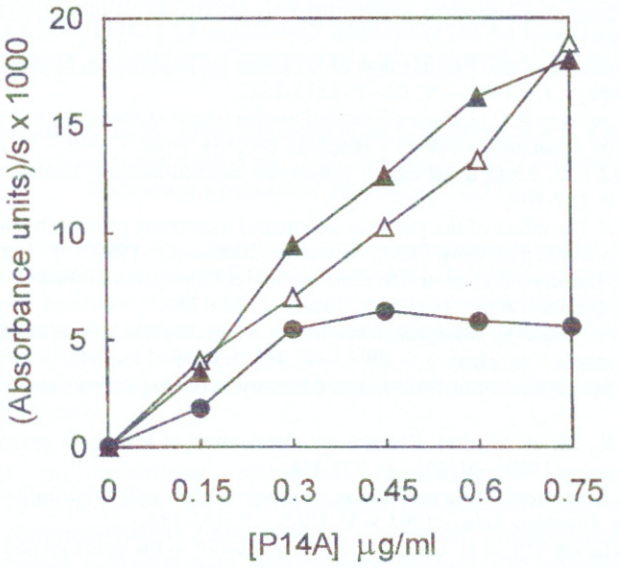

Figure 5. Dependence of the rate of hemolysis on the concentration of P14A in the absence (△) or presence of 0.3 mM chlorpromazine (▲) or presence of both chlorpromazine and melittin (3 μg/ml) (●) in the solution. RBC at final concentration ~10^6 cells/ml were introduced into cuvette with (●) or without (△,▲) melittin, and 25 s later chlorpromazine was added to block M-induced hemolysis. P14A at concentrations indicated was added 100 s after the cells.

In addition, P14A also produces different effect on peptide-induced volume changes of cell during hemolysis (Figs. 2 and 3). This effect depends on peptide structure and presence of additional substances (PLA_2). In this case both peptides act in synergism - P14A potentiates shrinking effect of bee venom, whereas melittin, in turn, potentiates corresponding action of P14A (Fig. 3).

CONCLUSIONS

Synergistic interaction of peptides in producing cell shrinkage and additivity in producing hemolysis show that both processes are not causally related in the sense that one of them is the result of the other one. Eventually cell swelling and lysis precedes shrinkage. Shrinkage is not due to osmotic effect during lysis because isolated ghosts demonstrate similar phenomenon in isotonic saline [29]. It is also not due to the fragmentation or micellizaton of lipid bilayer as far as the shrinkage is reversible and ghosts are able to restore spontaneously their volume close to initial value in the presence of peptides [29]. This may reflect that transmembrane redistribution of peptides over damaged cell membrane and interaction with intracellular constituents, presumably membrane cytoskeleton, is required to produce an effect. Melittin is known to induce rapid phospholipid flip-flop over M-induced pore and transmembrane movement of peptide molecules through the large pores formed during hemolysis using "edge" mechanism [30] can not be excluded. One can only speculate at this point that volume changes of membrane cytoskeleton are responsible for the effect, insofar it is known that cytoskeleton is able to shrink significantly under some circumstances [31,32]. However this particular phenomenon and other findings presented in this paper demonstrate unique ability of peptides from bee venom to interact with membrane of human red blood cells.

REFERENCES

1. Bashford C.L., Rodriguez L., Pasternak C.A. Protection of cells against membrane damage by hemolytic agents: divalent cations and protons act at the extracellular side of the plasma membrane // Biochim. Biophys. Acta. – 1989. – V. 983. – P. 56-64.
2. Bashford C.L., Alder G.M., Graham J.M. et al. Ion modulation of membrane permeability: effect of cations on intact cells and on cells and phospholipid bilayers treated with pore-forming agents // J. Membrane Biol. – 1988. – V. 103. – P. 79-94.
3. Dempsey C.E. The action of melittin on membranes // Biochim. Biophys. Acta. – 1990. – V. 1031. – P. 143-161.

4. Raghuraman H, Chattopadhyay A. Cholesterol inhibits the lytic activity of melittin in erythrocytes // Chem Phys Lipids. – 2005. – V. 134, N2. – P. 183-189.
5. Mahaney J.E., Kleinschmindt J., Marsh D., Thomas D.D. Effect of melittin on lipid-protein interaction in sarcoplasmic reticulum membranes // Biophys. J. – 1992. – V. 63.- P. 1513-1522.
6. Portlock S.H., Clague M.J., Cherry R.J. Leakage of internal markers from erythrocytes and lipid vesicles induced by melittin, gramicidin S and alamethicin: a comparative study // Biochim. Biophys. Acta. – 1990. – V. 1030. – P. 1-10.
7. Subbarao N.K., MacDonald R.C. Lipid unsaturation influences melittin-induced leakage of vesicles // Biochim. Biophys. Acta. – 1994. – V. 1189. – P. 101-107.
8. Van Veen M., Cherry R.J. // The effect of the presence of integral membrane protein (human band 3) on the membrane lytic properties of melittin in reconstituted systems, FEMS Microbiol. Immunol. – 1992. – V. 105. – P. 147-150.
9. Alder G.M., Arnold W.M., Bashford C.L. et al. Divalent cation-sensitive pores formed by natural and synthetic melittin and by triton X-100 // Biochim. Biophys. Acta. -1991. – V. 1061. – P. 111-120.
10. Rudenko S.V., Nipot E.E. Protection by chlorpromazine, albumin and divalent cations of hemolysis induced by melittin, [ala-14]melittin and whole bee venom // Biochem. J. – 1994. – V. 317, N3. – P. 747-754.
11. Rudenko S.V., Nipot E.E. Modulation of melittin-induced hemolysis of Red Blood Cells // Biochemistry (Moscow). – 1996. – V. 61. – P. 1524-1531.
12. De Grado W.F., Musso G.F., Lieber M. et al. Kinetics and mechanism of hemolysis induced by melittin and by a synthetic melittin analogue // Biophys. J. – 1982. – V. 37. – P. 329-338.
13. Dempsey C., Sternberg B. Reversible disc-micellization of dimyristoylphosphatidylcholine bilayers induced by melittin and [Ala-14]melittin // Biochim. Biophys. Acta. – 1991. – V. 1061. – P. 175-184.
14. Dempsey C.E., Bazzo R., Harvey T.S. et al. Contribution of proline-14 to the structure and action of melittin // FEBS Lett. - 1991 – V. 281. – P. 240-244.
15. Cornut I., Buttner K., Dasseux J.L., Dufourcq J. The amphipathic alpha-helix concept. Application to the de novo design of ideally amphipathic Leu, Lys peptides with hemolytic activity higher than that of melittin // FEBS. Lett. – 1994. – V. 349. – P. 29-33.
16. Asthana N., Yadav S.P., Ghosh J.K. Dissection of antibacterial and toxic activity of melittin: a leucine zipper motif plays a crucial role in determining its hemolytic activity but not antibacterial activity // J. Biol. Chem. – 2004. – V. 279. – P. 55042-55050.
17. Yan H., Li S., Sun X. et al. Individual substitution analogs of Mel(12-26), melittin's C-terminal 15-residue peptide: their antimicrobial and hemolytic actions // FEBS Lett. – 2003. - V. 554. – P. 100-104.
18. Hewish D.R., Barnham K.J., Werkmeister J.A. et al. Structure and activity of D-Pro14 melittin // J. Protein Chem. – 2002. - V. 21. – P. 243-253.
19. Unger T., Oren Z., Shai Y. The effect of cyclization of magainin 2 and melittin analogues on structure, function, and mode membrane interactions: implication to their mode of action // Biochemistry. – 2001. – V. 40. – P. 6388-6397.
20. Subbalakshmi C., Nagaraj R., Sitaram N. Biological activities of C-terminal 15-residue synthetic fragment of melittin: design of an analog with improved antibacterial activity // FEBS Lett. – 1999. – V. 448. – P. 62-66.
21. Shin S.Y., Kang J.H., Hahm K.S. Structure-antibacterial, antitumor and hemolytic activity relationships of cecropin A magainin 2 and cecropin A-melittin hybrid peptides // J. Pept. Res. – 1999. – V. 53. – P. 82-90.
22. Oren Z., Shai Y. Selective lysis of bacteria but not mammalian cells by diastereomers of melittin: structure-function study // Biochemistry. – 1997. – V. 36. – P. 1826-1835.
23. Habermann E. Bee and Wasp venom // Science. – 1972. – V. 177. – P. 314-322.
24. Akeson S.P., Mel H.C. Deformability and other rheological interactions of red blood cells in electronic cell sizing // Biorheology. – 1986. – V. 23. – P. 1-15.
25. Richiery G.V., Mel H.C. Membrane and cytoplasmic resistivity properties of normal and sickle red blood cells // Cell Biophysics. – 1986. – V. 8. – P. 243-259.
26. Tosteson M.T., Holmes S.J., Rasin M., Tosteson D.C. Melittin lysis of red cells // J. Membr. Biol. – 1985. – V.87. – P. 35-44
27. Tanaka Y., Mashino K., Inoue K., Nojima S. Mechanism of human erythrocyte hemolysis induced by short-chain phosphatidylcholines and lysophosphatidycholine // J. Biochem. – 1983. – V. 94. – P. 833-840.
28. Tang G.Q., Iida T., Yamamoto K., Honda T. A mutant toxin of Vibrio parahaemolyticus thermostable direct hemolysin which has lost hemolytic activity but retains ability to bind to erythrocytes // Infect. Immun. – 1994. – V. 62. – P. 3299-3304.
29. Rudenko S.V., Bojok G.A., Nipot E.E. Bee venom-induced shrinkage of erythrocyte ghosts // Biochemistry (Moscow). – 1997. – V. 62. – P. 104-109.
30. Fattal E., Nir S., Parente R.A., Szoka F.C. Pore-forming peptides induce rapid phospholipid flip-flop in membranes // Biochemistry. – 1994. – V. 33. – P. 6721-6731.
31. Svoboda K., Schmidt C.F., Branton D., Block S.M. Conformation and elasticity of the isolated red blood cell membrane skeleton // Biophys. J. – 1992. – V. 63. – P. 784-793.
32. Vertessy B.G., Steck T.L. Elasticity of the human red cell membrane skeleton. Effect of temperature and denaturants // Biophys. J. – 1989. – V. 55. – P. 255-262.