Protection by chlorpromazine, albumin and bivalent cations against haemolysis induced by melittin, [Ala-14]melittin and whole bee venom

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The ability of the peptides melittin, [Ala-14]melittin (P14A) and whole bee venom to lyse red blood cells (RBC) and to cause shape transformation, binding, partitioning and changes in volume of the cells during haemolysis, as well as the action of the bivalent cations Zn^{2+} and Ca^{2+} , chlorpromazine, albumin and plasma on the peptide-induced haemolysis of RBC in high ionic-strength solution, have been investigated. The protective effect of all inhibitors depends on whether they have been added to the media before or after the cells. When added before the cells they reduced significantly the rate of peptide-induced haemolysis and shape transformation. The effect was maximal when agents acted simultaneously after introduction of the cells into the media containing both inhibitors and peptides. Incubation of the cells in isotonic solution before the addition of peptides enhanced 2–3-fold the RBC susceptibility (i.e. rate of haemolysis) to lytic action of the same amount of peptides, and increased the order of the haemolytic reaction, although the power law coefficient did not exceed a value of 2 for all peptides, suggesting that haemolysis is attributable to the monomeric or dimeric forms of the peptides. Partition coefficients were of the order of $\sim 10^6 M^{-1}$, and P14A possessed a value 3-fold larger compared with melittin and bee venom, which correlated with its enhanced

haemolytic activity. The protective action of inhibitors against peptide-induced haemolysis has been explained on the basis of their ability to compete with peptide binding at an early stage of peptide–membrane interaction, and not as a result of inhibition of a pre-existing peptide-induced pore. Whereas melittin increased the volume of RBC during haemolysis, P14A, melittin in the presence of phospholipase A_2 or bee venom, reduced the volume in a concentration-dependent manner. The present data reveal the significant role of the initial stage of peptide–membrane interaction and peptide structure in the mechanism of haemolysis. These data are not consistent with a lipid-based mechanism of peptide-induced haemolysis, indicating that the mode of peptide–protein interaction is an important and decisive step in the haemolytic mechanism. It should be noted that data (in the form of three additional Tables) on the ability of inhibitors to protect cells from haemolysis when inhibitor and peptide act simultaneously are available. They are reported in Supplementary Publication SUP 50178, which has been deposited at the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in Biochem. J. (1996) **313**, 9.

INTRODUCTION

Melittin, the main component of bee venom, consisting of 26 amino acid residues, is widely used to study the mechanism of translocation, as well as incorporation, of peptides and proteins into model biological membranes [1,2]. Melittin has been shown to produce potential-dependent ion-channels [3,4] and an increase in the permeability and leakage of lipid vesicles [5–8]. At micromolar concentrations melittin causes cell crenation, release of membrane fragments [9,10] and lysis of red blood cells (RBC) [4,5,9–12]. The notion that haemolysis is due to melittin–lipid interaction is primarily based on melittin’s ability to disrupt the membranes of lipid vesicles. However, experiments with melittin derivatives showed that their haemolytic potency correlated well with their corresponding ability to aggregate intramembrane particles and to reduce the rotational mobility of band 3 protein in erythrocyte membranes [13,14] and bacteriorhodopsin in lipid vesicles [15]. It was surmised that besides melittin–phospholipid interactions, observed in lipid vesicles [5–8,16], interactions between melittin and membrane proteins [17–21] might be involved in the melittin-induced formation of lesions [5,13,14]. The latter might explain the discrepancies observed in the lytic action of melittin on RBC and lipid vesicles of various lipid compositions [5–7]. However, the relative contribution and inter-

relations between melittin–lipid and melittin–protein interactions in the haemolytic mechanism remain obscure. To clarify further the mechanism of melittin-induced haemolysis we compared the lytic action and binding ability of several peptides, melittin, the melittin analogue [Ala-14]melittin (P14A), in which the Pro residue at position 14 has been replaced by Ala [4,16], and whole bee venom where melittin acts in synergism with phospholipase A_2 (PLA $_2$) [2,22], as well as the ability of bivalent cations and other inhibitors, such as chlorpromazine, albumin and plasma proteins, to interfere with peptide–membrane interactions. Inhibitory analysis is useful for understanding the molecular mechanism of haemolysis because inhibitors can specifically or non-specifically interact with constituents of melittin-induced pores as well as affect the process of pore formation itself. We found that in addition to the well-known property of bivalent cations to close pre-existing melittin-induced pores [23–25], at an early stage of interaction they compete with and arrest melittin binding to the membrane. The rate of peptide binding in the presence of inhibitors is much slower than in their absence. Moreover, the present data suggest that in this case binding occurs to another type of membrane-binding site, different from those to which peptides bind in the absence of inhibitors. Our data provide additional evidence for the suggestion of Portlock et al. [5] for the existence of two types of peptide-binding sites

Abbreviations used: P14A, [Ala-14]melittin; RBC, red blood cells; TBS, Tris-buffered saline; HSA, human serum albumin; RPS, resistive pulse spectroscopy; MIC, membrane inhibitory component; PLA $_2$, phospholipase A_2 .

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(lytic and non-lytic) on RBC membranes. The role of non-lytic sites is especially important at the early stages of peptide-membrane interaction. Comparison of the results obtained with the known data regarding melittin-induced lysis of lipid vesicles shows that the two types of lysis have little in common, thus supporting the idea of a leading role of melittin-protein interactions in the haemolytic mechanism.

EXPERIMENTAL

Materials

In the present experiments only fresh blood was used. Some blood drops from a donor's 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). RBC pellet (30 μ l) was suspended into 0.5 ml of TBS and used over a period of hours as stock suspension. Melittin free of PLA₂, P14A and PLA₂ were gifts from Dr. C. Dempsey (Bristol University, U.K.). Whole bee venom was dissolved in distilled water and centrifuged subsequently to remove non-dissolved compounds. The concentration of bee venom in the solution was determined by its dry weight after evaporation. Chlorpromazine/HCl and human serum albumin (HSA) were from Sigma. The concentration of plasma proteins was determined according to the method of Bradford using HSA as a standard [26]. Other reagents were of the highest grade available.

Haemolysis assay

The dynamics of RBC haemolysis and alteration in their shape during interaction with peptides were measured spectrophotometrically [27–29]. RBC suspensions were constantly stirred in TBS and their apparent absorbance at 720 nm was recorded continuously. Stock RBC suspension (6–7 μ l) was placed into a spectrophotometer cuvette (2 ml), so that the absorbance was 0.12–0.13. This value corresponds to a concentration in the cuvette of $\sim 0.8 \times 10^6$ cells/ml, as detected by a Coulter counter. The photosignal was attributed solely to the low-angle scattering of light by RBC and not to absorption [27,28]. Aliquots of peptides from concentrated stock solutions

prepared in distilled water (0.5 mg/ml for melittin and bee venom and 0.2 mg/ml for P14A) or inhibitors (1 M for Ca²⁺, Zn²⁺ and EDTA, 1.4 M for sucrose, 3.3 mg/ml for chlorpromazine and 1 mg/ml for HSA and plasma) were added directly into a cuvette with or without the RBC suspension. The time of mixing was approximately 2 s. Because absorbance is proportional to cell concentration, the measured rate of absorbance change is proportional to the rate of haemolysis [27,28]. In all cases the rate of haemolysis was calculated from the kinetic curves as the tangent of α ($\text{tg}\alpha$), where α is the angle between linear part of the absorbance curve and the time axis (see Figure 1). The absorbance of a lysed suspension of cells at a given wavelength equals zero. All experiments were carried out at room temperature (20–22 °C).

Resistive pulse spectroscopy (RPS)

The specific of RPS technology, used for measuring volume distribution of RBC and ghosts, has been reported elsewhere [30,31]. Coulter-type sizing in RPS produces resistive pulses as particles pass through a current-limiting orifice with a constant current 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 analysed by computer. In the present study, a cylindrical, 50 μ m long and 50 μ m in diameter, orifice was used in a system of transducers provided for the almost complete hydrodynamic focusing of cells. To exclude significant deformation of cell membranes, the flow rate in the experiments was less than 1 m/s [31]. Each measurement cycle analysed 1×2^{15} cells and the current across the orifice did not exceed 0.2 mA. RPS is able to discriminate between 'leaky' and 'intact' ghosts. When ghosts became 'leaky', it resulted in a characteristic break in the dependence of the apparent volume on current through the orifice, indicating electrical breakdown of the membrane [30]. In all the present experiments we tested this hypothesis and found that dependences were linear, at least up to 0.4 mA, i.e. no electrical breakdown occurred, and the method provided a measure of the true volume of both RBC and their ghosts (particles) in suspension.

RESULTS

Influence of bivalent cations on kinetics of peptide-induced shape transformation

Figure 1 shows typical spectrophotometer traces obtained after addition of peptides and bivalent cations into an RBC suspension. The decrease in absorbance reflects RBC haemolysis [27,28], whereas the initial increase in absorbance, with the corresponding decrease in absorbance noise, reflects RBC shape transformation toward a symmetrical spherical form [29]. Hence, the initial rate of absorbance change can be used as a parameter reflecting the rate of peptide-induced RBC shape transformation. As follows from Figure 1, the addition of bivalent cations to an RBC suspension prior to the peptides results in a slowing in the shape transformation rate and the appearance of a delay (lag-time) between the addition of peptides and the onset of shape transformation. Figure 2 shows that Zn²⁺ significantly retards the onset of peptide-induced shape transformation as well as reducing the transformation rate. Comparison of shape transformation rate dependence on cation concentration for melittin, P14A and whole bee venom indicates that all peptides insert into RBC membranes in a similar manner, independent of the presence of PLA₂, a component of bee venom. At the same time the discrimination between peptides was evident from the lag-time

Figure 1 Typical changes in absorbance during peptide-induced haemolysis (A) and effect of bivalent cations on peptide-induced shape transformation (B)

The formulae give a definition of the rate of haemolysis (v) measured as the tangent of α ($\text{tg}\alpha$) and the rate of shape transformation (v_{sh}). A_0 , initial absorbance.

Figure 2 Effect of Zn^{2+} on relative rate of RBC shape transformation (A) and on delay-time between addition of peptides and on-set of shape transformation (B) induced by melittin (0.25 $\mu\text{g/ml}$) (○), P14A (0.12 $\mu\text{g/ml}$) (△) and bee venom (□) (0.25 $\mu\text{g/ml}$)

Values of v_{sh} and t_{lag} were calculated under the experimental conditions depicted in Figure 1. v_{sh}^0 is the rate of shape transformation after introduction of RBC into the media containing only peptides. Representative data from 3–4 experiments are shown. Lines were drawn by eye.

dependences on cation concentration, which indicated that bee venom is the most sensitive and P14A the least sensitive agent with respect to bivalent cation action. A similar result was obtained for Ca^{2+} ; however, Zn^{2+} was almost 50-fold more effective than Ca^{2+} (results not shown).

It has been stated previously [23–25] that bivalent cations exert their protective action by closing the pores formed by melittin in the cell membrane, which may be opened again after chelation or removal of cations by subsequent washing. The present kinetic data reveal that this mechanism does not operate here, because the cation concentration that reduced the rate of shape transformation 2-fold ($\sim 50 \mu\text{M}$ for Zn^{2+} and 5 mM for Ca^{2+}) was unable to inhibit haemolysis when added after haemolysis had commenced (results not shown). Therefore, in addition to the well-documented ability of bivalent cations to close haemolytic

Figure 3 Influence of Zn^{2+} ions on normalized rate of haemolysis induced by melittin (○, ●), P14A (△, ▲) and bee venom (□, ■)

Open symbols: RBC ($\sim 1 \times 10^6$ cells/ml) were introduced into media containing peptides and the indicated amount of Zn^{2+} ; closed symbols: RBC were introduced into media first and 25 s later aliquots of Zn^{2+} were added to obtain the required final concentrations. Peptides were added 50 s after the cells. Concentrations of peptides used: melittin, 1.0 $\mu\text{g/ml}$; P14A, 0.075 $\mu\text{g/ml}$; and bee venom, 1.25 $\mu\text{g/ml}$. All rates of haemolysis were normalized to the corresponding value obtained for control cells introduced into a cation-free medium containing only peptides. Representative data from 3–4 experiments are shown.

Figure 4 Effect of time of cell equilibration in the cuvette, preceding addition of melittin (1.25 $\mu\text{g/ml}$) (○) P14A (0.4 $\mu\text{g/ml}$) (△) and bee venom (1.25 $\mu\text{g/ml}$) (□) on the relative maximal rate of peptide-induced haemolysis

v_0 , haemolysis rate after introduction of RBC into the media containing peptides.

pores in the membranes, at the stage of peptide incorporation they protect the membrane acting via other mechanisms.

Effect of Zn^{2+} on the rate of peptide-induced haemolysis

Figure 3 shows that the influence of Zn^{2+} on the rate of haemolysis significantly depends on the order of addition of cations and peptides to the RBC suspension. Simultaneous action of cations and peptides results in a much slower rate of haemolysis compared with the case when they act one after the other. In the first case, Zn^{2+} ions reduce the rate of haemolysis 2-fold, acting at relatively low concentrations in the range 2–7 μM , suggesting that high-affinity binding to the membrane site is responsible for inhibition. In the second case, to reach the same rate of haemolysis a more than 10-fold increase in Zn^{2+} concentration is

Figure 5 Typical spectrophotometric traces obtaining during inhibition by Zn^{2+} (0.3 mM) and stimulation by an equimolar amount of EDTA of haemolysis induced by P14A (0.3 $\mu\text{g/ml}$)

Arrows indicate addition of a portion of RBC (final concentration $\sim 1 \times 10^6$ cells/ml), Zn^{2+} and EDTA into the cuvette as indicated. Labels without arrows denote that Zn^{2+} was added to the medium containing peptides prior to the cells.

required. An explanation of this unusual effect of bivalent cations came from the experiments presented in Figure 4. Here we see that the rate of haemolysis depends on the time between addition of the cells and the peptides into the cuvette. In other words, after dilution and equilibration in the cuvette, RBC became several times more sensitive to lytic action of the same amount of peptide. The magnitude of this effect is larger using bee venom and melittin as compared with P14A, and may be different in various blood samples. Repetitive washing of cells in TBS or washing in the presence of EDTA (1 mM) before the experiment reduces the time-dependent changes of RBC susceptibility to the lytic action of peptides (results not shown). Haemolysis induced by P14A is the least sensitive to dilution of cells among peptides tested. These findings are of importance in interpreting data regarding the inhibitory action of bivalent cations and other inhibitors (see below), and may also provide the basis for an explanation of some contradictions between the results obtained by various workers [5,6,32,33].

We hypothesize that the non-equal susceptibility of RBC to lytic action of the same amount of peptides, and the corresponding changes in the inhibitory ability of bivalent cations, are due to the existence of weakly bound surface-membrane components exerting a protective role against peptide-induced haemolysis. In this case the increase in cell susceptibility is easily explained by desorption of these membrane inhibitory components (MICs) during equilibration of a dilute cell suspension in physiological saline. The different inhibitory effects of bivalent cations (Figure 3) may be a result of interaction between the inhibitor and MICs. It seems that MICs possess the ability to increase significantly the inhibitory capacity of inhibitors when they are still linked with the RBC membrane, namely, at the very early stage of peptide-membrane interaction when desorption of MICs has not yet occurred. To verify this model, binding and partitioning experiments were performed in the presence

and absence of bivalent cations and the action of other inhibitors such as chlorpromazine, albumin and plasma was assessed.

Influence of bivalent cations on peptide-binding to RBC membranes

Because peptide-induced shape transformation toward echinocytic forms generally reflects insertion of peptides into the outer leaflet of the membrane, cation-induced delay in shape transformation and the beginning of haemolysis may be interpreted as the ability of cations to interfere with and arrest peptide incorporation. This may be due to initial occupation by cations of the same membrane sites (lytic sites) to which peptides normally bind. If so, one should expect changes in the mode of peptide binding in the presence or absence of inhibitors.

To resolve the problem of whether the inhibitory effect of bivalent cations is due to their influence on peptide binding or the inhibition of pre-formed pores without affecting peptide binding *per se*, kinetic experiments were performed. Figure 5 shows a typical example of experimental traces obtained during RBC haemolysis induced by P14A which has been blocked by Zn^{2+} and stimulated by an equimolar amount of EDTA under a variety of conditions. Panel (a) shows that addition of a new portion of cells (reference RBC) into the cuvette after complete haemolysis of the first, equal portion (test RBC) of cells, results in a much slower rate of subsequent haemolysis. This indicates that the majority of peptides are irreversibly bound to the test-cell portion. Haemolysis may be almost fully inhibited by a relatively high concentration of Zn^{2+} added after the onset of haemolysis (panel b), and subsequently liberated again by an equimolar amount of EDTA. The fact that in this latter case peptides have already been fully bound to RBC prior to the addition to Zn^{2+} is confirmed in panel (c) because after the

addition of reference RBC and stimulation of haemolysis by EDTA only the test cells undergo haemolysis, as in panel (b), leaving the reference RBC unimpaired. Therefore, after peptide binding, Zn^{2+} inhibits haemolysis from closing the pre-existing membrane pore. The results were different when bivalent cations interacted with RBC at an early stage of peptide-membrane interaction. In this case (panel d) EDTA also initiated Zn -inhibited haemolysis but at a rate significantly lower than in the previous case (compare panels b and d). Panel (e) shows that when RBC initially interact simultaneously with Zn^{2+} and peptides, both test and reference portions of cells lyse simultaneously, as confirmed by a control experiment when a double portion of cells was directly added to the cuvette containing the same amount of Zn^{2+} and P14A, followed by stimulation of haemolysis by EDTA (panel f). The rate of haemolysis of the double portion of cells does not depend significantly on the time of cell equilibration in physiological saline prior to stimulation by EDTA (panel f, curves 1 and 2). Hence, there was no binding of peptides to the test cells in the experiment shown in panel (e). This confirms that shape transformation correlates with peptide incorporation into the membrane. However, binding certainly occurred (panel g) with increasing time of cell equilibration in the presence of Zn^{2+} , in a manner similar to that of panel (c) where lysis of only the test cells is detected. The latter provides the possibility of reconciling our data regarding the absence of peptide binding in the presence of Zn^{2+} with that obtained earlier by Pasternak's group [23–25] that binding does not depend on the presence of cations. It is obvious that the presence of Zn^{2+} at an early stage of peptide-membrane interaction significantly retards the rate of peptide incorporation into the membrane. If the time required for peptide incorporation in the absence of Zn^{2+} is approx. ~ 15 s, (time of beginning of haemolysis, panels a–c, Figure 5), it became as much as 100 s in the presence of Zn^{2+} (panel e). These findings may be adequately explained by assuming competition between cations and peptides for common binding sites on the surface of the cell membrane. In favour of this suggestion are the facts that the rate of peptide binding could be increased by either decreasing the cation concentration or increasing the concentration of peptides in the media (results not shown). The present results cannot be explained by direct interaction between Zn^{2+} and peptides because: (1) Zn^{2+} does not interact directly with melittin in solution and in the presence of lipids [24]; and (2) practically identical kinetic curves were obtained using Ca^{2+} ions as protective agents instead of Zn^{2+} (results not shown).

Order of lytic reaction and partitioning of peptides between cells and solution

It is generally accepted that the rate of haemolysis should be proportional to the concentration of peptide monomer in the membrane, presumably in its lipid phase [12,34]. Hence, the above mentioned increase in RBC susceptibility to the same amount of peptide may reflect an enhanced ability of cells to bind peptides. The dependence of haemolysis rate on peptide concentration, as well as the rate of leakage of internal markers from lipid vesicles [6,12,34], may be described by the power function in the form $\nu = A \cdot c^n$, where c is peptide concentration in the medium, n is the power coefficient and A is a constant. The values of n shown in Table 1 indicate that equilibration of RBC in physiological saline increases the order of the reaction, although in both cases the power coefficient does not exceed a value of 2. Again, changes in the power coefficient for P14A were minimal, which is in good agreement with its relatively poor ability to change haemolytic activity with time (see Figure 4).

Table 1 Values of the power coefficient, n

Values of n for a function describing the dependence of haemolysis rate ($\nu = \text{tg}\alpha$) on peptide concentration in the form $\nu = A \cdot c^n$, where c is peptide concentration, n is the power coefficient and A is a constant obtained by least-squares fitting of the experimental results (mean $\pm$ S.D.). In Method 1, RBC were introduced into the media containing peptides and in Method 2 peptides were introduced into the RBC suspension 50 s after the cells.

Peptide	Method 1	No. of replicates	Method 2	No. of replicates
Melittin	1.35 ± 0.1	3	1.96 ± 0.5	4
P14A	1.6 ± 0.25	4	1.74 ± 0.17	3
Bee venom	1.12 ± 0.11	3	1.44 ± 0.29	4

The ratio of associated monomer peptide to lipid (mol per mol), r , can be related to the free (aqueous) concentration of monomeric peptide, c , by the 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 [34]. The above expression has been used to calculate the partition coefficient Γ in experiments in which aliquots of peptides were added to cell suspensions 50 s after the cells. Table 2 shows that the corresponding Γ/γ value for P14A is 3-fold larger compared with melittin or whole bee venom, thus providing a good explanation for the larger haemolytic activity of this agent [4]. On the other hand, binding of melittin in the presence of PLA₂ (bee venom) is approximately the same compared with melittin alone. The partition coefficient (Γ) of melittin between synthetic lipids and solution has an order of $\sim 10^4 \text{ M}^{-1}$ [34]. As seen, our estimate of the partition coefficient in the present experiments gives a value of the order of $\sim 10^6 \text{ M}^{-1}$, which is close to the value obtained by others in isotonic sucrose, i.e. $\sim 10^7 \text{ M}^{-1}$ [12]. This values clearly shows that membrane components others than lipids, presumably proteins, bind peptides, since the mellitin-protein binding constant, for instance to calmodulin, has an order $\sim 10^8 \text{ M}^{-1}$ [35].

It was also found that binding was less when the cells were incubated in the cuvette for 50 s before the addition of peptides, compared with experiments in which the cells were directly added to media containing the same amount of peptides (results not shown). These findings therefore show not a positive, but an inverse interrelation between peptide binding and their ability to lyse RBC (the more binding, the lower the rate of haemolysis). This strongly suggests that mechanisms other than simple peptide binding are involved in RBC haemolysis.

Table 2 Partition coefficients of peptides 10^{-6}

The concentration of free peptides in the media after complete haemolysis of a defined number of cells and, therefore, a known total amount of membrane lipids, was calculated on the basis of haemolysis of a second equal portion of cells in the same media using the same calibration as for dependence of haemolysis on peptide concentration described for Table 1. Results are means $\pm$ S.D.

Peptide	No. of replicates	$10^{-6} \times$ Partition co-efficient, $\Gamma/\gamma \text{ (M}^{-1}\text{)}$
Melittin	7	2.4 ± 0.4
P14A	8	7.5 ± 1.1
Bee venom	8	3.6 ± 1.0

Figure 6 Dependence of the mean relative volume of RBC on the concentrations of melittin (○), melittin in the presence of 1 µg/ml PLA₂ (◇), P14A (△) and bee venom (□)

The cells at concentrations of $\sim 1 \times 10^6$ cells/ml were incubated in TBS containing peptides for 5 min, pH 7.4, at room temperature. Their relative volume (V/V_0) was measured using the RPS technique. Representative data from 3–4 experiments are shown.

Inhibition of peptide-induced haemolysis by chlorpromazine, albumin and plasma

The important role of the initial stage of peptide–membrane interaction in the haemolytic mechanism was confirmed further by using other inhibitors of peptide-induced haemolysis. The general feature found in these experiments, in common with that of bivalent cations (Figure 3), consists of increased ability of inhibitors to protect cells when inhibitor and peptide act simultaneously (These results (three additional Tables) are reported in Supplementary Publication SUP 50178, which has been deposited at the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K.). On the other hand, the present experiments revealed additional significant differences in inhibition depending on both the type of inhibitor and peptide used. For example, all inhibitors significantly inhibited bee venom-induced haemolysis, but changed their efficacy relative to melittin and P14A. Chlorpromazine was less effective in the case of melittin, and especially as far as P14A-induced haemolysis was concerned. In fact, chlorpromazine is a very poor inhibitor of this latter type of haemolysis. Similarly, albumin was significantly less effective as an inhibitor of P14A-induced haemolysis compared with haemolysis induced by melittin and bee venom. In contrast, whole plasma proteins strongly inhibited haemolysis induced by all peptides, suggesting that components in plasma, other than albumin, are involved in inhibition. It is important to note that in contrast to chlorpromazine and bivalent cations (Figure 3), increasing concentrations of albumin or plasma in the medium, up to 15–20 µg/ml, eliminated the dependence of haemolysis on the order of addition of peptides and cells into the medium (see SUP 50178, Tables 4 and 5). This means that in the presence of these amounts of albumin or plasma, the cell susceptibility haemolysis does not depend on the time of cell equilibration in saline. These results may be explained on the basis that albumin or plasma components, in contrast to chlorpromazine and bivalent cations, prevent desorption of MICs from the membrane surface. Alternatively, one may suggest that albumin itself represents one of the weakly associated surface membrane proteins exerting a protective role. The fact that the concentration of albumin required to produce

Figure 7 Typical spectrophotometric traces obtaining during haemolysis induced by melittin (M; 1.5 µg/ml) or by P14A (0.3 µg/ml) in TBS (curves 1–3) and isotonic sucrose solution (curves 4)

Arrows indicate addition of sucrose from concentrated stock solution to obtain a final concentration of 45 mM. Labels without arrows denote that sucrose and peptides were added to the medium prior to the cells.

such an effect is close to its concentration in whole plasma (bearing in mind dilution of RBC) is in line with this suggestion.

Influence of peptides on changes in cell and ghost volume

The changes in particle (i.e. cells plus ghosts) volume occurring during haemolysis depended on the type of peptide used. Figure 6 shows that while melittin increases particle volume, venom and a mixture of melittin and PLA₂, which mimic the action of bee venom, reduce volume in a concentration-dependent manner. P14A produces a more complex response of initial swelling followed by shrinking as concentration is increased. Similar results were obtained using isolated ghost membranes (results not shown). Bearing in mind the difference in molecular structure of melittin ('hinged' molecule) and P14A (straight α -helix) [19], as well as the enhanced haemolytic potency of P14A [4] and its ability to form higher oligomers [36], one might expect that P14A should form larger holes in RBC membranes. The present results, however, show that P14A at low concentration initially produces larger swelling of RBC, indicating the formation of smaller pores compared with melittin. This was directly confirmed by a protective experiment using sucrose (Figure 7). In all cases sucrose completely inhibited P14A-induced haemolysis, and only to a moderate extent protected against melittin-induced haemolysis acting at the early stages. This is another example that even sucrose enhances its own protective ability at an early stage of peptide–membrane interaction, a property common to other inhibitors.

DISCUSSION

Data obtained over the last few years have presented circumstantial evidence that melittin interacts not only with lipids but also with the protein components of membranes. The main effect of these interactions results in protein aggregation, which was, as in the case of Ca-ATPase, partly due to indirect interaction of melittin with annular lipid surrounded protein molecules [19]. Other work has demonstrated direct melittin–protein interaction,

for example melittin-calmodulin interaction [38]. Generally speaking, membrane proteins may be included in melittin-induced leaky clusters and represent a constituent of a 'pore', or play a protective role by binding melittin and preventing its harmful interaction with the lipid bilayer [5,14]. It has been shown that incorporation of proteins into a pure lipid bilayer decreases the ability of melittin to produce lysis of such vesicles [30].

Another possibility is that surface or membrane protein can modulate interaction of peptides with the lipid component of the membrane. In any case, if the pore is finally formed within the lipid component, and does not include membrane proteins as a pore constituent or regulatory element, one should expect that at least some features of these pores would resemble the properties of pores formed in pure lipid systems.

However, a detailed comparison reveals many differences between peptide-induced pores in human RBC and lipid vesicles. It has been shown that the bivalent cations Ca^{2+} and Zn^{2+} were able either to activate or inhibit melittin-induced haemolysis, depending on the concentration and ionic strength, an effect not observed with lipid vesicles [5]. Phosphate ions were shown to inhibit haemolysis but were unable to prevent lysis of vesicles [5]. The concentration of cations required to inhibit or activate melittin-induced lysis of vesicles has been shown to be 10–100-fold larger compared with RBC [5,7]. This implies that cation-binding sites on the RBC membrane that are responsible for inhibition are not associated with membrane lipids.

The dependence of the rate of haemolysis on the concentration of peptides (Table 1) is a weak power function with a coefficient of less than 2, whereas a third or fourth power law was obtained in the case of lipid vesicles [6,34]. The rate of haemolysis depends on the concentration of RBC as $(1/c)^{-1/3}$ [39], whereas this value for lipid vesicles depends on vesicle concentration as $(1/c)^3$ [34]. Significant differences were observed in the values of partitioning coefficients of peptides between RBC or synthetic lipids and solution. The partitioning coefficient for RBC, which is of the order of 10^6 M^{-1} (Table 2), is two orders of magnitude larger compared with lipid vesicles (10^4 M^{-1}) [40,41]. These differences show that the molecular mechanism of peptide-induced haemolysis can hardly be explained on the basis of peptide-lipid interactions alone. It seems quite clear that membrane or surface proteins must play some part in the actual haemolytic mechanism.

The present study focused primarily on the stages of peptide insertion into RBC membrane to evaluate a possible role of proteins in the mechanism of haemolysis. On the basis of the activation of melittin-induced haemolysis by bivalent cations under certain conditions, Portlock et al. [5] have proposed the existence of non-lytic sites, presumably proteinaceous in nature, on RBC membranes. Although we were unable to confirm this particular result [33], the present data provide other evidence for this notion. We have shown that bivalent cations compete with peptides for a common binding site responsible for the initiation of haemolysis (Figures 2, 3 and 5). If there had been only one lytic site, bivalent cations at concentrations less than those required to inhibit pre-existing pores, would have either completely arrested subsequent peptide binding or, in the case of expulsion of bivalent cations by peptides from the lytic site, would not have prevented haemolysis. Figure 5 shows that prolonged incubation of RBC in the presence of peptides and Zn^{2+} permits peptide binding but does not cause haemolysis. The fact that the absence of haemolysis in this case is not due to inhibition of a pore already formed after peptide binding is confirmed by dramatic differences in the kinetics of stimulation of haemolysis by EDTA in both these cases (Figure 5). Therefore in the presence of Zn^{2+} , which initially prevents peptide binding,

peptides bind subsequently to other types of site (non-lytic), different from those responsible for cell haemolysis (lytic sites).

The next experimental indication confirming the existence of non-lytic membrane sites is the ability of RBC to enhance spontaneously their susceptibility to lytic action by the same amount of peptides (Figures 3 and 4; see also SUP 50178, Tables 3–5), which parallels the reduction in peptide binding and the enhancement of the order of lytic reaction. The absence of direct correlation between peptide binding and rate of haemolysis and the large values of the partition coefficients strongly suggest that a significant portion of peptides bind to membrane components other than lipids and do not directly participate in the initiation of haemolysis. Intrinsic changes in membrane structure, resulting in changes in RBC susceptibility, imply that non-lytic sites do not represent a uniform class of sites, but can be subdivided into at least two subclasses, strongly and weakly associated with the RBC membrane. Weakly associated sites provide a basis for an explanation of both time-dependent changes in RBC susceptibility and peculiarities in the action of bivalent cations and other inhibitors. According to this model, the time-dependent increase in RBC susceptibility is due to desorption of MICs from the membrane surface, whereas the increased protective ability of bivalent cations and other inhibitors at an early stage of peptide-membrane interaction is due to synergistic interaction of inhibitor and MICs that prevents their desorption. Indeed, Figure 3 (see also SUP 50178, Table 3) shows that bivalent cations and chlorpromazine do not prevent time-dependent changes in RBC susceptibility. In all cases the rate of haemolysis was larger after incubation of the cells for 200 s in physiological saline, both in the presence and absence of inhibitors. This result may be interpreted as an inability of these inhibitors to prevent desorption of MICs, and suggests further that cations and chlorpromazine are hardly involved in the structure of MICs. In contrast, albumin and plasma (SUP 50178, Tables 4 and 5) fully eliminated time-dependent susceptibility changes as their concentration was increased up to 15–20 $\mu\text{g}/\text{ml}$, suggesting that albumin, and possibly other plasma proteins, are either intrinsic elements of MICs or elements capable of interacting strongly with putative MICs, anchoring them to the membrane surface. In any case, a weak association makes it unlikely that these non-lytic sites are membrane lipids. Thus the present results show that the actual mechanism of peptide-induced haemolysis is complicated by interaction of peptides with a protein-based non-lytic class of sites, the role of which is especially important at an early stage of peptide-membrane interaction.

Comparison of the modes of lytic action of the two peptide analogues melittin and P14A revealed more differences than similarities in their action. In general, P14A is a more active lytic peptide, as was stated earlier [4]. The molecular basis of this is that the partition coefficient for P14A is 3-fold larger compared with melittin (Table 2), and probably, that P14A has a straight α -helix structure [4,19]. The susceptibility of RBC to the lytic action of P14A, depends weakly on the time of cell equilibration in physiological saline compared with melittin and bee venom (Figure 4). In contrast to melittin alone, P14A at a high concentration produces shrinkage of RBC in a manner similar to that of a mixture of melittin, PLA_2 and whole bee venom (Figure 6). This effect depends on peptide structure and the presence of additional enzymes (PLA_2). Assuming that shrinkage may be due to phase-transition of lipids from liquid to ordered state that reduces the surface area of lipid molecules by 20% [42–44], we obtained an estimate of the corresponding volume change as 0.7 relative to the initial volume. This value is larger than that found experimentally, especially in the case of bee venom, hence, cell

shrinkage cannot account for changes in the phase state of lipids. Peptide-induced contraction of the protein network might be responsible for this effect. As has been reported previously, the spectrin network shrinks significantly under some circumstances [45,46].

Despite the enhanced affinity for the membrane and the lytic activity of P14A, it forms smaller membrane pores, as judged from the larger initial swelling of cells and protection by sucrose (Figure 7). However, the most intriguing finding is that chlorpromazine and albumin, which are strong inhibitors of haemolysis induced by melittin and bee venom, are, in fact, poor inhibitors of P14A-induced haemolysis (SUP 50178, Tables 3 and 4). At the same time, plasma strongly inhibits P14A-induced haemolysis (SUP 50178, Table 5). All these differences permit the proposal that the actual haemolytic mechanisms of melittin and P14A may be different. It seems that MICs which are involved in the protection of haemolysis induced by melittin are relatively less important in the case of P14A-induced haemolysis. The structure of a peptide, which is responsible for its insertion, mutual association and interaction with membrane components, therefore, is a predominant factor underlying the lytic properties of a peptide. The present results show that substitution of only one amino acid in a peptide sequence can cause a dramatic change in its properties.

We thank Dr. C. Dempsey for providing melittin, P14A and phospholipase A₂.

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