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Changes in Human Erythrocyte Volume and Membrane Protein Spectrum Induced by Melittin, Phospholipase A_2, and Bee Venom

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Human erythrocytes and erythrocyte ghosts were treated with melittin (M), phospholipase A_2 (PLA_2), a whole bee venom. Treatment by whole bee venom or by M + PLA_2 resulted in significant decrease in volume, cell spherulation, and hemolysis. When applied separately, M produced an increase in cell volu during hemolysis, while treatment with PLA_2 stimulated M-induced hemolysis but did not affect cell sh or volume even at relative high concentration. Combined action of M and other agents showed: stimulation of M-induced hemolysis by Triton X-100, even at two orders of magnitude lower than l concentrations; 2) saponin acted similarly but at concentrations closer to those known to lyse cells; chlorpromazine inhibited hemolysis at sublytic concentrations. The action of these agents or PLA_2 M-induced hemolysis was not accompanied by decrease in cell volume. Erythrocytes or ghosts treated w M + PLA_2 or bee venom showed altered SDS-electrophoretic protein patterns; band 6 and 8 were marke reduced while new bands of relatively low molecular weight were identified. The absence of volume protein pattern changes during detergent-stimulated M-induced hemolysis, was taken an indication for specific nature of M-membrane interactions in the presence of PLA_2.

KEY WORDS: melittin, phospholipase A_2, Triton X-100, saponin, chlorpromazine, erythrocytes, membrane defect

The interaction of melittin, a low molecular weight peptide obtained from bee venom [1, 2], with the erythrocyte (RBC) membrane leads to breakdown of the permeability barrier, cell crenation, and the release of membrane fragments [3-8]. Erythrocyte lysis has been shown to be of colloidal-osmotic nature, its magnitude depending on melittin (M) concentrations [4, 5, 9]. Experiments with melittin derivatives showed that their hemolytic potency correlated well with their ability to aggregate intramembrane particles (IMPs) and to reduce rotational mobility of band 3 protein in erythrocyte membranes [10-11] and bacteriorhodopsin in lipid vesicles [12]. It was surmised that beside M–phospholipid interactions, observed in lipid vesicles [3, 6, 13, 14], interactions between melittin and membrane proteins might be directly involved in the melittin-induced formation of lesions [6, 10, 11]. The latter might explain the discrepancies observed in the lytic action of melittin on erythrocytes and lipid vesicles of various lipid compositic [6]. However, the cytotoxic action of bee venom, see ingly more complicated than the hemolytic action melittin and of phospholipase A_2, is probably due to content of various enzymes and peptides which can synergistically [2]. An apparent synergism of M and PL has been reported elsewhere [2, 15]. At present, it is clear whether the mechanism of bee venom action on c is colloidal-osmotic. Similarly, the role of PLA_2 and oth bee venom enzymes in supporting the lytic action melittin has also remained obscure. The aim of the pres study was to compare the lytic action and changes patterns of membrane proteins of erythrocytes produc by treatment with whole bee venom and with its t major components—M and PLA_2—taken either in co bination or separately. Mixtures of M and PLA_2 w used as a model of bee venom. Other well kno membrane perturbing agents such as Triton X-100, ponin, or chlorpromazine (ClPr) were also comparativ studied considering that polylysine [6] or alcohol are a to modulate melittin-induced lysis of lipid vesicle lysis [17].

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MATERIALS AND METHODS

Phospholipase A_2-free melittin and PLA_2 were the generous gift of Dr. C. Dempsey (Bristol University). Chlorpromazine, HCl, saponin, and L-α-lysolecithin were obtained from Calbiochem (USA), Triton X-100 from Merck (Germany), and lysolecithin (2-deoxy-1-lauroyl) from Sigma (USA). Whole bee venom was dissolved in distilled water and centrifuged to remove insoluble compounds. The concentration of the bee venom solution was determined by its dry weight after evaporation. Human erythrocytes were isolated from blood-bank blood by triple washing in a 10-fold excess of phosphate-buffered saline (150 mM NaCl, 5 mM sodium phosphate, pH 7.2) (PBS). The layer of leucocytes on the pellet was removed after each centrifugation by aspiration. Erythrocyte ghosts were obtained either by lysing cells with sodium phosphate buffer (5 mM, pH 8.0) at 0°C and subsequent triple washing in PBS according to [18] (Dodge ghosts, DG), or by incubating cells for 15 min in 1.5 M glycerol at 37°C with subsequent transfer into PBS (glycerol ghosts, GG) at 0°C and washing [19]. In both cases ghosts were collected by centrifugation (20,000*g*, 20 min). Membranes after treatment with lysing agents were pelleted and solubilized by adding 0.5 ml of 0.15 M Tris-HCl, 1% SDS, and 0.01% bromphenol blue (pH 8.8) to the pellet and incubating for 3 min in a boiling water bath. Erythrocyte ghosts not treated with a lysing agent were used as control samples. Electrophoresis of membrane proteins was performed on polyacrylamide gels (4-22% gradient) according to Gorg et al. [20].

Hemolysis Assay (HAS). The dynamics of erythrocyte hemolysis and alteration of their shape during interaction with melittin and other lysins were measured spectrophotometrically [21-23]. Erythrocyte suspensions were constantly stirred and the attenuance at 720 nm was recorded continuously. The photosignal is attributed solely to the low-angle scattering of light by erythrocytes and not to absorption [21-23]. Aliquots of lysins were added directly to a cuvette with erythrocyte suspension from concentrated stock solutions. Time of mixing was ~2 sec. All experiments were carried out at room temperature (20-22°C).

Resistive Pulse Spectroscopy (RPS). The details of RPS technology used for measuring volume distribution of erythrocytes and ghosts has been reported elsewhere [24, 25]. Cells were sorted by size using a Coulter-counter type device in which resistance pulse are induced by particles passing through a flow-limiting orifice with a constant flow. 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 a cylindrical, 50-μm long, and 50-μm diameter orifice was used in a system of transducers, providing practically complete hydrodynamic focusing of cells. The mean linear flow rate of the cell suspension through the orifice was below 1 m/sec. Each measurement cycle analyzed 32,000 cells, and the current across the orifice did not exceed 0.2 mA, so that no electrical breakdown [24] and no significant deformation of cell membrane [25] occurred, the method providing a measure of the true volume of both RBCs and their ghosts (particles) in suspension.

RESULTS

Figure 1 shows the time course of the attenuance of erythrocyte suspensions exposed to different concentrations of venom (a) and of melittin combined with PLA_2 (b). Increasing venom concentration increased the rate attenuance decrease due to hemolysis. As reported elsewhere [22, 23], the attenuance signal is proportional to the number of intact cells in suspension and represents fluctuations or noise in volume mean value. The amplitude of the noise is maximum for discoid cells, while for spheroidal ones it is zero. The amplitude of the noise is roughly proportional to the concentration of discoid shaped cells. If cells change their shape, for example, from discoid to spheroidal, the value of the noise decreases simultaneously with an increase in attenuance [22, 23]. It is clear from Fig. 1 that both venom and melittin either alone or in combination with PLA_2 initially reduce the noise toward zero. This corresponds to a shape change from discoid to spheroidal, followed by hemolysis. A similar change in signal, which is not followed by hemolysis, was identified when erythrocytes were treated with rose bengal [23]. Our microscopic analysis confirmed that noise disappearance corresponded to the acquisition of smooth spheroidal cell shape (data not shown). This was also the case for cells treated with detergents [4] or ethanol [26]. Increasing PLA_2 concentration accelerates the rate of M-induced hemolysis. The influence of PLA_2 on the time-course of hemolysis becomes, however, visible only when relatively high concentrations of PLA_2 are used, exceeding those present in bee venom [2]. PLA_2 alone at concentrations below 2 μM produces no changes in hemolysis or cell shape (not shown), as reported elsewhere [27, 28].

The changes in particle (i.e., cells plus ghosts) volume occurring during hemolysis were different when induced by bee venom, M + PLA_2, or melittin alone. The effect of venom or melittin concentration on volume changes in erythrocytes and their isolated ghosts (GG) are shown in Fig. 2. While melittin increases particle volume, venom and M + PLA_2 reduce volume in a concentration-dependent manner. In contrast to cells, melittin alone only

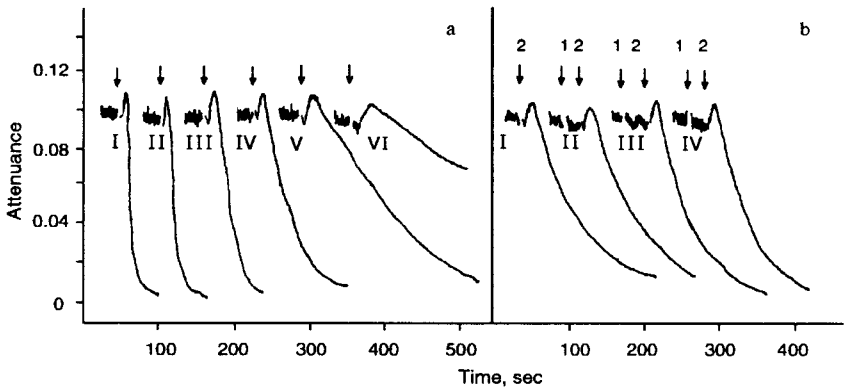


Fig. 1. Time courses of hemolysis induced by bee venom (a) or melittin and PLA_2 (b). Final concentrations of bee venom used: I) 1.5; II) 1.25; III) 1; IV) 0.75; V) 0.5; VI) 0.25 μg/ml. a) Arrows mark the addition of bee venom into the erythrocyte suspension ($7 \cdot 10^5$ cells/ml). b) Arrows 1 and 2 mark the additions of melittin (final concentration 1 μg/ml) and PLA_2. Final PLA_2 concentrations were as follows: I) 0; II) 0.05; III) 0.5; IV) 1 μg/ml.

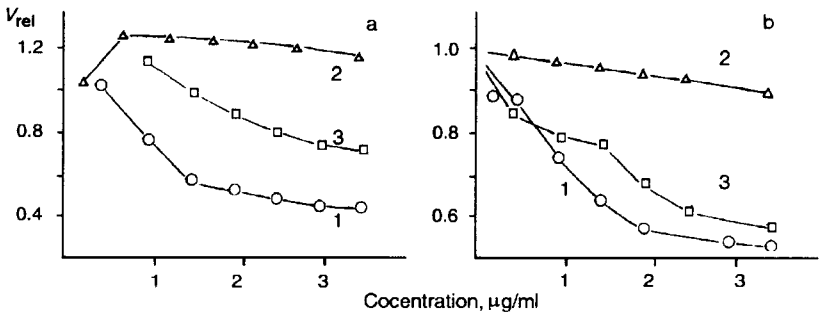


Fig. 2. Dependence of the mean relative volume V_{rel} of erythrocytes (a) and ghosts (b) on the concentrations of bee venom (1), melittin (2), and melittin in the presence of PLA_2 (1 μg/ml) (3). Cells or their ghosts at a concentration of $7 \cdot 10^5$ cells in 1 ml were incubated in PBS for 5 min (pH 7.2) at room temperature. Their relative volume was measured using the RPS technique. Representative data from 3-4 experiments.

slightly reduces the volume of ghosts, but in the presence of PLA_2 melittin leads to a significant volume decrease.

In order to eliminate possible artifacts of volume measurements or lysin-induced cell fragmentation, we performed a comparative study of the dynamics of cell volume changes during lysis elicited by classical agents such as ClPr, Triton X-100, and saponin. The time profiles of erythrocyte lysis as a function of concentration of the various agents were found to be close each other as well to those elicited by melittin (Fig. 3). Both chlorpromazine and Triton X-100 induced rapid transformations of cells into spherocytes. Then a lag-period between addition of the agent and the onset of hemolysis was observed. Saponin induced a slower shape transformation, resembling that caused by melittin. Lysis induced by chlorpromazine and saponin was preceded by an increase in erythrocyte volume, which indicates the colloidal-osmotic nature of this type of hemolysis. The extent of swelling was largest in the presence of ClPr. Triton X-100-induced lysis was accompanied by formation of highly deformed particles (Triton ghosts) [25], as found by changing the liquid flow rates when using the RPS method. A twofold increase in the flow rate was accompanied by a 5-6-fold decrease in the apparent volume. In contrast, ghosts formed during ClPr-, M-, M + PLA_2-, and venom-induced lysis had volumes not dependent on the flow rate, indicating higher rigidity than Triton ghosts. In the case of saponin-induced hemolysis, a significant decrease in particle (i.e., cells plus ghosts) concentration was observed. This resulted from the fact that saponin induces large pores in erythrocyte membranes [29] and reduces membrane electrical resistance to a level undetectable by the RPS method. In all other treatments including melittin or M + PLA_2, particle concentration did not change, apparently signifying lack of cell fragmentation.

Data shown in Fig. 1b indicated that PLA_2 at low concentrations acted synergistically with M in accelerating

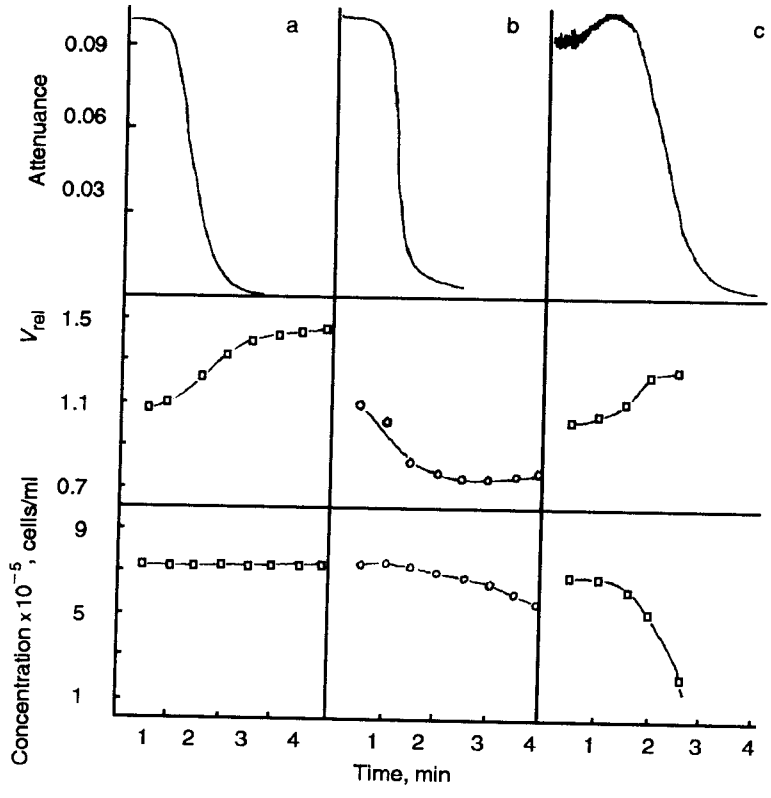


Fig. 3. Time courses of changes in attenuance, mean relative volume V_{rel}, and concentrations of particles in suspensions of erythrocytes induced by chlorpromazine (100 μM) (a), Triton X-100 (116 μg/ml) (b), and saponin (40 μg/ml) (c). Erythrocytes ($7 \cdot 10^5$ cells in 1 ml) were incubated in PBS at room temperature in the presence of Triton X-100 and saponin (pH 7.2) or chlorpromazine (pH 6.6).

hemolysis. On the other hand, other lysins diversely affect M-induced hemolysis. Triton X-100 strongly stimulated M-induced hemolysis at very low concentrations (Fig. 4b). Saponin also stimulated M-induced lysis, but at a relatively higher concentrations, whereas ClPr inhibited M-induced hemolysis. The latter occurred whether or not ClPr was added prior to or after melittin, showing that the ClPr-induced blockage is not the result of ClPr interaction with M, but is due to blocking of M-induced "pores", similar to that observed with divalent cations [7, 8, 17, 30]. It is noteworthy that decrease in erythrocyte volume, such as that caused by M + PLA_2 or bee venom was not observed with M combined with other agents.

In order to test possible roles of PLA_2 hydrolytic products in M-mediated hemolysis, we carried out studies with PLA_2 in the presence of Ca^{2+} or EDTA. In other experiments the action of hydrolysis products was modelled by addition to cell suspension of exogenous lysolipids before their exposure to M. As shown in Fig. 5, neither Ca^{2+} nor EDTA elicited noticeable changes in the time-course of hemolysis when added after bee venom. However, the rate of hemolysis induced by venom bee increased when the venom was added in the presence of EDTA (Fig. 5, curve III) and decreased in the presence of Ca^{2+}. Moreover, Ca^{2+} significantly retarded the shape transformation of RBCs induced by bee venom (curve V). The changes in maximal rate of hemolysis and mean relative volume of cells treated with PLA_2 in the presence or absence of Ca^{2+} and EDTA are summarized in Table 1. It is noteworthy that PLA_2 treatment in the presence of Ca^{2+}, while it significantly alters the rate of hemolysis, it does not decrease RBC volume. Hence, *in situ* formation of hydrolytic products by PLA_2 cannot account for the effect of cell shrinking induced by bee venom or M + PLA_2. Despite this fact, it was found that incorporation of exogenous lysolipids into RBC membranes, as determined by increase in attenuance and disappearance of the noise in the attenuance (i.e., transformation into smooth spheres) either stimulated or inhibited M-induced hemolysis depending on the type of lysolipid used. (In the last case there was observed shrinking of cells or ghosts.)

The effect of melittin, M + PLA_2, ClPr, Triton X-100, and saponin on protein content of erythrocyte and ghost membranes is shown in Fig. 6. It can be concluded that: 1) melittin, Triton X-100, and saponin produced no changes in protein composition with respect to control

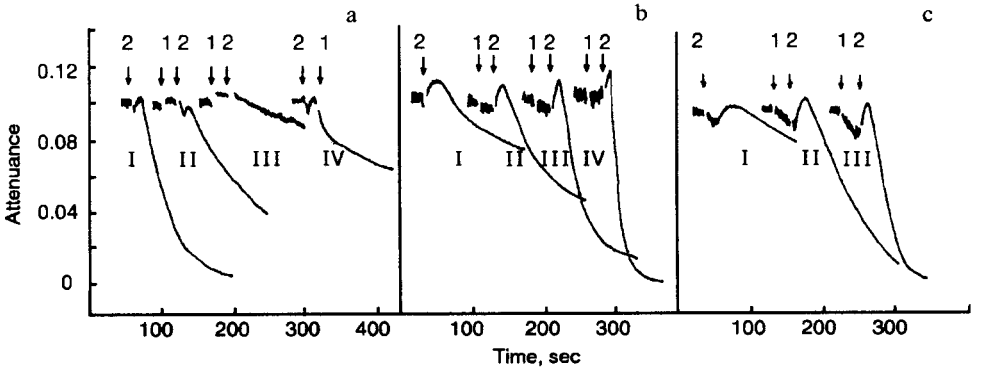


Fig. 4. Effects of chlorpromazine (a), Triton X-100 (b), and saponin (c) on melittin-induced hemolysis. Arrow 1 marks the addition of chlorpromazine, Triton X-100, or saponin to erythrocyte suspensions ($7 \cdot 10^5$ cells in 1 ml); arrow 2 indicates addition of melittin to final concentration of 2 (a) or 0.75 μg/ml (b, c). a) Final concentrations of chlorpromazine were: I) 0; II) 2.5; III, IV) 12.5 μM. b) Final concentrations of Triton X-100 were: I) 0; II) 0.5; III) 2; IV) 3.75 μg/ml. c) Final concentrations of saponin were: I) 0; II) 15; III) 25 μg/ml.

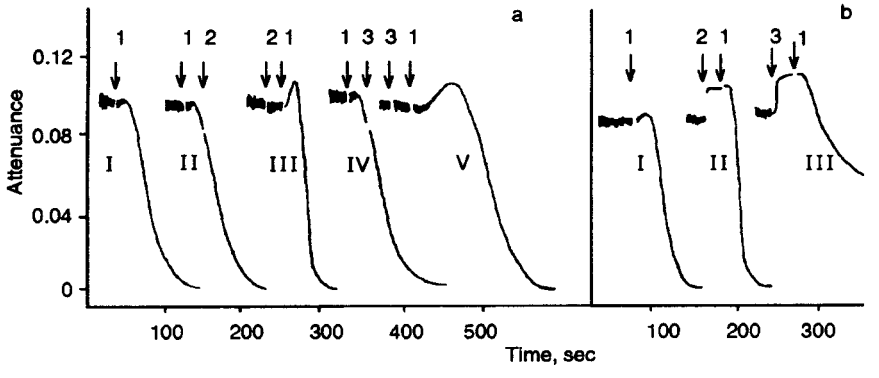


Fig. 5. Effect of Ca^{2+} and EDTA on bee venom hemolysis (a) and lysolipids on hemolysis induced by melittin (b) in Tris-buffered saline, pH 7.2. a) Arrow 1 marks the addition bee venom (final concentration 1 μg/ml); arrows 2 and 3 show addition of EDTA or Ca^{2+} (final concentrations 1 mM), respectively. b) Arrows indicate addition of: 1) melittin (final concentration 1 μg/ml); 2) L-α-lysolecithin (5 μg/ml); 3) lysolecithin (2-deoxy-1-lauroyl) (0.5 μg/ml).

ghosts, with the exception of band 6, which was not detected after either treatment; 2) M + PLA_2 and whole bee venom (in addition to band 6) eliminated band 8 proteins; 3) when applying M + PLA_2, ClPr, and whole bee venom, additional bands are found between band 8 and the band of Hb, with apparent molecular weight of 18 and 19 kD, respectively. All the changes are similar in erythrocytes and ghosts. Whole bee venom, however, eliminated residual hemoglobin which is found in ghosts. Treatment of RBCs with corresponding concentrations of PLA_2 alone did not cause hemolysis or changes in protein profiles compared to usual Dodge ghosts (not shown). Since changes in polypeptide pattern induced by bee venom were not abolished in the presence of EDTA (1 mM) in the incubation media, thus indicating that residual Ca^{2+} (probably originating from the bee venom) was not responsible for the effect. These data suggest that bee venom and its components (M + PLA_2), in contrast to melittin, Triton X-100, and saponin, synergistically influence erythrocyte membrane proteins.

DISCUSSION

It is well known that melittin, the main peptide of bee venom, is involved in lysis of erythrocytes and lipid vesicles [1-8, 16, 17]. The particular conformation of melittin responsible for bilayer disruption leading to RBC lysis remains unresolved. Some authors have invoked colloidal-osmotic properties in the mechanism of cell lysis [4, 5, 9]. The correlation found between lytic activities of melittin derivatives and their ability to aggregate intra-

TABLE 1. Influence of PLA_2 Treatment on Maximal Rate of M-Induced Hemolysis (V_{max}) and Mean Relative Volume (V_{rel}) of RBCs Prior to and After Interaction With Melittin[a]

Parameter	Control	EDTA	Ca^{2+}	PLA_2	PLA_2 + EDTA	PLA_2 + Ca^{2+}
$V_{max} \times 10^{-4}$, sec^{-1} (SD = 1.0)	8	18	12	8.4	14.8	116
V_{rel} (SD = 0.03)	1	1.01	1	1	1.01	1.1
V_{rel} after treatment with melittin (SD = 0.03)	1.17	1.04	1.23	1.16	1.05	1.18

[a]RBCs ($2 \cdot 10^8$ cells in 1 ml) were incubated in Tris-buffered saline (10 mM Tris-HCl, pH 7.2) in the absence or presence of either 5 mM EDTA or 5 mM Ca^{2+} with or without PLA_2 (3 μg/ml). Cells were incubated for 1.5 h at room temperature and the time course of hemolysis induced by melittin (0.5 μg/ml) was investigated using the HAS and RPS techniques. Relative volume of erythrocytes was determined after 5 min incubation of cells with melittin.

membrane particles has underlined the role of melittin–membrane protein interactions in lytic processes [10, 11, 15]. Thus, the process of erythrocyte membrane rupture may be the result of melittin interactions with both membrane lipids and membrane proteins, particularly with band 3 glycophorin complexes [10, 11]. The observed RBC spherulation and increase in the volume of erythrocytes caused by lytic concentrations of melittin (Fig. 2) support the idea of melittin insertion into the membrane and formation of pores. The latter are at first impermeable to Hb, but they grow in dimensions with increasing concentration of melittin [4, 5, 9]. In contrast to melittin, the lytic effect of bee venom, which in addition to melittin contains a variety range of enzymes [2], results in different changes in cell volume, despite similar time-courses of hemolysis (Figs. 1 and 2). To simplify, the cell shrinking can be considered as a manifestation of a more complex mechanism of melittin action when additional enzymes are present. This may be attributed to the hydrolytic properties of PLA_2, which together with M results in the acceleration of hemolysis and shape transformation (Fig. 1), i.e., changes similar to those produced by the action of bee venom. In addition, M + PLA_2 also results in shrinking of erythrocytes and their ghosts. In the present study, neither M nor PLA_2 by themselves produced shrinking of cells or ghosts contrary to those of Habermann [2], who reported ghosts shrinking in field phase contrast. Similar to the data of Tosteson et al. [9], those presented here indicate swelling of erythrocytes, while little affecting ghost volume. Moreover, treatment of erythrocytes with PLA_2 at concentrations higher than those used in the present study resulted in crenation and insignificant swelling of erythrocytes, which makes them sensitive to osmotic hemolysis [27, 28]. Freeze-fracture experiments have demonstrated that treatment with PLA_2 does not disturb the lipid bilayer organization [31] and produces no changes in protein pattern of membrane proteins as judged by SDS electrophoresis [27]. Significant hydrolysis of membrane lipids nevertheless is not accompanied by hemolysis [27, 28]. At the same time hemolysis induced by lysolecithins is of colloidal-osmotic nature, and it is accompanied by cell swelling [32].

Several facts support the notion that the synergistic action of PLA_2 with melittin is not related to its hydrolytic properties: 1) acceleration of melittin-induced cell shrinking and lysis in the presence of PLA_2 occurs virtually upon addition of the agents, prior to any substantial hydrolysis; 2) application of nominally Ca^{2+}-free media which does not support PLA_2 activity [33] caused similar effects; 3) addition of EDTA stimulated bee venom-induced hemolysis and while Ca^{2+} is inhibited it (Fig. 5); 4) the absence of PLA_2-induced volume and cell shape changes shows that only a small amount of lysolipid is formed because when significant hydrolysis occurs the cell volume increases and echinocytes form [27, 28]; 5) no cell shrinkage occurs after incorporation of exogenous lysolipids which either activate or inhibit M-induced hemolysis; 6) PLA_2 treatment of RBCs in Ca^{2+}-contained media while significantly accelerating hemolysis produced no cell shrinkage. The effect of M + PLA_2 on erythrocyte and ghost shrinkage correlated with disappearance of membrane protein bands 6 and 8 and appearance of some new low molecular weight proteins (Fig. 6). How changes in membrane proteins are related to the lytic phenomena remains unclear. Similar but not identical features in the activation of M-induced hemolysis are found in the combined action of melittin in the presence of sublytic concentrations of Triton X-100 (Fig. 4) and saponin. Triton X-100 is known to affect both erythrocyte lipids and proteins and to solubilize lipids and glycoproteins from erythrocyte membranes [34, 35]. It forms Triton X-100–glycophorin complexes at physiological pH values [36], increases membrane permeability with a minor selectivity for K^+ [37], and forms divalent cation-sensitive pores in cell membranes [8]. However, when added at sublytic concentrations no

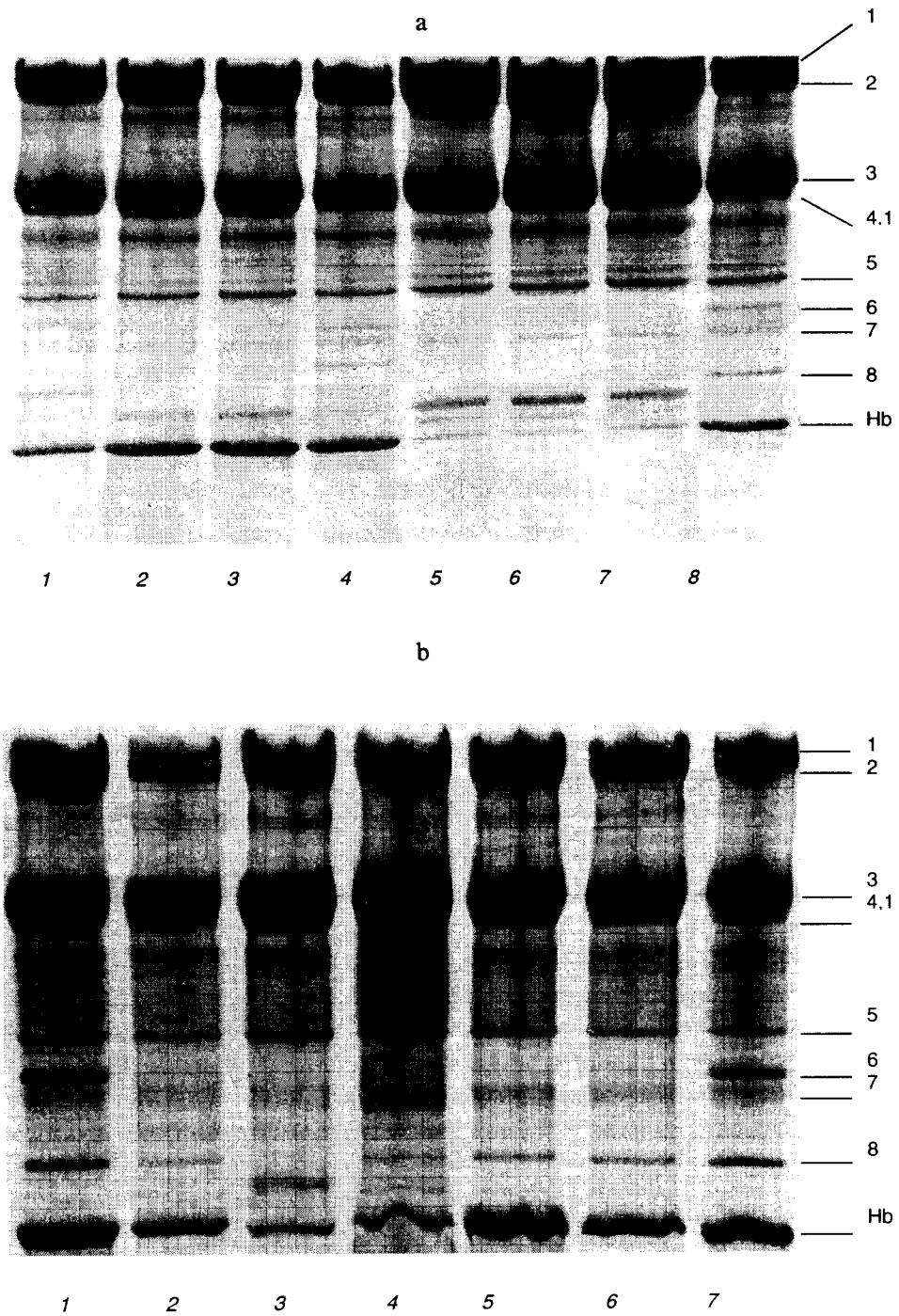


Fig. 6. SDS-gel electrophoresis of erythrocytes (lanes 2-4) and ghosts (DG) (lanes 5-7) treated with bee venom (a) or other agents (b). Erythrocytes and ghosts (~$1.3 \cdot 10^9$ cells in 1 ml) were incubated for 10 min in PBS (pH 7.2) with the following concentrations of agents. a: 1, 8) control ghosts; 2, 5) 94 μg/ml; 3, 6) 47 μg/ml; 4, 7) 12 μg/ml. b: 1, 7) control ghosts; 2) melittin (30 μg/ml); 3) melittin (15 μg/ml) + PLA_2 (7.5 μg/ml); 4) ClPr (2.5 μM); 5) saponin (50 μg/ml); 6) Triton X-100 (160 μg/ml). Hb, hemoglobin.

significant alteration occurred in the shape and volume of cells as monitored by HAS and RPS techniques, while short-term incubation with Triton X-100 prior to addition of melittin significantly increased the rate of hemolysis (Fig. 4). The mode of action of Triton X-100 on M-induced hemolysis differs from that of saponin, which activates hemolysis only at lytic concentrations. This implies that solubilization of cholesterol, with which saponin interacts, is not a crucial factor in M-induced hemolysis.

Finally, neither melittin nor Triton X-100 and saponin, taken in lytic concentrations, produced any alterations in the SDS protein pattern of the resulting ghosts (Fig. 6). Failure to detect shrinkage of cells or ghosts treated with M in the presence of these agents suggests that their stimulatory mechanism on M-induced hemolysis differs from that of PLA_2. The last lysin used, ClPr, is an amphipathic cation which induces invagination of cells and makes their shape stomatocytic [38]. ClPr inhibited M-induced hemolysis in a manner similar to cations such as Ca^{2+}, Zn^{2+} [7, 8, 30], or polylysine [6]. In contrast to other lysins used in the present work, ClPr affected the polypeptide pattern of erythrocytes, where the presence of the low molecular weight band, similar to those of M + PLA_2 and bee venom, has also been identified. This corresponds to a different action of ClPr on membrane proteins [39, 40]. It is worth mentioning that sublytic concentrations of chlorpromazine do not alter the RBC polypeptide pattern [41]. A correlation between hemolytic ability of melittin and its derivatives to aggregate IMPs [10, 11], and the characteristic discrepancies between the effect of melittin upon erythrocytes and lipid vesicles of various lipid composition [6], strongly suggest that melittin–protein interactions may play a significant role in the lytic process. Although the exact role of these interactions awaits elucidation, the data obtained here provides additional support for this idea. Apparently, PLA_2, besides its hydrolytic activity, possesses a specific ability to modulate M interactions with membrane proteins, and this is manifested in the contractile properties of erythrocytes and ghosts, and in the alteration of membrane protein structure, as detected by SDS gel electrophoresis. The data indicate that the band 8 protein, which exists in a tetrameric form in RBC membrane [42], is a likely candidate to take part in M–protein interactions. We suggest the mechanism of the M-induced lysis is altered by other agents from one based on colloidal-osmotic properties [3, 4, 13] to one based on structural changes [43], where formation of large-scale lesions may be due to intensified M–protein interactions. This mechanism is specific for PLA_2 and differ from that found with detergents, which does not affect M–protein interactions in membranes. It seems that PLA_2 and, probably, other bee venom enzymes may act as modulators of the melittin cytotoxic reaction.

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