J. Membrane Biol. 87, 35–44 (1985)

The Journal of
Membrane Biology

Melittin Lysis of Red Cells

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Summary. This paper describes experiments designed to explore interactions between human red blood cell membranes and melittin, the main component of bee venom. We found that melittin binds to human red cell membranes suspended in isotonic NaCl at room temperature, with an apparent dissociation constant of 3×10^{-8} M and maximum binding capacity of 1.8×10^7 molecules/cell. When about 1% of the melittin binding sites are occupied, cell lysis can be observed, and progressive, further increases in the fraction of the total sites occupied lead to progressively greater lysis in a graded manner. 50% lysis occurs when there are about 2×10^6 molecules bound to the cell membrane. For any particular extent of melittin binding, lysis proceeds rapidly during the first few minutes but then slows and stops so that no further lysis occurs after one hour of exposure of cells to melittin. The graded lysis of erythrocytes by melittin is due to complete lysis of some of the cells, since both the density and the hemoglobin content of surviving, intact cells in a suspension that has undergone graded melittin lysis are similar to the values observed in the same cells prior to the addition of melittin. The cells surviving graded melittin lysis have an increased Na and reduced K, proportional to the extent of occupation of the melittin binding sites. Like lysis, Na accumulation and K loss proceed rapidly during the first few minutes of exposure to melittin but then stops so that Na, K and hemoglobin content of the cells remain constant after the first hour. These kinetic characteristics of both lysis and cation movements suggest that melittin modifies the permeability of the red cell membrane only for the first few minutes after the start of the interaction. Direct observation of cells by Nomarsky optics revealed that they crenate, become swollen and lyse within 10 to 30 sec after these changes in morphology are first seen. Taken together, these results are consistent with the idea that melittin produces lysis of human red cells at room temperature by a colloid osmotic mechanism.

Key Words melittin-induced osmotic lysis · human red cells · permeability increases

Introduction

Melittin, the major toxin of the bee venom, is a small, cationic polypeptide composed of 26 amino acid residues. The secondary structure of the monomer has been established by X-ray crystallography [22] and NMR spectroscopy [3] to be a bent alpha-helical rod. Crystals grown from concentrated salt solutions show that melittin forms aggregates of four monomers with the four helices contributing their hydrophobic side chains to the center of the molecule. The polypeptide interacts with lipid monolayers, as judged by the changes in the surface pressure and surface potentials of lipid monolayers upon addition of melittin to the subphase [18]. Melittin also interacts with lipid vesicles [18] and lipid bilayers [6, 12, 13, 24], producing changes in their ionic permeability. It has further been shown that melittin has direct lytic action on cell membranes, inducing the release of hemoglobin from red cells and histamine from mast cells [10].

DeGrado et al. [7] have studied in detail the lytic effects of melittin in human red blood cells. Working at 4°C and low ionic strength (isotonic sucrose), they found that melittin binds rapidly to the outer surface of the membrane and that the time course of hemoglobin release is biphasic: a rapid, earlier phase, lasting a few minutes and a slower, later phase, lasting many hours. They interpreted their results as due to partial lysis of the cells, hemoglobin being lost through openings in the red cell membrane that are usually too brief to allow equilibration of intra- and extracellular hemoglobin during a single open cycle. They speculate that association of melittin with the membrane causes local expansion of the outer leaflet and subsequent deformation which, in turn, leads to further penetration of the polypeptide into the bilayer and to eventual disruption of the continuity of the bilayer. Once an opening is formed, melittin could be internalized and the hole closed before all of the hemoglobin could be lost from the cell. They have further suggested that, since melittin might be a tetramer in the aqueous solutions, the openings in the membrane might allow a melittin tetramer to be internalized.

In this paper we report observations of binding of melittin to human red cell membranes and melit-

tin-induced lysis of human red cells when the interaction takes place in isotonic saline at room temperature. Care was taken that the final melittin concentrations were always such that the peptide remained monomeric in the aqueous phase. The results of these measurements indicate that under these experimental conditions, graded melittin-induced lysis is not due to partial lysis of all the cells but to complete, all-or-none lysis of some of the cells. The mechanism of lysis probably involves colloid osmosis, as evidenced by the increase in red cell cation content and volume prior to lysis. Further, we also have found that the extent of lysis is uniquely defined by the amount of bound melittin/cell, 50% lysis occurring when the number of molecules of melittin bound per cell is about 2×10^6, a melittin/phospholipid ratio of *ca.* 0.06.

Materials and Methods

Freshly drawn, heparinized human blood from healthy donors was centrifuged and washed three times in cold (4°C) Na buffer (0.150 M NaCl, 0.01 M Tris-HEPES, 0.001 M EGTA, pH 7.4) to remove plasma and buffy coat. The final wash was made in the Na buffer or in other buffer as indicated in the text. After this last wash, the cells were resuspended 1 : 1 (vol/vol) in the buffer and the hematocrit and hemoglobin content determined in two samples. This 50% cell suspension was used to prepare all cell suspensions for lytic assays.

Melittin, purchased from Sigma Chemical Co. (St Louis, Mo.) was dissolved in distilled water (0.5 to 0.9 mM) and the resultant solution was frozen and stored at −54°C until used, within one week after it was dissolved. The exact concentration of the solution was determined on the day of each experiment, by measuring the absorbance at 280 nm (extinction coefficient 5570/M cm [16]). All salts used were reagent grade and were dissolved in double-distilled water. Inulin {^{14}C}-carboxylic acid, average molecular weight 5200, was purchased from Amersham Corp. (Arlington Heights, Ill.); H_2-DIDS (4,4′-diisothiocyano-1,2-diphenylethane-2,2′-disulfonate) was a gift of Dr. H. Passow (Frankfurt, Germany); bovine serum albumin, raffinose and polyethylene glycol (PEG, mol wt 1,000) were obtained from Sigma; Dextran T10 (mol wt 10,000) was obtained from Pharmacia Fina Chemicals (Piscataway, N.J.).

Hematocrits were determined in duplicate samples using an Autocrit II centrifuge (Clay Adams Div., Becton Dickinson, Rutherford, N.J.) and hemoglobin concentrations determined from the absorbance at 540 nm (or at 410 nm when the hemoglobin concentration was such that the OD at 540 nm was less than 0.1) in a Stasar II spectrophotometer (Gilford Instrument Laboratories Inc., Ohio). The values of hemoglobin released are expressed as a fraction of the total amount of hemoglobin released when all the cells in the assay system lysed.

Each experimental condition was repeated at least two different times in which each of the measurements were taken in duplicates and averaged. The results are reported either as mean values of two or more different measurements or as representative experiments, each point the average of the duplicates of one experiment.

A) Pretreatment of Tubes with Melittin

Since we found that melittin binds to the walls of tubes made of polypropylene, Teflon® and glass (both with and without treatment with silicone), we carried out experiments to establish the conditions to minimize such binding. The results of those experiments established that the binding of melittin to the walls of the tubes was minimized when the tubes were pretreated for a minimum of 2 hr (at room temperature) with a solution containing melittin at a concentration of 3 μM or higher. We also found that the results were very reproducible if the experiment was started within 15 min after preparation of the desired melittin concentration in the pretreated, rinsed tubes.

B) Kinetics of Hemolysis

The time course of the hemolysis of red cells exposed to melittin was determined through measurements of the hemoglobin content of the medium and/or in the cells after their separation by centrifugation through a layer of phthalate esters as follows. The 50% cell suspension was further diluted in appropriate buffer (as indicated) to a concentration 10× the final desired hematocrit. The cells were then added to the pretreated tubes where the final dilution took place. Cells and medium were mixed either using a vortex mixer or a rapid mixing chamber. In the former case the final dilution was 1 : 10, and the cells were added under constant stirring using a vortex mixer. When using the rapid mixing chamber, the final dilution was 1 : 2 and was obtained by loading 5-ml cell suspension (at a concentration twice the final one) in a plastic syringe and the buffer containing melittin into another syringe (pretreated with melittin). The solutions were then passed through the pretreated polyethylene chamber (50 μl) at approximately 10 ml/sec and the suspension collected in a pretreated tube. The tubes were then placed in the rotary mixer and, at the desired time after addition of the cells, 1.5 ml of the cell suspension (in duplicate) was withdrawn and carefully layered on top of 0.15 ml dibutyl-phthalate (specific gravity 1.0416, Fisher Scientific Co., Fair Lawn, N.J.) in Eppendorf tubes. The cells were then separated from the medium by centrifugation (Eppendorf Mod. 5413 or 5414). Hemoglobin was determined in the supernatant, above the phthalate layer and, in some cases, in the remaining cells, underneath this layer after lysis of the cells in the pellet using 1.5 ml of a 0.02% solution of Acationox (American Scientific Products, Bedford, Mass.). Since we found no significant difference in the time course of the lysis induced by melittin when using either of the two methods, the vortex method was used unless there was a specific need to mix cells and medium within milliseconds.

To study the kinetics of lysis of cells with varying resistance to osmotic lysis, cells were prepared by preincubation in hypotonic solutions for 30 min at room temperature. At the end of this period, the osmolarity of the suspension was brought to 300 mOsm by added an appropriate volume of concentrated NaCl (buffered to pH 7.4) and the tubes spun to separate the cells from the medium. The supernatant was used to determine the degree of hemolysis and the cells were washed two times in Na buffer and resuspended to 10% hematocrit. These cells were subse-

quently exposed to melittin and the kinetics of lysis determined as described above.

C) Determination of the Cell Density Distribution and of the Number of Cells at Each Density

The determination of the cell density distribution was done by separation of the cells in Eppendorf centrifuge tubes, each of which contained 0.15 ml of a mixture of methyl-phthalate (sp gr 1.189) and of dibutyl-phthalate (sp gr 1.0416) as indicated in [4]. The number of cells present in the supernatant (after centrifugation) of each of the tubes was determined using a Coulter Counter (mod. ZB1, Coulter Electronics Inc., Hialeah, Fla.) on an aliquot (50 μl) of the suspension on top of the phthalate. The hemoglobin content of both the top and bottom fractions was also determined as detailed in (B).

D) Ion Substitution Experiments

The kinetics of lysis of cells exposed to melittin in buffers where Cl (Na) was partially or totally replaced, was studied as detailed in (B), after treatment of the cells as follows. The packed, washed cells were washed once in the ion-substituted buffer and resuspended to 10% hematocrit in this buffer. When Cl was the ion replaced, the cell suspension was incubated at 37°C for 1 hr in a water bath (Precision, Fisher Scientific Co., Pittsburgh, Pa.), followed by gentle cooling of the cells to room temperature. The kinetics of lysis was started by adding an aliquot of the cell suspension to tubes containing the buffer with melittin.

E) Determination of Net Cation Movements

The content of Na and K in the cells, as well as the K content in the supernatant were determined before and after cells were treated with melittin, using a flame-spectrophotometer (Perkin-Elmer model 5000 Atomic Absorption Spectrophotometer, Perkin-Elmer Corp., Wellesley, Mass.) as follows.

i) Measurements of K in the Supernatant

Duplicate portions (1.5 ml) of a 1% cell suspension were transferred to Eppendorf centrifuge tubes at various times after addition of the cells to the melittin-containing buffer and spun to separate the cells from the medium. The top portion of the supernatant in each tube was carefully withdrawn and used to determine K and hemoglobin.

ii) Measurements of Na and K in the Cells

The remaining cells were washed three times with choline chloride buffer (0.15 M choline chloride, 0.001 M $MgCl_2$, 0.001 M EGTA, 0.01 M Tris-HEPES, pH 7.4) in the Eppendorf centrifuge tubes. After the last wash, the supernatant was withdrawn, the walls of the tubes carefully dried and the cells lysed with Acationox. After one more spin to remove the insoluble material, the supernatant was used to determine the Na, K and hemoglobin content of the cells. The change in ion content of cells was expressed in mmol/original liter cell using the measured values of the hematocrit and hemoglobin of the cell suspension before treatment with melittin.

F) Binding of Mellitin to Red Cell Membranes

The characteristics of the binding of melittin to the red cell membrane were determined by incubating red cell membranes (ghosts) with melittin, followed by a determination of the free melittin remaining in the aqueous phase.

i) Ghost Preparation

Red cell membranes were prepared following the procedure described by Bjerrum [1], adding ^{14}C-inulin after the last wash. The resealed ghosts were washed two times with Na buffer and stored at 4°C for up to a week. The integrity of the resealed membranes was determined by measuring the efflux of ^{14}C-inulin and the ghost concentration was measured by direct counting using the Coulter Counter.

ii) Efflux of ^{14}C-Inulin

A portion of the ghost suspension was brought to room temperature and washed twice with Na-buffer. After the last centrifugation, the ghosts were resuspended to 10% (vol/vol) and an aliquot (0.1 ml) was taken to determine the total counts at the initial time. At appropriate intervals, two samples of 0.1 ml were removed from the efflux tube to a centrifuge tube, spun and the supernatant counted in a liquid scintillation counter (Packard TriCarb, Packard Instruments Co., Downers Grove, Ill.). Measurements of inulin efflux in the presence of melittin indicated that the rate of efflux was unchanged in the presence of the toxin.

iii) Binding of Melittin to Ghosts

Melittin-containing Na buffer (10 to 20 ml) was prepared in pretreated plastic tubes. One minute later, a portion of the ghost suspension was added to make the desired final concentration of membranes. The tube was then placed in the rotary mixer for 3 to 5 min, since preliminary experiments indicated that the binding was completed after 30-sec incubation of the membranes with melittin (*cf.* also [8]). The ghost suspension was then centrifuged at 25,000 × *g* for 5 min, the supernatant (9 ml) added (under continuous stirring in a vortex mixer) to a melittin-pretreated tube containing 1.0 ml of a 10% red cell suspension and the kinetics of lysis followed as described in (B). The free melittin concentration was determined from interpolation using calibration curves obtained the same day as the binding experiment.

The bound melittin was calculated as the difference between the total and the free melittin. The data points were fitted using the Langmuir adsorption equation [5]:

$$B = \frac{(T + E + K)}{2} \pm \sqrt{\frac{(T + E + K)^2}{4} - E \cdot T}$$

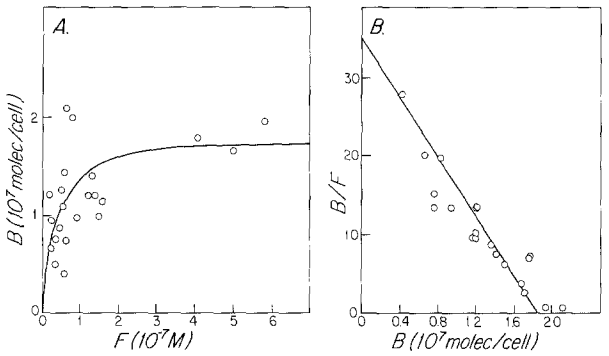

Fig. 1. Concentration dependence of melittin binding to resealed red cell membranes. (*A*) The data points are shown as molecules of melittin bound per cell (*B*) (ordinate) as a function of the free concentration of melittin (*F*) (abscissa). The points are experimental. The solid curve was calculated using the equation described in Materials and Methods, with $K = 3 \times 10^{-8}$ M and $E = 1.8 \times 10^7$ molecules/cell. (*B*) The data points are presented as a Scatchard plot where the bound melittin concentration (*B*) was determined as the difference between the total and the free (*F*)

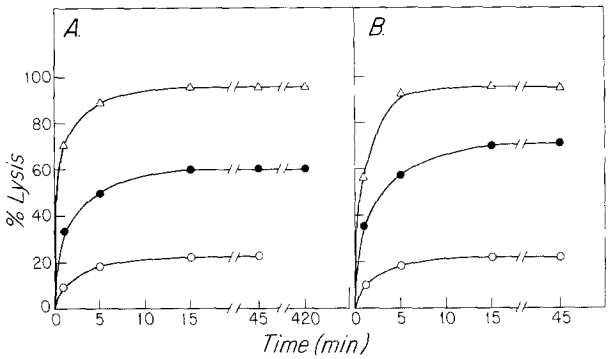

Fig. 2. Time dependence of the melittin-induced lysis. Red cells were mixed with Na buffer containing melittin and the extent of lysis determined at the indicated time periods after mixing, as detailed in Materials and Methods. (*A*) 1% cell suspension exposed to Na buffer containing the following melittin concentrations (in μM): 0.1 (○); 0.3 (●); 0.6 (△). (*B*) 1% (○); 0.1% (●) and 0.05% (△) cell suspension were exposed to Na buffer containing 0.1 μM melittin

with *B*: bound melittin; *T*: total melittin; *E*: maximum number of binding sites and *K*: apparent dissociation constant. *E* and *K* were obtained using a least-square fit of the data.

G) Cell Morphology

The morphology of the red cells incubated in the various solutions was first checked using a light microscope equipped with an oil immersion objective (American Optical, Buffalo, N.Y.). Video recordings of the melittin-treated cells were made in collaboration with Dr. D. Begg, from the Department of Anatomy, Harvard Medical School, using Nomarski differential-interference contrast optics on a Zeiss Universal microscope (Zeiss, W. Germany).

Twenty microliters of a 1% cell suspension were layered on top of a coverslip which had been pretreated with AquaSil® (Pierce Chemical Co., Rockford, Ill.) to prevent cell crenation by contact with glass. The cells were then covered with another AquaSil-treated coverslip which rested on a thin film of vacuum grease deposited on both sides of the blood sample and placed on the microscope stage, where they were illuminated with a 12V quartz-halogen light source, using a Zeiss 546 nm interference filter and a heat-absorbing filter. The cells were observed through a Zeiss 63×, N.A. 1.4 planapo objective. After choosing a field with enough red cells to observe, 100 μl of buffer containing 1.7 μM melittin was perfused through the slide. This procedure did not disturb the erythrocytes and made it possible to follow the change in the morphology of the cells in one field, as they were being modified through the interaction with melittin. Video recordings of the shape changes were made in real time using a Dage video camera equipped with a Newvicon tube (model 65, Dage-MTI, Michigan City, Ind.) and a NEC 3/4-inch cassette, time-lapse video recorder (model VC-9507, Nippon Electric Corp., Tokyo, Japan). The image was displayed on a 9-inch Panasonic monochromatic video monitor (model WV-5350, Panasonic, Secaucus, N.J.). Individual fields were photographed from the monitor on Kodak Plus-X 35 mm film using a Nikon F2 single lens, reflex camera, equipped with a Micro-Nikkor 55 mm f/12.8 macrolens. The exposure time used was 1/4 sec, with an aperture setting of f/8. The film was developed at 20°C in Kodak HC 110 developer, dilution B.

Results

Binding of Melittin to Red Cell Membranes

Figure 1 shows the results of experiments designed to determine the characteristics of the binding of melittin to resealed red cell membranes (ghosts). The amount of bound melittin increases with the concentration of free melittin in the medium until maximum binding is reached (Fig. 1*A*). Figure 1*B* shows the Scatchard plot of the data, and its linearity suggests that all of the binding sites have the same affinity for melittin. This notion is further supported by the fact that the data points can be well fitted by a binding curve calculated using the binding equation depicted in Materials and Methods with 1.8×10^7 molecules/cell as the maximum number of melittin molecules bound per cell and a value of 3×10^{-8} M for the apparent dissociation constant.

Lytic Effects of Melittin

A) Dependence on Melittin and Red Cell Concentration

Figure 2 shows the time course of the lysis induced by melittin in human red cells incubated at room

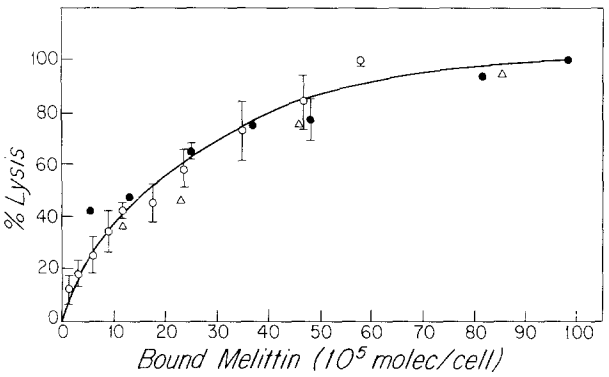

Fig. 3. Concentration dependence of the maximum level of lysis induced by melittin. The maximum level of lysis (±SD) which obtains at each melittin concentration and hematocrit is plotted on the ordinate as a function of the number of melittin molecules bound per cell. Bound melittin per cell was calculated from the known concentrations of total melittin and cells and the parameters estimated from the data shown in Fig. 1. The cell concentration in the several experiments used to prepare this figure were: 1% (○); 0.5% (△); 0.1% (●)

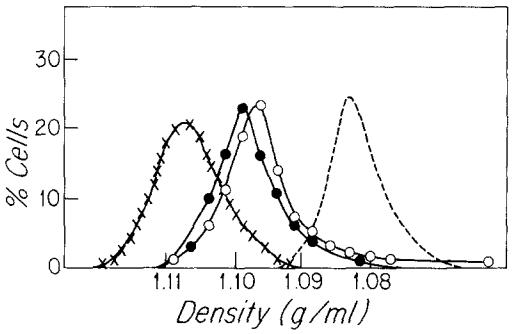

Fig. 4. Density distribution of cells. Cell density (g/ml) was determined by the phthalate method as described in Materials and Methods. Measurements were made on cells (1%) suspended in Na buffer at room temperature in the absence (●) and presence (○) of 0.2 μM melittin. The fraction of cells at each density is plotted on the ordinate as a function of the density, on the abscissa. In the case of cells exposed to melittin, 40% had lysed and the curve shows the density of the unlysed cells (60%). The mean densities of the cell population in the absence and presence of melittin were 1.098 and 1.096 (three different experiments). For comparison, density distributions are also shown for cells suspended in 0.11 (− −) and 0.19 (+ +) M NaCl, with relative volumes of 1.2 and 0.94, respectively

temperature in isotonic Na buffer. The curves in this figure show a rapid increase in the extent of lysis to a constant, maximum value which then remains unchanged for up to 7 hr. The fact that 20 to 100% of the maximum lysis occurs within the first minute of exposure to melittin indicates that the reaction of the melittin molecules with the red cell membrane leading to the hemoglobin loss is fast, as is the binding of melittin to the red cell membrane (*cf.* Materials and Methods and Fig. 7).

The maximum fraction of cells lysed at a given concentration of cells and total melittin concentration is seen in Fig. 2 to increase as the melittin concentration is increased (at constant hematocrit) and to decrease as the concentration of cells in the suspension increases (at constant melittin concentration). Figure 3 shows that this maximum fraction of cells lysed is a unique function of the melittin bound per cell. Worthy of notice is the fact that significant lysis occurs when less than 1% of the sites (or about 10^5 molecules/cell) are occupied and that 50% lysis is achieved at about 10% occupancy of the sites.

B) Partial vs. Complete Lysis of Cells

In order to determine if the graded lysis of red cells is due to partial lysis of all cells or complete lysis of some fraction of the cells, we have studied the density distribution of red cells before and after graded lysis by melittin, and the results are shown in Fig. 4. In the absence of melittin, the mean value of the distribution was found to be 1.098. In the presence

Table 1. Hemoglobin content of cells exposed to (but not lysed by) melittin[a]

Melittin (μM)	Extent of lysis	Relative hemoglobin/cell
0.1	20 ± 3	0.98 ± 0.09
0.2	40 ± 3	1.00 ± 0.10
0.6 (*)	75	0.99

[a] The hemoglobin content per cell was determined in the cells that did not lyse, after exposure to 0.1 and 0.2 μM melittin in Na buffer. The results are expressed as the ratio of the hemoglobin/cell in the cells that survived exposure to melittin to the hemoglobin/cell in cells not exposed to melittin. The results are mean values (±SD) from three different experiments, except for (*) which was only one experiment.

of 0.1 and 0.2 μM melittin, the unlysed cells were found to have a mean density of 1.096, not significantly different from the control value. Furthermore, as shown in the Table, the hemoglobin content per cell of these cells was not significantly different from that of cells which had not been exposed to melittin. These results strongly suggest that, under the conditions used in the experiments reported in this paper, graded lysis by melittin is due to complete lysis of some cells, rather than partial lysis of all cells. In the latter case, the distribution of cell densities would change, with the mean value of the distribution shifted to a lower mean density (since cells containing a lower concentration of hemoglobin would be less dense). Moreover, the value of the hemoglobin content per cell would

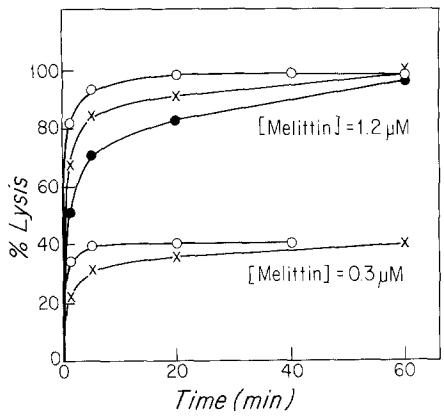

Fig. 5. Relative resistance to osmotic and melittin lysis. The figure shows the fraction of a cell suspension (1%) lysed in Na buffer containing melittin (ordinate) plotted as a function of time (min). Cells had been preincubated in Na buffer (300 mOsm, ○) or in Na buffer diluted to 143 mOsm (×) or to 138 mOsm (●). In the latter two cases, 30 and 78% of the original cells lysed osmotically, and the melittin lysis experiment was performed on the unlysed cells. See Materials and Methods and text for further details

also be reduced, since all cells would contain less hemoglobin after exposure to melittin.

C) Inhomogeneity in the Lytic Threshold of Melittin

In view of the results discussed in the previous section, the graded lytic response to melittin might arise either from a dispersion in the amount of melittin bound per unit area of membrane surface in different red cells, or from a difference in the susceptibility of cells to the action of melittin, with different cells in the suspension having different thresholds in the concentration of bound melittin necessary to produce lysis.

In this section we explore the possibility that the graded lytic response to melittin is due to a distribution in the susceptibility to lysis by melittin and will assume, for the sake of simplicity, that all the cells in the suspension can and do bind the same amount of melittin. In this case, changing the hematocrit at constant melittin/cell ratio should result in the lysis of the same proportion of cells, independent of the hematocrit. The results depicted in Fig. 3 indicate that this is what occurs when cells are lysed by melittin.

In order to determine if the sensitivity to lysis by melittin and by hypotonicity are correlated, we exposed cells to graded hypotonic lysis and tested the sensitivity of the surviving, more resistant cells to subsequent lysis by melittin. The results of this experiment, shown in Fig. 5 indicate that the graded lytic response to melittin is not related to the resistance of erythrocytes to lysis by hypotonic solutions, since the lytic threshold to melittin was the same for all of the different cell populations tested. Noticeable in Fig. 5 is the fact that the kinetics of lysis is changed, with the more resistant cells taking longer to lyse to the same level, perhaps because they are still shrunken. Bowdler *et al.* [2] have reported similar observations on the sensitivity of cells to hypotonic lysis.

D) Size of Melittin-Induced Lesion in the Red Cell Membrane

In order to determine the nature of the lesion produced in the red cell membranes as a consequence of the interaction with melittin and which leads to the lysis of cells, we have studied the movement of ions in cells exposed to various melittin concentrations. If melittin forms pores big enough for hemoglobin to go through, the cells should exchange their ionic content with the external medium at the same time or only shortly before the hemoglobin is lost from the cells. If, on the other hand, melittin induces an increase in the ionic permeability of the cell membrane via a small pore or some other pathway which excludes hemoglobin, leakage of the cell's small ions (Na, K, etc.) should precede lysis. Further, this increase in cation permeability could also lead to an increase in the cellular water content by colloid osmosis, thus resulting in the eventual lysis of the cell, if the cell volume reaches the critical hemolytic value.

The results shown in Fig. 6 indicate that, as a result of the interaction with melittin, there is an increase in the permeability of the red cell membrane to cations, as indicated by the net K loss and the net Na gain of the unlysed cells. Further, the permeability of the unlysed red cells for either of the cations was found increased 200- to 2000-fold in the first minute of exposure to melittin, depending on the concentration of melittin in the suspension. This increase in permeability of the unlysed cells was found to be transitory. The Na, K and total cation contents of the unlysed cells remained at the values obtained after the first minute for the remainder of the experiment (2 to 7 hr), while the lysis would take between 10 and 20 min to reach its maximum level. These results suggest that the melittin-induced lysis of red cells is an effect secondary to the formation of ion-permeable, hemoglobin impermeable pores. This conclusion is further supported by the observation that polyethylene glycol (mol wt 1,000, 10 mM) and dextran (10 kilodalton, 10 mM) but not raffinose or glucose inhibit melittin-induced

lysis (*data not shown*). Since resealed ghosts retain ^{14}C-inulin when exposed to melittin (*cf.* Materials and Methods), it appears that the channels produced by melittin in red cells have an effective sieve diameter that is smaller than the hydrodynamic diameter of inulin but larger than that of raffinose.

Direct observation of the erythrocytes when exposed to lytic concentrations of melittin confirmed the notion that the cells swell prior to lysis (*cf.* Fig. 7). Further, as illustrated in this figure, upon exposure to melittin, the red cells first seem to crenate, followed by what looks like a slight bulging of the membrane and swelling. Subsequently, the cells seem to burst on one side and to be simultaneously thrown aside by the reaction force and to lose the internal contents. Further seen in Fig. 7 is the fact that not all of the cells in one field lyse at the same time. Rather, some cells lyse early and others retain their spherical shape for several seconds and, in some instances, even minutes (*not shown*). Thus, the time it takes for all the cells that are going to lyse, to do so can be several minutes, consistent with the results shown in Fig. 2.

In the light of these observations, it is reasonable to propose that the amphiphile, melittin, first interacts with the outer half of the bilayer producing expansion of this layer relative to the cytoplasmic half, and thus inducing crenation. Subsequently, as melittin spans the bilayer forming ion-permeable pathways, it also interacts and expands the inner half of the bilayer and causes the cell to form a cup-shape. As the permeability to cations is increased, the cell loses K and gains Na and water, swells and, if the hemolytic volume is achieved, lyses (i.e. Fig. 7). Thus, it seems that melittin's interaction with the red cell membrane may be another example of an amphipath's interaction with the bilayer affecting the cell shape through a bilayer couple mechanism [20].

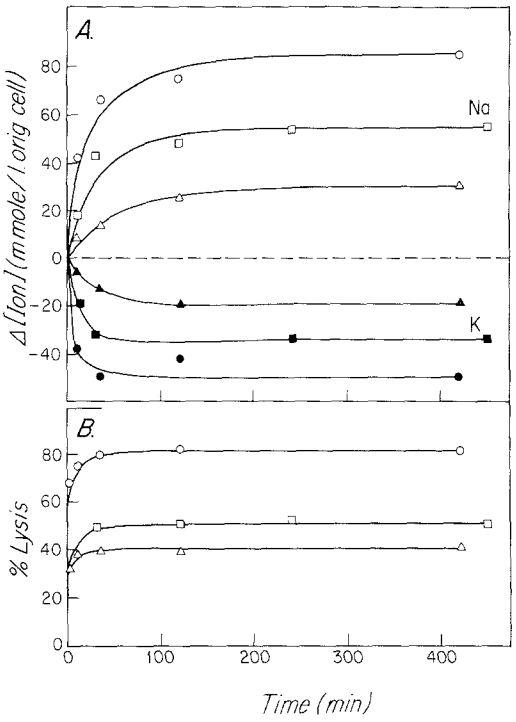

Fig. 6. Net ion movements induced by melittin. Cells (1%) were incubated in Na buffer containing 1.5 (△, ▲); 3.0 (□, ■) and 6.0 (○, ●) μM melittin for up to 7.5 hr. Two 1.5-ml samples were withdrawn at the indicated time points and the lysis and change in internal content of Na (open symbols) and K (closed symbols) were determined as indicated in Materials and Methods. (*A*) The change in ion content (ordinate) was calculated as the difference between the internal sodium (potassium) of the cells before treatment and is plotted as a function of the time the cells were incubated in the melittin-containing Na buffer. The curves were obtained in two different experiments. (*B*) The fraction of cells lysed is plotted as a function of the time they remained in the melittin-containing Na buffer

Effect of Ions on the Lysis Induced by Melittin

Figure 8 shows the effect that partial or total replacement of Cl by multivalent anions has on the lysis induced by melittin. Replacement by phosphate or citrate reduces the maximum extent of lysis, without much effect on the rate of lysis at a given membrane concentration of melittin. The reduction in the effect of melittin by these anions is probably due to their influence on the conformation of melittin in the aqueous phase, through their binding to the positively charged residues, as suggested by Podo *et al.* [15].

Shown further in Fig. 8 is the inhibition of the lysis induced by melittin due to the presence of large organic anions in the suspension. The most potent of these molecules seems to be H_2-DIDS, which when present in equimolar amounts produces 100% inhibition of the lytic effect of melittin. The effect of H_2-DIDS on the melittin-induced lysis was seen both in untreated cells as well as in cells which had been pre-exposed to 2×10^6 H_2-DIDS molecules per cell, to inhibit the anion transport system of the red cell [9]. The action of DIDS, as well as that of hemoglobin (Hb) and albumin (Alb) seems to be through their association with melittin, thus producing a substantial reduction in the free melittin concentration.

In contrast to the results described above, partial or total replacement of Na by choline or potassium (chloride salts) failed to produce an effect on the lysis produced by melittin. We also found no change in the toxin's effects when cells were de-

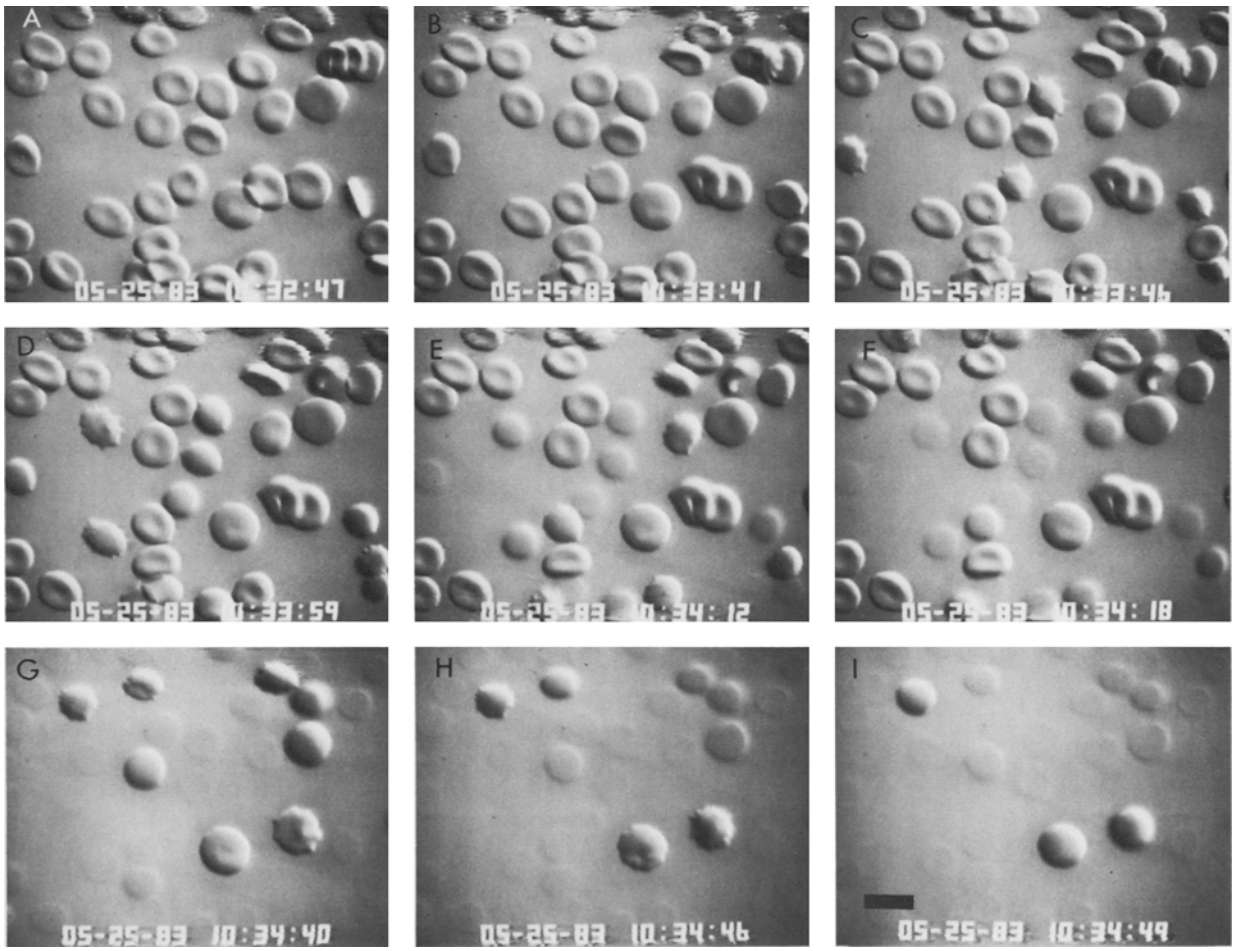

Fig. 7. Red cell shape changes induced by melittin. A 1% cell suspension in Na buffer was placed on the microscope stage and subsequently perfused with Na buffer containing melittin, as detailed in Materials and Methods. (*A*) Red cells just before exposure to melittin; (*B–I*) Show the same field after exposure of cells to 1.7 μM melittin. The time shown on the frames in actual time. The mellitin solution was made to flow past the cells at 10:32:50. Two minutes after, all cells in the field had lysed. Bar indicates 10 μm

pleted of internal Ca (by preincubation of cells in Na buffer containing the calcium ionophore A23187 and EGTA) and subsequently exposed to melittin.

Discussion

The experimental results reported in this paper confirm and extend previous reports that melittin binds tightly to the human red cell membrane. Bound melittin interacts with itself and/or with membrane molecules in such a way as to produce channels that become large enough to permit the passage of hemoglobin, resulting in lysis [7, 11, 18, 19].

Our measurements indicate that there are about 1.8×10^7 melittin binding sites per human red cell membrane, in agreement with previously reported estimates [7]. When all of these sites are occupied, this yields an area/bound melittin molecule of 79 nm, a value similar to that observed by Schoch and Sargent for melittin bound to planar lipid bilayers [17]. Under these conditions of maximum binding, the ratio of the number of membrane lipids to bound melittin molecule is about 10 (*cf.* [8] and [21]). The relatively large number of melittin binding sites on the red cell membrane and the similarity between the surface density of binding sites on red cell membranes and planar lipid bilayers, makes it unlikely that the melittin binding sites are specialized receptors, but, rather, regions of the lipid bilayer that are relatively nonspecific.

The apparent dissociation constant for melittin binding to the human red cell membrane, derived from our experiments is 30 nM. This figure agrees with previous estimates in red cells and lipid vesicles [7, 8, 21], further supporting the conclusion

that melittin's binding to the red cell membrane is an interaction between two kinds of amphiphilic molecules: melittin, on the one hand and the membrane lipids on the other.

Channels occur when only a small fraction of melittin binding sites are occupied. Evidence for this conclusion comes from the observation that lysis of red cells is observed when only about 1/100 of the sites are occupied and that 50% lysis occurs at 10% occupancy. Indeed, increased sodium permeability is observed when only 1/1000 of the sites are filled by melittin. Under these circumstances, about 2×10^4 molecules/cell are bound, comparable to the number of bound molecules required to produce increased permeability and lysis by other toxins [11].

Not only does channel formation occur when a small fraction of red cell membrane melittin binding sites are occupied, but also only a small fraction of bound melittin molecules are likely to be involved at a particular moment in the formation of the open channels through which salt and water can move. This conclusion is suggested by observations of the channels induced in planar phospholipid bilayers by melittin [12, 24]. In this system, single-channel conductances have been reported to vary from 0.01 to 2 nS, depending on experimental conditions, such as lipid/melittin ratio, ionic strength of the medium and lipid composition. This means that the ionic flux through one channel could vary from about 3×10^{-18} mole/sec to 6×10^{-16} mole/sec. Thus, if there were only one open channel/red cell, it would take from 2×10^4 to 25 sec for complete cation equilibration (1.5×10^{-14} moles). Consequently, if one cell were to have two of the largest-sized channels open for one sec, it would gain enough cations to reach hemolytic volume and lyse. Considerable evidence supports the idea that it requires at least 4 melittin molecules to form an open channel in planar phospholipid bilayers. Even if it required 100 melittin molecules to make lytic channel(s) in red cell membranes, less than 1% of the bound melittin need be involved in forming open channels.

Evidence reported in this paper supports the view that melittin induces lesions in the red cell membrane that permit the transport of molecules with an effective hydrodynamic radius equal to or smaller than raffinose, but do not allow movement of molecules larger than inulin. The retention of ^{14}C-inulin by resealed ghosts exposed to melittin and the inhibition of melittin-induced lysis of red cells support the conclusion that melittin-induced channels are small enough to exclude these molecules. The failure of raffinose or sucrose to prevent melittin-induced lysis and the partial equilibration of Na and K (*cf.* Fig. 6) indicate that these substances can penetrate melittin-induced channels, and is consistent with a colloid osmotic mechanism of melittin-induced lysis [23, 26]. This conclusion is also supported by the microscopic observations (Fig. 7) that cells exposed to melittin invariably swell prior to lysis.

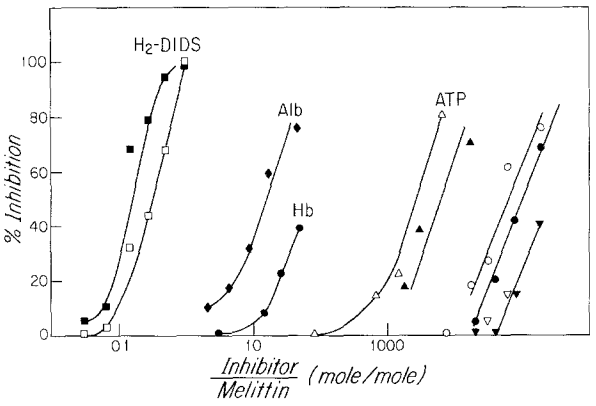

Fig. 8. Effect of anions on the melittin-induced lysis. Cells (1%) were exposed for 45 min to Na buffer containing melittin (0.4 μM, closed symbols; 0.8 μM, open symbols) and: H_2-DIDS (□,■); serum albumin (Alb, (♦); hemoglobin (Hb, ●) and ATP (△,▲) or to melittin-containing Na buffer in which Cl was partially replaced by citrate (○,●) or phosphate (melittin▽,▼) as detailed in Materials and Methods. The extent of lysis was measured at each molar ratio and plotted on the ordinate relative to the extent of lysis which obtains (at 45 min) when cells are incubated in Na buffer containing melittin. The abscissa, in logarithmic scale, indicates the molar ratio of anions to melittin to which the cells were exposed

The melittin-induced lesions in human red cell membranes are transient. Figure 2 and Fig. 6 taken together affirm that both lysis and increased permeability to Na and K occur during the first few minutes after red cells are exposed to melittin and then subside. The basis for this transient effect of melittin is not clear. It could be due to binding of melittin to hemoglobin and other intracellular anions (*cf.* Fig. 8 and [14]), or to enzymatic cleavage of melittin, or to some other mode of inactivation comparable to that observed in the effect of melittin on the conductance of planar phospholipid bilayers [25].

The graded lysis shown in Fig. 2 and the evidence shown in Fig. 4 and in the Table proving that the graded lysis is the result of complete lysis of some cells rather than partial lysis of all cells, means that human red cells vary in their susceptibility to melittin-induced lysis. The basis for this heterogeneity in the cell population is not clear. The fact that the same results were obtained with relatively slow (1 to 2 sec) and relatively fast (10 to 20 msec) mixing speaks against the possibility that inhomogenous mixing leads to differences in the magnitude of binding of melittin to different cells. Furthermore, resistance to melittin-induced lysis does

not seem to correlate with resistance to osmotic lysis (Fig. 5). Further investigation will be necessary to resolve this issue.

We thank Dr. D. Begg for the use of his microscope and his expertise. This work was supported by a National Institutes of Health grant GM 25277.

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Received 15 April 1985