KINETICS AND MECHANISM OF HEMOLYSIS INDUCED BY MELITTIN AND BY A SYNTHETIC MELITTIN ANALOGUE

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ABSTRACT   The cytotoxic peptide from honeybee venom, melittin, and a synthetic peptide analogue of it lyse human erythrocytes in a biphasic process. The kinetics of of the lysis in 0.30 M sucrose, 0.01 M sodium phosphate, pH 7.30 at 4°C were investigated. Our results show that melittin rapidly binds to the outer surface of the erythrocyte membrane, and the surface-bound monomers produce transient openings through which ~ 40 hemoglobin molecules can escape. Concomitantly, the melittin loses its ability to effect the process, presumably by translocation through the bilayer. The half-life for this process is 1.2 min. In a much slower process, dimers of this internalized melittin again produce transient membrane openings in a steady state. On a molar basis, the synthetic peptide analogue produces a fast process comparable to that caused by melittin, but is more efficient in the slow phase. Escape of hemoglobin and of carbonic anhydrase through the openings is diffusion controlled. These results suggest that the functional units necessary for the activity of melittin-like cytotoxic peptides are a 20 amino acid amphiphilic α-helix with a hydrophobic:hydrophilic ratio > 1 and a short segment with a high concentration of positive charges.

INTRODUCTION

We are currently engaged in assessing the role of the amphiphilic α-helix as a structural feature which effects or improves the binding of peptides and proteins to amphiphilic surfaces. The occurrence of this structural unit was first demonstrated in apolipoproteins, including apolipoprotein A-I (apo A-I) from human plasma high density lipoprotein-3. Apolipoproteins, the protein constituents of plasma circulating lipoproteins, stabilize the surface of the triglyceride/cholesterol ester core of these particles (1). Portions of these apolipoproteins are intercalated among the phospholipid head groups of the surface monolayer, which surrounds the lipid core (2), and, when in the surface-bound state, most apolipoproteins are devoid of tertiary structure (3). From the known amino acid sequence of apo A-I it has been proposed that the secondary structure responsible for its interfacial stability is the amphiphilic α-helix (4, 5). Indeed, at least six 20-amino acid segments have high helix-forming potential and, when in the α-helical form, would segregate the hydrophilic and hydrophobic amino acid side chains on opposite faces of the cylindrical helix, one-third of the residues being hydrophobic (6). To test this hypothesis, we have synthesized a docosapeptide of high amphiphilic α-helical potential with
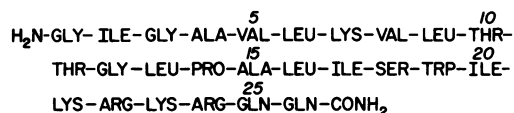
approximately one-third of the residues hydrophobic and with minimal homology to any of the proposed helical segments of apo A-I (7). This peptide's surface affinities and ability to activate lecithin:cholesterol acyltransferase (LCAT) (8) are comparable to those of apo A-I. Recent observations suggest that short amphiphilic α-helical segments are not unique to apolipoproteins, but may play an important structural role in many polypeptides with functions as diverse as those of the peptide hormones[1] (9, 10) and cytotoxins (11–13). This suggested to us that the amphiphilic α-helix may play a key role in the binding to cell membrane surfaces as well as to lipoprotein-water interfaces. In particular, the biologically active conformation of melittin, a cytotoxic peptide from bee (*Apis mellifera*), has been proposed to include an amphiphilic α-helix (Fig. 1) (14). The amphiphilic α-helical portion would include the N-terminal segment of the molecule, extending perhaps to residue 21 with a "kink" at the proline-14 residue. Most interestingly, two-thirds of the first 21 residues are hydrophobic.

Our method of testing the structural role of amphiphilic α-helical segments with synthetic nonhomologous analogues could be applied here to probe the function of the N-terminal region of melittin. At the same time an amphiphilic α-helical peptide with a hydrophobic:hydrophilic

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[1]Taylor, J., D. Osterman, R. Miller, and E. T. Kaiser. *J . Am. Chem. Soc.* In press.
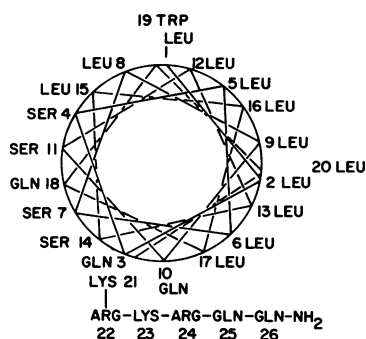
MELLITIN I

H₂N-GLY- ILE-GLY-ALA-VAL-LEU-LYS-VAL-LEU-THR-

Sequence numbers 5, 10 above

THR-GLY-LEU-PRO-ALA-LEU-ILE-SER-TRP-ILE-

Sequence numbers 15, 20 above

LYS-ARG-LYS-ARG-GLN-GLN-CONH₂

Sequence number 25 above

PEPTIDE I

H₂N-LEU-LEU-GLN-SER-LEU-LEU-SER-LEU-LEU-GLN-

Sequence numbers 5, 10 above

SER-LEU-LEU-SER-LEU-LEU-LEU-GLN-TRP-LEU-

Sequence numbers 15, 20 above

LYS-ARG-LYS-ARG-GLN-GLN-CONH₂
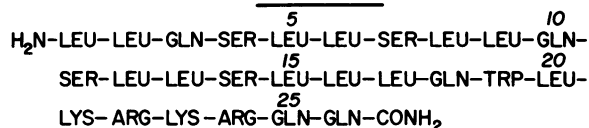
Sequence number 25 above

FIGURE 1 The amino acid sequences of melittin (from reference 20) and the amino acid sequence and helical projection for the melittin analogue, peptide I.

ratio of 2:1 could provide valuable information concerning the role of the hydrophobic-hydrophilic balance in the interaction of amphiphilic peptides with phospholipid mono- and bilayers. For the above reasons we designed the amphiphilic α-helical melittin analogue peptide I, shown in Fig. 1 (14). Residues 1–20 were chosen to form an amphiphilic α-helix with minimal homology to melittin, while maintaining the hydrophobic:hydrophilic ratio of 2. The hydrophobic face of the helix was composed of leucine residues chosen for their high helix-forming potential and hydrophobicity. While glutamine seemed ideal for the hydrophilic residues, some serine residues were also included, allowing us to match the hydrophilicity of native melittin. A tryptophan residue was left at position-19 for further studies of the intrinsic fluorescence of the peptide, and the C-terminal hexapeptide — thought to be essential for hemolytic action — was retained. This peptide was synthesized and purified as previously described (14). Its hemolytic activity, as measured by a 30-min incubation assay (15), was found to be somewhat greater than that of melittin. Peptide I has a higher surface affinity than native melittin, suggesting that the model peptide has a more extended helical segment than melittin does and that this is indeed important for cell lysis. Since the hemolysis must comprise at least a binding and a lytic step, the complete elucidation of the factors responsible for the higher specific activity of peptide I called for a kinetic analysis of the interaction of the peptides with erythrocyte

membranes. For the quantitation of the time-course of hemolysis we used the technique of rapid membrane filtration (16, 17). In the present paper we provide evidence that hemolysis occurs by the same mechanism for melittin and the melittin analogue, peptide I. Both peptides cause a biphasic release of hemoglobin (Hb) from the erythrocytes. Our results are consistent with a rapid partial release effected by the peptides bound on the surface of the erythrocyte, and this very same process is coupled to the penetration of melittin into the membrane; a second, slower release of the cytoplasmic contents is then caused by the dimer of membrane-bound melittin. On a molar basis, peptide I and melittin produce comparable fast phases, but the former is more efficient in the slow lysis.

MATERIALS AND METHODS

Melittin free from phospholipase A₂ (as measured by a sensitive enzymatic assay employing radioactivity) was the generous gift of Professor G. Kreil. It was further purified by reverse phase high pressure liquid chromatography (HPLC) using a DuPont Zorbax C₈ column (4.6 × 250 mm) (DuPont Instruments, Wilmington, DE) with 0.1% phosphoric acid, 0.1 M NaClO₄ and 56% (vol/vol) acetonitrile as the eluting solvent. Melittin purchased from Sigma (Sigma Chemical Co., St. Louis, MO) was also purified by HPLC as above (Fig. 2). The purified melittin was homogeneous by criteria of amino acid analysis, HPLC, and thin-layer chromatography using silica gel plates (Whatman LK5DF, Pierce Chemical Co., Rockford, IL) with n-butanol:acetic acid:pyridine:water (15:3:9:12 [vol/vol]) as the eluent and ninhydrin-collidine for detection. The synthesis and purification of the melittin model, peptide I, have been reported (14). Concentrations of melittin stock solutions were determined in duplicate by automated amino acid analysis.

Erythrocyte suspensions were prepared either from blood freshly drawn into Na₄EDTA from healthy subjects, or from recently outdated citrate-stabilized blood from the University of Chicago Blood Bank. In both cases, 5 ml whole blood was centrifuged for 10 min at 6,000 rpm employing a Sorvall-type M rotor (DuPont Instruments-Sorvall, Newtown, CT); the serum and buffy coat were removed by aspiration. The packed cells were resuspended in 0.30 M sucrose, 0.01 M sodium phosphate buffer (pH 7.30) and recentrifuged. This was repeated twice and then the washed cells were suspended in 0.30 M sucrose, 0.01 M sodium phosphate (pH 7.30) and their concentration determined using a
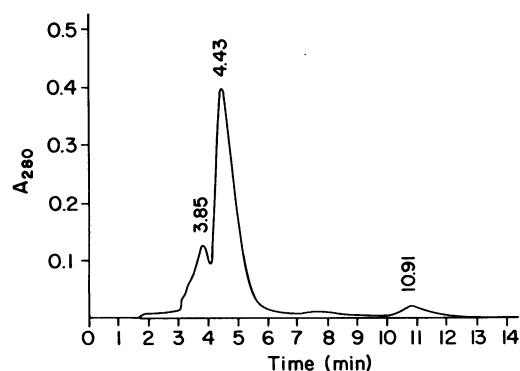

FIGURE 2 Purification of melittin by HPLC. A 10 μl aliquot of a solution of melittin (Sigma, 50 mg/ml) in a mixture of 0.1% phosphoric acid, 0.1 M NaClO₄ (44%) and acetonitrile (56%) (vol/vol) was injected using the conditions described in the Methods section.

Coulter counter model Z_b equipped with a Coulter Channelyzer (Coulter Electronics Inc., Healeah, FL). Sealed erythrocyte ghosts were prepared by the method of Steck and Kant (18).

Ficoll was purchased from Sigma Chemical Co., St. Louis, MO. Filters (2.5 cm, 3 μm pore), prefilters (2.5 cm), and Swinnex adapters for rapid membrane filtration were from Millipore (Millipore Corp., Bedford, MA). Water was deionized through a mixed bed ion exchanger. All other chemicals were of the finest quality commercially available. All buffers were filtered through a 0.45 μm pore α-Metricel GA6 filter immediately before use.

The kinetics of release of hemoglobin were measured by a rapid filtration method (16, 17). The hemoglobin content in the filtrate was determined spectrophotometrically from the absorbance at 578 nm. Melittin was monomeric (> 99% monomer) at the concentrations used in our experiments (14).[2] The concentration of carbonic anhydrase was measured by a stopped-flow acidimetric assay (19) using the indicator Bromthymol Blue at pH 7.3 and monitoring the absorbance at 630 nm using a Durrum-Gibson stopped-flow spectrophotometer (Durrum Instrument Corp., Sunnyvale, CA).

The extent of partial hemolysis was determined by monitoring the sedimentation velocity of the erythrocytes in a preformed linear density gradient of 0–40% Ficoll/0.30 M sucrose, 0.01 M sodium phosphate, pH 7.30 in 1 × 15-cm test tubes. Erythrocytes (1 × 10^{11} cells/liter) were incubated with melittin (9.2 × 10^{-7} M) for 10 min at 4°C. 3 ml of this suspension was layered on the Ficoll gradient, and the tubes were centrifuged at 1,600 rpm for 15 min at 4°C using a Sorvall RC-3 centrifuge equipped with an HG-4L rotor. The centrifuged solutions were eluted from tubes using a Buchler Auto Densiflow II (Buchler Instruments, Inc., Fort Lee, NJ), and the hemoglobin content was monitored after addition to a final volume of 100 μg/ml of melittin to cause complete lysis of the cells. The binding of melittin to erythrocyte ghosts was measured in 0.30 M sucrose, 0.01 M sodium phosphate, pH 7.30. The ghosts were incubated with melittin for 5–10 min at 4°C, then centrifuged at 18,000 rpm for 10 min at 4°C using a Sorvall RC-5B centrifuge equipped with an SS-34 rotor. The amount of melittin in the supernatant was determined by a calibrated hemolytic assay after a 45-min incubation with erythrocytes at 37°C.

To show that the time dependence of the hemoglobin release is not due to the slow binding of melittin to the erythrocytes, we incubated erythrocytes (5 × 10^{10} cells/liter) with melittin (3.3–6.7 × 10^{-7} M) for 15 s. 3 ml of the suspension was then filtered in the usual manner; the collected cells were rapidly washed with 2 ml of 0.30 M sucrose, 0.01 M sodium phosphate buffer, pH 7.30 and then incubated for an additional 5 min. The hemoglobin released during the latter period was eluted with 3 ml of the above buffer. The hemoglobin content in the combined washes was 80 ± 26% of that released without filtration.

RESULTS

Monitoring the time-course of hemoglobin release from erythrocytes treated with melittin or peptide I, we observed that the reaction is biphasic under a wide range of concentrations, ionic strengths, and temperatures (Fig. 3). We chose to study the reaction in an isotonic solution of 0.30 M sucrose, 0.01 M sodium phosphate, pH 7.30, at 4°C, as we found that this combination of low ionic strength and low temperature gave optimal reproducibility with the added advantage of slowing the reaction rate to one easily measured by our technique.

Observation of the erythrocytes exposed to melittin or peptide I under the light microscope indicated that the identity of the erythrocytes is maintained during the lytic process. Because all our experiments were carried out with dilute erythrocyte solutions, the total hemoglobin content
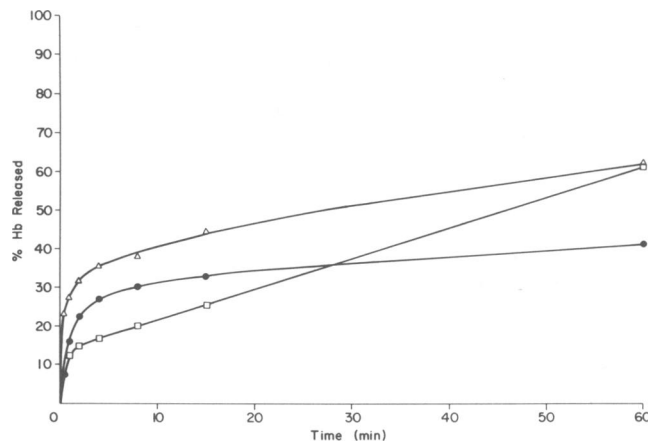

FIGURE 3 The time dependency of hemoglobin release induced by melittin at various temperatures. An erythrocyte suspension (5.21 × 10^{10} cells/l) was incubated with melittin (4.58 × 10^{-7} M) at 4°C(-●-); 22° (-△-), and 37°C (-□-).

of the cell was released at the end of the reaction, within our experimental error. To characterize the population of partially lysed cells at the end of the rapid phase, we determined, by rate zonal density gradient centrifugation, the density distribution of the erythrocytes exposed to an amount of melittin yielding 40% hemoglobin release in the fast phase (10 min). The average density of the cells decreased considerably and the variance of the density of the partially lysed cells was not any larger than that of the unlysed erythrocytes. From these data we conclude that (a) all erythrocytes are partially lysed at the end of the rapid phase; (b) the decay of the rate of hemoglobin released in the fast phase cannot be due to random closure of a single large opening in the membrane since the latter mechanism would result in broadening of the peak; (c) all erythrocytes must release approximately the same amount of hemoglobin during the fast phase. Thus, the membranes must have remained open to the same extent for each cell.

By varying the concentration of melittin and that of the erythrocytes, we observed that the initial rate of hemoglobin release expressed in mol liter^{-1} min^{-1} is directly proportional to the melittin concentration but independent of the erythrocyte concentration. The total amount of hemoglobin released in the fast phase is also proportional to the melittin concentration and independent of the erythrocyte concentration as long as the total cellular hemoglobin content is not greatly depleted during the fast phase. In contrast, the initial rate of the slow phase is proportional to the square of the melittin concentration and to the inverse of the erythrocyte concentration. The rate of release and the quantity of hemoglobin released per cell can be calculated from the rates and extents of release by dividing them by the erythrocyte concentration. Thus, the rate and extent of release of hemoglobin per cell in the fast phase are proportional to [melittin]/[erythrocyte] and the rate of release of hemoglobin per cell in the slow phase
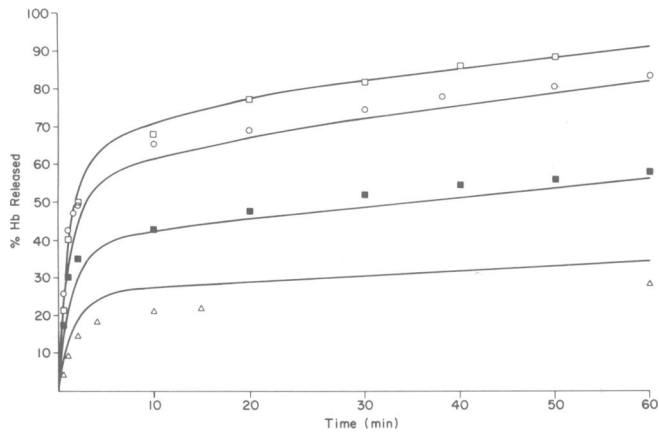
FIGURE 4 The time dependence of hemoglobin release caused by melittin at various concentrations at a fixed erythrocyte concentration. Erythrocytes (5×10^{10} cells/liter) were incubated with melittin; 2.20×10^{-7} M (-△-); 4.67×10^{-7} M (-■-); 7.01×10^{-7} M (-○-); and 9.34×10^{-7} M (-□-).

is proportional to ([melittin]/[erythrocyte])^2 (Figs. 4 and 5). The time dependency of the decay of the fast rate to the slow rate appears to correspond to a first order reaction. The slow phase also appears to be first order and must correspond to the depletion of hemoglobin as total release is exponentially approached at long times. Furthermore, the half life of the fast reaction seems to be independent of the melittin and the erythrocyte concentrations. We observed in almost all cases a slight induction period before the fast phase (of the order of ~ 15 s) which was too short to be explored by our method. At the end of the fast reaction the ghosts were collected by centrifugation, the pellet was dissolved in glacial acetic acid and analyzed for melittin by the thin layer chromatographic method described in Methods. By comparing the R_f and intensity of the bands obtained in this experiment with those of known quantities of melittin, we found that most of the
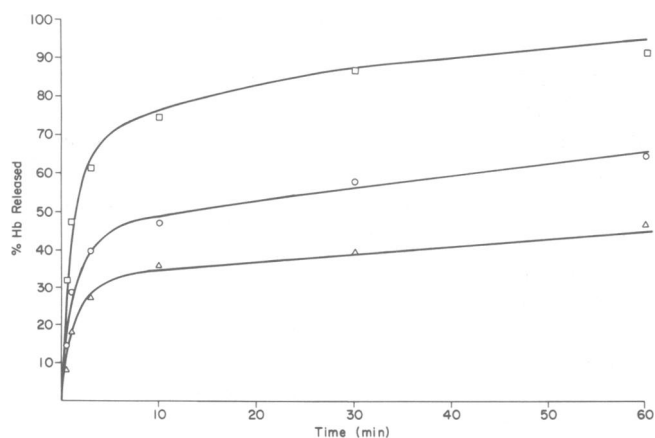

FIGURE 5 The time dependence of hemoglobin release induced by melittin at several erythrocyte concentrations. Incubation mixture containing 6.67×10^{10} cells/liter (-△-); 4.36×10^{10} cells/liter (-○-); and 2.20×10^{10} cells/liter (-□-); were treated with 4.64×10^{-7} M melittin.

melittin was recovered unchanged from the cell membrane.

The rate of hemoglobin release per cell in the fast and slow phases was dependent on the [melittin]/[erythrocyte] and ([melittin]/[erythrocyte])^2, respectively, indicating that perhaps surface bound melittin and its dimer were the species responsible for hemolysis. Indeed, melittin binds readily to the erythrocyte and to sealed erythrocyte ghosts. Under our experimental conditions, the binding to the ghosts could be described by a simple Langmuir isotherm (8), and analysis of the curve gave a dissociation constant on the order of 1×10^{-7} M and a limiting quantity of melittin bound of 2×10^7 molecules per ghost. Assuming that the area of the bound melittin is the same as that at the air-water interface, this corresponds to 45% of the outer erythrocyte surface. These values indicate that in all our kinetic experiments, at least 90% of the melittin became bound to the membrane. This binding appears to be very rapid with respect to hemoglobin release and essentially complete within 15 s, because erythrocytes which were treated with melittin for 15 s and then filtered and washed continued to release hemoglobin at $80 \pm 26\%$ of the rate of the unfiltered cells. Finally, that the melittin is quantitatively and tightly bound is further supported by the observation that addition of a second aliquot of erythrocytes after the fast phase produced no observable change in the reaction kinetics. On the other hand, addition of a second aliquot of melittin produced a second fast phase comparable to the first one and of the same half-life (Fig. 6).

The above observations suggest that (a) all of the melittin from the solution is rapidly bound to the erythrocyte surface and that a maximum total area available for the diffusion of Hb from the cell is proportional to this surface-bound melittin; (b) the surface-bound melittin and the permeable area associated with it disappear by a first-order decay reflected by the first phase. After losing its ability to lyse the erythrocyte rapidly, melittin is still chemically unchanged and associated with the erythrocyte. This then indicates that in the fast-phase melittin either undergoes a conformational and/or topographical change; (c) after translocation of the melittin in the fast phase, the membrane openings are proportional to the square of the membrane-associated melittin. Such a dependency is clearly observed in the slow phase shown in Fig. 6 when the doubling of the melittin concentration yields a fourfold increase in the rate of the slow phase.

The simplest reaction pathway able to account for all of the above observations (i.e., one with the minimum number of steps consistent with the experimental observations) is shown in Scheme 1 below, where M_s is the concentration in mol/cell of melittin bound to the outer

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[2]DeGrado, W. F., G. F. Musso, M. Lieber, E. T. Kaiser, and F. J. Kézdy. Unpublished results.
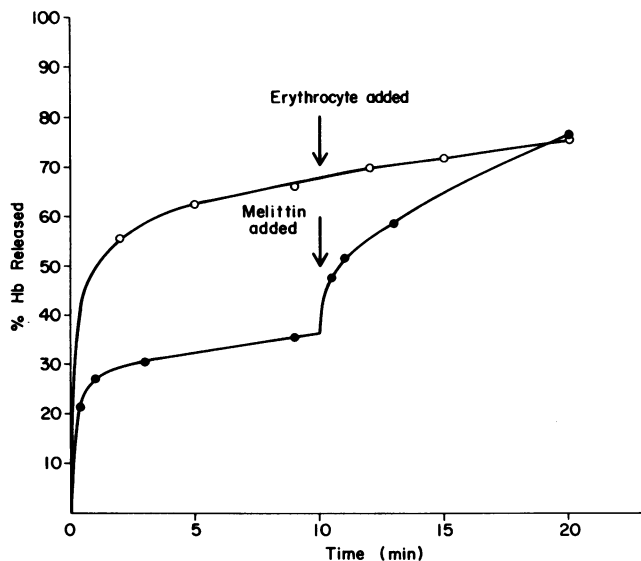
FIGURE 6 The effects of addition of erythrocytes and melittin during the "slow" phase. Melittin (2.93×10^{-7} M) was incubated with erythrocytes (5.4×10^{10} cells/liter) for 10 min. Melittin was then added to give a final concentration of 5.85×10^{-7} M (-●-). Erythrocytes (2.7×10^{10} cells/liter) were treated with melittin (5.85×10^{-7} M) for 10 min. Erythrocytes were then added to give a final concentration of 5.4×10^{10} cells/liter (-○-).

Scheme 1

$$M_s \xrightarrow[\text{slow}]{k_0} M_h \xrightarrow[\text{fast}]{k_z} M_i \xrightleftharpoons{K_2} M_{i_2} \xrightleftharpoons{K_3} M_{i_2}^h$$

In the derivation of the kinetic equations corresponding to the above scheme, the following assumptions will be made: (a) the rate of release of hemoglobin from the cell is a diffusion-controlled process, being proportional to the apparent area per cell of the openings in the membrane, S_{Tf}, the diffusion coefficient of hemoglobin, D, the concentration of hemoglobin in the cell, Hb_c, the inverse of the membrane thickness, δ, and that of the cell volume, V_c, i.e., $-dHb_c/dt = (D/V_c\delta) Hb_c \cdot S_{Tf}$ (21); (b) the area available for diffusion from the cell is proportional to both M_h and to $M_{i_2}^h$, i.e., $S_{Tf} = \alpha M_h + \beta M_{i_2}^h$; (c) we assume a steady-state for M_h, i.e., the openings generated by the melittin monomer on the outer surface have a very short life-time. From this assumption it follows that $M_h = k_0/k_z M_s$; (d) because the slow phase is second-order with respect to melittin, we also assume that M_{i_2} and $M_{i_2}^h$ are both much smaller than M_i. In other words, $K_2K_3 = M_i^2/M_{i_2}^h >> 1$. With the help of these assumptions we find that $M_s = M_{s_0}e^{-k_0t}$ and $M_i = M_{s_0}(1 - e^{-k_0t})$. Therefore,

$$S_{Tf} = \alpha \frac{k_0}{k_z} M_{s_0}e^{-k_0t} + \beta \frac{M_{s_0}^2}{K_2K_3} [1 - e^{-k_0t}]^2.$$

Because the initial rate of the fast phase is much larger than that of the slow phase, $\alpha k_0/k_z M_{s_0}$ is between one to three orders of magnitude larger than $\beta M_{s_0}^2/K_2K_3$. Then, the square of the exponential in the second term can be approximated by $1 - 2e^{-k_0t}$. With this simplification, the integration of the equation expressing the velocity of the

surface of the erythrocytes, M_h is the concentration in mol/cell of melittin associated with openings in the membrane, M_i is the concentration of melittin which has penetrated the membrane, M_{i_2} is the concentration of the reversible dimer thereof, and $M_{i_2}^h$ is the concentration of the pentrated melittin dimers associated with openings in the membrane.
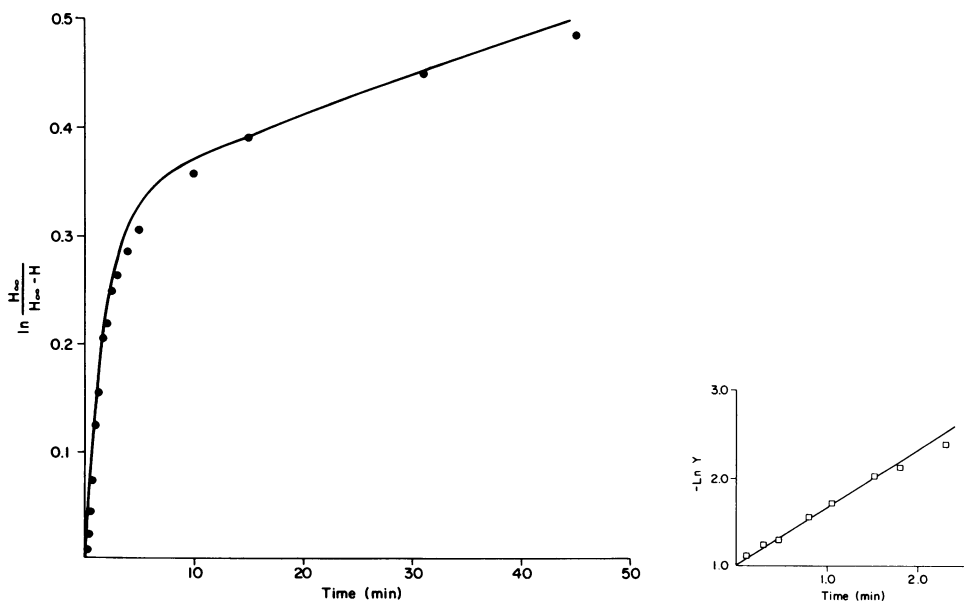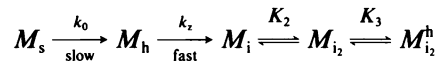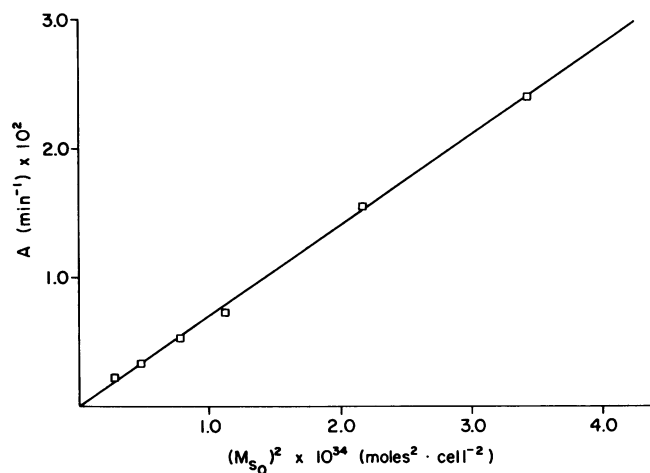

FIGURE 7 Plot of ln $(Hb_\infty/[Hb_\infty - Hb_t])$ as a function of time. The inset shows ln Y as a function of time.

FIGURE 8   The parameter A as a function of $M_{s_0}^2$.

hemoglobin release yielded:

$$-\ln \frac{Hb_c}{Hb_{c_0}} = \left(\frac{D}{\delta V_c}\right)\left(\frac{\beta M_{s_0}^{2\beta}}{K_2 K_3}\right)t$$

$$+ \left(\frac{D}{\delta V_c}\right)\left[\frac{M_{s0}}{k_z} - \frac{2\beta M_{s_0}^2}{k_0 K_2 K_3}\right](1 - e^{-k_0 t}),\quad (1)$$

where M_{s_0} is the surface concentration of melittin in the reaction mixture at $t = 0$. This equation is of the form

$$\ln \frac{Hb_{c_0}}{Hb_c} = At + B - Be^{-k_0 t}$$

With Hb and Hb_∞ being current and final concentration of hemoglobin observed in solution, respectively, the latter equation predicts that if $\ln[Hb_\infty/(Hb_\infty - Hb)]$ is plotted as a function of time, a straight line should be obtained at $t \gg 1/k_0$ and an exponential portion should be present at lower time values. As shown in Fig. 7, the experimental data do adhere to the equation predicted by the kinetic scheme (Scheme 1), and from the straight line at high values of t, A can be calculated. The value of B is then

determined by first extrapolating this line to $t = 0$. Subtracting the experimental points in the fast phase from this extrapolated line, we obtain a variable, Y, which should obey the relationship $Y = Be^{-k_0 t}$. A plot of $-\ln Y$ vs. t does indeed yield a straight line for at least two half-lives of the fast phase (Fig. 7), again showing that the experimental data are consistent with Scheme 1. Values of A, B, and k_0 determined from experiments at varying melittin and erythrocyte concentrations are shown in Table I. In agreement with Eq. 1, A is directly proportional to $M_{s_0}^2$, i.e., proportional to the square of the total melittin concentration at any given erythrocyte concentration and inversely proportional to the square of the erythrocyte concentration at any given melittin concentration (Fig. 8). Fig. 9 shows that the value of B, as a function of M_{s_0}, gives a straight line at low values of the indepedent variable but shows a slight negative deviation from the straight line at high values of M_{s_0}. Eq. 1 predicts that $B = DM_{s_0}\alpha/\delta k_z V_c - 2(A)/k_0$. From the experimental data we can thus calculate the value of $DM_{s_0}\alpha/\delta k_z V_c$ for each experiment. Plotting this value as a function of melittin concentration, we obtain a straight line, as shown in Fig. 9. Thus, Eq. 1 correctly describes the time dependency and the concentration dependency of the melittin-induced lysis, and the three parameters governing the kinetics are $k_0 = 0.57\ \text{min}^{-1}$;

$$D\beta/\delta K_2 K_3 V_c = 7.14 \times 10^{31}\ \text{cell}^2\ \text{mol}^{-2}\ \text{min}^{-1};$$

and

$$D\alpha/\delta k_z V_c = 5.82 \times 10^{16}\ \text{cell mol}^{-1}.$$

Using these values and Eq. 1, we have calculated theoretical curves for the experiments shown in Figs. 4 and 5. The excellent agreement between the theoretical curves and all of our experimental data firmly supports our analysis and validates all the assumptions used in our derivation. Fig. 10 shows data using peptide I, under conditions identical to those of the melittin experiment. The data can again be analyzed using Eq. 1, and it appears that while k_0 is
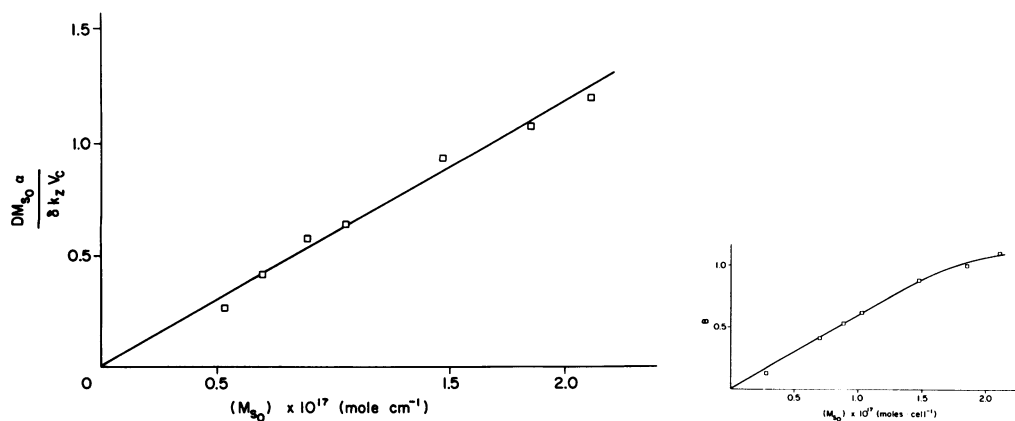

FIGURE 9   The parameter $(D/\delta V_c) \cdot M_{s_0}\alpha/k_z$ as a function of M_{s_0}. The inset shows B vs. $M_{s_0}^2$.
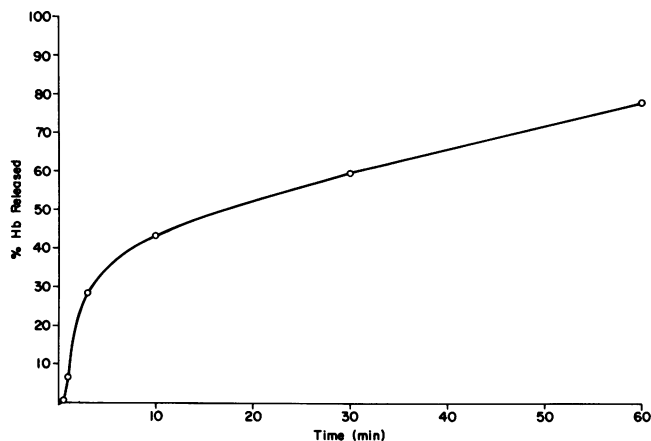
FIGURE 10 The time dependence of hemoglobin release induced by peptide I. A suspension of erythrocytes (4.43×10^{10} cells/liter) was treated with peptide I (5.75×10^{-7} M).

identical to that for melittin and $\alpha/k_z \ M_{s_0}$ is slightly smaller but comparable to that of melittin, $\beta/K_2K_3M_{s_0}^2$ is significantly larger than that observed for melittin (Table I). Eq. 1 also correctly describes the conventional dosage-response curves (15) and allows one to determine the values of parameters A and B for both melittin and its synthetic analogue.

Finally, the kinetics of carbonic anhydrase release also follow Scheme 1. Analysis of data according to Eq. 1 yields kinetic parameters very similar to those found for hemoglobin release (Table I).

DISCUSSION

Our results establish that the kinetics of hemolysis are correctly described by Eq. 1, and thus the pathway shown in Scheme 1 is consistent with all our experimental observations. Since the translocation of melittin according to Scheme 1 occurs by a system of two consecutive reactions,

pure first-order kinetics could be observed either when $k_0 \gg k_z$ or when $k_z \gg k_0$. To decide between these alternatives one has to take into consideration the time necessary for hemoglobin to diffuse through a membrane opening which should be of a size at least comparable to that of the hemoglobin molecule. Calculations using the equation $dHb_c/dt = D/V_c\delta \ Hb_c \cdot S_{T_t}$ show that for δ of the order of 100 Å even a single hole per cell should result in complete lysis within < 1 min. This is clearly not the case. Since the density gradient centrifugation indicates that there has to be a large number of holes, we can, therefore, rule out the possibility that $k_z \ll k_0$. Thus, the membrane openings associated with the translocation of the melittin must be extremely short-lived. The stoichiometry of the reaction in fact shows that at the most 40 hemoglobin molecules are released when one melittin is "internalized." Since carbonic anhydrase, with a molecular weight one-half of that of hemoglobin, is released at a rate comparable to that of hemoglobin, the openings in the membrane must be appreciably larger than the cross section of the hemoglobin molecule.

At this time we can only speculate as to the mechanisms by which melittin can create transient openings in the membrane. Preliminary observations in our laboratory showed that in mixed monolayers of Peptide I and lecithin the Peptide I is intercalated between the lecithin side-chains, whereas an apolipoprotein model peptide with a hydrophobic:hydrophilic ratio of 0.5 was inserted only between the headgroups of the phospholipid.[3] The large increase in the area caused by the melittin inserted into erythrocyte membranes should result in local expansion of the outer leaflet of the membrane. Because of the viscoelastic nature of the erythrocyte membrane, we should first obtain a local outward bulge with the concomitant flaring

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[3]Cho, M. J. Unpublished results.

TABLE I
PARAMETERS MEASURED FOR ERYTHROCYTE LYSIS BY MELITTIN AND MELITTIN ANALOGUE

Melittin conc. $\times 10^7$ (M)	Erythrocyte conc. $\times 10^{-10}$ cell liter^{-1}	$M_{s_0} \times 10^{18}$ (mol/cell^{-1})	k_0 (min^{-1})	$A \times 10^3$ (min^{-1})	$\dfrac{D}{\delta V_c} \dfrac{\alpha}{k_z} M_{s_0}$	B
4.64	6.67	6.96	0.49	3.37	0.42	0.41
4.64	4.36	10.6	0.47	7.27	0.645	0.62
4.64	2.20	21.1	0.63 ± 0.2	22 ± 9	1.2	1.1
2.86	5.4	5.30	0.36	1.27	0.286	0.282
4.67	5.29	8.83	0.77	5.29	0.574	0.556
7.01	4.77	14.7	0.72	15.5	0.933	0.879
9.34	5.05	18.5	0.54	24	1.08	0.994
3.77	5.21	4.5	0.54	3.6	0.345	0.332*
				7 ± 3	0.312	0.287‡
5.75§	4.43	12.98	0.609	18.9	0.428	0.366

*Values calculated from hemoglobin release, monitored spectrophotometrically.
‡Values calculated from carbonic anhydrase release, monitored by the stopped flow method (19).
§Results for melittin analogue, peptide I.

of the hydrophobic portion of the inner leaflet. This deformation could then lead to further penetration of the melittin and ultimate destruction of the continuity of the membrane. Once an opening is formed, melittin could be internalized and the hole closed. We feel that the positively charged C-terminus of the melittin would be eminently suitable to attract the molecule toward the negatively charged inner leaflet of the membrane. Once internalized, only melittin dimers are conducive to reopening the membrane. Presumably, the helical portions of two melittin molecules can form a totally hydrophobic cluster which then could reintercalate into the bilayer. That a hydrophobic dimer is involved in this step is further supported by the fact that peptide I, which is more prone to aggregate than melittin because it can form a longer cylindrical α-helix (14), produces a faster second phase than melittin. Such a mechanism would account for the structural requirements for cytotoxins, i.e., an amphiphilic α-helix with at least a 2:1 hydrophobic:hydrophilic ratio and a strongly positively charged moiety. We feel that the particular amino acid sequences of the naturally-occurring cytotoxins of the melittin type are of no relevance as long as the above requirements are fulfilled.

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DISCUSSION

Session Chairman: John N. Weinstein *Scribe:* Gradimir Georgevich

TERWILLIGER: You present a very detailed kinetic analysis of the lysis of erythrocytes by melittin. If you were to assume that the slow portion of the lysis is first-order instead of second-order, would the agreement between your model and your experimental data be substantially different?

DEGRADO: Yes. The rate of the second phase is first-order with respect to hemoglobin and second-order with respect to melittin over the erythrocyte concentration.

TERWILLIGER: According to Fig. 3, the initial rate of leakage of hemoglobin from erythrocytes at a fixed melittin concentration depends strongly on temperature. The rate is higher at 22°C than at either 4° or 37°C. Have you repeated your kinetic analysis at either 37° or 22°C?

DEGRADO: Yes, we expected to see different results at different