ELSEVIER

Biochimica et Biophysica Acta 1514 (2001) 253–260

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

'Detergent-like' permeabilization of anionic lipid vesicles by melittin

Alexey S. Ladokhin *, Stephen H. White

Department of Physiology and Biophysics, University of California, 364-D Medical Sciences 1, Irvine, CA 92697-4560, USA

Received 23 April 2001; received in revised form 25 June 2001; accepted 28 June 2001

Abstract

Melittin (MLT), the 26-residue toxic peptide from the European honeybee *Apis mellifera*, is widely used for studying the principles of membrane permeabilization by antimicrobial and other host-defense peptides. A striking property of MLT is that its ability to permeabilize zwitterionic phospholipid vesicles is dramatically reduced upon the addition of anionic lipids. Because the mechanism of permeabilization may be fundamentally different for the two types of lipids, we examined MLT-induced release of entrapped fluorescent dextran markers of two different molecular masses (4 and 50 kDa) from anionic palmitoyloleoylphosphatidylglycerol (POPG) vesicles. Unlike release from palmitoyloleoylphosphatidylcholine (POPC) vesicles, which is highly selective for the 4 kDa marker, implying release through pores of about 25 Å diameter [Ladokhin et al., Biophys. J. 72 (1997) 1762], release from POPG vesicles was found to be non-selective, i.e., 'detergent-like'. Oriented circular dichroism measurements of MLT in oriented POPG and POPC multilayers disclosed that α-helical MLT can be induced to adopt a transbilayer orientation in POPC multilayers, but not in POPG multilayers. The apparent inhibition of MLT permeabilization by anionic membranes may thus be due to suppression of translocation ability.

Keywords: Pore formation in membranes; Detergent-like action; Melittin; Antimicrobial peptide; Oriented circular dichroism

1. Introduction

Many host-defense peptides, such as toxins and antimicrobial peptides, exert their action through permeabilization of cellular membranes [1–4]. The mode and effectiveness of this disruption of normal membrane barrier function depend upon membrane lipid composition [5–9]. This lipid, and hence membrane, specificity is important to understand in order to design effective antimicrobial peptides that can distinguish between host and invader membranes. A common strategy is to seek peptides that simultaneously have low hemolytic and high antibacterial activity [8,10]. The archetypal membrane-disrupting peptide used as a model for hemolytic activity [11,12] is melittin (MLT), the 26-residue toxic peptide from the European honeybee *Apis mellifera* [13,14]. It too shows lipid specificity. For example, compared to release from pure zwitterionic phosphocholine

Abbreviations: POPC, palmitoyloleoylphosphatidylcholine; POPG, palmitoyloleoylphosphatidylglycerol; LUV, extruded large unilamellar vesicles of 100 nm diameter; FD-4 and FD-70, fluorescein isothiocyanate dextrans with molecular masses of 4400 and 50 700 Da, respectively; Triton, reduced Triton X-100; MLT, melittin; cmc, critical micelle concentration; R_i, lipid-to-peptide ratio; R_{Tx}, Triton-to-lipid ratio; HEPES, *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; OCD, oriented circular dichroism

* Corresponding author. Permanent address: Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine, Kiev 252143, Ukraine. Fax: +1-949-824-8540.

E-mail address: ladokhin@uci.edu (A.S. Ladokhin).

0005-2736/01/$ – see front matter
PII: S0005-2736(01)00382-0

vesicles, a threefold increase in MLT membrane concentration is required to achieve equivalent release of small fluorescent markers from vesicles comprised of 50% anionic phospholipids such as diacylphosphatidylglycerol [5,6]. This result is puzzling because cationic MLT partitions much more strongly into anionic membranes [15]. This raises the possibility, examined here, that the mechanism of release may be fundamentally different for zwitterionic and anionic phospholipid vesicles.

Two permeabilization mechanisms that are frequently invoked for explaining release of vesicle contents are 'pore-like' [16–20] and 'detergent-like' leakage [5,9,21–23], illustrated schematically in Fig. 1. Pore-like leakage from vesicles implies that release-of-contents depends upon the size of an entrapped solute, whereas detergent-like leakage implies release that is independent of solute size. Using a method based upon detecting the differential release of co-encapsulated markers of different sizes, we demonstrated [20] that MLT forms pores in zwitterionic POPC vesicles. The pores were estimated to have effective diameters of 25–30 Å at a lipid-to-peptide ratio (R_i) of 50, consistent with models in which MLT forms barrel-stave aggregates of transmembrane α-helices [24]. In order to address the question of whether MLT acts similarly in anionic lipid vesicles, we have now used the same method to study release from POPG vesicles. The results, described here, revealed that MLT caused detergent-like release, in the sense that release was non-selective. The amount of MLT required for a given amount of release was much higher than for POPC vesicles, as expected. In order to investigate possible structural differences between MLT-induced release from POPC and POPG vesicles, we used oriented circular dichroism (OCD) measurements [25–27] of MLT in oriented POPC and POPG multilayers at high hydrations. These measurements showed that α-helical MLT could be induced to adopt a transmembrane configuration only in POPC multilayers, suggesting that the ability of melittin to form transmembrane pores in membranes is related to its ability to adopt a transbilayer orientation, which in turn depends upon lipid membrane lipid composition.

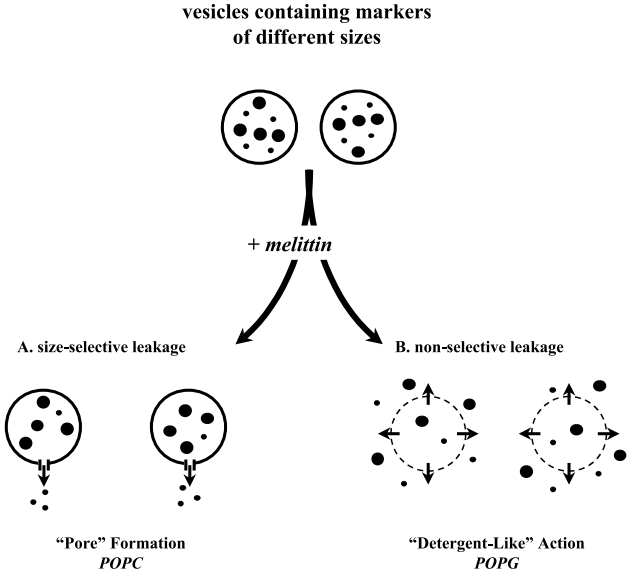

Fig. 1. A schematic representation of vesicle permeabilization by (A) pore-like and (B) detergent-like leakage mechanisms. The principal distinction is selectivity in the release contents, although detergent-like can mean complete solubilization of vesicle membranes. The data of Table 1 show that melittin causes non-selective 'detergent-like' leakage from POPG membranes. In contrast, melittin-induced release from POPC vesicles is pore-like [20].

2. Materials and methods

2.1. Materials

Lipids were obtained from Avanti Polar Lipids (Alabaster, AL). Melittin (sequencing grade), Triton X-100, FD-4 dextran, and FD-70 dextran were obtained from Sigma (St. Louis, MO) and Sephacryl S-400 HR from Pharmacia Biotech (Uppsala, Sweden). The buffer solution (pH 7.0) contained 10 mM HEPES, 50 mM KCl, 4 mM EDTA, and 3 mM NaN_3. The buffer was carefully degassed before each run to avoid formation of air bubbles on the column.

Concentrations of the stock solutions of melittin were determined spectrophotometrically and those of phospholipid LUV by means of a standard phosphate assay [28]. Purity of lipids was checked by thin layer chromatography. Even after prolonged incubations with melittin, the lipids remained free of lyso derivatives.

2.2. Preparation of dextran-loaded vesicles and leakage experiments

The experimental procedures have been described in detail in Ladokhin et al. [20]. Large unilamellar vesicles (LUV) of approximately 0.1 μm diameter were formed by extrusion under N_2 pressure through Nucleopore polycarbonate membranes using the method of Mayer et al. [29]. To prepare LUV with entrapped solutes, the lipid was first suspended in buffer containing 2 mg/ml of FD-4 and 4 mg/ml of FD-70 and then frozen and thawed 20 times prior to extrusion and several additional times during the extrusion process. The increased loading of the FD-70 compared to FD-4 was done in order to improve the resolution in the elution profile (see Section 2.3). Untrapped dextran was removed by gel filtration using Sephacryl S-400 HR packed into a 38×1 cm column. Fluorescein-dextran containing LUV were treated with either 2% Triton (for determination of the ratio of entrapped dextrans) or melittin. Samples were applied to a column whose output was connected to a flow-through fluorescence cuvette placed in a SLM-8100 fluorimeter (SLM/Aminco, Urbana, IL). Excitation and emission wavelengths were 490 and 530 nm, respectively. Because the exciting light tends to be vertically polarized, a horizontally oriented polarizer was introduced into the emission path in order to minimize artifacts associated with scattering. All experiments were carried out at room temperature (23°C).

In order to trap the vesicle samples with partially released contents, we used the method of Schwarz and Arbuzova [30]. Specifically, after incubation of dextran-loaded LUV with a leakage-inducing agent for a given time, an overwhelming amount of unloaded LUV was added (0.1 ml of a 100 mM LUV suspension). The sample was then loaded onto the column and the elution profile analyzed. Unloaded LUV do not interfere with the analysis because they do not exhibit a fluorescence signal and scattering was effectively suppressed by the monochromator and polarizer.

2.3. Data analysis

The elution profiles were fitted by the sum of three log-normal distributions as described in detail previously [20]. The only minor improvement of the original procedure was that no fixed parameters were used in the fitting procedure. This became possible because of the increased signal from the FD-70 peak resulting from the increase in the initial loading (see above). The areas under the peaks were calculated numerically using the software package Origin 6.0 (MicroCal, Northampton, MA). The fractions of individual markers released were calculated from the ratios of areas of individual peaks for samples with partially and completely released contents. Complete release was achieved by solubilizing vesicles with 2% Triton. The uncertainties in fractions were estimated to be ± 0.03. The selectivity of leakage was calculated as a ratio of release of FD-4 over FD-70.

2.4. Oriented circular dichroism (OCD) measurements

OCD has been described in detail by several groups [25–27,31]. Oriented multilayers with incorporated melittin were formed on a quartz slide by deposition from methanol solutions containing 0.2 mM melittin and 10 mM of POPC or POPG. These were added drop-wise to the slide and then dried. Traces of methanol were removed under vacuum. A small drop of twice-distilled water was carefully placed onto the surface of the sample to increase the hydration without disruption of the multilamellar structure. The sample was then sealed in a tube containing an additional drop of water and allowed to equilibrate overnight. The tube was placed in a home-built holder [32] designed to fit into the sample compartment of a Jasco J720 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan). The signal was collected and averaged for four 90 degree rotations of the slide, each maintaining a perpendicular orientation of the slide to the beam. All four orientations exhibited nearly identical signal magnitudes. After initial data collection, the sample was subjected to a heating procedure in which the sample and holder were placed in an oven maintained at 45°C for 60 h. High humidity was maintained by placing a reservoir with water into the oven with the sample tube temporarily unsealed. The tube was re-sealed following incubation, brought to the ambient temperature, and allowed to equilibrate for 2 h prior to any additional measurements. Because the actual

amount of melittin in the beam could not be determined, the spectra were normalized with respect to the maximum negative peak value for consistency of presentation.

2.5. Simulations of OCD spectra

Helical basis spectra for the surface (helix axis parallel to bilayer plane) and inserted (helix axis normal to bilayer plane) forms were computed assuming an ideal helix [26]. A basis spectrum for a random-coil component was obtained by using a single Gaussian with 16 nm dispersion and molar ellipticity of -16×10^3 deg cm^{-2} dmol^{-1} centered at 197 nm. This choice was consistent with the CD spectrum observed for monomeric melittin free in solution. Equal amounts of this random coil spectrum were added to both the ideal surface and inserted basis spectra to arrive at new basis spectra with 80% helical content. Finally, these basis spectra were re-normalized to account for the finite length of the helix according to Rohl and Baldwin [33]. These final basis spectra were mixed in different proportions to simulate various combinations of surface and inserted peptides. Although these modifications were made in order to obtain simulated spectra that were as close as possible to the experimentally observed spectra, the analysis was largely unaffected by simply using the unmodified spectra of Huang and coworkers [26].

3. Results and discussion

3.1. Melittin-induced leakage from POPG vesicles

The size-selectivity of the leakage pathway was determined using a method based on the differential release of co-encapsulated markers of different sizes from lipid LUV [20]. Briefly, vesicles preloaded with fluorescently labeled dextrans FD-4 (4 kDa) and FD-70 (50 kDa) were treated with a leakage-inducing agent (MLT or Triton) and then passed down a Sephacryl S-400 gel-filtration column connected to a flow-through cuvette placed in a fluorescent spectrometer. The elution profile allowed the relative release of the two markers to be determined, as described in detail elsewhere [20] (see Section 2). If the inducing agent forms pores of limited diameter in the vesicle membrane, the extent of leakage will depend upon solute size. If, however, the agent micellizes the membrane, causes large perturbations in membrane integrity, or forms very large pores, leakage will not depend upon solute size. If the leakage pathways are very narrow, then of course there will be no leakage of large solutes.

Typical time-traces of the elution of POPG LUV vesicles with co-encapsulated FD-4 and FD-70 are shown in Fig. 2. The early peak corresponds to unreleased dextrans still contained within the LUV, while the two later peaks correspond to released dextrans. The gray trace in Fig. 2, obtained in the presence of solubilizing concentrations of Triton (2% v/v; $R_{Tx}=15.6$), demonstrates complete release of the dextrans. The other traces show the release of dextrans in the presence of several different melittin concentrations (corresponding to $R_i=35$, 20, and 10 lipids per MLT). In all cases, the FD-70 peak is larger than the FD-4 peak because of the heavier loading of the vesicles with FD-70 (see Section 2). It is apparent even from these raw data that the MLT-induced leakage of POPG vesicles lacks the strong size-depen-

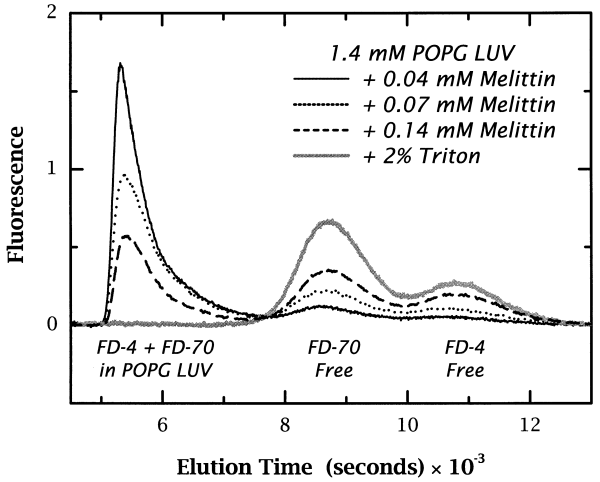

Fig. 2. Release of co-encapsulated FD-4 and FD-70 dextrans from large unilamellar POPG vesicles induced by melittin. Samples containing the same concentrations of POPG vesicles preloaded with the fluorescently labeled dextrans were incubated with different amounts of melittin for 20 h and then applied to the gel filtration column to determine the amount of released dextrans, as described by Ladokhin et al. [20]. As a control, complete release was achieved by solubilizing the membranes with Triton ($R_{Tx}=15.6$). The size selectivity of the leakage is presented in Table 1.

Table 1
Release of co-encapsulated fluorescently labeled dextrans FD-4 and FD-70 from POPG LUV induced by melittin

POPG/melittin[a] (mol/mol)	Incubation time (h)	FD-4_{out} (%)	FD-70_{out} (%)	Selectivity[b]
35	20	15	14	1.1 ± 0.3
20	20	37	32	1.2 ± 0.2
10	4	69	50	1.4 ± 0.1
10	20	81	53	1.5 ± 0.1

[a]POPG concentration was 1.4 mM.
[b]Selectivity was calculated as the ratio of release of the smaller to larger dextran. Error in selectivity was calculated by assuming that the errors in determination of the release of two dextrans were independent of each other and equal to 3%.

dence of the leakage reported with POPC vesicles [20]. The size-selectivity of the leakage was quantitated using the fitting procedure of Ladokhin et al. [20] in order to determine the relative areas of individual elution peaks (see Section 2). The fractions of the released dextrans obtained for several MLT concentrations are presented in Table 1. The selectivity, defined as a ratio of the released fraction of the smaller dextran to that of the larger dextran, of ~1–1.5 is low compared to values of about 4 observed for POPC vesicles [20]. Considering experimental errors, release was largely non-selective. In contrast to POPC vesicles for which 88% of the FD-4 was released after 3 h of incubation at $R_i = 50$, POPG vesicles released 69% after 4 h incubation at a fivefold higher melittin content ($R_i = 10$, Table 1). Pure POPG LUV therefore had a reduced sensitivity to MLT, similar to that observed for vesicles formed from mixtures of POPC with POPG and other anionic phospholipids [5,6]. The membrane interactions of melittin follow simple partitioning equilibrium. Consequently, the fraction of bound peptide is largely independent of R_i [34]. From the free energies of transfer from water to bilayer reported earlier [35], we estimate that at least 95% of melittin was bound to POPC and at least 99% was bound to POPG under the conditions of our leakage experiments.

3.2. Topology of melittin in oriented POPC and POPG multilayers

The ability of melittin to adopt a transbilayer orientation and thence a barrel-stave aggregation state has been invoked as the basis for pore formation in phosphocholine vesicles [24,36]. Similar correlations between pore formation and transbilayer orientations have been reported for alamethicin and magainin [37,38]. A crucial question is thus the ability of MLT to adopt a transbilayer configuration. Huang and coworkers [26,39,40] have demonstrated that the orientation of an α-helix with respect to membranes can be deduced from CD measurements of helical peptides incorporated into lipid multilayers oriented on a quartz slide (oriented CD). For slides oriented normal to the optical axis, helical peptides oriented parallel to the membrane surface have spectra with minima at 208 and 222 nm. Random-coil components with a minimum at 197 nm, probably present in the samples, can cause an apparent increase in the 208 nm component relative to the one at 222 nm. Peptides oriented normal to the surface have a spectrum with a single minimum at 222 nm.

The spectra of MLT in oriented POPC and POPG multilayers at high hydration are shown in Fig. 3A,C, respectively. For samples prepared and equilibrated at 23°C (dashed curves, designated as unheated), the OCD spectra of MLT in both POPC and POPG are those expected of helices oriented parallel to the bilayer surface, as judged by dominant minima at 208 nm. After heating the samples at 45°C for 60 h and then re-equilibrating at 23°C, the MLT spectrum for POPG was unaffected whereas the spectrum for POPC changed to one with a dominant 222 nm minimum (solid curves in Fig. 3A,C). The simplest explanation of the data is that some of the MLT in POPC, but not in POPG, could be induced to adopt a transbilayer orientation. Even without heating, prolonged incubation of the POPC sample results in progressive changes in the ratio of the peaks at 222 and 208 nm (data not shown). We attributed these changes to slow conformational equilibration upon hydration of the lipid/protein film dried from organic solvent. The heating procedure

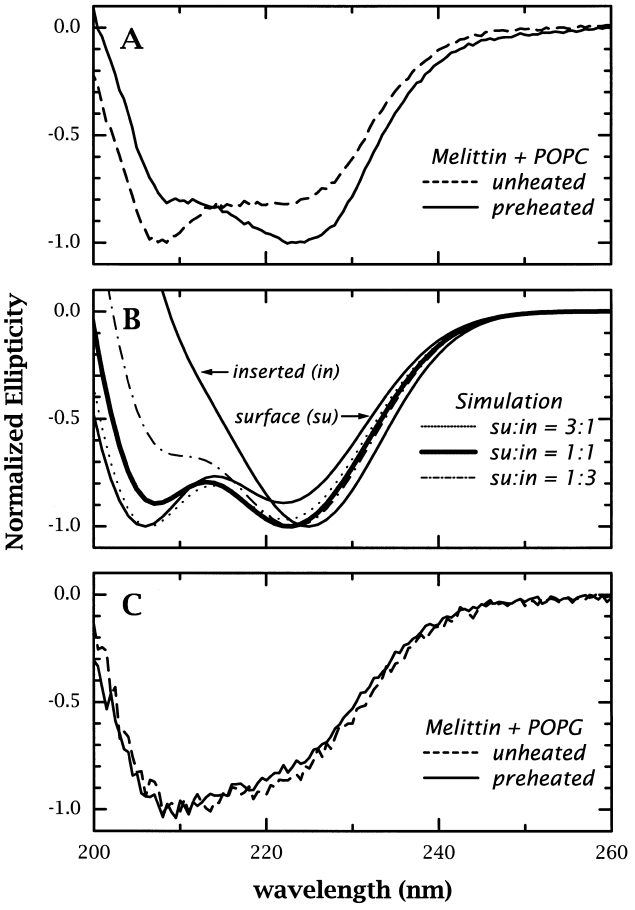

Fig. 3. Normalized CD spectra of melittin in POPC and POPG oriented multilayers deposited on the quartz slide. The plane of the oriented bilayers was oriented perpendicular to the optical axis of the spectropolarimeter. Lipid-to-melittin mole ratio $R_i = 50$ in both cases. All experimentally determined spectra were collected at room temperature (23°C). (A) Melittin in POPC multilayers without heat treatment (dashed curve) and after preheating to 45°C for 60 h (solid curve). The characteristic changes in the ellipticity at 208 and 222 nm observed after preheating is consistent with a change in the orientation of the α-helices from a parallel to perpendicular orientation relative to the membrane plane [26], as shown in B. This transition is observed in POPC but not in POPG samples. (B) Simulated preheated spectra of melittin in POPC. See text for details. (C) Melittin in POPG multilayers without heat treatment (dashed curve) and after preheating to 45°C for 60 h (solid curve). There is little change in the spectrum, which is that expected for helices parallel to the membrane plane.

employed here was used to accelerate the equilibration process. We explored through simulations the relative populations of surface (*su*) and inserted (*in*) MLT required to describe the 'preheated' spectrum for POPC in panel A (see Section 2.3). Fig. 3B shows that $su:in = 1:1$ explains the spectrum satisfactorily. We can thus reasonably conclude that MLT can be induced to adopt a transmembrane orientation in POPC multilayers, but not in POPG multilayers. That MLT is oriented only parallel to the POPC multilayers in the absence of preheating is consistent with the existence of a kinetic barrier to insertion which is no doubt elevated in the highly viscous environment of oriented multilayers. The apparent failure of MLT to insert into POPG multilayers is consistent with the lack of insertion of alamethicin and protegrin into charged multilayers [41]. Together, our OCD data suggest, but do not prove, that helical MLT can be transbilayer in POPC vesicles, but not POPG vesicles.

3.3. What is a 'detergent-like' action of peptides?

Non-selective leakage of large markers is often referred to as detergent-like [3,21,42,43]. Indeed, solubilizing concentrations of Triton ($R_{Tx} = 15.6$), which result in phospholipid–Triton mixed micelles [44], caused complete loss of the dextrans, as shown in Fig. 2. The idea of membrane solubilization as a means of leakage induction has been formally extended to peptides by Shai and colleagues (reviewed in [22]) by means of their so-called carpet mechanism. They propose that some peptides, folded or unfolded, can form destructive peptide–lipid micelle-like structures after accumulating on the surface of membranes in a parallel configuration.

Even though MLT-induced release of dextrans from POPG vesicles is non-selective, and thus consistent with detergent-like terminology, we think it is unlikely that MLT is acting by a micellization mechanism, at least at the concentrations used in our experiments. Unfolded (monomeric) MLT partitions into membrane interfaces and adopts an α-helical conformation [45,15,34] through a process referred to as partitioning–folding coupling [46], which is driven by the reduction of the high cost of peptide bond partitioning accompanying intrahelical H-bond formation [35]. At low surface concentrations, helical MLT is oriented parallel to the membrane interface with its non-polar surface facing toward the bilayer interior [47–49]. With increases in the surface concentration of MLT, as the peptide moves toward its disruptive configuration in the membrane, dimeriza-

tion appears to be an important step [50–52]. The next steps toward permeabilization with further increases in concentration are only vaguely understood. In zwitterionic POPC bilayers, most data [20,24,53,54] are consistent with the ultimate formation of pores by barrel-stave aggregates of transbilayer α-helices. Translocation of MLT across the bilayer is obviously an important step, but it is not yet clear how translocation is coupled to aggregation. As discussed elsewhere [20], the size of the pores is not fixed but probably increases with increases in membrane concentration. The OCD measurements reported here and our earlier measurements of the selective release of dextran markers of different sizes [20] agree entirely with this general scheme for the permeabilization of zwitterionic membranes by melittin.

The apparent Stokes radii of the 4 and 50 kDa dextrans are ~23 and ~50 Å, respectively, based upon the measurements of Laurent and Granath [55]. Therefore, if the MLT-induced leakage from POPG vesicles seen here is due to pore formation, the inner diameter of the pores would have to exceed ~50 Å, or twice the estimated diameter of the pores formed in POPC vesicles. Our OCD measurements suggest that MLT cannot readily adopt a transmembrane configuration in POPG bilayers, but that might simply be a consequence of a heightened kinetic barrier due to strong electrostatic interactions of MLT with POPG. Alternatively, perhaps a new type of pore with helices parallel to the membrane plane is being formed. X-ray diffraction studies [32,56] indicate that surface-bound α-helical peptides reduce the thickness of bilayers, even at very low peptide/lipid ratios. Even very small peptides have this ability [57]. One can speculate that aggregation of surface-bound helices might have an exaggerated effect on local bilayer thickness. Non-selective detergent-like leakage may thus occur because surface-bound MLT aggregates reduce bilayer thickness and consequently cause local structural instabilities and fluctuations equivalent to transient pores. Another possibility could be a 'leaky' fusion, in which part of the contents is released during MLT-induced vesicular fusion. Indeed, we have observed increased scattering of POPG vesicles upon addition of melittin (not shown). In either case, the much higher membrane concentration of MLT required for permeabilization of anionic vesicles points toward large aggregates with low association constants. Regardless of the exact nature of MLT permeabilization of POPG vesicles, this study clearly demonstrates that the *mechanism* of membrane disruption is not an inherent feature of a given peptide but depends strongly on the lipid bilayer.

Acknowledgements

The research was supported by grant GM-46823 from the National Institutes of Health. We thank Dr. William C. Wimley for constructing the sample holder for oriented CD measurements. We also thank Mr. Michael A. Myers for his editorial assistance.

References

[1] S.H. White, W.C. Wimley, M.E. Selsted, Curr. Opin. Struct. Biol. 5 (1995) 521–527.
[2] W.L. Maloy, U.P. Kari, Biopolymers 37 (1995) 105–122.
[3] B. Bechinger, J. Membr. Biol. 156 (1997) 197–211.
[4] D. Andreu, L. Rivas, Biopolymers 47 (1998) 415–433.
[5] T. Benachir, M. Lafleur, Biochim. Biophys. Acta 1235 (1995) 452–460.
[6] D.K. Hincha, J.H. Crowe, Biochim. Biophys. Acta 1284 (1996) 162–170.
[7] A.K. Ghosh, R. Rukmini, A. Chattopadhyay, Biochemistry 36 (1997) 14291–14305.
[8] E.M. Tytler, G.M. Anantharamaiah, D.E. Walker, V.K. Mishra, M.N. Palgunachari, J.P. Segrest, Biochemistry 34 (1995) 4393–4401.
[9] K. Hristova, M.E. Selsted, S.H. White, J. Biol. Chem. 272 (1997) 24224–24233.
[10] M. Dathe, T. Wieprecht, H. Nikolenko, L. Handel, W.L. Maloy, D.L. MacDonald, M. Beyermann, M. Bienert, FEBS Lett. 403 (1997) 208–212.
[11] T. Katsu, C. Ninomiya, M. Kuroko, H. Kobayashi, T. Hirota, Y. Fujita, Biochim. Biophys. Acta 939 (1988) 57–63.
[12] T. Katsu, M. Kuroko, T. Morikawa, K. Sanchika, Y. Fujita, H. Yamamura, M. Uda, Biochim. Biophys. Acta 983 (1989) 135–141.
[13] E. Habermann, Science 177 (1972) 314–322.
[14] C.E. Dempsey, Biochim. Biophys. Acta 1031 (1990) 143–161.
[15] G. Beschiaschvili, J. Seelig, Biochemistry 29 (1990) 52–58.
[16] K. Matsuzaki, O. Murase, N. Fujii, K. Miyajima, Biochemistry 34 (1995) 6521–6526.
[17] E. Fattal, S. Nir, R.A. Parente, F.C. Szoka, Biochemistry 33 (1994) 6721–6731.
[18] K. He, S.J. Ludtke, H.W. Huang, D.L. Worcester, Biochemistry 34 (1995) 15614–15618.

[19] W.C. Wimley, M.E. Selsted, S.H. White, Protein Sci. 3 (1994) 1362–1373.
[20] A.S. Ladokhin, M.E. Selsted, S.H. White, Biophys. J. 72 (1997) 1762–1766.
[21] H. Ostolaza, B. Bartolomé, I. Ortiz de Zárate, F. de la Cruz, F.M. Goñi, Biochim. Biophys. Acta 1147 (1993) 81–88.
[22] Z. Oren, Y. Shai, Biopolymers 47 (1998) 451–463.
[23] E. Gazit, A. Boman, H.G. Boman, Y. Shai, Biochemistry 34 (1995) 11479–11488.
[24] H. Vogel, F. Jähnig, Biophys. J. 50 (1986) 573–582.
[25] H. Vogel, Biochemistry 26 (1987) 4562–4572.
[26] Y. Wu, H.W. Huang, G.A. Olah, Biophys. J. 57 (1990) 797–806.
[27] H.H.J. de Jongh, E. Goormaghtigh, J.A. Killian, Biochemistry 33 (1994) 14521–14528.
[28] G.R. Bartlett, J. Biol. Chem. 234 (1959) 466–468.
[29] L.D. Mayer, M.J. Hope, P.R. Cullis, Biochim. Biophys. Acta 858 (1986) 161–168.
[30] G. Schwarz, A. Arbuzova, Biochim. Biophys. Acta 1239 (1995) 51–57.
[31] W.C. Wimley, S.H. White, Biochemistry 39 (2000) 4432–4442.
[32] K. Hristova, W.C. Wimley, V.K. Mishra, G.M. Anantharamaiah, J.P. Segrest, S.H. White, J. Mol. Biol. 290 (1999) 99–117.
[33] C.A. Rohl, R.L. Baldwin, Biochemistry 36 (1997) 8435–8442.
[34] S.H. White, W.C. Wimley, A.S. Ladokhin, K. Hristova, Methods Enzymol. 295 (1998) 62–87.
[35] A.S. Ladokhin, S.H. White, J. Mol. Biol. 285 (1999) 1363–1369.
[36] T.A. Harroun, W.T. Heller, L. Yang, T.M. Weiss, H.W. Huang, Biophys. J. 76 (1999) A124.
[37] H.W. Huang, Y. Wu, Biophys. J. 60 (1991) 1079–1087.
[38] S.J. Ludtke, K. He, Y.L. Wu, H.W. Huang, Biochim. Biophys. Acta 1190 (1994) 181–184.
[39] G.A. Olah, H.W. Huang, J. Chem. Phys. 89 (1988) 2531–2538.
[40] G.A. Olah, H.W. Huang, J. Chem. Phys. 89 (1988) 6956–6962.
[41] W.T. Heller, A.J. Waring, R.I. Lehrer, H.W. Huang, Biochemistry 37 (1998) 17331–17338.
[42] Z. Oren, Y. Shai, Biochemistry 36 (1997) 1826–1835.
[43] T.C.B. Vogt, B. Bechinger, J. Biol. Chem. 274 (1999) 29115–29121.
[44] E.A. Dennis, Arch. Biochem. Biophys. 165 (1974) 764–773.
[45] H. Vogel, FEBS Lett. 134 (1981) 37–42.
[46] W.C. Wimley, S.H. White, Nat. Struct. Biol. 3 (1996) 842–848.
[47] C. Altenbach, W.L. Hubbell, Proteins 3 (1988) 230–243.
[48] C.E. Dempsey, G.S. Butler, Biochemistry 31 (1992) 11973–11977.
[49] A. Okada, K. Wakamatsu, T. Miyazawa, T. Higashijima, Biochemistry 33 (1994) 9438–9446.
[50] G. Schwarz, R.T. Zong, T. Popescu, Biochim. Biophys. Acta 1110 (1992) 97–104.
[51] J. Takei, A. Remenyi, C.E. Dempsey, FEBS Lett. 442 (1999) 11–14.
[52] K. Hristova, C.E. Dempsey, S.H. White, Biophys. J. 80 (2001) 801–811.
[53] M.T. Tosteson, D.C. Tosteson, Biophys. J. 36 (1981) 109–116.
[54] S. Rex, Biophys. Chem. 58 (1996) 75–85.
[55] T.C. Laurent, K.A. Granath, Biochim. Biophys. Acta 136 (1967) 191–198.
[56] Y. Wu, K. He, S.J. Ludtke, H.W. Huang, Biophys. J. 68 (1995) 2361–2369.
[57] R.E. Jacobs, S.H. White, Biochemistry 28 (1989) 3421–3437.