Effect of Phospholipid Composition on an Amphipathic Peptide-Mediated Pore Formation in Bilayer Vesicles

François Nicol,*† Shlomo Nir,‡ and Francis C. Szoka, Jr.*

*School of Pharmacy, University of California, San Francisco, California 94143-0446 USA; †Centre de Recherche Paul Pascal, CNRS, 33600 Pessac, France; and ‡Seagram Center for Soil and Water Sciences, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, Rehovot 76100, Israel

ABSTRACT To better understand the influence of phospholipid acyl-chain composition on the formation of pores by cytotoxic amphipathic helices in biological membranes, the leakage of aqueous contents induced by the synthetic peptide GALA (WEAALAEALAE ALAEHLAEALAEALEALAA) from large unilamellar phospholipid vesicles of various compositions has been studied. Peptide-mediated leakage was examined at pH 5.0 from vesicles made of phosphatidylcholine (PC) and phosphatidylglycerol (PG) with the following acyl-chain compositions: 1-palmitoyl-2-oleoyl (PO), 1,2-dioleoyl (DO), 1,2-dielaidoyl (DE), and 1,2-dipetroselinoyl (DPe). A mathematical model predicts and simulates the final extents of GALA-mediated leakage of 1-aminonaphthalene-3,6,8-trisulfonic acid (ANTS) and *p*-xylene-bis-pyridinium bromide (DPX) from 1-palmitoyl-2-oleoyl-phosphatidylcholine/1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPC/POPG) and 1,2-dielaidoyl-sn-glycero-3-phosphocholine/1,2-dielaidoyl-phosphatidylglycerol (DEPC/DEPG) liposomes at pH 5.0 as a function of peptide concentration in the bilayer, by considering that GALA pores responsible for this leakage have a minimum size of 10 ± 2 monomers and are formed by quasiirreversible aggregation of the peptide. With the phospholipid acyl-chain compositions tested, GALA-induced ANTS/DPX leakage follows the rank order POPC/POPG ≈ DEPC/DEPG > DPePC/DPePG > DOPC/DOPG. Results from binding experiments reveal that this reduced leakage from DOPC/DOPG vesicles cannot be explained by a reduced binding affinity of the peptide to these membranes. As shown by monitoring the leakage of a fluorescent dextran, an increase in the minimum pore size also does not explain the reduction in ANTS/DPX leakage. The data suggest that surface-associated GALA monomers or aggregates are stabilized in bilayers composed of phospholipids containing a *cis* unsaturation per acyl chain (DO and DPe), while transbilayer peptide insertion is reduced. GALA-induced ANTS/DPX leakage is also decreased when the vesicles contain phosphatidylethanolamine (PE). This lends further support to the suggestion that factors stabilizing the surface state of the peptide reduce its insertion and subsequent pore formation in the bilayer.

INTRODUCTION

The synthetic peptide GALA (WEAALAEALAEALAE-HLAEALAEALEALAA) has been designed to undergo a pH-sensitive conformational change (Subbarao et al., 1987) that results in a ~100-fold increase in the apparent egg PC bilayer/water partition coefficient of the peptide (Parente et al., 1990a). In aqueous solution at neutral pH the formation of an α-helix is disfavored because of the electrostatic repulsions between the carboxylic acid moieties of the glutamic acid residues, whereas at pH 5.0 the neutralization of these groups promotes the formation of an amphipathic α-helix and the binding to phosphatidylcholine (PC) bilayers (Subbarao et al., 1987; Parente et al., 1990a). In the optimal pH range of 5.0 and below, GALA induces the leakage of liposome-encapsulated 1-aminonaphthalene-3,6,8-trisulfonic acid/p-xylene-bis-pyridinium bromide (ANTS/DPX) at very low ratios of membrane-bound peptide per vesicle and induces rapid flip-flop of membrane phospholipids (Subbarao et al., 1987; Parente et al., 1990b; Fattal et al., 1994). Interpretation by a mathematical model of GALA-induced leakage of ANTS/DPX (Parente et al., 1990b; Nicol et al., 1996) or flip-flop of phospholipids (Fattal et al., 1994) from 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-choline (POPC) and egg PC liposomes at pH 5.0 has led to the proposition that the peptides aggregate in a quasiirreversible fashion in the bilayer to form size-selective pores. Only the aggregates that contain 10 ± 2 or more helices induce ANTS/DPX leakage and pore-mediated lipid flip-flop (Parente et al., 1990b; Fattal et al., 1994; Nicol et al., 1996). The addition of the anionic phospholipid 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) (up to 66.6 mol%) to a POPC bilayer does not modify the peptide efficiency to induce leakage of ANTS/DPX from vesicles at pH 5.0 (Nicol et al., 1996). However, the presence of cholesterol in the bilayer (≥20 mol%) decreases GALA-induced leakage of ANTS/DPX, because of a reduced binding affinity of GALA to cholesterol-containing vesicles and an increased reversibility of peptide aggregation in these membranes (Nicol et al., 1996). These phenomena are accentuated when the cholesterol content is increased from 20 to 40 mol%.

The cytotoxic potency of natural amphipathic α-helical toxins is also modulated by the lipid composition of the target cell membrane(s). These peptides are believed to exert their cytotoxic activities directly on the lipid matrix of the cell membrane (Boman, 1994), by inducing its permeabilization, disruption, or disintegration. The proposed

Received for publication 2 September 1998 and in final form 29 October 1999.

Address reprint requests to Dr. Francis C. Szoka, School of Pharmacy, University of California, San Francisco, CA 94143-0446. Tel.: 415-476-3895; Fax: 415-476-0688; E-mail: szoka@cgl.ucsf.edu.

0006-3495/00/02/818/12 $2.00

mechanisms of permeabilization of model lipid bilayers by these peptides, including melittin, δ-toxin, magainins, alamethicin, pardaxins, and mastoparans, is the formation of aqueous pores (Schwarz and Arbuzova, 1995; Matsuzaki et al., 1995a, 1996, 1997a,c; Rapaport et al., 1996; Rex, 1996; Ludtke et al., 1996; He et al., 1996a,b; Ladokhin et al., 1997). Studies that investigate the effect of lipid composition on peptide-induced perturbations of model membranes (liposomes or planar lipid films) or biomembranes can elucidate the principles that govern the cell selectivities displayed by natural α-helical toxins (Matsuzaki et al., 1995b; Tytler et al., 1995; Silvestro et al., 1997). The cytotoxic activities of these peptides can vary significantly in efficacy if the target cells are of bacterial or mammalian origin (Steiner et al., 1981; Zasloff, 1987; Chen et al., 1988; Katsu et al., 1989, 1990; Matsuzaki et al., 1997b). Thus the focus of many studies on peptide-mediated bilayer perturbations is based on the differences in lipid composition between erythrocytes and bacterial membranes (Christensen et al., 1988; Portlock et al., 1990; Matsuzaki et al., 1991, 1995b; Gazit et al., 1994, 1995; Silvestro et al., 1997). The outer leaflet of human erythrocyte membranes is exclusively composed of zwitterionic phospholipids (Verkleij et al., 1973), whereas bacterial membranes abundantly contain acidic phospholipids as well as lipopolysaccharides (LPSs) in the outer membrane of Gram-negative bacteria (Duckworth et al., 1974; Rothman and Kennedy, 1977). Cholesterol is very abundant in erythrocyte membranes (Turner and Rouser, 1970) but is absent in bacterial membranes.

Studies on cecropin- and magainin-induced permeabilization of liposomes have deduced that the reduced hemolytic activity of these peptides, in comparison to their antibacterial activities, arises primarily from the difference in anionic phospholipids between the erythrocyte and the bacterial membranes (Gazit et al., 1994, 1995; Matsuzaki et al., 1995b). Although to a lower extent than the absence of anionic lipids, the high cholesterol content of erythrocyte membranes is responsible for the low hemolytic activity of magainin-2 (Matsuzaki et al., 1995a). The same study concluded that cholesterol does not affect melittin-induced permeabilization of vesicles. However, Portlock et al. (1990) and Benachir et al. (1996) reported a reduction in melittin's efficacy in inducing dye leakage from cholesterol-containing liposomes. This effect was due primarily to a reduction in the membrane-binding affinity of the peptide (Benachir et al., 1996).

The membrane-perturbing activities of amphipathic α-helical peptides may also depend on the lipid acyl-chain structure, which affects membrane fluidity. However, this lipid-structure/peptide-function relationship is poorly understood. Peptide-membrane interactions can vary significantly with acyl-chain length, because of the effect of this length on membrane thickness. Membrane insertion of hydrophobic α-helices has been shown to be regulated by the degree of hydrophobic mismatch between the length of the helix and the thickness of the bilayer hydrophobic core (Ren et al., 1997). It was reported that cecropin AD aggregation in model bilayers and alamethicin membrane-binding affinity vary with the length of the lipid acyl-chains (Stankowski et al., 1988; Stankowski and Schwarz, 1989; Mchaourab et al., 1994). Changes in membrane fluidity can modify membrane permeabilization and morphological changes of vesicles induced by amphipathic α-helical peptides. Matsuzaki et al. (1991) showed that magainin is more efficient in inducing calcein leakage from vesicles in the liquid-crystalline state (1,2-dioleoyl-phosphatidylglycerol, DOPG) than in the gel state (DPPG). It was suggested that melittin-induced fragmentation of multilamellar DPPC vesicles into small disks occurs preferentially with gel-phase phospholipids, because of a deeper peptide insertion into the core of the bilayer (Dufourcq et al., 1986; Dufourc et al., 1986). With the bacterial peptide δ-toxin, vesicle fragmentation is favored for phospholipids in the liquid-crystalline state, which was explained by an expulsion of the peptide from the core of gel-phase bilayers (Dufourc et al., 1990). Interestingly, acyl-chain length modulates the stability of the discoidal structures induced by melittin (Faucon et al., 1995) and GALA (Subbarao et al., 1988). In addition, the inclusion of unsaturated lipids, cholesterol, or negatively charged lipids in DPPC vesicles reduces the extent of micellization induced by melittin (Monette et al., 1993; Monette and Lafleur, 1995, 1996; Pott and Dufourc, 1995).

Only a few studies, however, have focused on the effect of varying the degree of unsaturation of the lipid acyl chains with membranes in the liquid-crystalline state. Rex (1996) reported that, for the same number of membrane-bound peptides per vesicle, melittin is less efficient at inducing carboxyfluorescein leakage by pore formation from liposomes composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (18:1n-9*cis*)(18:1n-9*cis*) than from vesicles made of POPC (16:0)(18:1n-9*cis*). The critical peptide/lipid molar ratio (P/L)* above which alamethicin is membrane-inserted in a pore structure was shown to vary considerably with the phospholipid acyl-chain structure (Huang and Wu, 1991; Wu et al., 1995). Interestingly, the structure of the membrane pores formed by alamethicin also depends on the phospholipid composition. Indeed, the pore is constituted of 11 transmembrane helices in DPhPC membranes and eight helices in DLPC membranes (He et al. 1995, 1996a,b).

The current study examines the influence of phospholipid acyl-chain structure and the role of phospholipid unsaturation in pore formation by GALA in liposomes, using a combination of leakage and binding experiments and model calculations.

MATERIALS AND METHODS

Reagents

1,2-Dielaidoyl-sn-glycero-3-phosphocholine (DEPC), 1,2-dielaidoyl-phosphatidylglycerol (DEPG), DOPC, 1,2-dioleoyl-sn-glycero-3-phospho-eth-

anolamine (DOPE), DOPG, 1,2-dipetroselinoyl-sn-glycero-3-phosphocholine (DPePC), 1,2-dipetroselinoyl-phosphatidylglycerol (DPePG), POPC, and POPG in chloroform were purchased from Avanti Polar Lipids (Alabaster, AL). 1-Aminonaphthalene-3,6,8-trisulfonic acid (ANTS), *p*-xylene-bis-pyridinium bromide (DPX), 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole (NBD fluoride), and neutral Texas red dextran (MW 3000) were purchased from Molecular Probes (Eugene, OR).

Vesicle preparation and sizing

Reverse-phase evaporation vesicles (REVs) were prepared as described previously (Szoka and Papahadjopoulos, 1978) in 1) 5 mM *N*-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES), 100 mM KCl at pH 7.0, or in 2) 5 mM TES, 12.5 mM ANTS, 45 mM DPX, 20 mM KCl at pH 7.0. To study the leakage of a neutral Texas red dextran (MW 3000), this molecule was included in 1) or 2) at a concentration of 0.5 mM. The vesicles were extruded five times through a 0.1-μm polycarbonate membrane (Nuclepore Corp., Pleasanton, CA) with a hand-held extrusion device (Avestin, Ottawa, Canada). When ANTS/DPX and/or the neutral Texas red dextran was encapsulated in liposomes, a Sepharose 4-B column (1×15 cm) was used to separate vesicles from unencapsulated material with the elution buffer, 5 mM TES, 100 mM KCl, pH 7.0. Lipid phosphorus was determined by a modification of the Bartlett (1959) method. The size distribution of the liposomes was determined by quasielastic light scattering with a Coulter N4 apparatus.

GALA synthesis and NBD labeling

The detailed synthesis, purification, and analysis of the peptide GALA has been described in a number of publications (Subbarao et al., 1987; Parente et al., 1990a,b; Nicol et al., 1996, 1999). The NBD labeling of the peptide has been presented by Nicol et al. (1996).

Fluorescence measurements

Measurements were made on a Spex Fluorolog photon-counting instrument (Edison, NJ) under the control of a IBM PC equipped with the software DM3000, using a 150-W xenon light source, as previously described (Parente et al., 1990a,b).

NBD-GALA binding experiments

The procedure, essentially based on that of Frey and Tamm (1990), is described elsewhere (Nicol et al., 1996). Briefly, a peptide mixture composed of GALA and NBD-labeled GALA, in a stock solution at pH 8.0 containing 5 mM TES and 50 mM KCl, was added to the stirred vesicles at 20°C in a solution at pH 5.0 containing 20 mM sodium acetate and 90 mM KCl. The fluorescence signal was monitored at the peak of the NBD emission, $\lambda = 525$ nm (5 mm slits), when the sample was irradiated at $\lambda = 467$ nm. The intensity was recorded at its maximum, 100 s after peptide addition to the liposome suspension. The contribution of the liposomes alone was subtracted from this intensity, and the dilution factor corresponding to the addition of an aliquot (μl) of peptide was accounted for, to compute a fluorescence intensity, F, corresponding to the NBD fluorescence. We checked that the addition of unlabeled GALA to a liposome suspension does not modify the intensity at $\lambda = 525$ nm. For a particular peptide concentration, the fraction of membrane-bound peptides (f_{bp}) was determined by $f_{bp} = (F - F_0)/(F_{100} - F_0)$, where F_0 is the NBD fluorescence intensity observed when all of the peptide is in solution without liposomes and F_{100} is the NBD fluorescence intensity acquired when 100% of the peptide is bound. F_{100} was determined when, for the same amount of peptide in a separate experiment, a large increase in the liposome concentration did not result in further increase in the NBD fluorescence intensity. In both the binding and leakage experiments, the absence of vesicle aggregation and fusion was checked by determining the turbidity of the preparation.

Measurements of ANTS/DPX leakage

The ANTS/DPX assay (Ellens et al., 1984) was used to measure the ability of the peptide GALA to induce leakage of ANTS/DPX preencapsulated in liposomes of various compositions. Details of this assay can be found in Nicol et al. (1996) and Parente et al. (1990b). To initiate a leakage experiment, the peptide GALA, in a stock solution at pH 8.0 containing 5 mM TES and 50 mM KCl, was added to the stirred vesicle suspension (0.1 mM lipid) at 20°C in the appropriate buffer. The reaction buffer consisted of 5 mM sodium acetate, 100 mM KCl, and the pH was adjusted to 5.0 with sodium hydroxide.

Measurements of leakage of neutral 3000 MW Texas red dextran

REV liposomes containing neutral 3000 MW Texas red dextran were prepared as indicated above. The determination of GALA-induced dextran leakage, when the dextran is encapsulated alone or when it is coencapsulated with ANTS/DPX, is described elsewhere (Nicol et al., 1996). This leakage was not affected by the presence of ANTS/DPX and was terminated within 45 min after the addition of GALA to the vesicles. Vesicles without encapsulated material were incubated with the Texas red dextran and fractionated on a Sepharose 4-B column (1×15 cm) to determine if any correction was necessary for binding of this compound to the lipid. No correction was needed.

Theoretical analysis of pore formation

The model assumes that once added to a vesicle suspension, the peptide binds rapidly to the bilayer of the vesicles, where it aggregates with a degree of reversibility that can be characterized by a coefficient denoted K_s (Nicol et al., 1996). When an aggregate within a bilayer has reached a critical size (i.e., it consists of M peptides), a pore is created and leakage of liposome-encapsulated molecules occurs. The size of the pore depends on the number of peptides forming it and dictates the upper bound on the size and shape of the molecules that can leak. Detailed derivations of the model can be found in Parente et al. (1990b), Rapaport et al. (1996), and Nicol et al. (1996).

RESULTS

Effect of acyl-chain composition on GALA-mediated leakage of ANTS/DPX from liposomes

To determine if GALA-induced leakage of ANTS/DPX from large unilamellar PC and PC/PG vesicles is influenced by modifications in the structure of the phospholipid acyl chains, we examined four acyl-chain compositions: 1-palmitoyl 2-oleoyl (PO), 1,2-dioleoyl (DO), 1,2-dielaidoyl (DE), and 1,2-dipetroselinoyl (DPe). The sn-1 position of the PO phospholipids is saturated (16:0), and the sn-2 position contains a *cis* unsaturation on the ninth carbon (18:1n-9). Both acyl chains of the DO and the DE phospholipids carry an unsaturation on the ninth carbon (18:1n-9)(18:1n-9), a *cis* unsaturation for the DO lipids and a *trans* unsaturation for the DE lipids. The DPe phospholipids con-

tain a *cis* unsaturation on both chains, like the DO lipids, but on the sixth carbon (18:1n-6)(18:1n-6). The gel-to-liquid crystalline phase transition temperatures (T_m) of the four phospholipids as fully hydrated vesicles are −20°C (DOPC), −2°C (POPC), +1°C (DPePC), and +12°C (DEPC).

GALA-induced leakage from POPC liposomes at pH 5.0 was not modified when the negatively charged lipid POPG was added up to a POPC/POPG molar ratio of 1:1 (Nicol et al., 1996). The same result was obtained for DEPC/DEPG, DPePC/DPePG, and DOPC/DOPG liposomes (data not shown). Furthermore, GALA-induced leakage from the above vesicles was an "all-or-none" process, similar to what was previously observed with POPC, POPC/POPG, POPC/POPG/cholesterol, and egg PC liposomes (Parente et al., 1990b; Nicol et al., 1996).

In the next experiments the anionic phospholipid PG was included in the vesicle composition at a PC/PG molar ratio of 5:1 to avoid vesicle aggregation at high lipid and peptide concentrations, as well as to better mimic the composition of biological membranes. Release of ANTS/DPX from POPC/POPG (5:1), DEPC/DEPG (5:1), DOPC/DOPG (5:1), and DPePC/DPePG (5:1) liposomes followed a similar pattern after the addition of GALA to the liposomal suspension at pH 5.0 (Fig. 1). However, the leakage kinetics depended on the phospholipid composition of the vesicles. Indeed, ANTS/DPX leakage from DOPC/DOPG vesicles reached a plateau at longer times after GALA addition.

The extent of ANTS/DPX leakage at the plateau was monitored at pH 5.0 as a function of the lipid/peptide molar ratio, for the four liposome compositions mentioned above, and a lipid concentration of 0.1 mM (Fig. 2). A decrease in the lipid/peptide molar ratio, i.e., an increase in the average number of peptides per liposome, resulted in an increased extent of ANTS/DPX leakage at the plateau, for each liposome composition assayed.

GALA efficiency in inducing ANTS/DPX leakage by pore formation from PC/PG liposomes was highly dependent on the conformation of the acyl chains (Fig. 2). The final extents of leakage were similar in the case of POPC/POPG and DEPC/DEPG liposomes. Thus a modification of the acyl-chain structure from DE (18:1n-9*trans*)(18:1n-9*trans*) to PO (16:0)(18:1n-9*cis*) did not alter the ability of GALA to form pores in PC/PG vesicles.

However, pore formation was significantly reduced with phospholipids that contain a *cis* unsaturation on both chains. In comparison to POPC/POPG liposomes, a lower lipid/peptide molar ratio, i.e., a higher peptide concentration, was required with DPePC/DPePG (18:1n-6*cis*)(18:1n-6*cis*) liposomes to obtain the same extent of ANTS/DPX leakage. This phenomenon was accentuated with DOPC/DOPG (18:1n-9*cis*)(18:1n-9*cis*) liposomes, where between five- and sixfold more peptide was needed to yield the same extent of leakage than with POPC/POPG liposomes.

The reduced efficiency of the peptide in forming pores might be directly related to a decrease in the mean molecular order of the phospholipid acyl chains. Indeed, the overall flexibility of the *cis* unsaturated chains of the DO lipids is higher than that of the PO and DE phospholipid

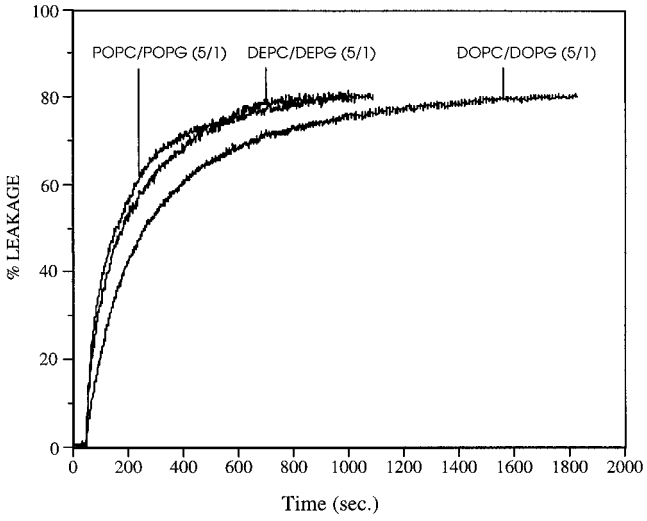

FIGURE 1 Kinetics of GALA-induced ANTS/DPX leakage from POPC/POPG (5:1), DEPC/DEPG (5:1), and DOPC/DOPG (5:1) liposomes at pH 5.0. A final extent of leakage of 80% was obtained for a lipid concentration of 0.1 mM and peptide concentrations of 15.4 nM (lipid/peptide molar ratio of 6500), 15.9 nM (lipid/peptide molar ratio of 6300), and 66.7 nM (lipid/peptide molar ratio of 1500), respectively.

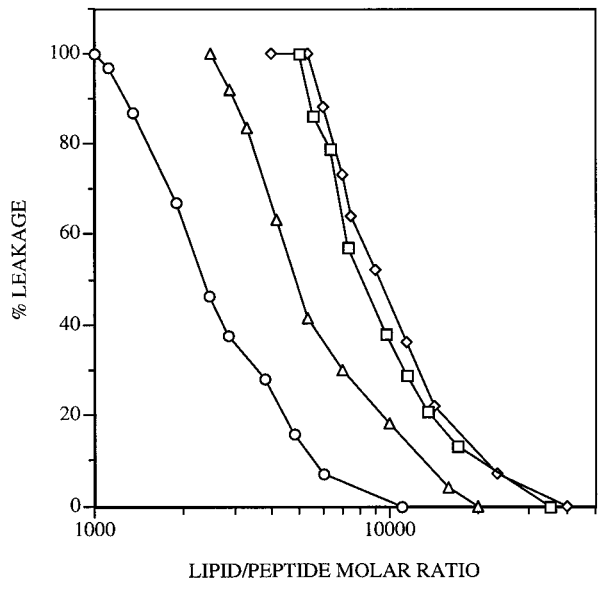

FIGURE 2 Final extents of ANTS/DPX leakage from liposomes as a function of lipid/peptide ratio at pH 5.0. Curves are given for DOPC/DOPG (5:1) (○), DPePC/DPePG (5:1) (△), DEPC/DEPG (5:1) (□), and POPC/POPG (5:1) (◇) liposomes. The lipid concentration was kept constant at 0.1 mM. The final extents of leakage at a given lipid/peptide ratio and liposome composition varied by no more than ±4% when the extents of leakage were above 20% and by no more than ±3% below 20% leakage.

acyl chains. However, the packing of the DPe lipids in the vesicles bilayer is similar to that of the PO lipids, as indicated by their comparable gel-to-liquid crystalline phase transition temperature (T_m), but GALA was less active in inducing ANTS/DPX leakage by pore formation from DPePC/DPePG vesicles (Fig. 2). Moreover, the mean molecular order of the acyl chains of the DE phospholipids is higher than that of the PO lipids, but GALA-induced leakage was similar with the two lipids. Lipid packing can also be increased by decreasing the temperature (Korstanje et al., 1989). However, GALA-induced ANTS/DPX leakage from POPC/POPG and DOPC/DOPG vesicles at pH 5.0 was not modified by a decrease in the temperature from 20°C to 2°C (data not shown).

Thus the process of ANTS/DPX leakage by pore formation is highly modulated by the physical properties of the bilayer, but differences in the peptide activity cannot be explained only by modifications of the mean order parameter of the lipid acyl chains.

Membrane-binding affinity of GALA

Membrane-binding studies were performed with a mixture of unlabeled and NBD-labeled GALA, at a molar ratio where NBD self-quenching in solution and in bilayers is insignificant (Nicol et al., 1996). Modifying GALA with NBD did not alter its ability to induce leakage from any of the vesicles studied here. In addition, no change in the turbidity of the liposome suspension was detected after addition of the peptide under the conditions tested, suggesting that neither aggregation nor fusion of vesicles occurred (data not shown).

Two peptide concentrations (0.125 μM and 0.025 μM) were used to determine the fraction of membrane-bound peptides as a function of the lipid concentration at pH 5.0, for the four liposome compositions mentioned above (Fig. 3, *a* and *b*). The fraction of membrane-bound GALA increased when the lipid concentration was raised, for each peptide concentration and liposome formulation assayed (Fig. 3, *a* and *b*). At all peptide and lipid concentrations tested, the rank order of the membrane-bound fraction was DEPC/DEPG $<$ DPePC/DPePG $\approx$ POPC/POPG $<$ DOPC/DOPG.

These results support the idea that the flexibility of the lipid acyl chains modulates the membrane-binding affinity of GALA, because a reduced chain flexibility corresponded to a smaller fraction of membrane-bound peptides (from the ordered DE acyl chains to the flexible DO acyl chains). It has been suggested that *cis* unsaturations cause the acyl chains to occupy a slightly wedge-shaped space because of an inhomogeneous decrease in chain order that is largest toward the end of the chain (Holte et al., 1995). This loosens the packing of the phospholipids at the membrane surface. Consequently, a "free volume" exists at the lipid-water interface between neighboring molecules (Holte et al., 1995, 1996). An increase in free volume expands the interface and results in an increased membrane-binding affinity of the amphipathic α-helical GALA.

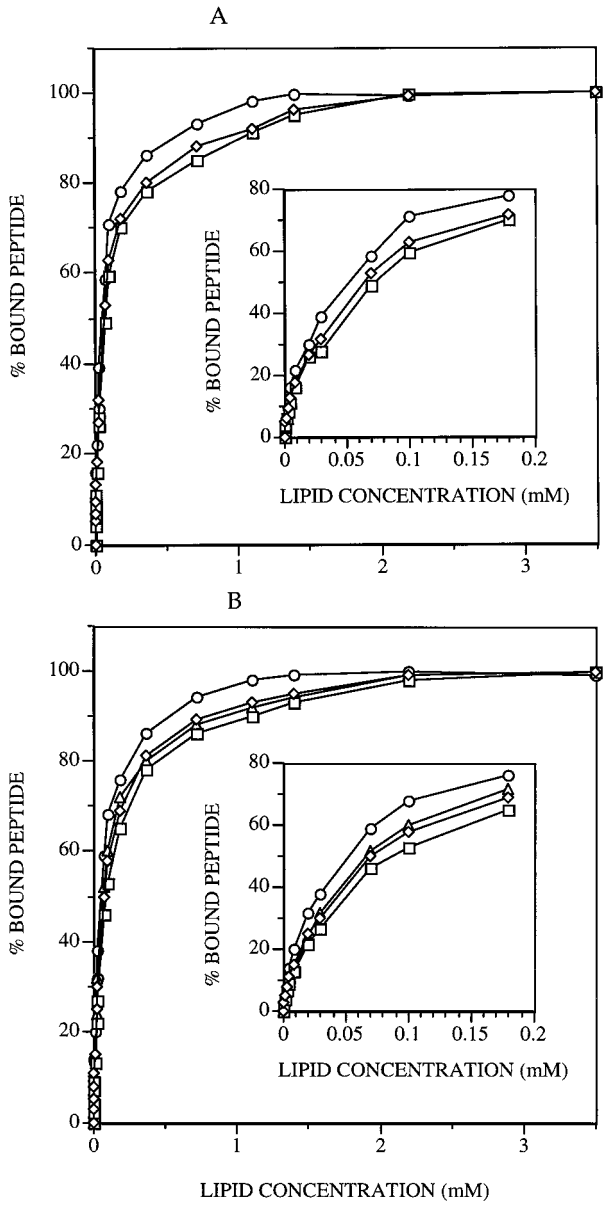

FIGURE 3 Effect of liposome composition on percentage of binding of NBD-labeled GALA to liposomes at pH 5.0. ○, DOPC/DOPG (5:1); △, DPePD/DPePG (5:1); ◇, POPC/POPG (5:1); □, DEPC/DEPG (5:1). A mixture of NBD-GALA/unlabeled GALA of 1:9 was used for a total peptide concentration of 0.125 μM (*A*), and a mixture at a 1:5 ratio was used for a total peptide concentration of 0.025 μM (*B*). The increase in NBD fluorescence intensity was monitored with excitation at $\lambda = 467$ nm and emission at $\lambda = 525$ nm. The fraction of membrane-bound GALA was determined as described in Materials and Methods. Each data point is an average of three experiments. The largest deviations were within 6% of the average value. The insets emphasize the range of lipid concentrations up to 0.2 mM.

To perform the theoretical analysis of GALA-induced ANTS/DPX leakage at a lipid concentration of 0.1 mM

(Fig. 2), we needed to determine the fraction of membrane-bound peptides at this lipid concentration. For a peptide concentration of 0.125 μM, which corresponded to a lipid/total peptide ratio of 800, the percentages of membrane-bound peptides were 63, 59.5, 61, and 71 with POPC/POPG (5:1), DEPC/DEPG (5:1), DPePC/DPePG (5:1), and DOPC/DOPG (5:1) liposomes, respectively. For a peptide concentration of 0.025 μM (lipid/total peptide ratio of 4000 with 0.1 mM lipid) the percentages of bound-peptides were 58, 53, 60, and 68, respectively. Furthermore, by combining these data with those in Fig. 2 we plotted in Fig. 4 the percentage of ANTS/DPX leakage as a function of the lipid to membrane-bound peptide ratio. It is clear that the number of peptides required on a vesicle to form a pore yielding ANTS/DPX leakage increases when the bilayer composition is modified from POPC/POPG and DEPC/DEPG to DOPC/DOPG vesicles, and to a smaller extent when the modification is to DPePC/DPePG liposomes. Thus the reduced ANTS/DPX leakage from DpePC/DpePG and DOPC/DOPG liposomes (Fig. 2) cannot be explained by a reduced membrane-binding affinity of the peptide.

Leakage when all of the peptide is membrane-bound

We monitored the leakage of ANTS/DPX induced by GALA (pH 5.0) at a number of peptide concentrations (from 0.208 μM to 0.835 μM) and a lipid concentration of 2.5 mM, at which all of the added peptide was bound to the liposomes, regardless of composition (see Fig. 3). For each peptide concentration tested our results demonstrated a reduction of ANTS/DPX leakage with DPePC/DPePG (5:1) and DOPC/DOPG (5:1) liposomes, in comparison to the case of POPC/POPG (5:1) and DEPC/DEPG (5:1) liposomes (Table 1). This reduction was more pronounced with DOPC/DOPG liposomes. Under these conditions, the number of membrane-bound peptides per liposome is similar for each liposome composition. Thus the leakage results indicated that in DPePC/DPePG (5:1) and DOPC/DOPG (5:1) bilayers, a higher number of membrane-bound peptides was required to yield a pore. These results confirmed that the reduction in GALA activity observed with DPePC/DPePG (5:1) and DOPC/DOPG (5:1) vesicles at a lipid concentration of 0.1 mM was not related to a decrease in the fraction of peptide that is membrane-bound.

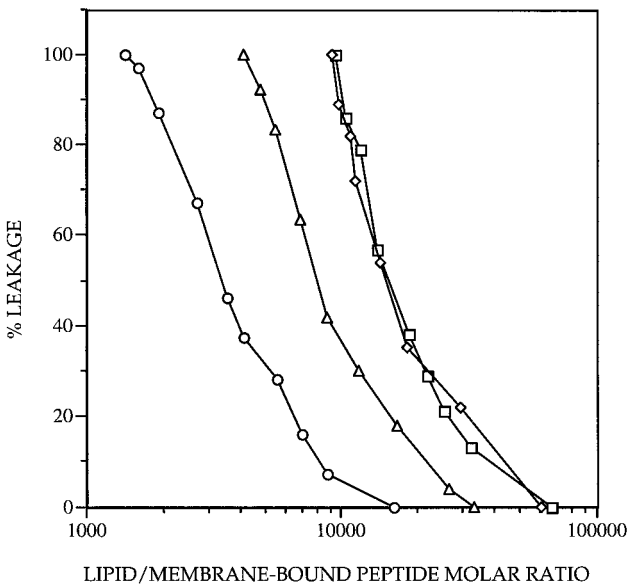

FIGURE 4 Final extents of ANTS/DPX leakage from liposomes as a function of lipid/membrane-bound peptide ratio at pH 5.0. Curves are given for DOPC/DOPG (5:1) (○), DPePC/DPePG (5:1) (△), DEPC/DEPG (5:1) (□), and POPC/POPG (5:1) (◇) liposomes. The data are derived from Fig. 2, using the fraction of membrane-bound peptides at a lipid concentration of 0.1 mM from Fig. 3.

Leakage of dextran from liposomes

For each liposome composition, there was a minimal peptide concentration or a maximum lipid/peptide molar ratio for which complete ANTS/DPX leakage was obtained. This ratio depended on the liposome composition; it was 5300, 5000, 2500, and 1000 with POPC/POPG (5:1), DEPC/DEPG (5:1), DPePC/DPePG (5:1), and DOPC/DOPG (5:1) liposomes, respectively (Figs. 2 and 5). When a neutral 3000 MW Texas red dextran was coencapsulated with ANTS/DPX, it did not leak at this lipid/peptide molar ratio (Fig. 5). In the case of POPC/POPG and DEPC/DEPG liposomes, about sevenfold more peptide (lipid/peptide molar ratio of 750) was necessary, for the same lipid concentration of 0.1 mM, to initiate the leakage of dextran, whereas with DOPC/DOPG and DPePC/DPePG liposomes, fourfold and sevenfold more peptide (lipid/peptide molar ratios of 250 and 375) was needed, respectively. The size of the pore

TABLE 1 Final extents of ANTS/DPX leakage under conditions of complete binding of the peptide to liposomes (2.5 mM lipid)

	Lipid acyl chains			
	PO	DE	DPe	DO
Lipid/peptide molar ratio*	Percentage leakage†			
12,000	68.5	64	27	0
9,000	100	100	45	5
6,000	100	100	85	21
3,000	100	100	100	68

*GALA concentrations tested ranged from 0.208 μM (lipid/peptide molar ratio of 12,000:1) to 0.833 μM (lipid/peptide molar ratio of 3000:1).
†Percentage leakage of ANTS/DPX at pH 5.0 from POPC/POPG (5:1) (mol/mol) (PO), DEPC/DEPG (5:1) (DE), DPePC/DPePG (5:1) (DPe), and DOPC/DOPG (5:1) (DO) liposomes. The lipid concentration was kept constant at 2.5 mM. Final extents of leakage were obtained from the average of three measurements. The variation in final extents of leakage was no more than ±4%.

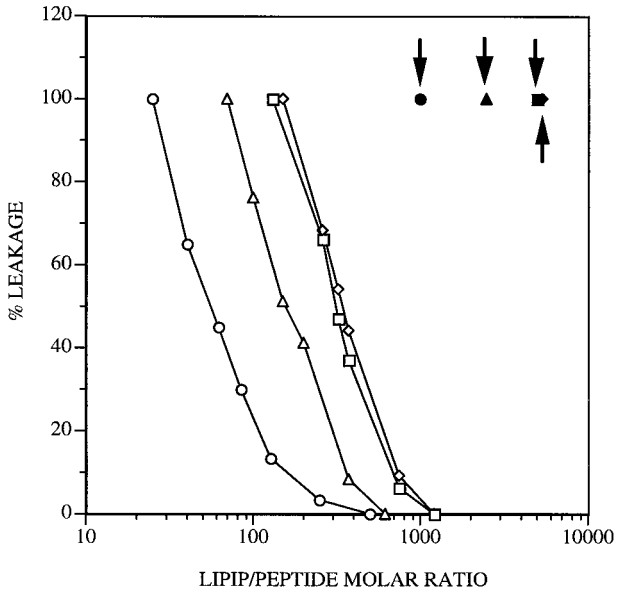

FIGURE 5 Percentage leakage of neutral Texas red dextran (MW 3000) from liposomes as a function of lipid/peptide molar ratio at pH 5.0. ○, DOPC/DOPG (5:1); △, DPePC/DPePG (5:1); □, DEPC/DEPG (5:1); ◇, POPC/POPG (5:1). The lipid concentration was kept constant at 0.1 mM. Each data point is an average of three experiments. The final extents of leakage at a given lipid/peptide molar ratio varied by no more than ±6%. The maximum lipid/peptide molar ratio where 100% leakage of ANTS/DPX is obtained (indicated by an *arrow*) is 1000, 2500, 5000, and 5300 for DOPC/DOPG (5:1) (●), DPePC/DPePG (5:1) (▲), DEPC/DEPG (5:1) (■), and POPC/POPG (5:1) (♦) liposomes, respectively.

depends on the number of peptides forming it and dictates the size of the aqueous marker that can permeate. Given the Stokes radius of 3000 MW dextran (11–12 Å), its leakage would require a pore composed of at least 12–14 transmembrane GALA molecules. Absence of dextran leakage in the range of lipid/peptide molar ratios where the ANTS/DPX leakage was monitored demonstrated that the pore structure is restrained to less than 12–14 peptides for each liposome formulation tested. We verified, with a separate encapsulation of ANTS/DPX or dextran, that the coencapsulation did not affect the leakage efficiency for the two dyes (data not shown).

The reduction of ANTS/DPX leakage from DPePC/DPePG and DOPC/DOPG vesicles might arise from an increase in the size of the GALA pores, because larger pores require the assembly of more transmembrane GALA molecules. The minimum size of the pores formed in POPC/POPG vesicles that induce ANTS/DPX permeation is $M = 10 \pm 2$ peptides (Parente et al., 1990b; Nicol et al., 1996). If only pores composed of at least x-fold more peptides were formed in DPePC/DPePG and DOPC/DOPG liposomes, the formation of a single pore in such a liposome would require at least x-fold more membrane-bound peptides, assuming the reversibility of GALA aggregation is similar in all membrane types. The restrained size of the pore causing ANTS/DPX leakage from DPePC/DPePG and DOPC/DOPG vesicles (limited to $M = 12$–14 peptides) disproves this explanation of the acyl-chain effect on GALA-induced leakage.

Leakage of ANTS/DPX from PE-containing liposomes

In addition to the presence of *cis* unsaturations, the shape of a phospholipid molecule and its area at the lipid/water interface can be modified by changing the size of its headgroup. The slightly wedge-shaped space occupied by the acyl chains near the glycerol backbone when *cis* double bonds are introduced in both acyl chains (as in the DO and the DPe phospholipids) can be accentuated by reducing the size of the headgroup (Lee et al., 1993). Because of this inverted-cone shape, *cis* unsaturated phosphatidylethanolamine (PE) phospholipids, which frequently constitute a substantial fraction of biological membranes (Cullis et al., 1985), possess a large spontaneous curvature responsible for the formation of H_{II} phases at relatively low temperatures (Gruner, 1989).

When POPE was added to POPC and DOPE was added to DOPC in the liposome composition at 40 mol%, GALA-induced ANTS/DPX leakage was reduced by about three- and fivefold, respectively (Fig. 6), while an incorporation of

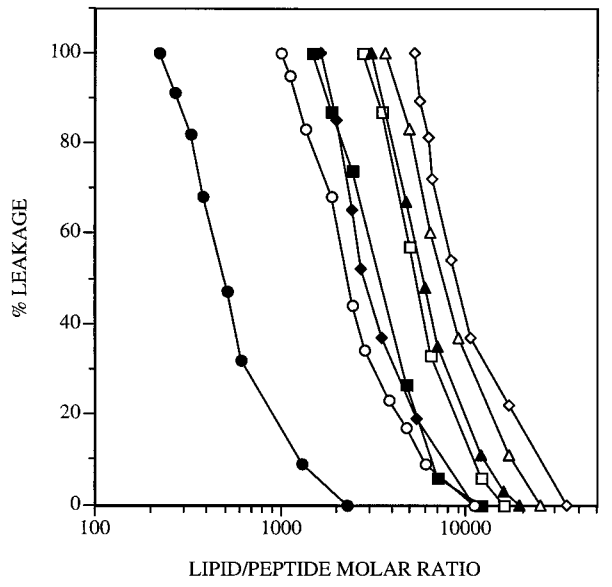

FIGURE 6 Effect of the phospholipid headgroup on final extents of ANTS/DPX leakage from liposomes as a function of lipid/peptide ratio at pH 5.0. Curves are given for POPC (△), POPC/POPE (60:40) (♦), POPC/DOPC (85:15) (△), POPC/DOPC (75:25) (▲), POPC/DOPE (85:15) (□), POPC/DOPE (75:25) (■), DOPC (○), and DOPC/DOPE (60:40) (●) liposomes. The lipid concentration was kept constant at 0.1 mM. The final extents of leakage at a given lipid/peptide ratio and liposome composition varied by no more than ±4% when the extents of leakage were above 20% and by no more than ±3% below 20% leakage.

15 mol% POPE into a POPC bilayer did not affect the leakage efficiency of the peptide (data not shown).

We also compared the effects of the inclusion in a POPC bilayer of either the unsaturated phosphatidylcholine DOPC or the unsaturated phosphatidylethanolamine DOPE on GALA-induced ANTS/DPX leakage (Fig. 6). GALA leakage activity was already reduced when 15 mol% DOPC was included in the POPC vesicle. There was a further decrease in leakage as the DOPC content was increased to 25 mol%. When DOPC was replaced by DOPE at the same mol%, the peptide leakage activity was further reduced, so that an inclusion of 15 mol% DOPE had an effect similar to that of 25 mol% DOPC. Moreover, the membrane-bound fraction of GALA increased slightly with the DOPC or DOPE content of the membrane (data not shown). Thus both the presence of a *cis* unsaturation on each phospholipid acyl chain and a decrease in the size of the headgroup reduce pore formation by GALA.

Theoretical analysis of GALA-induced ANTS/DPX leakage

Following the procedure described by Rapaport et al. (1996) and Nicol et al. (1996), the analysis of final extents of leakage employs two parameters, M, the minimal number of peptides required to form a pore that permits the leakage of ANTS/DPX, and K_s, which measures the degree of reversibility of the peptide in the bilayer. The experimental results of final extents of ANTS/DPX leakage from POPC/POPG and DEPC/DEPG liposomes are predicted very well by the model calculations by setting $M = 10$ and $K_s = 15$, which amounts to a very small degree of reversibility (Fig. 7 and Table 2). The data could also be fitted by setting an infinite value for K_s, i.e., ignoring the reversibility of surface aggregation of the peptide. Thus the values of K_s and M deduced previously for POPC/POPG liposomes (Nicol et al., 1996) could be extended to the case of DEPC/DEPG liposomes. In Nicol et al. (1996), a small degree of reversibility in POPC/POPG liposomes was demonstrated by fluorescence energy transfer measurements using NBD- and rhodamine-labeled peptides. A larger degree of surface aggregation was deduced in cholesterol-containing liposomes, in accord with the findings of the model calculations. Hence K_s is an essential parameter for the description of pore formation by the peptide. The comparison between experimental and calculated values of final extents of ANTS/DPX leakage from POPC/POPG and DEPC/DEPG liposomes is shown in Fig. 7. Table 2 presents a summary of these fits, which give $R^2 = 0.98$ in both cases. For the case of DPePC/DPePG liposomes, the best fit to the data was obtained by setting $M = 12$ and $K_s = 1.3$. As is seen in Fig. 7 and Table 2 ($R^2 = 0.94$), the fit is not as good as in the previous cases, but the model can still yield a reasonable simulation of the data. Setting $M = 12$ for the pore size is still in agreement with Parente et al. (1990b) and Nicol et al. (1996), where $M = 10 \pm 2$. As is shown in Fig. 7 and Table 2, the current model gives a rather poor fit to the experimental values of final extents of leakage from DOPC/DOPG liposomes when M is set at 10 ($R^2 = 0.75$), yielding overestimates for high lipid/peptide molar ratios and underestimates for low lipid/peptide ratios. In a separate calculation we set $M = 10$ and determined variable values of K_s that would give a perfect fit to the experimental results. These values varied monotonically from 0.01 for a lipid/peptide molar ratio of 11,000 to 15 for a lipid/peptide molar ratio of 1000.

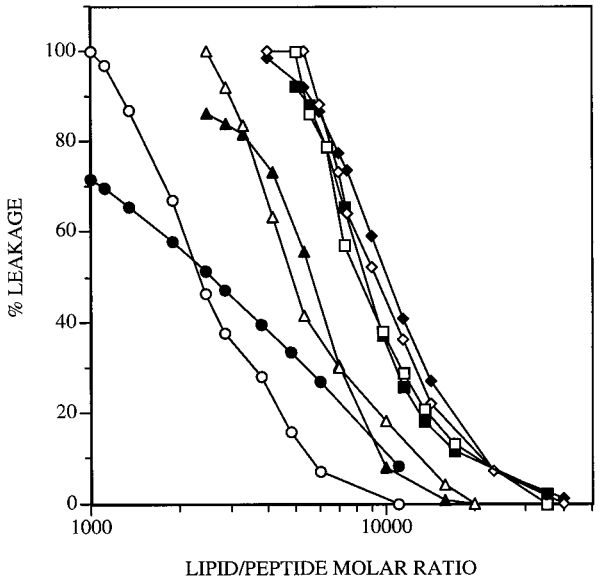

FIGURE 7 Experimental and calculated final extents of leakage from liposomes at pH 5.0 as a function of lipid/peptide molar ratio. Curves are given for DOPC/DOPG (5:1) (exp. (○), cal. (●)), DPePC/DPePG (5:1) (exp. (△), cal. (▲)), DEPC/DEPG (5:1) (exp. (□), cal. (■)), and POPC/POPG (5:1) (exp. (◇), cal. (◆)). The parameters used in the calculations are listed in Table 2.

DISCUSSION

Natural amphipathic α-helical toxins exert their cytotoxic activities in a selective manner that depends upon the lipid

TABLE 2 Summary of calculations to obtain the best fits of final extents of ANTS/DPX leakage

Liposome composition	M	K_s	R^2	RMSE (%)*
POPC/POPG (5:1) (16:0)(18:1n-9*cis*)	10	15	0.98	5.1
DEPC/DEPG (5:1) (18:1n-9*trans*)(18:1n-9*trans*)	10	15	0.98	4.5
DPePC/DPePG (5:1) (18:1n-6*cis*)(18:1n-6*cis*)	12	1.3	0.94	9.1
DOPC/DOPG (5:1) (18:1n-9*cis*)(18:1n-9*cis*)	10	0.12	0.75	18.6

*RMSE (%) is defined by RMSE $= [\sum_{i=1}^{n} (Y_i - Y_{ci})^2/(n-1)]^{1/2}$, in which Y_i and Y_{ci} are experimental and calculated final extents of leakage (%), respectively.

composition of the target cell membrane. The understanding of the mechanisms of action of such peptides was significantly advanced by biophysical studies with lipid vesicles that mimic the lipid composition of relevant biomembranes (Gazit et al., 1994, 1995; Matsuzaki et al., 1995b; Silvestro et al., 1997). Studies with analogs of natural toxins (Matsuzaki et al., 1997a,b; Shai, 1994) and with model amphipathic α-helices that consist of optimized or simplified sequences, such as GALA (Subbarao et al., 1987; Parente et al., 1990a,b; Nicol et al., 1996), have contributed to a better understanding of selected aspects of peptide-bilayer interactions and the mechanisms of pore formation. In the case of the synthetic pH-sensitive peptide GALA, the lipid composition of liposomes modulates the propensity of the peptide to form pores in their bilayer. At low peptide concentrations, GALA aggregates quasiirreversibly in POPC and egg PC bilayers at pH 5.0 to form pores composed of 10 ± 2 peptides (Parente et al., 1990b; Nicol et al., 1996). An increase of the membrane negative surface potential, generated by inclusion of the negatively charged phospholipid POPG in the bilayer, does not alter GALA's pore-forming capacity. However, the addition of cholesterol to the vesicle composition reduces GALA activity, because of a decreased membrane-binding affinity and an increased reversibility of peptide self-association in the bilayer (Nicol et al., 1996).

Reduced pore formation capacity by GALA in a bilayer composed of [(18:1 *cis*) (18:1 *cis*)] phospholipids

The efficiency of GALA in inducing ANTS/DPX leakage at pH 5.0 followed the rank order of POPC/POPG $\approx$ DEPC/DEPG $>$ DPePC/DPePG $>$ DOPC/DOPG (Fig. 2), whereas the binding affinity of GALA to liposomes of these compositions followed the rank order DOPC/DOPG $>$ DPePC/DPePG $\approx$ POPC/POPG $>$ DEPC/DEPG (Fig. 3). Thus under conditions of equal average number of membrane-bound peptides per liposome, the extent of leakage was significantly higher in POPC/POPG and DEPC/DEPG liposomes than in DOPC/DOPG vesicles (Fig. 4 and Table 1). This means that the number of bound GALA peptides required to form a pore yielding ANTS/DPX leakage in the bilayer of a DOPC/DOPG vesicle was appreciably higher than that needed in a POPC/POPG bilayer.

Rex (1996) reported that, for the same number of membrane-bound peptides per vesicle, melittin is less efficient at inducing carboxyfluorescein leakage from DOPC liposomes than from POPC liposomes. A kinetic analysis of the transient dye leakage concluded that the rate of pore openings is larger with the latter vesicles (Rex and Schwarz, 1998).

The configuration of the phospholipid packing in the bilayer can influence the membrane binding of an amphipathic α-helical peptide. Melittin (Dasseux et al., 1984) and the 18L model peptide, which features the consensus sequence of class L (lytic) peptides (Polozov et al., 1997), preferentially associate with bilayers in the liquid-crystalline state. We observed a decreased affinity of GALA for POPC/POPG membranes that contain cholesterol (Nicol et al., 1996). This effect might arise from a decrease in the hydrophobic area accessible to peptide binding at the bilayer interface. When PO phospholipids are replaced by DO phospholipids, the presence of a *cis* unsaturation on both acyl chains increases the chain flexibility, particularly in the hydrophobic core of the bilayer. Consequently, the hydrophobic region of the bilayer exposed to the aqueous phase and accessible for the binding of GALA amphipathic α-helix is expanded, which results in an increase in the membrane-binding affinity of the peptide.

We consider several explanations for the higher number of bound peptides needed to generate a pore that permits ANTS/DPX leakage in DOPC/DOPG and DPePC/DPePG bilayers than in the POPC/DOPG and DEPC/DEPG bilayers.

1. Pores formed in DOPC/DOPG and DPePC/DPePG bilayers contain more peptide monomers (larger pores) than pores formed in POPC/POPG bilayers. The minimum number of α-helices that compose the pores required for ANTS/DPX leakage from POPC/POPG liposomes is $M = 10 \pm 2$. The lack of leakage of a neutral 3000 MW dextran at the maximum lipid/peptide molar ratio where ANTS/DPX leakage is complete demonstrates that the size of the pores is restrained to less than 14 peptides for all liposome compositions (Fig. 5). Thus larger pores do not account for the effect of the phospholipid acyl chain structure on GALA-induced ANTS/DPX leakage.

2. There is an increased reversibility of peptide aggregation in DOPC/DOPG membranes. This has been proposed to be a major factor in reducing ANTS/DPX leakage from POPC/POPG vesicles that contain cholesterol (Nicol et al., 1996). In that case, model calculations could fit experimental leakage extents, retaining a minimum pore size similar to that of POPC/POPG vesicles ($M = 10 \pm 2$), but using a reduced K_s (higher reversibility of peptide aggregation). In the present analysis, however, we were unable to obtain a satisfactory fit to the data on leakage of ANTS/DPX from DOPC/DOPG vesicles when the minimum pore size of 10 peptides was retained and K_s was changed (Fig. 7 and Table 2). Even when M was set at 12 or 14, which is the maximum realistic value for the pore size given the lack of leakage of dextran, no satisfactory fit was obtained. These results suggest that the mathematical model does not properly describe the situation in the case of this liposome composition (Fig. 7 and Table 2). At this stage, we prefer to discard the possibility of allowing K_s to vary with the size of peptide aggregates because too many parameters, i.e., multiple K_s values, would have to be included in the analysis.

3. There is a phospholipid-dependent insertion of surface aggregates in the bilayer core. This possibility extends the original model of pore formation by GALA with an additional constraint and requires a modification of the mathematical analysis, i.e., the inclusion of a probability of inser-

tion for each surface aggregate size. We did not carry out this extension of the model because, again, too many parameters would have to be included, and, therefore, the calculations would not necessarily give an adequate picture of the reality. Precedents for lipid-dependent insertion of surface-adsorbed peptides were reported with alamethicin and magainin-2 (Huang and Wu, 1991; Ludtke et al., 1994, 1995; He et al., 1995, 1996a,b; Wu et al., 1995). These peptides are either adsorbed on the membrane surface at low peptide/lipid molar ratios or inserted parallel to the bilayer normal at high peptide/lipid molar ratios (Huang and Wu, 1991; Ludtke et al., 1994), with a coexisting region in between. There is a critical peptide/lipid molar ratio for insertion, (P/L)*, marking the beginning of the coexistence region from the low peptide concentration side. In DPhPC bilayers (P/L)* $\approx$ 1/40, whereas in membranes composed of the phospholipids DOPC and DLPC, (P/L)* $\approx$ 1/200 and (P/L)* $<$ 1/300, respectively. X-ray diffraction measurements demonstrated that the main effect of peptide adsorption is to reduce the thickness of the hydrocarbon region of the bilayer proportionally to the peptide concentration (Wu et al., 1995; Ludtke et al., 1995).

When the peptide is adsorbed on the surface of the bilayer, it displaces the lipid headgroups. Because the total volume of the acyl chains is constant, peptide adsorption causes the membrane to become thinner. As a result of this deformation of the bilayer, the free energy of peptide adsorption increases when the peptide concentration is raised in the membrane, such that peptide insertion becomes energetically favorable above a critical peptide/lipid molar ratio (He et al., 1996a). It was found that alamethicin and magainin-2 adsorbed on the surface of the bilayer are dispersed rather than aggregated. In the inserted state, neutron scattering measurements in the plane of the membrane revealed that these peptides form pores.

In the case of GALA, the formation of a pore that allows ANTS/DPX leakage in POPC/POPG and DEPC/DEPG liposomes occurs at much lower peptide concentrations than for alamethicin, because it requires only 10 $\pm$ 2 peptides bound to a vesicle. Thus unlike the cases of alamethicin and magainin-2, global thinning of the membrane can be ignored in our case.

Our model (Parente et al., 1990b; Nicol et al., 1996, 1999) suggests that GALA forms aggregates at the surface of the POPC/POPG bilayer before it inserts into the hydrophobic core. Thus, in comparison to alamethicin, the bilayer surface has to accommodate aggregates instead of monomers. We suggest that, with certain bilayer compositions, when GALA surface aggregates reach a critical size, they create a energetically high local deformation of the membrane that increases their instability and promotes their insertion and subsequent pore formation. This interpretation would explain why a pore that permits leakage of ANTS/DPX is formed when only 10 $\pm$ 2 peptides are bound to a vesicle.

This qualitative concept can also be applied to understanding the effect of acyl-chain composition on pore formation. When GALA is bound to a DOPC/DOPG bilayer, the surface state is more favored energetically relative to a POPC/POPG bilayer, and the inserted state is less favored, which reduces the probability of insertion and subsequent pore formation in DOPC/DOPG liposomes. The DO phospholipids, which contain less ordered acyl chains than the PO phospholipids and have a slightly wedge-like shape, can better accommodate the headgroup displacement because of the GALA surface aggregate than the PO phospholipids. In this regard we note a higher binding affinity of the peptide for DOPC/DOPG liposomes.

Conversely, the inserted state of the peptide might be less favored in DOPC/DOPG vesicles because of the configuration of the acyl chains. The short-range interactions between the transmembrane α-helix and the phospholipid acyl chains might be reduced in this case because the helix can less easily accommodate the angle imposed on the acyl chains by the two *cis* unsaturations (one per chain). We suggest that insertion and pore formation occur in DOPC/DOPG vesicles at higher peptide concentrations than in POPC/POPG vesicles, i.e., when several surface aggregates cause a cooperative local deformation of the membrane.

However, we cannot discard the possibility that transmembrane aggregates are formed in a similar manner in DOPC/DOPG and POPC/POPG liposomes, but these aggregates cannot grow further in DOPC/DOPG vesicles to reach a size that allows ANTS/DPX leakage.

The fact that the lipid/peptide molar ratio had to be reduced to achieve comparable ANTS/DPX leakage from POPC/DOPE liposomes than from POPC/DOPC liposomes (Fig. 6) is consistent with hypothesis 3. Indeed, it suggests that every factor that stabilizes the surface state of the peptide, in this case a smaller headgroup, reduces peptide insertion and subsequent pore formation. The reduction in GALA-induced ANTS/DPX leakage from DOPC/DOPE (60:40) liposomes, in comparison to the case of DOPC liposomes, and from POPC/POPE (60:40) liposomes, in comparison to the case of POPC liposomes (Fig. 6), also supports this hypothesis. In the case of alamethicin, the partial replacement of DPhPC by DPhPE lipids resulted in an increase in the peptide/lipid molar ratio required for insertion. Heller and co-workers (1997) proposed that the onset of insertion occurred at higher peptide concentrations when DPhPE was included because of the small size of the PE headgroup in comparison to the phospholipid cross section in the acyl-chain region (Heller et al., 1997). When DPhPE was present, peptide adsorption was accommodated to a certain extent by displacement of the bound water of the PE headgroup. Therefore, the energy of bilayer deformation induced by surface-adsorbed peptides was lower at a given peptide concentration, which resulted in an increase in the peptide/lipid molar ratio required for insertion.

The same argument can be made in the case of GALA. The bilayer deformation energy caused by GALA aggregate adsorbed on the membrane surface is lower when PE lipids are present. Therefore, PE lipids stabilize this surface state and reduce peptide insertion and pore formation.

CONCLUSION

GALA has proved to be an interesting peptide to use for the exploration of peptide-induced perturbations of bilayer vesicles. It is a pH-sensitive peptide that forms stable transbilayer pores at low peptide/lipid ratios in certain membrane compositions (egg PC, POPC/POPG, and DEPC/DEPG). We have shown here that phospholipid acyl-chain (*cis*-unsaturated DO and DPe) and headgroup (PE) compositions that increase the hydrophobic area at the bilayer-aqueous interfacial region decrease leakage of liposomal aqueous contents at low pH induced by the peptide GALA. The peptide efficiency in inducing ANTS/DPX leakage is reduced by about fivefold when the acyl-chain composition is varied from POPC to DOPC and further decreased by fivefold when the headgroup composition is varied from DOPC to DOPC/DOPE (60:40). This decrease in leakage occurs under conditions where peptide binding increases. We attribute the reduced pore formation in these bilayers to a stabilization of GALA binding in an orientation parallel to the vesicle surface.

This work was supported by a grant from the National Institutes of Health (DK 46052).

REFERENCES

Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* 234:466–468.

Benachir, T., M. Monette, J. Grenier, and M. Lafleur. 1996. Study of membrane targeting with melittin. *Biophys. J.* 70:425a.

Boman, H. G. 1994. Antimicrobial peptides. Chairman's opening remarks. *Ciba Found. Symp.* 186:1–4.

Chen, H. C., J. H. Brown, J. L. Morell, and C. M. Huang. 1988. Synthetic magainin analogues with improved antimicrobial activity. *FEBS Lett.* 236:462–466.

Christensen, B., J. Fink, R. B. Merrifield, and D. Mauzerall. 1988. Channel-forming properties of cecropins and related model compounds incorporated into planar lipid membranes. *Proc. Natl. Acad. Sci. USA.* 85:5072–5076.

Cullis, P. R., M. J. Hope, B. De Kruijff, A. J. Verkleij, and C. P. S. Tilcock. 1985. *In* Phospholipids and Cellular Regulation. J. F. Kuo, editor. Chap. 1 CRC Press, Boca Raton, FL. 1–59.

Dasseux, J. L., J. F. Faucon, M. Lafleur, M. Pezolet, and J. Dufourcq. 1984. A restatement of melittin-induced effects on the thermotropism of zwitterionic phospholipids. *Biochim. Biophys. Acta.* 755:37–50.

Duckworth, D. H., E. M. Bevers, A. J. Verkleij, J. A. F. O. D. Kamp, and L. L. M. V. Deenen. 1974. Action of phospholipase A2 and phospholipase C on *Escherichia coli*. *Arch. Biochem. Biophys.* 165:379–387.

Dufourc, E. J., I. C. Smith, and J. Dufourcq. 1986. Molecular details of melittin-induced lysis of phospholipid membranes as revealed by deuterium and phosphorus NMR. *Biochemistry.* 25:6448–6455.

Dufourc, E. J., J. Dufourcq, T. H. Birkbeck, and J. H. Freer. 1990. δ-Haemolysin from *Staphylococcus aureus* and model membranes. A solid-state ^{2}H-NMR and ^{31}P-NMR study. *Eur. J. Biochem.* 187: 581–587.

Dufourcq, J., J. F. Faucon, G. Fourche, J. L. Dasseux, M. Le Maire, and T. Gulik-Krzywicki. 1986. Morphological changes of phosphatidylcholine bilayers induced by melittin: vesicularization, fusion, discoidal particles. *Biochim. Biophys. Acta.* 859:33–48.

Ellens, H., J. Bentz, and F. C. Szoka, Jr. 1984. pH-induced destabilization of phosphatidylethanolamine-containing liposomes: role of bilayer contact. *Biochemistry.* 23:1532–1538.

Fattal, E., S. Nir, R. A. Parente, and F. C. Szoka, Jr. 1994. Pore-forming peptides induce rapid phospholipid flip-flop in membranes. *Biochemistry.* 33:6721–6731.

Faucon, J. F., J. M. Bonmatin, J. Dufourcq, and E. J. Dufourcq. 1995. Acyl chain length dependence in the stability of melittin-phosphatidylcholine complexes. A light scattering and. ^{31}P-NMR study. *Biochim. Biophys. Acta.* 1234:235–243.

Frey, S., and L. K. Tamm. 1990. Membrane insertion and lateral diffusion of fluorescence-labelled cytochrome *c* oxidase subunit IV signal peptide in charged and uncharged phospholipid bilayers. *Biochem. J.* 272: 713–719.

Gazit, E., A. Boman, H. G. Boman, and Y. Shai. 1995. Interaction of the mammalian peptide cecropin P1 with phospholipid vesicles. *Biochemistry.* 34:11479–11488.

Gazit, E., W. J. Lee, P. T. Brey, and Y. Shai. 1994. Mode of action of the antibacterial cecropin B2: a spectrofluorometric study. *Biochemistry.* 33:10681–10692.

Gruner, S. M. 1989. Stability of lyotropic phases with curved interfaces. *J. Phys. Chem.* 93:7562–7570.

He, K., S. J. Ludtke, W. T. Heller, and H. W. Huang. 1996a. Mechanism of alamethicin insertion into lipid bilayers. *Biophys. J.* 71:2669–2679.

He, K., S. J. Ludtke, D. L. Worcester, and H. W. Huang. 1995. Antimicrobial peptide pores in membranes detected by neutron in-plane scattering. *Biochemistry.* 34:15614–15618.

He, K., S. J. Ludtke, D. L. Worcester, and H. W. Huang. 1996b. Neutron scattering in the plane of membranes: structure of alamethicin pores. *Biophys. J.* 70:2659–2666.

Heller, W. T., S. T. Ludtke, T. A. Harroun, and H. W. Huang. 1997. Effect of changing the size of lipid headgroup on peptide insertion in membranes. *Biophys. J.* 73:239–244.

Holte, L. L., S. A. Peter, T. A. Sinnwell, and K. Gawrisch. 1995. ^{2}H nuclear resonance order parameter profiles suggest a change of molecular shape for phosphatidylcholines containing a polyunsaturated acyl chain. *Biophys. J.* 68:2396–2403.

Holte, L. L., F. Separovic, and K. Gawrisch. 1996. Nuclear magnetic resonance investigation of hydrocarbon chain packing in bilayers of polyunsaturated phospholipids. *Lipids.* 31:S199–S203.

Huang, H. W., and Y. Wu. 1991. Lipid-alamethicin interactions influence alamethicin orientation. *Biophys. J.* 60:1079–1087.

Katsu, T., M. Kuroko, T. Morikawa, K. Sanchika, Y. Fujita, H. Yamamura, and M. Uda. 1989. Mechanism of membrane damage induced by the amphipathic peptides gramicidin S and melittin. *Biochim. Biophys. Acta.* 983:135–141.

Katsu, T., M. Kuroko, T. Morikawa, K. Sanchika, H. Yamanaka, S. Shinoda, and Y. Fujita. 1990. Interaction of wasp venom mastoparan with biomembranes. *Biochim. Biophys. Acta.* 1027:185–190.

Korstanje, L. J., E. E. van Faassen, and Y. K. Levine. 1989. Reorientational dynamics in lipid vesicles and liposomes studied with ESR: effect of hydration, curvature and unsaturation. *Biochim. Biophys. Acta.* 982: 196–204.

Ladokhin, A. S., M. E. Selsted, and S. H. White. 1997. Sizing membrane pores in lipid vesicles by leakage of co-encapsulated markers: pore formation by melittin. *Biophys. J.* 72:1762–1766.

Lee, Y. C., T. F. Taraschi, and N. Janes. 1993. Support for the shape concept of lipid structure based on a headgroup volume approach. *Biophys. J.* 65:1429–1432.

Ludtke, S. J., K. S. He, W. T. Heller, T. A. Harroun, L. Yang, and H. W. Huang. 1996. Membrane pores induced by magainin. *Biochemistry.* 35:13723–13728.

Ludtke, S. J., K. S. He, and H. W. Huang. 1995. Membrane thinning caused by magainin 2. *Biochemistry.* 34:16764–16769.

Ludtke, S. J., K. S. He, Y. Wu, and H. W. Huang. 1994. Cooperative membrane insertion of magainin correlated with its cytolytic activity. *Biochim. Biophys. Acta.* 1190:181–184.

Matsuzaki, K., M. Harada, S. Funakoshi, N. Fujii, and K. Miyajima. 1991. Physiochemical determinants for the interactions of magainins 1 and 2 with acidic lipid bilayers. *Biochim. Biophys. Acta.* 1063:162–170.

Matsuzaki, K., O. Murase, and K. Miyajima. 1995a. Kinetics of pore formation by an antimicrobial peptide, magainin 2, in phospholipid bilayers. *Biochemistry.* 34:12553–12559.

Matsuzaki, K., A. Nakamura, O. Murase, K. Sugishita, N. Fujii, and K. Miyajima. 1997a. Modulation of magainin 2-lipid bilayer interactions by peptide charge. *Biochemistry.* 36:2104–2111.

Matsuzaki, K., K. Sugishita, N. Fujii, and K. Miyajima. 1995b. Molecular basis for membrane selectivity of an antimicrobial peptide, magainin 2. *Biochemistry.* 34:3423–3429.

Matsuzaki, K., K. Sugishita, M. Harada, N. Fujii, and K. Miyajima. 1997b. Interactions of an antimicrobial peptide, magainin 2, with outer and inner membranes of Gram-negative bacteria. *Biochim. Biophys. Acta.* 1327:119–130.

Matsuzaki, K., S. Yoneyama, and K. Miyajima. 1997c. Pore formation and translocation of melittin. *Biophys. J.* 73:831–838.

Matsuzaki, K., S. Yoneyama, O. Murase, and K. Miyajima. 1996. Transbilayer transport of ions and lipids coupled with mastoparan X translocation. *Biochemistry.* 35:8450–8456.

Mchaourab, H. S., J. S. Hyde, and J. B. Feix. 1994. Binding and state of aggregation of spin-labeled cecropin AD in phospholipid bilayers: effects of surface charge and fatty acyl chain length. *Biochemistry.* 33: 6691–6699.

Monette, M., and M. Lafleur. 1995. Modulation of melittin-induced lysis by surface charge density of membranes. *Biophys. J.* 68:187–195.

Monette, M., and M. Lafleur. 1996. Influence of lipid chain unsaturation on melittin-induced micellization. *Biophys. J.* 70:2195–2202.

Monette, M., M.-R. Van Calsteren, and M. Lafleur. 1993. Effect of cholesterol on the polymorphism of dipalmitoylphosphatidylcholine/melittin complexes: an NMR study. *Biochim. Biophys. Acta.* 1149: 319–328.

Nicol, F., S. Nir, and F. C. Szoka, Jr. 1996. Effect of cholesterol and charge on pore formation in bilayer vesicles by a pH sensitive peptide. *Biophys. J.* 71:3288–3301.

Nicol, F., S. Nir, and F. C. Szoka, Jr. 1999. Orientation of the pore-forming peptide GALA in POPC vesicles determined by a BODIPY-avidin/biotin binding assay. *Biophys. J.* 76:2121–2141.

Parente, R. A., L. Nadasdi, N. K. Subbarao, and F. C. Szoka, Jr. 1990a. Association of a pH-sensitive peptide with membrane vesicles: role of amino acid sequence. *Biochemistry.* 29:8720–8728.

Parente, R. A., S. Nir, and F. C. Szoka, Jr. 1990b. Mechanism of leakage of phospholipid vesicle contents induced by the peptide GALA. *Biochemistry.* 29:8713–8719.

Polozov, I. V., A. I. Polozova, J. G. Molotkovsky, and R. M. Epand. 1997. Amphipathic peptide affects the lateral domain organization of lipid bilayers. *Biochim. Biophys. Acta.* 1328:125–139.

Portlock, S. H., M. J. Clague, and R. J. Cherry. 1990. Leakage of internal markers from erythrocytes and lipid vesicles induced by melittin, gramicidin S and alamethicin: a comparative study. *Biochim. Biophys. Acta.* 1030:1–10.

Pott, T., and E. J. Dufourc. 1995. Action of melittin on the DPPC-cholesterol liquid-ordered phase: a solid state ^{2}H- and ^{31}P-NMR study. *Biophys. J.* 68:965–977.

Rapaport, D., R. Peled, S. Nir, and Y. Shai. 1996. Reversible surface aggregation in pore formation by pardaxin. *Biophys. J.* 70:2502–2512.

Ren, J., S. Lew, Z. Wang, and E. London. 1997. Transmembrane orientation of hydrophobic alpha-helices is regulated both by the relationship of helix length to bilayer thickness and by the cholesterol concentration. *Biochemistry.* 36:10213–10220.

Rex, S. 1996. Pore formation induced by the peptide melittin in different lipid vesicle membranes. *Biophys. Chem.* 58:75–85.

Rex, S., and G. Schwarz. 1998. Quantitative studies on the melittin-induced leakage mechanism of lipid vesicles. *Biochemistry.* 37: 2336–2345.

Rothman, J. E., and E. P. Kennedy. 1977. Asymmetrical distribution of phospholipids in the membrane of *Bacillus megaterium. J. Mol. Biol.* 110:603–618.

Schwarz, G., and A. Arbuzova. 1995. Pore kinetics reflected in the dequenching of a lipid vesicle entrapped fluorescent dye. *Biochim. Biophys. Acta.* 1239:51–57.

Silvestro, L., K. Gupta, J. N. Weiser, and P. H. Axelsen. 1997. The concentration-dependent membrane activity of cecropin A. *Biochemistry.* 36:11452–11460.

Stankowski, S., and G. Schwarz. 1989. Lipid dependence of peptide-membrane interactions. Bilayer affinity and aggregation of the peptide alamethicin. *FEBS Lett.* 250:556–560.

Stankowski, S., U. D. Schwarz, and G. Schwarz. 1988. Voltage-dependent pore activity of the peptide alamethicin correlated with incorporation in the membrane: salt and cholesterol effects. *Biochim. Biophys. Acta.* 941:11–18.

Steiner, H., D. Hultmark, Å. Engström, H. Bennich, and H. G. Boman. 1981. Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature.* 292:246–248.

Subbarao, N. K., C. J. Fielding, R. L. Hamilton, and F. C. Szoka, Jr. 1988. Lecithin: cholesterol transferase activation by synthetic amphipathic peptides. *Proteins.* 3:187–198.

Subbarao, N. K., R. A. Parente, F. C. Szoka, Jr., L. Nadasdi, and K. Pongracz. 1987. pH-dependent bilayer destabilization by an amphipathic peptide. *Biochemistry.* 26:2964–2972.

Szoka, F. C., Jr., and D. Papahadjopoulos. 1978. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl. Acad. Sci. USA.* 75:4194–4198.

Turner, J. D., and G. Rouser. 1970. Precise quantitative determination of human blood lipids by thin-layer and triethylaminoethylcellulose column chromatography. I. Erythrocyte lipids. *Anal. Biochem.* 38:423–436.

Tytler, E. M., G. M. Anantharamaiah, D. E. Walker, V. K. Mishra, M. N. Palgunachari, and J. P. Segrest. 1995. Molecular basis for prokaryotic specificity of magainin-induced lysis. *Biochemistry.* 34:4393–4401.

Verkleij, A. J., R. F. A. Zwaal, B. Roelofsen, P. Comfurius, D. Kastelijn, and L. L. M. Van Deenen. 1973. The asymmetric distribution of phospholipids in the human red cell membrane. A combined study using phospholipases and freeze-etch electron microscopy. *Biochim. Biophys. Acta.* 323:178–193.

Wu, Y., K. He, S. J. Ludtke, and H. W. Huang. 1995. X-ray diffraction study of lipid bilayer membrane interacting with amphiphilic helical peptides: diphytanoyl phosphatidylcholine with alamethicin at low concentrations. *Biophys. J.* 68:2361–2369.

Zasloff, M. 1987. Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc. Natl. Acad. Sci. USA.* 84: 5449–5453.