Effect of Cholesterol and Charge on Pore Formation in Bilayer Vesicles by a pH-Sensitive Peptide

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ABSTRACT The effect of cholesterol on the bilayer partitioning of the peptide GALA (WEAALAEALAEALAEHLAEALAEALEALAA) and its assembly into a pore in large unilamellar vesicles composed of neutral and negatively charged phospholipids has been determined. GALA undergoes a conformational change from a random coil to an amphipathic α-helix when the pH is reduced from 7.0 to 5.0, inducing at low pH leakage of contents from vesicles. Leakage from neutral or negatively charged vesicles at pH 5.0 was similar and could be adequately explained by the mathematical model (Parente, R. A., S. Nir, and F. C. Szoka, Jr., 1990. Mechanism of leakage of phospholipid vesicle contents induced by the peptide GALA. *Biochemistry.* 29:8720–8728) which assumed that GALA becomes incorporated into the vesicle bilayer and irreversibly aggregates to form a pore consisting of 10 ± 2 peptides. Increasing cholesterol content in the membranes resulted in a reduced efficiency of the peptide to induce leakage. Part of the cholesterol effect was due to reduced binding of the peptide to cholesterol-containing membranes. An additional effect of cholesterol was to increase reversibility of surface aggregation of the peptide in the membrane. Results could be explained and predicted with a model that retains the same pore size, i.e., 10 ± 2 peptides, but includes reversible aggregation of the monomers to form the pore. Resonance energy transfer experiments using fluorescently labeled peptides confirmed that the degree of reversibility of surface aggregation of GALA was significantly larger in cholesterol-containing liposomes, thus reducing the efficiency of pore formation.

INTRODUCTION

The understanding of lipid-peptide and lipid-protein interactions has been greatly advanced by the study of cytolytic peptides, such as gramicidin, alamethicin, melittin, and pardaxin, as well as synthetic peptides, such as GALA or pardaxin analogs. Leakage and pore formation have been observed in cells, liposomes, and bilayer membranes upon addition of cytolytic peptides or polypeptides to the medium (DeGrado et al., 1982; Tosteson et al., 1985; Harshman et al., 1989; Wu and Aronson, 1992; Ostolaza et al., 1993; Ghosh et al., 1993; Shai, 1994; Kerr et al., 1995) or during interactions of certain viruses with liposomes and cells (Spruce et al., 1989; Cheetham et al., 1994; White, 1992).

In the current study we investigate the mechanism of leakage induced by the peptide GALA from liposomes of a variety of compositions with a particular emphasis on cholesterol-containing bilayers. Because many biological membranes, such as the plasma membranes of living cells, and hence the endosomal membranes, include cholesterol, it has been of importance to study the effect of this component on the leakage. In fact, we think this is the first systematic study of this issue where the peptide concentration in the bilayer is known. We will show that the presence of cholesterol in membranes requires a significantly larger number of peptides per vesicle for the initiation of leakage. By combining leakage and binding studies with theoretical analysis, we elucidate a mechanism of leakage by pore formation in cholesterol-containing membranes.

The synthetic amphipathic peptide GALA is water-soluble at neutral pH where it has extremely low binding affinity to membranes (Subbarao et al., 1987; Parente et al., 1990). At low pH the neutralization of the glutamic acid residues results in a conversion of the peptide conformation to an amphipathic α-helix, induces high affinity binding to neutral and negatively charged membranes, and causes aqueous content release (Subbarao et al., 1987).

The peptide mediates aggregation and fusion of small unilamellar neutral liposomes (Parente et al., 1988), up to a radius of 50 nm, beyond which fusion terminated. Furthermore, the peptide did not induce aggregation of large unilamellar liposomes. On the other hand, Parente et al. (1990) showed that GALA forms pores consisting of 10 ± 2 peptides in neutral egg phosphatidylcholine (egg PC) large unilamellar vesicles (LUV).

Several peptides, known to induce leakage and membrane destabilization, such as GALA (Haensler and Szoka,

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Abbreviations used: GALA, peptide with the sequence WEAALAEALAEALAEHLAEALAEALEALAA; ANTS, 1-aminonaphthalene-3, 6, 8-trisulfonic acid; DPX, *p*-xylene-bis-pyridinium bromide; Chol, cholesterol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl- 2-oleoyl-phosphatidylglycerol; egg PC, egg phosphatidylcholine; REV, reverse-phase evaporation vesicles; LUV, large unilamellar vesicles; RMSE, root-mean-squared error; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; MES, 2[*N*-morpholino]ethanesulfonic acid; NBD fluoride, 4-fluoro-7-nitrobenz-2-oxa-1, 3-diazole; rhodamine, lissamine rhodamine B sulfonyl chloride.

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1993), HA2 (Wagner et al., 1992), and others (Plank et al., 1994) have been utilized in enhancing the efficiency of gene delivery to cells, the idea being that these peptides promoted the release of DNA from endosomes.

Leakage from liposomes due to pore formation by peptides that do not redistribute between vesicles can be characterized by (Parente et al., 1990): 1) The extent of leakage increases with time until it reaches its final extent, which is dependent on the lipid/peptide ratio; 2) The leakage of vesicle contents follows an all-or-none mechanism (Weinstein et al., 1981; Ladokhin et al., 1995); i.e., some of the vesicles release all of their contents, whereas others retain all their contents. 3) The leakage depends on the size of the encapsulated molecules. The leakage induced by several peptides, among them the model peptide GALA (Parente et al., 1990), and the fusion peptide of HIV (Nieva et al., 1994) fulfills these requirements, and therefore may occur via pore formation. The all-or-none mechanism of leakage was also reported in studies using melittin (Benachir and Lafleur, 1995) and pardaxin and a few of its analogs (Rapaport et al., 1994). Interestingly, melittin-induced lysis of red blood cells was also deduced to follow an all-or-none mechanism (Tosteson et al., 1985).

The mathematical model of pore formation includes the assumption that, after a rapid stage of peptide binding to the vesicle membranes (Parente et al., 1990; Schwarz et al., 1987), surface aggregation of peptide occurs. When the structure and conformation of the peptides used are appropriate, the aggregates that have reached a critical size, i.e., include M or more peptides, will form a pore. If surface aggregation of the peptide is irreversible, then vesicles that include M or more bound peptides will eventually leak all their encapsulated contents, whereas the other vesicles will not leak at all. The kinetics of leakage is dictated by the kinetics of surface aggregation, because once a pore has formed, the leakage of all the contents occurs within less than 1 s (Lewis and McConnell, 1978; Parente et al., 1990; Schwarz and Robert, 1990), thus yielding a finite final extent. The model correctly simulated the kinetics of leakage, the pore size, and yielded quantitative predictions of the increase in the final extent of leakage with an increase in the sizes of the liposomes (Parente et al., 1990).

When surface aggregation is reversible, as was recently found for the peptide pardaxin (Rapaport et al., 1996), only a fraction of the vesicles containing more than M peptides will include a pore, and consequently leak all their contents. In cases where induction of leakage requires the presence of a relatively large number of bound peptides in a liposomal membrane (Benachir and Lafleur, 1995), there might be uncertainty whether the mechanism is due to pore formation or micellization induced by the peptide, which is known to occur at higher peptide/lipid ratios. We will show that in the case of leakage induced by the peptide GALA from cholesterol-containing liposomes, the assumption of reversible surface aggregation of the peptide can explain the leakage observed by pore formation with the same pore size as needed in neutral or negatively charged liposomes without cholesterol ($M = 10$).

MATERIALS AND METHODS

Reagents

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) in chloroform were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Cholesterol was obtained from Sigma. 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), lissamine rhodamine B sulfonyl chloride, and neutral Texas-red dextran (3000 MW) were purchased from Molecular Probes (Eugene, OR).

Vesicle preparation and sizing

Reverse-phase evaporation vesicles (REV) were prepared as described previously (Szoka and Papahadjopoulos, 1978) in 1) 5 mM TES, 100 mM KCl, or in 2) 5 mM TES, 12.5 mM ANTS, 45 mM DPX, 20 mM KCl at pH 7.0. In certain cases, when the leakage of dextran was studied, 0.5 mM neutral Texas-red dextran, 3000 MW was included in 1) or 2). The vesicles were extruded three times through a 0.1-μm polycarbonate membrane (Nucleopore Corp., Pleasanton, CA) through a hand held extrusion device (Avestin, Ottawa, CA). When ANTS/DPX or/and neutral Texas-red dextran 3000 MW were encapsulated in liposomes, a Sephadex G-75 column (1 × 20 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. Quasi-elastic light scattering of solutions of liposomes was measured with a Coulter apparatus N4 to determine the size distribution of the liposomes.

GALA synthesis and fluorescent labeling

GALA 30 was synthesized with an automatic synthesizer and Merrifield resin. Details of the synthesis and purification have been described previously (Subbarao et al., 1987). Briefly, purification was carried out by reverse phase HPLC on a Perkin-Elmer instrument equipped with a Dynamax C_{18} column. GALA was eluted with a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid. Analysis of purified material on a Vydac analytical C_{18} column showed that the final peptide fractions were greater than 99% pure.

For NBD or rhodamine labeling of GALA, the peptide was dissolved in a 0.1 M sodium bicarbonate pH 9.0 solution. NBD fluoride or lissamine rhodamine B sulfonyl chloride in dimethylformamide were added to the stirred peptide solution with a final fluorophore/peptide molar ratio of 8. A Sephadex G-10 column (1 × 20 cm) was used to separate fluorophore-labeled and unlabeled GALA from the free fluorophore with the elution buffer 0.1 M ammonium acetate pH 8.0. Separation of the fluorophore-labeled GALA from the unlabeled peptide was carried out by reverse phase HPLC on a Perkin-Elmer instrument equipped with a Dynamax C_{18} column. GALA was eluted with a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid. The NBD modified GALA eluted at 73% acetonitrile and the rhodamine modified GALA eluted at 71% acetonitrile.

Fluorescence measurements

Measurements were made on a Spex Fluorolog photon counting instrument (Edison, NJ) using a 150-W xenon light source as previously described (Parente et al., 1990).

Leakage of ANTS/DPX

The ANTS/DPX assay (Ellens et al., 1984) was used to monitor leakage of ANTS induced by the peptide GALA from liposomes. The fluorescence signal resulting from the dequenching of ANTS released into the medium was observed through a Schott GG 435-nm cutoff filter (50% transmittance at λ = 435 nm) while samples were irradiated at λ = 360 nm. For some experiments, 90° light scattering was simultaneously recorded through a monochrometer at λ = 360 nm to check for vesicle integrity and absence of vesicle aggregation. Fluorescence intensity from intact vesicles in buffer, F_0, was set to 0% leakage. To begin an experiment, the peptide GALA in 5 mM TES, 50 mM KCl at pH 7.5 was added to the stirred vesicle suspensions (the lipid concentration was 0.1 mM unless specifically stated) at 20°C in the appropriate buffer. Reaction buffers consisted of 5 mM MES, 100 mM KCl, pH 6.4, 6.0, 5.85, 5.7 or 5.5, 5 mM sodium acetate, 100 mM KCl, pH 4.5, 5.0 or 5.3. Data points were recorded at 1-sec intervals over a 14-min period. The plateau in fluorescence intensity was obtained in 12 min after peptide addition. Then, vesicles were lysed with the detergent dodecyloctaethylene glycol monoether ($C_{12}E_8$) (Calbochiem, La Jolla, CA) to obtain the maximal fluorescence intensity, F_{100}, corresponding to 100% ANTS leakage. The final leakage extent of ANTS due to the interaction of the peptide with liposomes was then determined by the formula: Final % leakage = $(F - F_0)/(F_{100} - F_0)$, where F corresponds to the fluorescence intensity observed at the plateau. When leakage experiments were performed with 15 mM lipid, 2% of the liposomes (0.3 mM) were loaded with ANTS/DPX and 98% were empty. The fluorescence intensity that corresponded to 100% release of ANTS was determined for the 0.3 mM of loaded vesicles alone. Addition of the peptide to empty vesicles did not change recorded intensity. To differentiate if leakage of contents was an all-or-none event (some of the vesicles release all of their contents) as opposed as a graded event (all the vesicles release some of their contents), we used the method developed by Parente et al. (1990), which is a slight modification of the fluorescence dequenching method of Weinstein et al. (1981).

NBD-GALA membrane binding

The procedure was essentially based on that in Frey and Tamm (1990) who utilized the fact that fluorescence intensity of NBD-labeled peptide increases upon transfer from a solution to the hydrophobic environment in the membrane. The degree of peptide association with liposomes was measured by adding a particular amount of the mixture of unlabeled GALA/NBD-labeled GALA to liposomes of different concentrations. The peptide, in a stock solution of 5 mM TES, 50 mM KCl, pH 7.5, was added to the stirred vesicles at 20°C in a 20 mM sodium acetate, 90 mM KCl, pH 5.0 solution. The fluorescence signal was monitored at the peak of the NBD fluorescence, λ = 525 nm (5-mm slits), when the sample was irradiated at λ = 467 nm. After subtraction of the contribution of the liposomes alone and taking into account the dilution factor corresponding to the addition of microliter amounts of peptides, the fluorescence intensity, F, was recorded 100 s after peptide addition, which corresponded to maximum intensity. We checked that the addition of unlabeled peptide to liposomes did not modify recorded intensity. For a particular peptide concentration, the fraction of membrane-bound peptide (f_{bp}) is determined by: $f_{bp} = (F - F_0)/(F_{100} - F_0)$, where F_0 is the fluorescence intensity observed when all the peptide is in solution without liposomes and F_{100} is the fluorescence intensity observed 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 of the liposome concentration did not result in further increase in fluorescence intensity. As in leakage experiments, vesicle integrity and absence of vesicle aggregation were checked.

Leakage of neutral Texas-red dextran 3000 MW

Liposomes (REV) were made in the presence of neutral Texas-red dextran 3000 MW (0.5 mM) in 5 mM TES, 12.5 mM ANTS, 45 mM DPX, 20 mM KCl at pH 7.0, or in 5 mM TES, 100 mM KCl at pH 7.0. After elution on a Sephadex G-75 column (1 × 20 cm) to remove unencapsulated material, these liposomes were used for typical leakage experiments in a pH 5.0 buffer at 20°C (the lipid concentration was always 0.1 mM). In the case of ANTS containing liposomes (coencapsulated with dextran), the fluorescence signal corresponding to ANTS leakage was recorded at the plateau, 12 min after peptide addition, to determine the final extent of ANTS leakage. At this time, the liposomes were either lysed with the detergent dodecyloctaethylene glycol monoether ($C_{12}E_8$) to obtain the fluorescence intensity that corresponds to 100% leakage of ANTS, or were not lysed. Dextran leakage was determined from the unlysed liposomes; 30 min after peptide addition, the pH of the solution was raised to 8.0 with addition of a few microliters of 0.5 M TES, pH 8.0 buffer to stop possible further leakage. The mixture was placed on a Sepharose 4-B column (0.5 × 10 cm) and eluted with 20 mM TES, 80 mM KCl, pH 8.0 buffer to separate the free dextran that has leaked from the dextran that was still encapsulated into liposomes. For each fraction, $C_{12}E_8$ was added to lyse the vesicles. Fluorescence emission of each fraction was measured at λ = 610 nm when excited at λ = 570 nm along with lipid phosphorus, and from a standard curve after substraction of the lipid contribution, the percentage of Texas-red dextran released was determined. We checked that leakage of dextran terminated within 30 min after peptide addition and that we obtained the same extents of dextran leakage with encapsulation of dextran alone. Vesicles without encapsulated material were incubated with Texas-red dextran and passed through the column to determine if any correction was necessary for binding of this compound to the lipid. No correction was needed.

Theoretical analysis of leakage

The model assumes that 1) the peptides bind and become incorporated into the bilayer of the vesicles, and 2) peptide aggregation occurs within the membranes. When an aggregate within a membrane has reached a critical size, i.e., it consists of M peptides, a channel or pore can be created within the membrane, and leakage of encapsulated molecules can occur. The size of the pore dictates the upper bound on the size (and shape) of molecules that can leak. The size of the pore depends on the number of peptides forming it.

As in previous studies (Parente et al., 1987; Schwarz et al., 1987; Schwarz and Robert, 1990), we assume that the process of peptide incorporation into the vesicle membranes is rapid, and that once a pore has been formed in a vesicle all its contents will leak rather quickly. Thus, the rate of leakage is assumed to be limited by the rate of formation of aggregates consisting of M or more peptides in the membrane. The kinetics of irreversible aggegation was first investigated by Smoluchowski (1917) and later extended to reversible aggregation by Blatz and Tobolsky (1945). The general scheme as treated in Bentz and Nir (1981) and Nir et al. (1983) is:

$$X_1 + X_1 \underset{D_{11}}{\overset{C_{11}}{\longleftrightarrow}} X_2$$
$$X_i + X_j \underset{D_{ij}}{\overset{C_{ij}}{\longleftrightarrow}} X_{i+j} \qquad (1)$$

where X_i are molar concentrations of aggregates of order i. Here we have used the same scheme, but in our case X_i denotes surface concentrations.

Previous treatments of the pore model (Parente et al., 1990; Fattal et al., 1994; Nieva et al., 1994) ignored the possibility of aggregate dissociation, setting $D_{ij} = 0$. Here, reversibility of surface aggregation of the peptide GALA in cholesterol-containing membranes has been considered as in Rapaport et al. (1996). We have used the same simplifications made by Smoluchowski (1917) and by Blatz and Tobolsky (1945), i.e., that $C_{ij} = C$ and $D_{ij} = D$ for all i and j. It has been shown (Peled et al., 1995) that with these assumptions the analytical solution of Blatz and Tobolsky (1945) yields essentially similar results to the numeric solutions (Bentz and Nir, 1981; Nir et al., 1983).

The fraction of encapsulated material that has leaked at the plateau level is denoted by L. Due to the dependence of the encapsulated volume on the third power of the inner diameter, a small fraction of large vesicles can contribute significantly to L. As in Fattal et al. (1994), we considered the vesicles to consist of $j = 1, 2, \ldots, S$ size classes. The distribution of vesicles according to their diameters was determined by dynamic light scattering. We set $S = 10$ and we denote the fraction of encapsulated volume in vesicles of type j by f_j, $1 \leq j \leq 10$.

Let A_{ij} be the normalized fraction of vesicles of size class j that contain i bound peptides, i.e.,

$$\sum_{i=0}^{N_j} A_{ij} = 1 \tag{2}$$

in which N_j is the largest number of peptides that can bind to a vesicle of size class j. The procedures for calculating the quantities A_{ij} from binding data have been described before (Nir et al., 1986; Bentz et al., 1988; Parente et al., 1990; Nir et al., 1994). In the simpler case of irreversible surface aggregation of the peptide (Parente et al., 1990) the expression for L is given by

$$L = \sum_{j=1}^{s} \sum_{i=M}^{N_j} A_{i,j} f_j \tag{3}$$

Equation 3 reflects the assumption that when aggregation is irreversible, all the peptides within the membrane will eventually be incorporated into a single aggregate so that all vesicles with at least M bound peptides will eventually leak.

In the more general case of reversible surface aggregation, the expression for L becomes

$$L = \sum_{j=1}^{S} \sum_{i=M}^{N_j} Z(M, i, j, K_s) A_{i,j} f_j \tag{4}$$

in which $Z(M, i, j, K_s)$ is the probability that a vesicle of size class j that contains i bound peptides will include an aggregate consisting of M or more peptides, and K_s is given by

$$K_s = C/D \tag{5}$$

In the case of irreversible surface aggregation K_s tends to infinity. For the calculation of final extents of aggregation, only K_s and the surface concentration of the peptides, X_0, are required, the latter being known from the binding measurements. As in Rapaport et al. (1996)

$$Z = p^{M-1} \times (M - Mp + p) \tag{6}$$

in which p is given by (Blatz and Tobolsky, 1945)

$$p = 1/(K + \sqrt{K^2 - 1}) \tag{7}$$

where K is given by

$$K = 1 + 1/(4K_s X_0) \tag{8}$$

The quantity p is given in the formalism of Flory (1939) by

$$p = (X_0 - X)/X_0 \tag{9}$$

in which X_0 and X are total surface concentrations of peptides and aggregates (including peptides remaining as monomers), respectively. For an infinite K_s the quantities K, p, and Z equal unity and Eq. 4 reduces to Eq. 3, which corresponds to irreversible surface aggregation.

Reversibility of surface aggregation

Aggregation of peptides in membranes and reversibility of this surface aggregation were determined by observing the fluorescence resonance energy transfer (FRET) from the NBD-labeled GALA (donor) to the rhodamine-labeled GALA (acceptor) that were coinjected to a liposomal suspension including 20 mM sodium acetate, 90 mM KCl, at pH 5.0 and 20°C. We used lipid and peptide concentrations where complete binding of the peptide to the liposomes was determined. We monitored the fluorescence of NBD at $\lambda = 525$ nm (5-mm slits), when the sample was irradiated at $\lambda = 467$ nm. Quenching of NBD fluorescence was correlated to an increase of rhodamine fluorescence. Aggregation of the peptide in solution at pH 5.0 in absence of liposomes was observed by comparing NBD fluorescence intensities of mixtures containing either NBD-GALA and unlabeled GALA or NBD-GALA and rhodamine-GALA, for the same total peptide concentration. Due to massive aggregation of the peptide at pH 5.0, the liposomes were always added first in leakage or binding experiments. No aggregation and fluorescence energy transfer were observed at the pH of the stock solution (7.5) at the peptide concentrations studied here.

RESULTS AND DISCUSSION

Our focus has been on studying the effect of membrane composition, and in particular the effect of cholesterol on the extent and mode of leakage of an encapsulated compound induced by the amphipathic peptide GALA from large unilamellar vesicles. For that purpose we have used the ANTS/DPX leakage assay at various pH. At pH 5.0, where most of the studies were performed, the lipid/peptide ratios tested varied from 40,000 to 4000 for POPC and POPC/POPG liposomes, and up to 20 for vesicles containing 40% Chol. We verified that under these conditions GALA does not induce vesicle aggregation or fusion to a measurable extent. This was already shown for neutral egg PC liposomes in Parente et al. (1988) and (1990) using a fluorescence resonance energy transfer assay (Struck et al., 1981) which showed that no membrane mixing occurred in these ranges of lipid/peptide ratios. Turbidity measurements indicated that peptide-induced liposome aggregation did not occur under these conditions. These tests imply that the leakage observed was independent of interactions between vesicles. By using the same procedure described in Parente et al. (1990), we found that the leakage of the entrapped ANTS occurred by an all-or-none mechanism for all liposome compositions tested, irrespective of the cholesterol content; vesicles either leaked or retained all of their contents.

pH-Dependence of the leakage induced by GALA from POPC and POPC/Chol liposomes

The drastic pH dependence of the leakage induced by the peptide GALA from large unilamellar neutral POPC vesicles has already been studied (Subbarao et al., 1987; Parente et al., 1990), and has been explained by the conformational change of the peptide from a random coil at pH 7.0 to an amphipathic α-helix at pH 5.0, due to the neutralization of the seven glutamic acid residues along the peptide. In this study we first compared the pH dependence of the final extent of ANTS leakage from POPC liposomes with that

from POPC/Chol 2:1 liposomes (33.3 mol % Chol) (Fig. 1, *a* and *b*) to determine if membrane composition changes the pH profile of the interactions of the peptide with the vesicles. In the case of POPC liposomes (Fig. 1 *a*), the increase of the pH from 5.0 to 6.4 resulted in a steep increase in the peptide concentration needed to obtain a particular final extent of leakage (indicated by a decrease of the lipid/peptide ratio needed for the same lipid concentration of 0.1 mM). At pH 5.0 GALA is remarkably active to induce leakage of ANTS, as a ratio of 5300 is sufficient to induce complete leakage of vesicle contents. With POPC/Chol 2:1 liposomes the ability of GALA to destabilize the vesicles was considerably reduced at each pH tested. At pH 5.0, a lipid/peptide ratio of 120 was needed to induce complete leakage of ANTS, i.e., 44-fold more peptide was required. Nevertheless, the same dramatic effect of the pH increase was observed, indicating that the pH does not affect the relative efficiency of GALA to induce leakage from membranes of different compositions. As shown in Fig. 2, the final extents of ANTS release at pH 5.0 were reached in 12 min after peptide addition, with or without cholesterol.

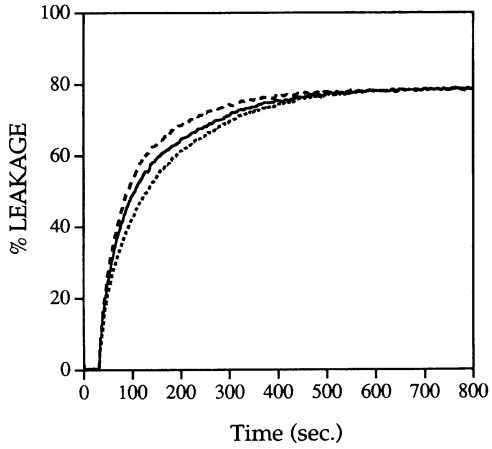

FIGURE 2 Kinetics of GALA-induced leakage from liposomes at pH 5.0. POPC (——); POPC/POPG 1:1 (· · · · ·); and POPC/Chol 2:1 (- - - -). A final extent of leakage of 78.5% was obtained for peptide concentrations of 15.2 nM (lipid/peptide molar ratio of 6600), 15.6 nM (lipid/peptide molar ratio of 6400), and 0.25 μM (lipid/peptide molar ratio of 400), respectively.

Effect of liposome charge on leakage

To test whether the charge of the membrane constitutes an important factor that modulates the peptide ability to destabilize liposomes, we determined at pH 5.0 the effect of the addition of the acidic phospholipid POPG in POPC-based liposomes and in POPC/Chol-based liposomes where the cholesterol content is kept constant at 33.3 mol %. Our results indicated for both lipid matrices (Fig. 3, *a* and *b*) that an increase in the negative charge density of the liposomal membrane does not modify the ability of the peptide to induce leakage. This confirms that the net charge of GALA at pH 5.0, which is close to 0 (Subbarao et al., 1987), allows the peptide to act similarly on neutral and negatively charged membranes. Furthermore, the kinetics of leakage observed with negatively charged liposomes was similar to that observed for neutral POPC liposomes, as shown in Fig. 2, where a final leakage extent of 78.5% was reached.

Effect of cholesterol on leakage

The results in Fig. 3, *a* and *b* show a large decrease in the efficiency of GALA to induce leakage at pH 5.0 when cholesterol is included in POPC/POPG liposomes at 33.3 mol %. We studied the change in the capacity of GALA to induce ANTS leakage when increasing the cholesterol content in POPC/POPG 5:1-based liposomes from 15 to 40 mol % (Fig. 4). A cholesterol content of 15% did not affect the capacity of GALA to destabilize liposomes, but at 20% or more, the addition of cholesterol reduced the final leakage extents of ANTS. As the cholesterol content was increased in the liposome formulation, the lipid/peptide ratios needed to reach a particular final leakage extent had to be reduced. As will be shown, two major causes are responsible for this reduced ability of GALA to permeabilize liposomes in which the cholesterol content is increased. One factor is a decrease in the membrane-binding affinity of the peptide,

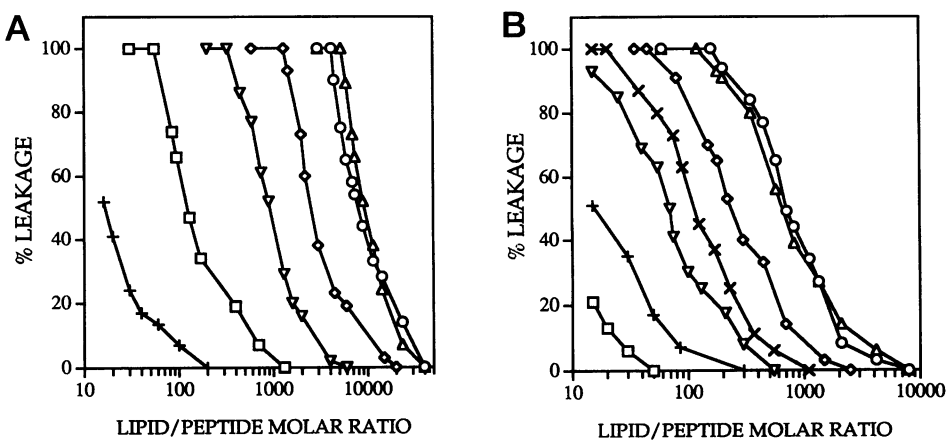

FIGURE 1 Final extents of leakage from neutral liposomes as a function of lipid/peptide ratio and pH. (*a*) POPC; (*b*) POPC/Chol 2:1. Each data point is an average of three experiments. The final extents of leakage at a given lipid/peptide ratio and pH varied no more than ±4% when the extents of leakage were above 20% and no more than ±2% below 20% leakage. The lipid concentration was kept constant at 0.1 mM. Curves are for pH values of (*a*) 4.5 (○), 5.0 (△), 5.3 (◇), 5.7 (▽), 6.0 (□), and 6.4 (+); and (*b*) 4.5 (○), 5.0 (△), 5.3 (◇), 5.5 (X), 5.7 (▽), 5.85 (+), and 6.0 (□).

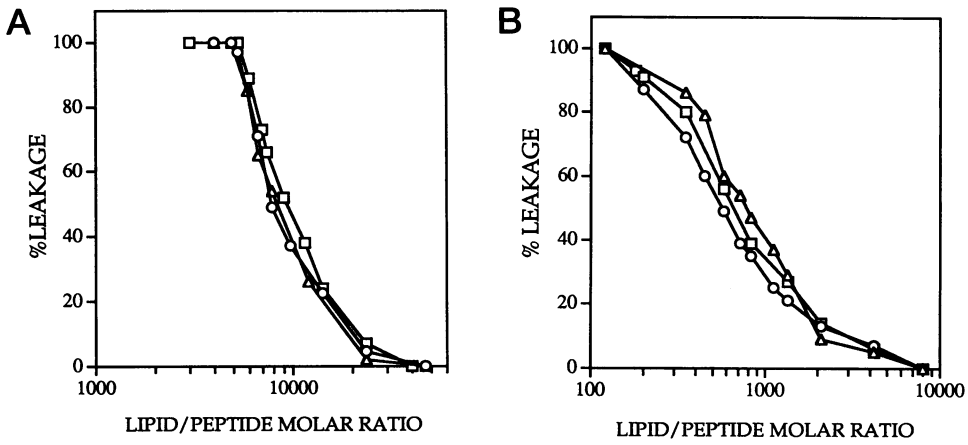

FIGURE 3 Final extents of leakage from liposomes as a function of lipid/peptide ratio at pH 5.0. (*a*) POPC/POPG; (*b*) POPC/POPG/Chol containing 33.3 mol % Chol. Each data point is an average of three experiments. The reproducibility of the final leakage extents is similar to that in Figure 1. Curves are drawn for various molar ratios of POPC/POPG, i.e., 1:0 (□), 2:1 (△), 1:2 (○).

the other factor being a modification in the pore formation process. We have designed experiments to elucidate the contribution of each factor to the effect of cholesterol.

Membrane-binding affinity of GALA

We have labeled GALA with the fluorophore NBD to determine the membrane-bound (Frey and Tamm, 1990) and free fractions of the peptide for a large range of lipid and peptide concentrations and various cholesterol contents. First, we have shown with 0, 25 and 33.3 mol % Chol that the labeled peptide has a similar ability to induce leakage as the native one (data not shown) from POPC/POPG/Chol liposomes. No change in turbidity was observed under the conditions tested, suggesting that no aggregation nor fusion of vesicles occurred. Binding experiments were performed with a mixture of native and NBD-labeled GALA in a molar ratio sufficient to avoid the phenomenom of NBD self-quenching in solution and in the membrane that would bias the evaluation of the membrane-bound fraction. First, we checked that for the lipid and peptide concentrations tested, a modification of the ratio NBD-labeled/unlabeled peptide in the chosen range did not affect the quantum yield in the membrane or in solution. Secondly, under conditions where all the peptide was bound (evidenced by no increase in fluorescence when the lipid concentration was further increased in a separate experiment for the same peptide concentration), we checked that a modification of the total concentration of peptide for the same lipid concentration did not affect the quantum yield of the bound peptide. Finally, we checked for the peptide in solution without lipid that an increase of the peptide concentration did not affect the quantum yield (data not shown).

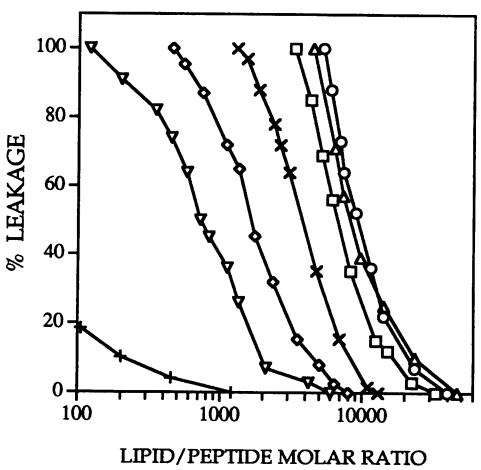

FIGURE 4 Percent leakage from POPC/POPG/Chol liposomes at pH 5.0 as a function of lipid/peptide ratio. Each data point is an average of three experiments with three different vesicle preparations of the same liposome formulation. The final extents of leakage at a given lipid/peptide ratio and cholesterol content varied no more than ±5.5%. Curves are given for POPC/POPG/Chol liposomes whose POPC/POPG molar ratio is 5:1 and the mol % of cholesterol is 0% (○), 15% (△), 20% (□), 25% (X), 29.4% (◇), 33.3% (▽), and 40% (+).

We chose three peptide concentrations (0.55 μM, 0.11 μM, and 0.022 μM) in the range used for leakage experiments with 0.1 mM lipid, and determined the membrane-bound fraction as a function of the lipid concentration for POPC/POPG 5:1, POPC/POPG/Chol 5:1:2 and 5:1:3 liposomes. Results obtained for 0.55 μM and 0.022 μM are displayed in Figure 5, *a* and *b*. The assay sensitivity set a limit on studying very low concentrations. For a particular peptide concentration and a fixed liposome formulation, the membrane-bound fraction of GALA increases with the lipid concentration tested, as expected (Fig. 5, *a* and *b*). Our results show, for each lipid and peptide concentration tested, a decrease in the membrane-bound fraction of GALA upon increasing the cholesterol content in the membrane. A direct consequence is that the lipid concentration required for complete peptide binding is higher with cholesterol-containing liposomes. From these experiments we extracted the bound fraction of the peptide when we used 0.1 mM lipid, and used these results in the analysis of leakage. In the absence of cholesterol, we found with POPC/POPG 5:1 liposomes 64% membrane-bound peptide for 0.55 μM peptide (lipid/total peptide ratio of 180 with 0.1 mM lipid), 58.5% for 0.11 μM (lipid/total peptide ratio of 900), and 56% for 0.022 μM (lipid/total peptide ratio of 4500). When cholesterol was introduced into the liposome composition as

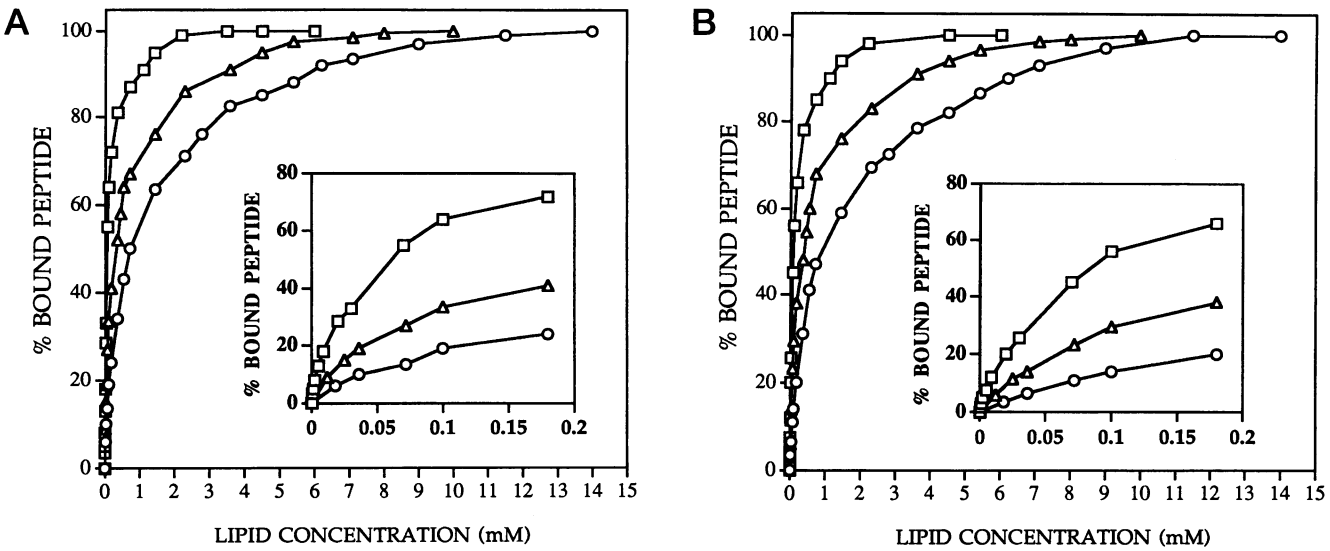

FIGURE 5 Effect of cholesterol on percent of binding of NBD-labeled GALA to liposomes. POPC/POPG 5:1 (□), POPC/POPG/Chol 5:1:2 (25 mol % Chol) (△), POPC/POPG/Chol 5:1:3 (33.3 mol % Chol) (○). A mixture of NBD-GALA/unlabeled GALA at a molar ratio of 1:19 was used for a total peptide concentration of 0.55 μM (*a*) and a mixture at a 1:4 ratio was used for a total peptide concentration of 0.022 μM (*b*). The increase of NBD fluorescence intensity was monitored with excitation at 467 nm and emission at 525 nm. The fraction of bound GALA was determined as described in the Methods section. 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.

with POPC/POPG/Chol 5:1:2 liposomes (25 mol % Chol), binding was 33.5%, 31%, and 29%, respectively. Increasing the cholesterol content to 33.3 mol % (POPC/POPG/Chol 5:1:3) further decreased peptide binding to 19%, 16%, and 14%, respectively.

Leakage when all the peptide is membrane-bound

We tested directly whether, when the cholesterol content is raised in the membrane, there is an additional factor that affects the decrease in leakage efficiency beyond the decreased bound fraction of the peptide for the same lipid concentration. To test this possibility, the lipid concentration was set to 15 mM, a concentration at which all the added peptide is bound to the liposomes regardless of composition. Because the liposome diameters and size distribution are similar for all liposome compositions, the number of peptides per vesicle will be comparable. We then determined at pH 5.0 the final extent of ANTS leakage from POPC/POPG 5:1, POPC/POPG/Chol 5:1:2, and POPC/POPG/Chol 5:1:3 liposomes, at a number of peptide concentrations (from 0.28 μM to 2.22 μM). Our results (Table 1) indicate that for each peptide concentration investigated, the final extents of ANTS leakage were reduced when the cholesterol content in the membrane was increased under conditions where the average number of peptides bound per liposome was the same. These results indicate that more membrane-bound peptides per liposome are necessary to form a pore when the cholesterol content is increased in the membrane. That means that, apart from the reduced binding affinity of the peptide, there is an additional effect that affects the pore formation and consequently reduces the efficiency of the peptide to induce leakage. For every peptide concentration examined in this experiment (from 0.28 μM to 2.22 μM), the lipid/peptide ratio needed to get a particular final leakage extent was in good correlation with the ratio lipid/membrane-bound peptide (calculated from the membrane-bound fraction determined in Fig. 5) needed to get the same leakage extent when we used 0.1 mM lipid (Fig. 4).

TABLE 1 Final extents of ANTS/DPX leakage under conditions of complete binding of the peptide to liposomes (15 mM lipid)

GALA[a] concentration (in multiples of 0.55 μM)	Cholesterol (mol %)			
	0	25	29.4	33.3
	Percent leakage[b]			
0.5	3.2	0	0	0
1	24.3	10.0	0	0
2	68.2	44.6	15.0	5.0
3	98.5	69.4	29.3	22.1
4	100	83.9	42.1	34.8

[a]GALA concentrations tested ranged from 0.28 μM to 2.2 μM.
[b]Percent leakage of ANTS/DPX at pH 5.0 from POPC/POPG 5/1 (mol/mol), POPC/POPG/Chol 5:1:2, POPC/POPG/Chol 5:1:2.5 or POPC/POPG/Chol 5:1:3 liposomes. The lipid concentration was kept constant at 15 mM.
Final extents of leakage were obtained from the average of three experiments. The variation in final extents of leakage was no more than ±4%.

Theoretical determination of pore size

The determination of the value of M, the minimal number of peptides required for formation of a pore in a liposomal membrane, (which would yield complete leakage from that liposome, in accord with the all-or-none mechanism of leakage) is described in the Methods section. We first tested the adequacy of the prediction that setting $M = 10$ in Eq. 3 (as in Parente et al. (1990) for neutral egg PC liposomes) can explain the final extents of leakage from the negatively charged POPC/POPG liposomes. The results of these calculations, in which no parameter was adjusted, are shown in Figure 6 *a* for POPC/POPG 5:1 liposomes. Hence, the experimental results of leakage from the negatively charged liposomes are adequately explained by using the same pore size as in the case of the neutral egg PC vesicles. We have used the same statistical criteria (Efron, 1982) as explained in Parente et al. (1990) according to which $M = 10 \pm 2$. Other statistical criteria considered were R^2 (0.98) and

$$\mathrm{RMSE} = \left[\sum_{i=1}^{n}(Y_i - Y_{ci})^2/(n-1)\right]^{1/2}$$

The value of RMSE was 5.5% in this case, i.e., within the experimental error.

The application of this model for the simulation of the leakage from cholesterol-containing liposomes could not yield any satisfactory fit to the data. Determination of the values of M that would give an exact fit to the final extents of leakage from POPC/POPG/Chol 5:1:3 vesicles resulted in a progressive increase from 9 to 40, the higher values corresponding to a lipid/peptide ratio of 120.

We reasoned that a reversible aggregation of the peptide in the membrane could account for the apparent decreased efficacy for peptide-induced contents leakage in the presence of cholesterol. As is seen in Figure 6 *b*, the use of the more general model (Eqs. 4–8), that allows for reversibility of surface aggregation of the bound peptide, could yield a reasonable fit ($R^2 = 0.96$; RMSE = 6.8). In these calculations we used $M = 10$ for the pore size and $K_s = 0.24$ for the degree of reversibility of the peptide surface aggregation (see Eq. 5). No further improvement in the fit was achieved by varying M. Hence, the same pore size deduced for leakage induced by GALA from neutral POPC and negatively charged POPC/POPG liposomes could also explain the results of leakage from liposomes containing 33.3% Chol, despite the order of magnitude reduction in leakage efficiency in this case. As will be elaborated later, the value obtained for K_s corresponds to a small degree of reversibility of surface aggregation. It should be recalled that our calculations used the experimental values of 0.14–0.19 for the fraction of peptide bound to these POPC/POPG/Chol 5:1:3 liposomes, whereas the corresponding value for POPC/POPG 5:1 liposomes was 0.56. The results of leakage from POPC/POPG/Chol 5:1:2 liposomes (25 mol % Chol) were intermediate between those with 0% and 33.3% Chol (see Fig. 6 *b*). The fraction of peptide bound was 0.29–0.31, and the calculations used, again, $M = 10$. The value $K_s = 1.3$ corresponds to a smaller degree of reversibility of surface aggregates of the bound peptides than obtained for liposomes containing 33.3% Chol. Leakage from liposomes containing 15% Chol could still be explained by ignoring reversibility of surface aggregation. Table 2 gives a summary of the results of the simulations. We have also repeated the calculations for the leakage from POPC/POPG vesicles by looking for a value of K_s that would yield the best fit. The value of K_s found in this case (15), which gave a marginal improvement of the fit (Table 2), corresponds to a very small degree of reversibility of surface aggregation of the peptide.

Leakage of dextran

The main purpose of these experiments was to test whether the leakage induced by GALA from liposomes used in this study, i.e., with or without cholesterol, is selective to the size of preencapsulated molecules in a similar fashion to the leakage from egg PC liposomes, where a pore size $M = 10 \pm 2$ was shown to be compatible with the Stokes radii of molecules that could leak (Parente et al., 1990).

Neutral Texas-red dextran 3000 MW has been coencapsulated with ANTS/DPX in POPC/POPG 5:1 (no choles

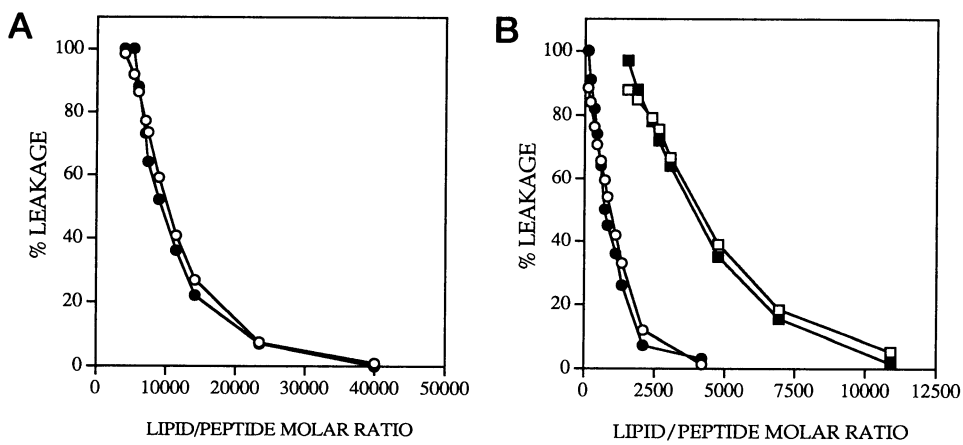

FIGURE 6 Experimental and calculated final extents of leakage from liposomes at pH 5.0 as a function of lipid/peptide ratios. (*a*) POPC/POPG 5:1 [exp. (●), cal. (○)]. (*b*) POPC/POPG/Chol 5:1:2 [exp. (■), cal. (□)] and POPC/POPG/Chol 5:1:3 [exp. (▲), cal. (△)]. The parameters used in the calculations are listed in Table 2.

TABLE 2 Summary of calculations for the fit of final extents of ANTS/DPX leakage with a pore consisting of 10 GALA molecules

Cholesterol (mol %)	K_s	R^2	RMSE (%)
	Infinite	0.98	5.5
0	15	0.98	5.1
25	1.3	0.98	4.7
29.4	0.34	0.93	10
33.3	0.24	0.96	6.8

terol) and POPC/POPG/Chol 5:1:3 (33.3 mol % Chol) liposomes. Leakage experiments were performed to compare the efficiency of GALA to induce leakage of dextran 3000 MW to that of ANTS. We checked with a separate encapsulation of ANTS, or dextran, that the coencapsulation did not affect the leakage efficiency for any one of the two dyes (data not shown).

Our results (Fig. 7) demonstrate that no leakage of dextran occurred from both liposome formulations under conditions where complete leakage of ANTS was achieved, i.e., 18.9 nM peptide with POPC/POPG 5:1 liposomes (lipid/peptide ratio of 5300 with 0.1 mM lipid) and 0.83 μM peptide with POPC/POPG/Chol 5:1:3 liposomes (lipid/peptide ratio of 120 with 0.1 mM lipid). In the case of POPC/POPG liposomes, sevenfold more peptide (0.13 μM) was necessary to initiate leakage of dextran, whereas with POPC/POPG/Chol 5:1:3 liposomes, 2.4-fold more peptide (1.99 μM) was needed. Because the Stokes radius of dextran 3000 MW is about 12 Å, our calculations indicate that dextran leakage would imply a pore constituted of 12–14

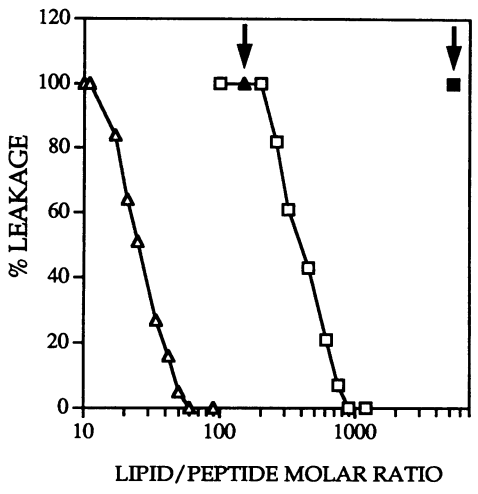

FIGURE 7 Percent leakage of neutral Texas-red dextran 3000 MW from liposomes as a function of lipid/peptide ratio at pH 5.0. POPC/POPG 5:1 (□); POPC/POPG/Chol 5:1:3 (33.3% Chol) (△). Each data point is an average of three experiments. The lipid peptide ratios corresponding to 100% leakage of ANTS/DPX are indicated by the arrows and are 120 and 5300 for POPC/POPG/Chol 5:1:3 (▲) and POPC/POPG 5:1 (■) liposomes, respectively. The lipid concentration was kept constant at 0.1 mM. The final extents of leakage at a given lipid/peptide ratio varied no more than ±6%.

peptides. The results show that in the range of peptide concentrations used to follow the evolution of ANTS leakage, the pore formed with both liposome formulations is not large enough to allow leakage of dextran 3000 MW. These results further confirm that when cholesterol is included in the membrane, the size of the pore formed by GALA is similar to that formed in neutral egg PC or POPC/POPG liposomes. This implies that the number of peptides required in an aggregate to form a pore is the same in all cases, despite the dramatic differences in binding and leakage efficiencies when cholesterol is included in the membrane. We will elaborate later on the observation that a further increase in the amount of peptide added did result in increased leakage of dextran.

Reversibility of surface aggregation

We tested the hypothesis that the degree of reversibility of aggregation of the peptide in the membrane increases with the percentage of cholesterol in liposomes. In order to test this hypothesis, we used fluorescently labeled peptides under conditions of complete binding to the liposomes (15 mM) at pH 5.0. Control experiments (not shown) showed that the efficiency of GALA to induce ANTS leakage from the liposomes tested was not modified by the peptide labeling with rhodamine or NBD. We followed the fluorescence resonance energy transfer (FRET) from NBD-labeled GALA to the coinjected rhodamine labeled peptide, due to aggregation of the peptides in membranes. Rhodamine was selected as the FRET acceptor because of its efficiency in similar assays. If reversibility of surface aggregation of the peptide occurs, then the addition of a large excess of unlabeled peptide should result in a certain degree of recovery of NBD fluorescence due to a partial dissociation of aggregates formed by the first injected labeled peptides, which would form new aggregates with the unlabeled peptide.

We injected a mixture of NBD-GALA/unlabeled GALA at a molar ratio of 4:76 (Fig. 8 *a*, *trace 1*, with POPC/POPG 5:1 liposomes and a total peptide concentration of 13.3 μM) into a suspension containing either POPC/POPG 5:1, POPC/POPG/Chol 5:1:2, or POPC/POPG/Chol 5:1:3 liposomes, and we observed a similar fluorescence intensity for each cholesterol content tested (0 mol %, 25 mol %, or 33.3 mol %). In separate experiments we injected, for the same total peptide concentrations of 13.3 μM, a mixture of NBD-GALA/rhodamine-GALA/unlabeled GALA at a ratio of 4:6:70 (Fig. 8 *a*, *trace 2*, with POPC/POPG 5:1 liposomes). In comparison to the fluorescence observed with the mixture of NBD-GALA/unlabeled GALA, we observed 26% and 23% decreases of the fluorescence intensity of NBD-GALA at the plateau (fluorescence intensity FR_1) due to energy transfer to the rhodamine-labeled peptide in POPC/POPG 5:1 liposomes and POPC/POPG/Chol 5:1:3 liposomes, respectively.

In separate experiments, a mixture of NBD-GALA/rhodamine-GALA was injected at a molar ratio of 4:6 (total

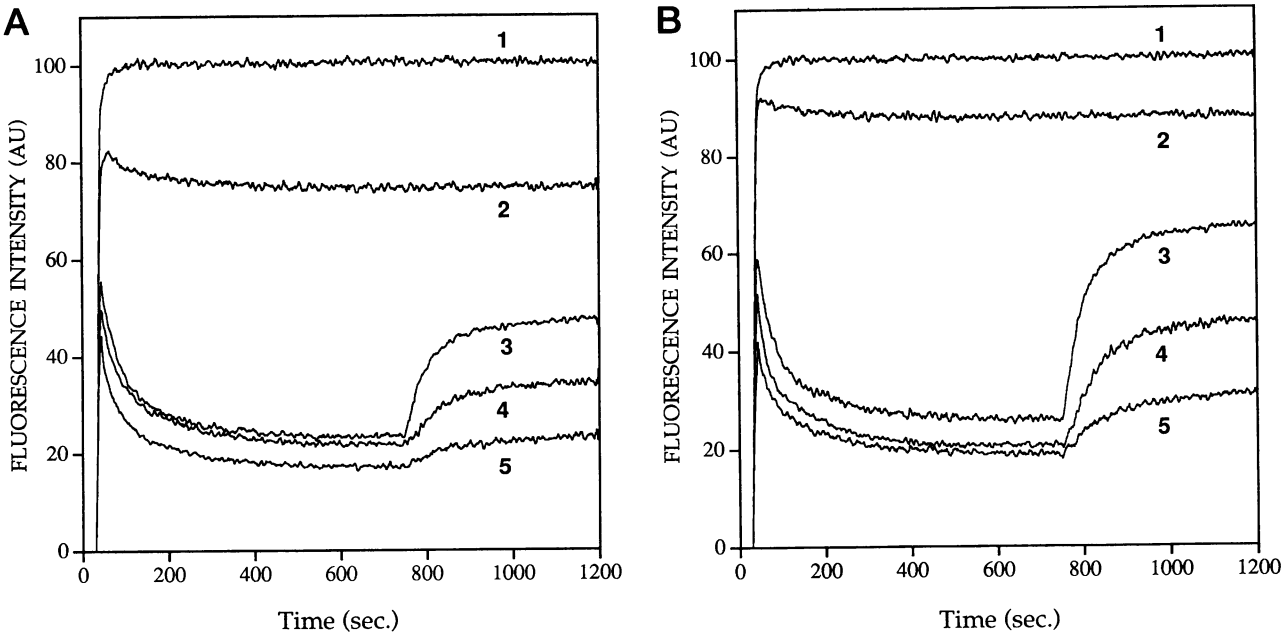

FIGURE 8 Reversibility of surface aggregation of the peptide in the absence and presence of cholesterol. (*a*) a mixture of NBD-GALA/unlabeled GALA at molar ratios of 4:76 (*trace 1*) or a mixture of NBD-GALA/rhodamine-GALA/native GALA at ratios 4:6:70 (*trace 2*) were added to a suspension of POPC/POPG 5:1 liposomes, at a total peptide concentration of 13.3 μM. Essentially the same results as in traces 1 and 2 were obtained with cholesterol-containing liposomes. In traces 3–5, a mixture of NBD-GALA/rhodamine-GALA was injected at a molar ratio of 4:6 (total peptide concentration of 1.67 μM and lipid/peptide ratio 9000) to a suspension of either POPC/POPG/Chol 5:1:3, POPC/POPG/Chol 5:1:2, or POPC/POPG 5:1 liposomes, respectively; then, at 720 s after the first peptide injection, sevenfold (11.67 μM) unlabeled peptide was added to reach, for every component of the peptide mixture, the same final concentration as in trace 2. (*b*) mixtures of NBD-GALA/unlabeled GALA at a molar ratio of 4:146 (*trace 1*) and NBD-GALA/rhodamine-GALA/native GALA at a ratio of 4:6:140 (*trace 2*) were added to a suspension of POPC/POPG 5:1 liposomes at a total peptide concentration of 25 μM. The same mixture of NBD-GALA/rhodamine-GALA as in (*a*) was injected to a liposomal suspension of POPC/POPG/Chol 5:1:3, POPC/POPG/Chol 5:1:2, or POPC/POPG 5:1 liposomes (*traces 3–5*, respectively); then, at 720 s after the first peptide injection, 14-fold (23.3 μM) unlabeled peptide was added instead of sevenfold in (*a*). In all cases lipid concentration was 15 mM.

peptide concentration of 1.67 μM and lipid/peptide ratio 9000) to POPC/POPG/Chol 5:1:3, POPC/POPG/Chol 5:1:2, or POPC/POPG 5:1 liposomes (Fig. 8 *a*, *traces 3–5*, respectively). In comparison with the fluorescence intensity of the mixture NBD-GALA/unlabeled GALA with the same amount of NBD-GALA (Fig. 8 *a*, *trace 1*), we observed a much lower fluorescence intensity (F_0) that depended on the cholesterol content in the membrane. This reduced intensity corresponds to a high degree of energy transfer, indicating the ability of the peptide to self-associate in the membrane. Then, at 720 s after the first injection (time at which the plateau of ANTS leakage is reached), we added sevenfold (11.67 μM) unlabeled peptide to reach the same final peptide concentrations as for the coinjection of NBD-GALA/unlabeled GALA or NBD-GALA/rhodamine-GALA/native GALA. Upon this addition of unlabeled peptide, we observed a recovery of the NBD fluorescence (up to the fluorescence intensity F), corresponding to dissociation of aggregates of the first injected labeled peptides, that now form new aggregates with the unlabeled peptide. A complete dissociation of these aggregates would dequench the NBD fluorescence to the fluorescence level (FR_1) obtained with coinjection of NBD-GALA/rhodamine-GALA/native GALA (*trace 2* with POPC/POPG 5:1 liposomes). The fraction of NBD fluorescence recovery (R) is defined by $R = (F - F_0)/(FR - F_0)$. Complete dissociation implies $F = FR$, or $R = 1$, and no dissociation implies $F = F_0$, $R = 0$. We obtained, 450 s after the addition of sevenfold unlabeled peptides, $R = 0.45$, 0.24, and 0.11 with POPC/POPG/Chol 5:1:3, POPC/POPG/Chol 5:1:2, and POPC/POPG 5:1 liposomes, respectively.

We repeated the same sequence of experiments with the same amount and ratio of labeled peptides (1.67 μM and 4:6 respectively) but with an addition of 14-fold unlabeled peptide (23.3 μM) instead of sevenfold, to see if, in the time frame of the experiment, the degree of dissociation of the preformed aggregates of labeled peptides is a function of the amount of unlabeled peptide added (Fig. 8 *b*, *traces 3–5*). For each cholesterol content we obtained a fraction of NBD fluorescence recovery (R) higher than with the addition of sevenfold peptide ($R = 0.63$, 0.37, and 0.175 with POPC/POPG/Chol 5:1:3, POPC/POPG/Chol 5:1:2, and POPC/POPG 5:1 liposomes, respectively).

In both experiments we did not observe complete reversal of energy transfer to the level corresponding to complete dissociation of the preformed aggregates in the time frame of the experiment. This may be due to the fact that the rate constant of dissociation of surface aggregates, D, in Eqs. 1 and 5, is relatively small. It is also possible that a subset of the preformed aggregates, i.e., those aggregates that have

formed a pore, are locked in an irreversible configuration in the time frame of the experiment described in Figure 8.

Clearly there is a significantly larger degree of reversibilty of surface aggregation of the peptide in liposomes containing larger cholesterol percentage in the membranes. Thus, for the same number of bound peptides per liposome, the reduced efficiency of the peptide to induce leakage when the cholesterol content is raised in the membrane (see Table 1) stems from the larger degree of surface reversibility, or smaller average aggregate size in cholesterol-containing liposomes. This is a strong support that cholesterol has two effects: it reduces the membrane-binding affinity of the peptide and increases the reversibility of its surface aggregation in the bilayer.

GENERAL DISCUSSION

Efficiency of GALA as a pore forming agent

GALA at pH 5.0 induces complete leakage of ANTS/DPX from neutral or negatively charged vesicles at a lipid/peptide ratio of about 5000 at 0.1 mM lipid. The presence of physiologically relevant cholesterol molar ratios (0.33) in the bilayer reduces the efficacy (lipid/peptide ratio required for 100% leakage) to 120. This brings it into the same range as the majority of other peptides that induce leakage, e.g., melittin, magainin, cecropin, pardaxin, and HIVarg, although these also act at neutral pH. It may be possible to take advantage of the differential effect of membrane composition on GALA-induced leakage to selectively lyse membranes with low cholesterol content. GALA is also a promising starting point for the design of triggerable lytic peptides that are less affected by the presence of cholesterol in the membrane, which could improve cytoplasmic delivery of molecules into cells subsequent to their internalization by endocytosis (Haensler and Szoka, 1993). Regardless of practical considerations, GALA has been useful in elucidating peptide-membrane interactions (Subbarao et al., 1987; Parente et al., 1988; Parente et al., 1990; Fattal et al., 1994) and in this study its application has revealed a subtle effect of cholesterol on reversibility of peptide aggregation in bilayers as well as reinforced previous notions concerning cholesterol effects on partitioning into bilayers.

The cholesterol effect

Effects of cholesterol on membrane leakiness induced by peptides have been reported. The inclusion of cholesterol in egg PC vesicles resulted in a reduced efficiency of leakage by the 17-amino acid peptide corresponding to the N-terminus of influenza virus hemagglutinin (HA2.17), but had little effect on the leakage induced by the N-terminal peptide of Sendai virus F-protein (Düzgünes and Shavnin, 1992). A significant decrease in the conductance caused by the peptides cecropin AD or MP3 was observed in planar lipid membranes by Christensen et al. (1988) upon addition of cholesterol, and it was suggested that the weak hemolytic activity of cecropin was due to the presence of cholesterol in erythrocytes. Matsuzaki et al. (1995a) observed an increase in hemolytic activity of the antimicrobial peptide magainin upon cholesterol depletion of erythrocytes and a decrease of destabilization of phosphatidylserine liposomes upon inclusion of cholesterol, whereas none of these effects was observed with the peptide melittin. Interestingly, Tytler et al. (1995) who designed and synthesized analogs of magainin, whose lytic effects were significantly reduced by cholesterol, proposed that these peptides had direct interactions with cholesterol that inhibit the formation of peptide structure capable of lysis.

Our results in Figures 1–4 demonstrate that induction of leakage by the peptide GALA from liposomes containing more than 15% Chol required increased numbers of peptides per liposome depending on the cholesterol content. Part of this reduction in the efficiency of induction of leakage is due to a significant drop in the affinity of binding of the peptide to liposomes containing cholesterol, but for the same number of membrane-bound peptides per vesicle the leakage from liposomes containing cholesterol was severalfold less than that from POPC or POPC/POPG liposomes (see Table 1). Furthermore, to explain the leakage data with cholesterol-containing liposomes, we encountered the failure of the pore model used for POPC and POPC/POPG liposomes, which assumes irreversible surface aggregation of the peptide. We had to use a larger degree of reversibility of peptide aggregates in cholesterol-containing vesicles to explain quantitatively the reduction in leakage efficiency for the same amount of bound peptide per liposome. We have tested several hypotheses to explain leakage obtained from cholesterol-containing liposomes.

1. We examined the possibility that the leakage from cholesterol-containing liposomes occurred by micellization, because larger numbers of bound peptides per vesicle were needed. This possibility can be discarded on the basis of the sharp drop in the extent of leakage of dextran 3000 MW in comparison to the leakage of ANTS/DPX. Thus, for liposomes containing 33.3% cholesterol at a lipid/peptide ratio of 120, where the leakage of ANTS/DPX was 100%, no leakage of dextran was observed.
2. Another possibility, which was proposed for the leakage induced by several peptides (Schwarz and Robert, 1992) that leakage can occur via formation of transient pores, can not be substantiated. The application of this proposal to the inhibitory effect of cholesterol on the leakage would imply shorter lifetimes of the pores in cholesterol-containing vesicles. However, the leakage from cholesterol-containing liposomes occurred according to an all-or-none mechanism, whereas transient pores would exhibit graded release.
3. *Reduced fluidity.* We examined to what extent reduced membrane fluidity could explain the effect of cholesterol on the efficiency of GALA to induce leakage. The possibility of the effect of fluidity was proposed by Matsuzaki et al. (1995a) who noted reduced leakage induced

by the antibacterial peptide magainin from cholesterol-containing liposomes. Clearly, the penetration of the peptide into more rigid membranes is less favorable (Dufourcq and Faucon, 1977). Hence, the three- to four-fold reduction in the binding of GALA to POPC/POPG/Chol 5:1:3 liposomes, relative to POPC/POPG 5:1 ones, might be due to this effect. Our results follow the general trend observed by De Young and Dill (1988) of decreasing bilayer partition coefficients with an increase of cholesterol content and surface density of phospholipids in the membrane. However, a certain reduction in affinity for the same degree of fluidity can not be ruled out. We analyzed whether the larger degree of reversibility of surface aggregates in cholesterol-containing liposomes could arise directly from the reduced fluidity. In considering the final extents of aggregation of colloidal particles, the viscosity of the medium has no effect, because both C and D in Eq. 5 depend inversely on the viscosity, which cancels out in the expression of K_s. Furthermore, as was pointed out in Nir et al. (1983), the average aggregate size is usually reduced at elevated temperatures despite the increase in fluidity. We will point out a few observations that further complicate the simple correlation between the effect of cholesterol and fluidity on pore formation and leakage. One result is the similar kinetics of leakage (Fig. 2) for the two types of liposomes, with or without cholesterol. The other result is that with 15% Chol there is no mesurable inhibition of leakage efficiency, despite the expected reduction in fluidity. Inhibition of leakage rises steeply from 20% to 40% Chol in the liposomes.

We have pointed out that the application of a model that allowed for reversibility of surface aggregation of GALA could adequately explain (with the same pore size) the final extents of leakage from cholesterol-containing liposomes (Fig. 6 *b*) for the whole range of lipid/peptide ratios. The use of peptides labeled with NBD and rhodamine showed (Fig. 8) that there was a smaller degree of energy transfer between the peptides in cholesterol-containing liposomes for the same amount of bound peptide per vesicle. This might indicate reduced extent of peptide aggregation. Furthermore, we found a larger reduction of energy transfer in cholesterol-containing liposomes upon addition of unlabeled peptide (Fig. 8) to preformed aggregates of donor (NBD) and acceptor (rhodamine) peptides in the membrane. This larger extent of dissociation of preformed aggregates directly demonstrated a higher degree of reversibility of surface aggregates of the peptide. A recent study with the amphipathic and positively charged peptide pardaxin (Rapaport et al., 1996) led to the proposal of this model that includes reversibility of surface aggregation. In the latter study this reversibility was demonstrated by using fluorescein-labeled pardaxin. Because our studies were conducted at pH 5.0, we avoided such labeling in the case of GALA. As expected from the model, we also note that the extents of leakage induced by pardaxin at 30°C were larger than at 43°C, despite the increased binding at the higher temperature, where the fluidity is larger.

Reversibility of surface aggregation

In the study with pardaxin the value deduced at 30°C for K_s (which measures the degree of reversibility; see Eq. 5) was 0.002, which is two orders of magnitude below the value used here for liposomes containing 33.3% Chol. To illustrate what this difference in K_s implies, let us consider the case of a liposome of a diameter of 100 nm, which includes 15 bound peptides (i.e., $X_o = 15$ in Eq. 8). We note that the values of K_s were given for vesicles of 200 nm in diameter. For a vesicle of 100 nm in diameter the values of K_s should be multiplied by 4.17. According to Eqs. 6–8, the probability of leakage from such a liposome would be unity for $K_s = 1000$, which implies irreversible surface aggregation. The probabilities for leakage from such a vesicle (Z values in Eqs. 4–8) would be $4.3\ 10^{-5}$, and 0.53 for K_s values of 0.002 and 0.24, respectively. The corresponding probability would be 0.84 for 25% Chol ($K_s = 1.3$) and 0.98 for POPC/POPG vesicles where we use $K_s = 15$ (see Table 2).

According to the formalism of Flory (1939) the probability of finding a free monomer is $(1 - p)^2$. In the case of POPC/POPG vesicles, the value of p (Eq. 7) is 0.98, which implies that only 0.04% of the peptides in the surface remain as monomers. With 33.3% Chol the calculations imply that the percent of monomers would be 2.4. This example illustrates that reversibility of surface aggregation of pore-forming peptides can play a dominant role in the probability of pore formation, and can imply that the number of peptides bound per vesicle might be severalfold larger than that required for forming a pore. This effect can be operative in addition to the existence of transient pores (Schwarz and Robert, 1992), translocation of peptides (Matsuzaki et al., 1995b) or other mechanisms.

Fixed or growing pore size

The results of Parente et al. (1990) for neutral egg PC liposomes at pH 5.0 support a model where GALA forms pores consisting of 10 ± 2 peptides. This was deduced from the analysis of final extents of leakage and was further confirmed by the selectivity of the leakage to the size of the encapsulated molecules. Such selectivity was also demonstrated with human neutrophil defensins (Wimley et al., 1994). Our current results demonstrate that the same pore size also applies for negatively charged and cholesterol-containing liposomes. The other possibility (Baumann and Mueller, 1974) is that the size of the pore can increase by the addition of more peptides in a liposome. This possibility has been denoted the barrel stave model (Ehrenstein and Lecar, 1977; Shai, 1994). A comparison between the leakage of ANTS/DPX and dextran 3000 MW from POPC/POPG liposomes at a lipid/peptide ratio of 5300, where there is 100% leakage of ANTS, yet no leakage at all of

dextran, might lead to the conclusion that the pore formed by GALA can not incorporate more than 10 ± 2 peptides. The Stokes radius of dextran 3000 MW is ≈ 12 Å, which would require a circular pore consisting of 12–14 peptides to induce leakage. However, a further increase in the peptide content in POPC/POPG membranes resulted in a certain degree of leakage of dextran 3000 MW. Application of Eq. 3 for this leakage by using $M = 14$ (number of bound peptides required per vesicle to obtain leakage) and irreversible surface aggregation would yield much more leakage than the observed values at the lipid and peptide concentrations tested. Our calculations indicate that the probability of formation of a pore larger than 10 ± 2 is significantly reduced. The final extents of leakage of dextran 3000 MW from POPC/POPG liposomes require that the critical number of peptides per vesicle would be 180 rather than 14. Consequently, a pore consisting of 10 peptides or more would always form if the vesicle contains 10 peptides or more, but the probability of formation of a pore consisting of 14 peptides is 10-fold smaller. Although this issue requires more detailed study, our conclusion at this stage is that the pore size $M = 10 \pm 2$ is not strictly fixed, but the probability for the occurrence of a larger pore decreases steeply with the size of the pore.

CONCLUSIONS

Pore formation by GALA is unaffected by negative surface charge in the bilayer. However, cholesterol reduces GALA pore formation by decreasing the partitioning of the peptide and by increasing the reversibility of its aggregation in the membrane. This effect of cholesterol on the behavior of GALA can be explained by a simple model whose predictions are consistent with both the concentration dependence of contents leakage and the reversibility of energy transfer between fluorescently labeled peptides in the bilayer.

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