Pore-Forming Peptides Induce Rapid Phospholipid Flip-Flop in Membranes†

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ABSTRACT: A kinetic model for pore-mediated and perturbation-mediated flip-flop is presented and used to characterize the mechanism of peptide-induced phospholipid flip-flop in bilayers. The model assumes that certain peptides can bind to and aggregate within the membrane. When the aggregate attains a critical size (*M* peptides), a channel is created that results in a fast flip-flop of phospholipids. In addition, certain peptides induce flip-flop through perturbation of the membrane without forming a pore. Donor phospholipid vesicles with an asymmetrical distribution of the fluorescent phospholipid 1-oleoyl-2-[12-[(7-nitro-1,2,3-benzoxadiazol-4-yl)amino]dodecanoyl]phosphatidylcholine (NBD-PC) were used to measure the extent of flip-flop by quantitating the decrease in fluorescence as the NBD-PC exchanged from the donor vesicles to acceptor vesicles that contained a quencher of the NBD fluorescence. Flip-flop curves generated at lipid/peptide ratios ranging from 30/1 to 300000/1 could be well-simulated by the model. Pore-forming peptides, such as melittin or the synthetic peptide GALA (WEAALAEALAEALAEHLAEALAEALEALAA), induce rapid phospholipid flip-flop with half-times for flip-flop of seconds at low peptide/vesicle ratios. The deduced pore sizes are $M = 10 \pm 2$ for GALA and $M = 2$–4 for melittin. The synthetic peptide LAGA (WEAALAEAEALALAEHEALALAEAELALAA) can catalyze flip-flop via bilayer perturbation. In contrast, hydrophobic peptides such as gramicidin A and valinomycin intercalate into the membrane, but induce little flip-flop. Modeling of the kinetics of phospholipid translocation supports pore formation as the key factor in accelerating phospholipid flip-flop. Thus, amphipathic segments from membrane proteins may account for non-energy-dependent phospholipid flip-flop in biological membranes.

The formation and maintenance of phospholipid asymmetry in biological membranes are crucial for membrane function (Thompson, 1978; Op den Kamp, 1979; Herrman *et al.*, 1991). Under most conditions the transbilayer flip-flop of phospholipids is exceptionally slow, with half-times for flip-flop ranging from hours to weeks (Kornberg & McConnell, 1971; Homan & Pownall, 1988; Lipka *et al.*, 1991). Membrane proteins termed flippases have been identified that catalyze the transbilayer flip-flop of certain phospholipids in both an ATP-dependent and an ATP-independent fashion, yielding half-times of less than 5 min (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985; Backer & Davidowicz, 1987; Schroit *et al.*, 1987, 1990; Devaux *et al.*, 1990). Recently, the asymmetrical distribution of phospholipids in rod outer segments has been explained on the basis of an asymmetrical distribution of charged residues on membrane proteins (Hubbell, 1990).

In membranes where the asymmetrical distribution of phospholipids is an equilibrium property, flippase proteins may employ simple mechanisms to mediate rapid transbilayer phospholipid flip-flop. The low rates of transbilayer reorientation of phospholipids can be increased significantly by the incorporation of channel-forming antibiotics in the membrane such as amphotericin B (Schneider *et al.*, 1986). Schneider and co-workers have suggested that the enhancement of flip rates by the channel results from a thinning of the bilayer at the channel–lipid interface and a reduction of the hydrophobic barrier for the polar phospholipid headgroups. Since the flip-flop of a phospholipid across a bilayer has a similarity to the permeation of a water-soluble molecule equivalent to the headgroup of the phospholipid (Ganong & Bell, 1984), we tested whether peptides that interact with membranes and self-associate to form aqueous pores could mediate rapid phospholipid flip-flop. We used a previously described 30 amino acid, pH-sensitive, α-helical amphipathic peptide designated GALA[1] (Subbarao et al., 1987) to investigate the role of channel-forming peptides in phospholipid flip-flop. GALA undergoes a transition from a random coil at pH 7.5 to an amphipathic α-helix at pH 5.0, which strongly interacts with the membrane to induce fusion and contents leakage (Subbarao *et al.*, 1987; Parente *et al.*, 1988, 1990a,b). GALA induces leakage of liposomal contents from large vesicles without the induction of their aggregation or fusion (Parente *et al.*, 1990b). We also employed melittin and three other peptides. The ability of these five different peptides to mediate transbilayer phospholipid flip-flop as a function of peptide/lipid ratios has been compared under conditions where vesicle integrity is retained.

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[1] Abbreviations: DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; GALA, peptide with the sequence WEAALAEALAEALAEHLAEALAEALEALAA; LAGA, peptide with the sequence WEAALAEAEALALAEHEALALAEAELALAA; LUV, large unilamellar vesicles; NBD-PC, 1-oleoyl-2-[12-[(7-nitro-1,2,3-benzoxadiazol-4-yl)amino]dodecanoyl]phosphatidylcholine; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; RMSE, root-mean-squared error; POPC, 1-palmitoyl-2-oleoyl-3-phosphatidylcholine; SUV, small unilamellar vesicles; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

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A kinetic model that employs mass-action was used to simulate and predict the extent and kinetics of flip-flop induced by pore-forming peptides, as well as peptides that merely perturb the membrane. The model assumes that certain peptides bind and become incorporated into the bilayer of the vesicles. Within the membranes, the peptide can aggregate. When an aggregate within a membrane reaches a critical size of M peptides, a channel or a pore is created within the membrane, which results in a fast flip-flop of probe molecules (as well as other molecules in the area of the pore and its vicinity). Thus, a relatively fast flip-flop occurs, leading to equilibration of the lipid composition on the two monolayers. We denote this process as pore-mediated flip-flop. In the case where the probe can transfer between vesicles, the presence of acceptor vesicles results in the removal of the probe molecules from the outer monolayer of prelabeled vesicles. For the purpose of generality, we consider that, in addition to this major mode of flip-flop, a slower flip-flop mode can occur in vesicles including less than M peptides in an aggregate. This mode of flip-flop can reflect a perturbation in the membrane that proceeds for a limited duration or for as long as the peptide resides in the membrane and will be denoted perturbation-mediated flip-flop. Ordinarily, in unperturbed phospholipid vesicles, including those employed in the current study, the flip-flop process is extremely slow in the absence of peptides or other perturbants (Kornberg & McConnell, 1971; Backer & Davidowicz, 1987; Devaux *et al.*, 1990; Schroit *et al.*, 1990). Times required for equilibration by such flip-flop are on the order of several hours to weeks. Hence on a time scale of minutes to hours, we ignore this type of intrinsic flip-flop. In the current calculations, we have considered two types of flip-flop as described above, but in certain ideal cases, only the pore-mediated flip-flop has to be considered on a time scale of up to several minutes.

The assumption that a critical number of aggregating peptides is required for the induction of fast flip-flop emanates from our previous studies on the mechanism of leakage induced by the peptide GALA (Parente *et al.*, 1990b). In the latter study, we explained and predicted the kinetics and final extents of leakage by employing a model according to which GALA forms a transbilayer channel composed of M = 8–12 monomers. The kinetics of leakage was shown to reflect the kinetics of self-aggregation of peptides in the membrane.

In addition to demonstrating that pore-forming peptides such as GALA or melittin can induce very rapid flip-flop with half-times on the order of seconds, we also demonstrate the existence of two other classes of peptides: those that mediate flip-flop by a perturbation mode and those that intercalate into the bilayer with little effect on phospholipid flip-flop.

MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-oleoyl-3-phosphatidylcholine (POPC), 1-oleoyl-2-[12-[(7-nitro-1,2,3-benzoxadiazol-4-yl)-amino]dodecanoyl]-3-phosphatidylcholine (NBD-PC), and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (Rh-PE) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). The peptides GALA and LAGA were synthesized and purified as described (Subbarao *et al.*, 1987; Parente *et al.*, 1990a). Gramicidin D and valinomycin were purchased from Sigma (St. Louis, MO). Melittin was purchased from Sigma and further purified as described (King *et al.*, 1976).

Preparation of Asymmetrically Labeled Large Unilamellar Vesicles (LUV). Asymmetrically labeled LUV were prepared by a slight modification of the method of Pagano *et al.* (1981), as indicated below.

Preparation of Symmetrically Labeled LUV. LUV (20 mM) composed of NBD-PC/POPC (3/97 molar ratio) were prepared according to the reverse-phase evaporation method (Szoka *et al.*, 1980). A dried lipid film, composed of 38.8 mmol of POPC and 1.2 mmol of NBD-PC, was dissolved in 5 mL of diethyl ether, and 1.5 mL of 5 mM TES/NaOH, 100 mM KCl, and 0.21 M sucrose (pH 7.5) (TS buffer) was added to the organic solution. After complete evaporation of the diethyl ether, the volume of the liposomal suspension was adjusted to 2 mL using TS buffer. The liposomes were extruded five times through a 0.4-μm polycarbonate membrane. In order to separate the unencapsulated sucrose, the suspension was dialyzed against 600 mL of 5 mM TES/NaOH and 100 mM KCl (pH 7.5) (TK buffer) and centrifuged (80000g 1 h, 4 °C). The pellet was resuspended in a total volume of 2 mL of TK buffer. LUV composed of POPC were prepared using the same procedure.

Preparation of Symmetrically Labeled Small Unilamellar Vesicles (SUV). A concentrated suspension of 120 mM NBD-PC/POPC (molar ratio, 6/94) was prepared from a phospholipid dispersion in 2 mL of TK buffer. The liposomes were sonicated until they are optically clear (45 min using a bath sonicator; Laboratory Supplies Company Inc., Hicksville, NY). The vesicles were centrifuged (80000g, 1 h, 4 °C), and the SUV in the supernatant were recovered. This resulted in a vesicle preparation with a diameter of about 45 ± 0.5 nm and a narrow polydispersity index (0.2 ± 0.5). SUV composed of POPC were prepared using the same procedure.

Preparation of Asymmetrically Labeled LUV Containing the Probe on the Inner Monolayer. Donor (LUV containing 3% NBD-PC) and acceptor SUV were mixed at a donor/acceptor ratio of 1/10 and incubated for 1 h at 37 °C. The mixture was then centrifuged (80000g, 1 h, 4 °C). The pellet was resuspended with TK buffer and mixed again with SUV acceptor vesicles (1 h, 37 °C) at an average ratio of donor/acceptor of 1/1.7. The mixture was centrifuged (80000g, 1 h, 4 °C), the pellet containing the LUV was resuspended in TK buffer, and the centrifugation and resuspension process was repeated two additional times. After the final centrifugation, the asymmetrically labeled LUV were resuspended in TK buffer.

Preparation of Asymmetrically Labeled LUV Containing the Probe on the Outer Monolayer. Donor (SUV containing 6% NBD-PC) and acceptor LUV were mixed at a donor/acceptor ratio of 2/1 and incubated for 1 h at 37 °C. The mixture was centrifuged (80000g, 1 h, 4 °C). The pellet was resuspended with TK buffer and mixed again with SUV donor vesicles at an average donor/acceptor ratio of 1/2 for 1 h at 37 °C. The mixture was centrifuged three times. Each time, the pellet containing the LUV was resuspended in TK buffer.

Lipid Phosphorus and Fluorescence Measurements. Lipid phosphorus was determined by a modification of the Bartlett (1959) method. Fluorescence measurements were made on a Spex Fluorolog photon-counting instrument (Edison, NJ) using a 150-W xenon light source.

Characterization of the Asymmetrically Labeled Vesicles. Vesicle diameter was measured by laser light scattering (Malvern), and the fluorescence intensity of NBD-PC in the vesicles was measured at 535 nm with excitation at 450 nm. The amount of NBD-PC was determined from a standard curve of NBD-PC in vesicles. The asymmetry of the vesicles was characterized by using KI as an aqueous quencher. Potassium iodide was added at different concentrations (0.05–0.7 M) from a stock solution of 1 M KI and 0.001 M sodium bisulfite to 0.8 mmol of symmetric or asymmetric vesicles in

2 mL of 5 mM TES/NaOH buffer, and sufficient KCl was added to keep the ionic strength constant and equal to a 1 M KI solution. The excitation slits were 1.25 and 2.5 mm. The emission slits were 0.5 and 1.25 mm. Data were analyzed according to the Stern–Volmer equation for collisional quenching:

$$F_0/F = 1 + K_{sv}Q$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, Q is the molecular concentration of quencher, and K_{sv} is the Stern–Volmer quenching constant. K_{sv} is a reliable indication of the bimolecular rate constant for collisional quenching of NBD through the aqueous phase (Lehrer, 1971; Chattopadhyay, 1990).

Assay of Transbilayer Translocation of NBD-PC. The ability of NBD-PC located on the external monolayer to transfer rapidly between vesicles (Pagano *et al.*, 1981; Nichols & Pagano, 1982) was the basis for measuring the peptide-induced flip-flop of NBD-PC. The NBD-PC on the inner monolayer of asymmetrical vesicles does not transfer between vesicles in the time frame of these experiments (4 h). However, if the NBD-PC is induced to flip-flop across the bilayer, it can rapidly transfer between vesicles. We used acceptor vesicles that contained Rh-PE, a nontransferrable fluorescent lipid, to quench the fluorescence of transferred NBD-PC (Struck *et al.*, 1981). In a typical flip-flop assay, asymmetrical vesicles (500 nmol of lipids) were mixed with the test peptide in a volume of 0.5–0.55 mL. The pH was lowered to 5.0 by adding an aliquot (*ca.* 10 μL) of a concentrated acetate buffer solution (2 M, pH 5.0), and the incubation was continued for 30 min at room temperature. This mixture (100 nmol) was then incubated with 1 mmol of Rh-PE/POPC (1/99 molar ratio) vesicles at pH 5.0 at 37 °C for various times. When NBD-PC was transferred to RH-PE vesicles, its fluorescence was quenched. The fluorescence of NBD-PC (excitation = 450 nm, emission = 535 nm) was measured at different times (0, 20, 40, 60, 90, 120, 180, and 240 min). The excitation slits were 1.25 and 2.5 mm. The emission slits were both 2.5 mm. Control experiments with or without peptide were carried out at pH 7.5 and 5.0 when appropriate. Under conditions where flip-flop is observed, we have shown that LUV remain intact and do not fuse in the presence of peptides (Parente et al., 1988).

Peptide Concentration Effect on Flip-Flop. The peptides GALA, LAGA, melittin, gramicidin, and valinomycin were tested for their effect on flip-flop in large unilamellar vesicles containing NBD-PC on the inner monolayer. The lipid/peptide ratio varied from 30 to 30 000 for LAGA, from 30 to 300 000 for GALA and melittin, and from 30 to 100 for gramicidin and valinomycin. These latter two peptides were dissolved in DMSO and added to lipid suspensions so that the final DMSO concentration was 1%. DMSO induced no measurable flip-flop at this concentration. Gramicidin and valinomycin were also dissolved in ethanol and added to lipid suspensions. The final ethanol concentration was 1% in these experiments. We observed a level of flip-flop in the ethanol only control similar to that in the ethanolic peptide solution (equal to the DMSO peptide solution). Therefore, ethanol solutions were not used to introduce peptides in the flip-flop assay. Melittin was dissolved in TK buffer containing 10 mM EDTA. The percent of transfer can be related to the fluorescence intensity with the following expression:

$$\% \text{ transfer} = \frac{1}{0.71}(1 - F_\infty/F_0)100$$

where F_∞ is the C-12-NBD-PC fluorescence intensity after 240 min, and F_0 is the initial fluorescence. The correction factor 0.71 corresponds to the value of $1 - F_\infty/F_0$ when NBD-PC is on the outer monolayer of asymmetrical vesicles after 240 min of incubation with Rh-PE vesicles. This correction factor is the residual NBD-PC fluorescence when the probe transfers among all of the vesicles in the assay medium.

pH-Jump Experiment. The association of GALA with bilayers is dependent upon the pH (Subbarao *et al.*, 1987; Parente *et al.*, 1990a). This characteristic permitted a lower bound to be placed upon the rate of flip-flop induced by GALA when it intercalates into the membrane. GALA is displaced from the membrane by jumping the pH back to 7.5 after the peptide has been allowed to interact with the LUV at pH 5.0. In this experiment, asymmetrical LUV with the probe on the outer monolayer were mixed with GALA at a lipid/peptide ratio of 1/100 and a lipid concentration of 1 mM. The pH was lowered to 5.0 for 60 s. The pH was raised to pH 7.5 by adding a small aliquot of a 1 M solution of TES/NaOH (pH 7.5), and the mixture was then incubated for 30 min at room temperature. Then the Rh-PE acceptor vesicles were added, and the percent flip-flop was quantitated by measuring the amount of NBD-PC that could exchange, as described above. The amount of NBD-PC flip-flop is equivalent to 100% transfer.

THEORETICAL ANALYSIS OF FLIP-FLOP KINETICS AND EXTENT. FINAL EXTENTS OF FLIP-FLOP

Consider identical vesicles in which the fractions of probe molecules in the internal and external monolayers are denoted by n_1 and n_2, respectively, such that

$$n_1 + n_2 = 1 \tag{1}$$

The process of flip-flop is described by the differential equation

$$dn_2/dt = k_1 n_1 - k_2 n_2 \tag{2}$$

in which k_1 and k_2 (s^{-1}) are first-order rate constants. The same equation will still hold for a distribution of vesicles, provided that all vesicles are intrinsically similar. The solution of eq 2 is

$$n_2(t) = k_1/k + (n_2(0) - k_1/k)\exp(-kt) \tag{3}$$

in which $k = k_1 + k_2$. If $n_2(0) = 0$, then

$$n_2(t) = (k_1/k)(1 - \exp(-kt)) \tag{4}$$

At equilibrium, $n_2(\infty) \equiv n_2$, so

$$\bar{n}_2 = k_1/k \tag{5}$$

Hence,

$$n_2(t) = \bar{n}_2(1 - \exp(-kt)) \tag{6}$$

$\bar{n}_2$ will equal $^1/_2$ when $k_1 = k_2$. Equation 6 describes the process of natural or intrinsic flip-flop (Kornberg & McConnell, 1971).

For a situation where initially all probe molecules are in one monolayer, e.g., in the internal one,

$$FL(t) = n_2(t) \tag{7}$$

and at equilibrium

$$\overline{FL} = \bar{n}_2 \tag{8}$$

where FL can be defined as the net fraction of probe that has flipped. According to this definition, when $k_1 = k_2$, a net fraction of transfer that equals 0.5 implies complete equilibration or 100% flip-flop. If the flip-flop is *essentially a pore-mediated process*, then the final extent of the net fraction of probe that has flippped is given by

$$\overline{FL} = \overline{FL}_1 = \bar{n}_2 \sum_{i=M}^{N} A_i \tag{9}$$

in which N is the number of peptides that can bind to a single vesicle, and A_i is the fraction of vesicles containing i incorporated peptides. The procedure for calculating the function A_i was derived by Nir *et al.* (1986) and Bentz *et al.* (1988) and was further described in Parente *et al.* (1990b). The calculation requires the value of the binding constant, which in turn was obtained from measuring the amount of peptide bound.

In the current calculations, we have considered the vesicle population to have a size distribution given by our measurements of dynamic light scattering. We used the vesicle number distribution for S size classes, with fractions a_j converted to lipid fractions according to

$$Q_j = a_j N_j / (\sum_{j=1}^{S} a_j N_j) \tag{10}$$

in which N_j is the number of lipid molecules per vesicle. Hence, FL in eq 9 is calculated by

$$\overline{FL} = \overline{FL}_1 = \sum_{j=1}^{S} \bar{n}_{2j} \sum_{i=M}^{N} A_{ij} \tag{11}$$

which for large vesicles gives

$$\overline{FL} = \bar{n}_2 \sum_{j=1,i=M}^{S,N} Q_j A_{ij} \tag{12}$$

To simplify the notation, we will omit summations arising from the vesicle size distribution.

If there is a contribution to the flip-flop from a perturbation mode, the final extent value of $\overline{FL}$ is increased by a term $\bar{F}L_2$ arising only from vesicles containing 1 to $M - 1$ peptides, since in vesicles containing $\geq M$ peptides the flip-flop is complete. If flip-flop results in a fraction, f, of probe transfer from vesicles containing a single peptide, then the term $\overline{FL}$ is given by

$$\bar{F}L_2 = \bar{n}_2 \sum_{i=1}^{M-1} f_i A_i \tag{13}$$

$$\overline{FL} = \bar{F}L_1 + \bar{F}L_2 \tag{14}$$

A comparison between calculated and experimental fluorescence values for several lipid/peptide ratios enables one to find the parameter M, the number of peptides required to form a "pore" that results in fast flip-flop, as well as the parameter f, the fraction of flip-flop that can occur from vesicles containing one peptide. Of course, this formalism implicitly implies that

$$f(M-1) < 1$$

The equations of final extent are also obtained from those describing the kinetics of flip-flop.

Kinetics of Flip-Flop. In the case of the noncatalyzed or intrinsic mode of flip-flop, eqs 3 or 6 yields a simple description that requires essentially a single parameter. We note that the relation $k_1 = k_2$ can strictly hold only if the two monolayers are essentially equivalent and of equal area. If the areas are not equal, a single rate constant can still be employed, but the distribution of vesicle sizes has to be considered explicitly.

In the case of pore-mediated flip-flop, the process of flip-flop from a particular vesicle can start only after a pore has been formed. However, if a perturbation mode of flip-flop exists, albeit at a slower rate, some flip-flop can occur instantaneously upon peptide binding, which is usually a very fast process. In the following, we present the general solution for the kinetics of flip-flop. The details of the derivation are given in the Appendix.

$$\bar{F}L(t) = \bar{n}_2 \{ \sum_{i=1}^{m} (1 - \exp(-ik_{pt})) A_i + \sum_{i=M}^{N} (1 - \exp(-g_i(t))) A_i \} \tag{15}$$

where $m = M - 1$. The analytical solution includes k_p, the perturbation mode rate constant of flip-flop, and k, the pore-mediated rate constant, which appears in $g_i(t)$. The function $g_i(t)$ is given in eq A.27. It includes terms due to both the pore-mediated and perturbation-mediated modes of flip-flop.

RESULTS

Characterization of the Asymmetrically Labeled Vesicles. The diameter of the vesicles was unaffected by the procedure used to generate the asymmetrical distribution of probe in the bilayer (Table 1). The mole percents of NBD-PC in the asymmetrical vesicles were 1.62% for vesicles with probe on the inner monolayer and 1.7% for vesicles with probe on the outer monolayer. The difference between the experimental and theoretical probe level in the inner labeled vesicles is due to incomplete transfer of the probe from the outer monolayer, as well as from a slight contamination by the vesicles used to deplete the probe from the outside. The reason for the difference when the probe was added to the outside is that the system did not have sufficient time to equilibrate. These differences are small and do not interfere with our ability to measure flip-flop.

The NBD group on the acyl chain of NBD-PC is accessible to the water/lipid interface (Chattopadhyay, 1990). Therefore, we used a water-soluble quencher, KI, which does not rapidly permeate through the bilayer, to measure the extent of exposure of the NBD-PC in the various preparations. The Stern–Volmer plots are represented in Figure 1. The Stern–Volmer constant is small for asymmetrical vesicles (K_{sv} =0.43 M^{-1}) with the probe inside, whereas K_{sv} = 4.51 M^{-1} when the probe is outside. In the case of symmetrical vesicles, K_{sv} = 1.1 M^{-1}. Consequently, the probe is almost inaccessible to the quencher when it is located in the inner monolayer and is highly accessible in the outer monolayer, substantiating the fact that the vesicles used to detect flip-flop are asymmetrical with respect to NBD-PC.

Transbilayer Flip-Flop of NBD-PC. To quantitate the extent of NBD-PC flip-flop, we monitored the loss of fluorescence when the probe exchanged from the asymmetrical vesicles into the acceptor vesicles, which contained a quencher (Rh-PE) of the NBD-PC fluorescence. The NBD-PC could

Table 1: Characteristics of Asymmetrically Labeled LUV

type of vesicle	diameter (nm) of symmetrical LUV (polydispersity)	diameter (nm) of asymmetrical LUV (polydispersity)	found NBD-PC in asymmetric LUV (mol %)	expected NBD-PC in asymmetric LUV (mol %)
LUV probe inside	185 ± 3.6 (0.2 ± 0.02)	182 ± 1.7 (0.2 ± 0.02)	1.62	1.5
LUV probe outside	178 ± 2.6 (0.3 ± 0.01)	178 ± 3 (0.3 ± 0.01)	1.7	2.0

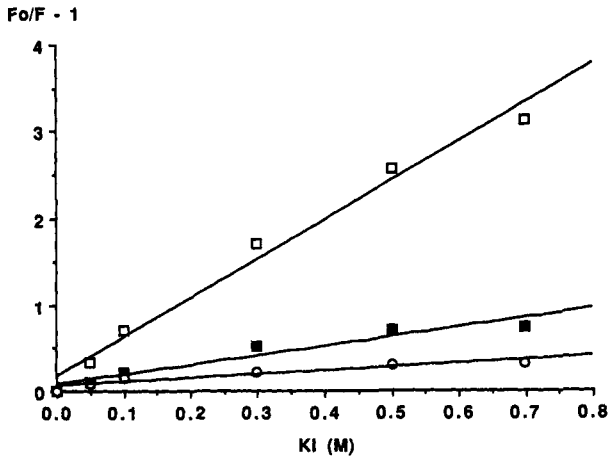

FIGURE 1: Potassium iodide Stern–Volmer quenching of NBD-PC in labeled vesicles: asymmetrical vesicles with the NBD-PC on the inner monolayer (○); asymmetrical vesicles with the NBD-PC on the outer monolayer (□); symmetrical vesicles (■).

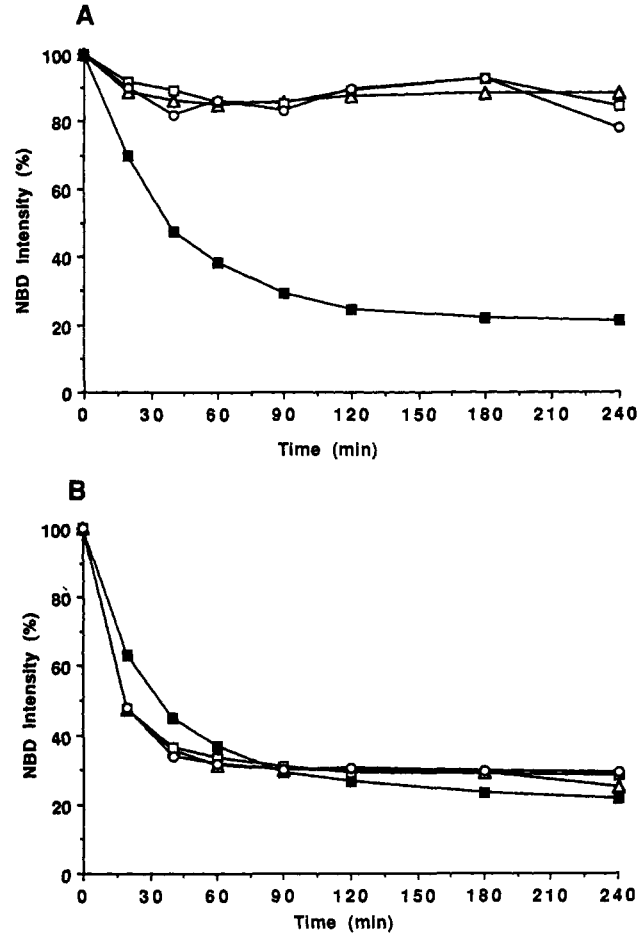

FIGURE 2: Measurement of peptide-induced phospholipid flip-flop by quenching of a fluorescent phospholipid. Large unilamellar vesicles (LUV) composed of POPC containing 1.5–1.7 mol % of the exchangeable phospholipid NBD-PC in either the inner (A) or outer (B) monolayer were prepared as described in Materials and Methods. The NBD-PC probe is on the inner monolayer (panel A) or outer monolayer (panel B): ■, in the presence of GALA (lipid/GALA = 100/1) at pH 5.0; △, in the presence of GALA (lipid/GALA = 100/1) at pH 7.5; □, in the absence of peptide at pH 5.0; ○, in the absence of peptide at pH 7.5. The result is from a single experiment that is representative of three independent experiments that agreed in the final extent to within ±5%.

only transfer when it was on the outer monolayer of the donor vesicles. In Figure 2A we show the results for vesicles with probe on the inner monolayer. When no peptide was present (buffer at pH 5.0 or 7.5) there was less than a maximum of 8% quenching of fluorescence, which was due to the residual quantity of NBD-PC on the outer monolayer.

The synthetic pH-sensitive amphipathic peptide GALA (Subbarao et al., 1987) is water-soluble and only weakly interacts with bilayers at pH 7.5, but partitions into membranes when the pH is lowered to 5.0 (Parente *et al.*, 1990a). In the bilayer, GALA assumes an α-helical secondary structure that is perpendicular to the surface of the bilayer, as measured by oriented FTIR (Goormaghtigh *et al.*, 1991). Within the membrane GALA assembles into an oligomeric pore. This membrane insertion causes leakage of vesicle contents (Parente *et al.*, 1990b). When GALA was present at pH 7.5, there was no decrease in NBD fluorescence (Figure 2A). This indicates that NBD-PC on the inner monolayer did not translocate across the bilayer. This was observed in three independent experiments and is consistent with the inability of GALA to interact with membrane at this pH (Subbarao *et al.*, 1987; Parente *et al.*, 1990a,b). However, in the presence of GALA at pH 5.0, the fluorescence decreased to a minimum value, which indicates that the NBD-PC translocated into the outer monolayer and distributed to the acceptor vesicles. At pH 5.0, GALA caused a maximal decrease in fluorescence (71%). This decrease is equivalent to what occurs when NBD-PC and Rh-PE are mixed in the same vesicle population, such as occurs when the two vesicle populations are sonicated together. These data confirm that NBD-PC is a good choice to evaluate peptide-induced flip-flop, since it has a slow transbilayer redistribution in the absence of a perturbation to the membrane (Figure 2A).

When the NBD-PC was originally only in the outer monolayer (Figure 2B), it transferred in all conditions tested. Transfer of the NBD-PC resulted in a minimal value for NBD-PC fluorescence by 240 min. Again, this is the same value that can be reached if the two vesicle preparations are mixed together and sonicated. Interestingly, when GALA was added at pH 5.0 to vesicles with the probe on the outside, the rate of transfer of the probe to the Rh-PE vesicles was significantly slower than the rate in the other conditions (Figure 2B). This suggests that GALA at pH 5.0 caused a redistribution of the probe to the interior of the donor vesicles, so that its transfer to the acceptor vesicles was delayed. Indeed, when GALA was present at pH 5.0 the rate of NBD-PC transfer to the Rh-PE vesicles was similar whether the probe was on the inner or outer monolayer, which implies that transbilayer flip-flop is fast and is not the rate-limiting step in these circumstances.

Kinetic Analyses of the Effect of Lipid/Peptide Ratios on Flip-Flop. GALA induces flip-flop in a dose-dependent fashion. The explanation for the observed dose dependence

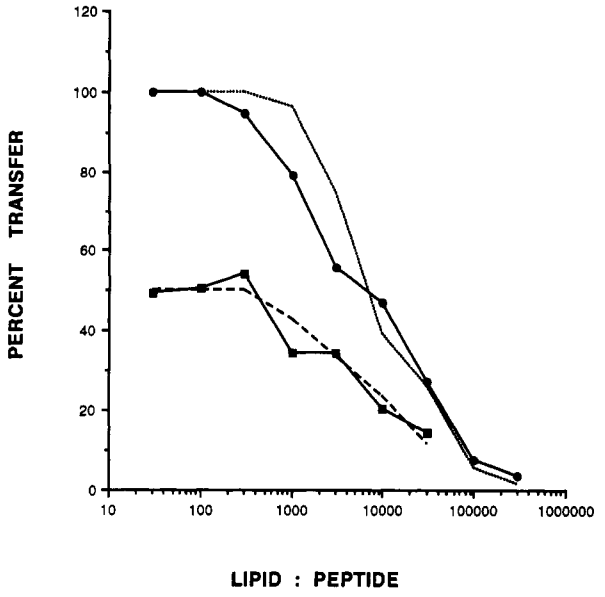

FIGURE 3: Concentration dependence of GALA-induced phospholipid flip-flop at pH 5.0. Large unilamellar vesicles containing NBD-PC asymmetrically in either the inner monolayer (●) or outer monolayer (■) were prepared as described in Materials and Methods. The upper curve displays the results when various amounts of GALA were added to the vesicles with the probe on the inner monolayer at pH 5.0, and the extent of probe transfer was measured after 240 min as described in Materials and Methods. This experiment is one of two independent dose response experiments that agreed to within 7% along the entire dose response curve. The lower curve is the result when various amounts of GALA were added at pH 5.0 to vesicles with the probe in the outer monolayer, and then the pH was increased to pH 7.5 after 60 s to remove GALA from the outer membrane. The vesicles were subsequently incubated at pH 7.5 for 30 min, and then the extent of probe transfer was measured as described above. The maximum extent of transfer in this case is 50%. This experiment is one of two independent dose response experiments that agreed to within 5% along the entire dose response curve. The dotted line in the upper curve and the dashed line in the lower curve are the calculated values with the parameters given in Table 2.

is that GALA partitions into the bilayer and assembles into an oligomeric pore (Parente *et al.*, 1990b). The extent of flip-flop is consistent with the presence of at least one oligomeric pore per vesicle (Parente *et al.*, 1990b) and complete flip-flop is observed at a lipid/peptide ratio of 300/1 (Figure 3).

The flip-flop induced by GALA is rapid. This was shown by taking advantage of the fact that GALA interacts with the bilayer only at low pH. By increasing the pH, GALA is removed from the membrane at defined times after its addition (Figure 3, lower curve). In this experiment, asymmetrical vesicles with NBD-PC on the outer monolayer were prepared and placed at pH 5.0. GALA was added and incubated for 60 s at pH 5.0, and then the pH was adjusted to 7.5, which causes the dissociation of GALA from the membrane (Parente *et al.*, 1990a,b). NBD-PC that has translocated to the inner monolayer is unable to exchange from the vesicles. In these conditions, the NBD-PC equilibrates across the bilayer at low lipid/peptide ratios, as indicated by the fact that 50% of the NBD-PC can transfer (Figure 3, lower curve). We have observed that in small vesicles equilibration of the asymmetrical probe could be achieved within 10 s (data not shown). When the concentration of GALA added to asymmetrical vesicles with NBD-PC on the inner monolayer is increased from a lipid/peptide ratio of 300000/1 to 300/1, the percent transfer increases from 3% to 100% (Figure 3, upper curve).

Determination of Pore Size and Rate Constants for Pore-Mediated and Perturbation-Mediated Flip-Flop. A kinetic model was developed that describes peptide-induced phospholipid flip-flop by means of two processes: (i) assembly of peptides into a pore in the membrane and (ii) a perturbation of the bilayer by the inserted or associated peptides (see Materials and Methods and Appendix). The experimentally determined parameters of the model include the final extent of flip-flop, the apparent partition coefficient of the peptide into the membrane, the concentration of the peptide and lipid, and the number diameter distribution of the vesicles. GALA has been shown to orient in an α-helical secondary structure perpendicularly in the membrane (Goormaghtigh *et al.*, 1991); as such, GALA should span the bilayer. Hence, peptide concentration in the two monolayers would be the same, and different concentrations in each monolayer would not complicate the computations. The adjustable parameters of the model include the minimal number of monomers required for pore formation and the rate constants for pore- and perturbation-mediated flip-flop. The parameters for GALA and the other peptides examined in these studies are given in Table 2. In the case of GALA, the observed concentration dependence of final extents of flip-flop can be modeled using a minimal pore size of 10, as previously found for GALA-induced contents leakage (Parente *et al.*, 1990b).

Table 2: Parameters of Peptides Used in Phospholipid Flip-Flop Studies

peptide	apparent partition coefficient and reference	M	k_{pore} (s^{-1})	$k_{perturbation}$ (s^{-1})
GALA	10^6 at pH 5.0 Parente *et al.*, 1990a	10	≥1	3×10^{-6}
melittin[a]	2×10^3 Kuchinka & Seelig, 1989	2	1	ND[b]
melittin	3×10^4 Stankowski & Schwarz, 1990	4	1	ND
LAGA	10^4 at pH 5.0 Parente *et al.*, 1990a	0	0	1.5×10^{-6}
gramicidin	10^5 Classen *et al.*, 1987	0	0	$<10^{-6}$
valinomycin	10^6 Stark *et al.*, 1972	0	0	$<10^{-6}$

[a] The rate constant for melittin is computed using the same surface aggregation rate as was used for GALA. [b] ND, not determined.

As in that study, we have carried out a statistical test for the goodness of fit using the bootstrap estimate (Efron, 1982) for the standard deviation of M, SD(M), according to

$$\mathrm{SD}(M) = \sum_{j=1}^{P} [(10 - M_j)^2/P]^{1/2} \qquad (16)$$

with $P = 256$ (16 points), in which M_j are other estimates chosen to yield the best fit upon the omission of two points at random. The calculation of R^2 gave 0.98. These indices indicate a good statistical fit of the calculated to experimental values for the final extent of flip-flop and a small variance in the estimate of M. Hence, for GALA-induced flip-flop $M = 10 \pm 2$, as was previously found from leakage studies.

In these calculations, we used a partition relation that gives $B = 0.645$ for the fraction of peptide bound to 100 μM liposomes and 3000/1 for the lipid/peptide ratio. From this binding ratio, we determined the binding constants for each vesicle size. As previously found, the assumption of a constant partition was adequate over a wide range of lipid/peptide ratios, but our current procedure is more precise. In the current study, we also had a wider range of lipid/peptide ratios than in the leakage studies.

Having determined M, we obtained an estimate for k, the rate constant for pore-mediated flip-flop, by assuming that

the final extent had been reached within 60 s. This gave $k \geq 1\ s^{-1}$. In these calculations, we employed the same value of C (eq A13) as used in Parente *et al.* (1990b). The fit of the results of GALA-induced flip-flop, where the probe was in the inner monolayer and exchanged after transfer to the outer monolayer, required that we take into account the contribution of the perturbation mode as well, since this experiment proceeded for 4 h. As we have pointed out, 1 min was sufficient for the termination of flip-flop by means of the pore-mediated mode. Hence, fixation of M from the pH-jump experiments enabled us to determine k_p, the rate constant for the perturbation-mediated mode. There is, however, uncertainty in the assignment of the time t because of the added delay due to the transfer of the probe to the acceptor vesicles. We used the estimate $t = 10^4$ s, i.e., = 3 h. If the relevant time is one-half of this value, then k_p is twice the value given in Table 2. The pore-mediated rate constant of flip-flop is more than 4 orders of magnitude faster than that due to perturbation. Consequently, the perturbation mode did not contribute to the final extent after 60 s, but perturbation has a small contribution for longer incubation times (Figure 3). Another statistical criterion for the fit is given by the root-mean-squared error (RMSE), defined by

$$\mathrm{RMSE} = \sum_{i}^{n}(Y_i - Y_{ci})^2/(n-2)^{1/2} \qquad (17)$$

in which Y_i and Y_{ci} are experimental and calculated values, respectively, and n is the number of points. The (percent) value of RMSE was 7.8, i.e., within experimental error.

Other Peptides. To learn whether rapid flip-flop is induced by other membrane-associated peptides, three well-studied peptides, gramicidin A, valinomycin, and melittin, were examined for flip-flop activity. When added to asymmetrical vesicles, melittin caused extensive flip-flop even at low lipid/peptide ratios (Figure 4). The concentration dependence could be modeled by a pore size of 2–4 peptides, depending on the apparent partition coefficient, K_a (Table 2). The RMSE was 6.9, whereas the experimental standard deviation was 6 and $R^2 \geq 0.99$. Gramicidin or valinomycin did not induce any appreciable flip-flop even at high peptide/lipid ratios (Figure 4). This was the case when these peptides were added from DMSO (Figure 4) or from ethanol solutions (data not shown). Finally, a peptide designated LAGA, with the same amino acid composition as GALA, but with a nonamphipathic sequence, mediated a significant extent of flip-flop at lipid/peptide ratios of 3000/1. This peptide, whose K_a is 100-fold lower than that of GALA (Table 2), does not induce contents leakage even at a ratio of 50/1 (Parente *et al.*, 1990b). We were unable to fit the observed flip-flop kinetics by a pore model, but instead found that a first-order rate constant for perturbation, proportional to the number of vesicle-associated peptides, gave a reasonable fit to the observed flip-flop (Figure 4). The RMSE was 3.9 vs 10 for the experimental standard deviation and $R^2 \geq 0.99$.

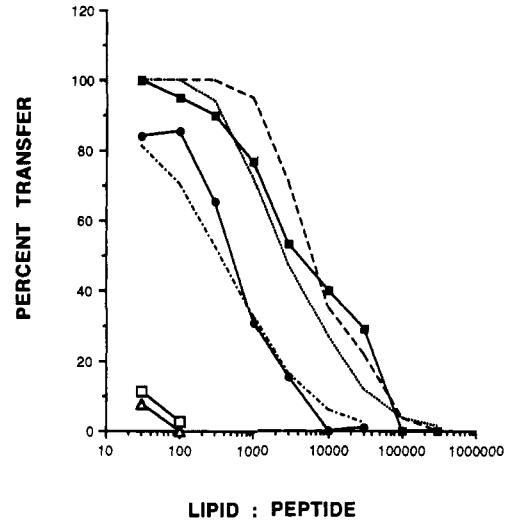

FIGURE 4: Concentration dependence of peptide-mediated phospholipid flip-flop for melittin and for peptides that do not form oligomeric pores in the bilayer. Peptide-induced flip-flop of NBD-PC from the inner monolayer of large unilamellar vesicles was measured; melittin (■) induced flip-flop calculated with a pore size $M = 2$ (···) or $M = 4$ (- - -) and the other parameters indicated in Table 2. LAGA (●) was calculated with perturbation kinetics (····); valinomycin (△) and gramicidin A (□). Flip-flop caused by the latter two peptides had a rate attributed to membrane perturbation that was less than $10^{-6}\ s^{-1}$ per peptide per vesicle. The results are from single experiments that were replicated twice in the case of LAGA, three times in the case of melittin, and four times in the cases of valinomycin and gramicidin. The error ranged from 5% at the 30/1 lipid/peptide ratio to 10% at the 10 000 lipid/peptide ratio.

DISCUSSION

We have characterized peptide-mediated phosphatidylcholine flip-flop in bilayers and present a theoretical treatment for analyzing flip-flop kinetics. Amphipathic peptides, such as melittin or GALA, that can organize into transmembrane oligomeric pores mediate rapid flip-flop (Figure 5A). Pores formed from peptides can be due to a transient aggregation of monomers in the membrane that form openings much like those observed in flickering conductance measurements in alamethicin channels; if phospholipids are to flip across the membrane through these aggregated structures, there must be a continuous pathway across the membrane filled with water molecules. Therefore, amphiphilic peptides are required, and peptides composed strictly of hydrophobic residues, which might aggregate in the membrane, would not mediate phospholipid flip-flop. Membrane-associating peptides such as LAGA, which are unable to form discrete pores, catalyze flip-flop through bilayer perturbations (Figure 5B). Finally, hydrophobic peptides such as valinomycin (Stark *et al.*, 1972) and gramicidin (Wallace, 1990), which intercalate into the bilayer but do not form water-filled channels, are unable to catalyze flip-flop except at extremely high peptide/lipid concentrations (Figure 5C).

In plasma membranes, lipid asymmetry can be induced by ATP-dependent proteins that catalyze the unidirectional translocation of aminophospholipids (flippase proteins) (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985; Schroit *et al.*, 1987; Devaux *et al.*, 1990). In the case of subcellular organelles, lipid flip-flop appears to be catalyzed by flippases with little phospholipid headgroup specificity, which do not require ATP (Bishop & Bell, 1985; Backer & Davidowicz, 1987; Hermann *et al.*, 1990; Wu & Hubbell, 1993). The involvement of ATP in aminophospholipid translocation may indicate a complex process, whereas simple mechanisms may explain ATP-independent flip-flop. Hall (1981), using black lipid membranes, demonstrated a voltage-dependent phospholipid flip-flop induced by alamethicin. Since alamethicin only assembles into a pore under an applied voltage, Hall showed that the flip-flop depended upon the assembly of a conducting pore. He pointed out some of the potential biological ramifications of this voltage-regulated flip-flop. Our results demonstrate that other pore-forming peptides, such as GALA and melittin, can induce very fast flip-flop wit'ı half-times on the order of minutes or less, in comparison with

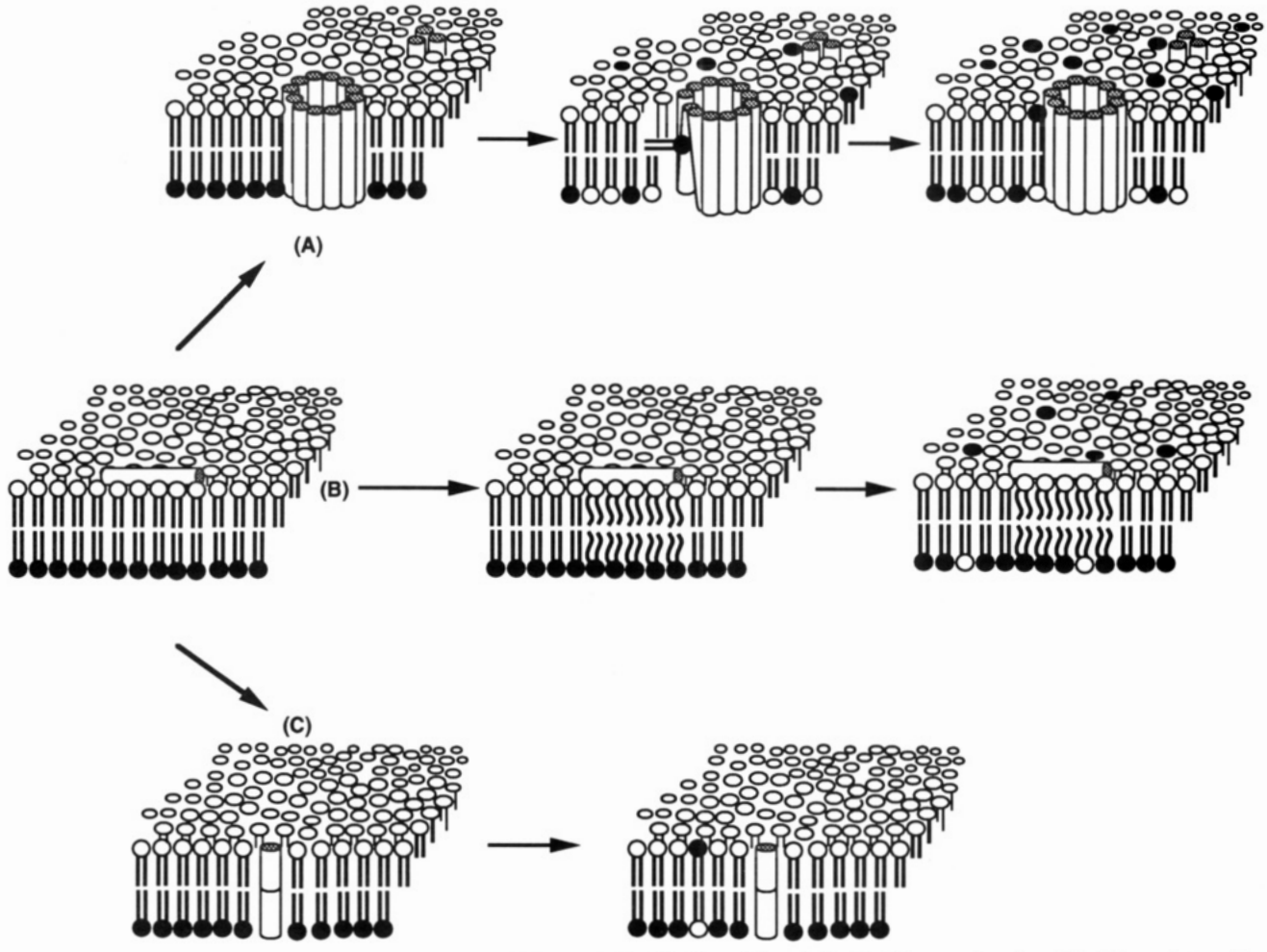

FIGURE 5: Modes of peptide–bilayer interaction and their influence on the flip-flop of phospholipids. Three modes of peptide–bilayer interactions corresponding to the peptides studied in this work: (A) pore-mediated flip-flop; (B) perturbation-mediated flip-flop; (C) stable intercalation of hydrophobic peptides resulting in little flip-flop. The perturbation mode of flip-flop may also occur in cases A or C, but is much slower compared to the rate induced by the pore (case A) and not detectable in the conditions employed in these experiments (case C).

hours to weeks for the spontaneous process in unperturbed membranes (Op den Kamp, 1979). In the case of GALA, where the flip-flop could be arrested upon raising the pH, the half-times are in the range of seconds, and the rate constant for pore-mediated flip-flop is at least 1 s^{-1}. The alamethicin-induced flip-flop rate is within an order of magnitude of this value (Hall, 1981).

A mechanism to account for the rapid flip-flop induced by the pore-forming peptides is that the phospholipid headgroup resides in the water-filled pore, while the translocating acyl chains remain within the hydrocarbon interior of the bilayer. The translocating phospholipid can be accommodated by a small fluctuation in the packing of the helices, permitting both the headgroup as well as the acyl chains to be in a favorable environment. Amino acid composition and residue location in the pore could influence the rate of phospholipid flip-flop. This may be particularly important for acidic phospholipids, since they are negatively charged and interaction with charged residues lining the pore could facilitate (positively charged residues) or hinder (negatively charged residues) flip-flop rates. The effect of peptide charge on phosphatidylcholine flip rates is more difficult to predict, since PC is zwitterionic and both the phosphate and the quaternary ammonium moiety could interact with charged amino acids in the pore. In this regard, melittin is positively charged, with four lysines and one arginine, yet mediates rapid phosphatidylcholine flip-flop under conditions where the peptides must be positively charged. Even though GALA contains seven glutamates, at pH 5.0 GALA might also have a slight positive charge due to the histidine residue in the middle of the sequence. Thus, a positively charged amphiphilic helix can mediate PC flip-flop. On the basis of the results with melittin, making a global change of E to K in GALA and running the flip-flop at pH 10 probably would not influence the flip-flop rate of PC. The potential for flip-flop modulation by charged residues in the peptide is certainly worthy of further investigation.

An additional implication of this finding relates to the action of cytolytic peptides and proteins on membranes: damage to cells by pore-forming toxins may not only be due to the dissipation of ion/osmotic gradients [reviewed by Ojcius and Young (1991)] but may also be caused by the loss of lipid asymmetry. The involvement of flip-flop in the action of the complementary attack on membranes has been previously discussed (Bevers *et al.*, 1983; Weidmer *et al.*, 1986). Complementary binding to membranes displays both a perturbation mode when components C5–C8 bind (Van der Meer *et al.*, 1989) and possibly also a pore-mediated flip-flop when C9 forms the pore in the membrane (Weidmer *et al.*, 1986). The resulting aminophospholipid flip-flop in platelets (Bevers *et al.*, 1983) ultimately leads to accelerated clotting (Weidmer *et al.*, 1986).

The loss of lipid asymmetry may disrupt other cell functions, such as membrane fusion (Hermann *et al.*, 1991) and vesicle trafficking, or promote enzymatic degradation of phospholipids

in the inner monolayer of the membrane. For instance, melittin can bring about an osmotic lysis of membranes (Dempsey, 1990) and also can stimulate phospholipase-catalyzed hydrolysis of lipids (Mollay *et al.*, 1976). Melittin-catalyzed flip-flop would ensure that all of the phospholipids in the cell membrane are substrates for the phospholipase.

The general model for peptide-induced flip-flop of phospholipids presented here is useful to characterize the two different modes of flip-flop. We could simulate and predict flip-flop results for lipid/peptide ratios varying from 30 to 300 000 (see Figures 3 and 4). The application of the model was essential in establishing that the observed rapid flip-flop was due to a pore-mediated process. In the case of GALA, the pore size deduced is $M = 10 \pm 2$, i.e., the same as that previously determined from leakage studies (Parente *et al.*, 1990b). The same conclusions were deduced irrespective of the initial residence of probe molecules in the external or internal monolayers. In the case of melittin, the pore size deduced, $M = 2$–4, depended on the magnitudes of the binding constants used, which were calculated from the apparent partition coefficients reported (see Table 2). This deduced pore size is in general accord with previous physicochemical studies [Degrado *et al.*, 1982; Tosteson *et al.*, 1987; Vogel, 1987; reviewed in Dempsey (1990)].

The rate constants for pore-mediated flip-flop are several orders of magnitude larger than those for the perturbation-mediated process (Table 2), which in turn are at least an order of magnitude larger than those for flip-flop in unperturbed vesicles. It should be noted that the values of k_p given in Table 2 pertain to one bound peptide per vesicle, whereas for n bound peptides the actual rate values are n-fold larger. The perturbation mode may be responsible for the phospholipid flip-flop induced by cytochrome b_5 in sonicated vesicles (Greenhut & Roseman, 1985) and, as noted above, is responsible for flip-flop when the C5–C8 complementary components insert into bilayers (Van der Meer *et al.*, 1989). Despite the extensive flip-flop induced by the peptide LAGA (Parente *et al.*, 1990b), only the perturbation mode of flip-flop could simulate the results (Figure 4). Remarkably, this nonamphipathic peptide with the same composition and length as GALA is ineffective in inducing leakage (Parente *et al.*, 1990b).

The observation with LAGA raises an interesting question: which type of peptide sequence is more appropriate for a flippase, one that forms a water-filled pore or one that creates a membrane perturbation without forming a pore? Flippase proteins containing a membrane-perturbing sequence that do not cause the loss of water-soluble components might be preferred for cellular phospholipid flip-flop; phospholipids could move, but cellular contents would be retained. In either case, hydrophilic residues in the proteins would be involved; only their orientation in the membrane would differ.

APPENDIX

We will present the derivation of the equations of flip-flop kinetics.

Perturbation Mode. When only the perturbation mode exists, we will assume that the flip-flop rate constants in a vesicle containing i peptides are proportional to i:

$$k_{1i} = ik_{11}$$
$$k_{2i} = ik_{21} \qquad \text{(A1)}$$
$$k_i = ik$$

Hence,

$$\mathrm{FL}(t) = \mathrm{FL}_2(t) \qquad \text{(A2)}$$

where from eqs 9 and A1 it follows that

$$\mathrm{FL}_2(t) = \sum_{i=1}^{N}[(k_{11}/k) - k_{11}\exp(-ikt)/k]A_i = \bar{n}_2\sum_{i=1}^{N}(1 - \exp(-ikt))A_i \qquad \text{(A3)}$$

where $\bar{n}_2$ is given by eq 5. At short times, such that $ikt \ll 1$,

$$\mathrm{FL}_2(t) \sim \sum_{i=1}^{N} ik_{11}tA_i = \bar{n}_2\sum_{i=1}^{N} iktA_i \qquad \text{(A4)}$$

which has the same form as eq 13, if the perturbation terminates at a certain time, $t = t_1$. If the perturbation proceeds, then eventually

$$\bar{\mathrm{FL}}_2 = \sum_{i=1}^{N}\bar{n}_2A_i = \bar{n}_2(1 - A_o) \qquad \text{(A5)}$$

Pore-Mediated Mode. (i) Rapid Pore Formation. In the limit of very rapid pore formation by the aggregating peptides relative to the flip-flop,

$$\bar{\mathrm{FL}}_2(t) = \sum_{i=M}^{N} n_2(t)A_i \qquad \text{(A6)}$$

in which $n_2(t)$ is given by eq 6. By employing eq A1

$$\mathrm{FL}_2(t) = \bar{n}_2\sum_{i=M}^{N}(1 - \exp(-ikt))A_i \qquad \text{(A7)}$$

It should be recalled that the lowest rate constant that appears in eq A7 is Mk. Since $\bar{n}_2$ is assumed to be known and M can be determined from eq 9, it follows that only the parameter k has to be determined.

(ii) Slow Pore Formation. We will sum up the contribution to $n_2(t)$ as

$$n_2(t) = \sum_{i=M}^{N} n_{2i}(t)A_i \qquad \text{(A8)}$$

For $i \geq M$,

$$dn_{2i}(t)/dt = -k_{2i}n_{2i}(t) + k_{1i}n_{1i}(t)Q_{iM}(t) \qquad \text{(A9)}$$

in which $Q_{iM}(t)$ is the fraction of vesicles in which aggregates of order $\geq M$ have been formed out of the subpopulation of vesicles containing i peptides. Treatments of the problem of particle dynamical aggregation are given in Bentz and Nir (1981) and Nir *et al.* (1983). In Parente *et al.* (1990b), we treated the problem of the surface aggregation of peptides by employing the Smoluchowski (1917) solution that ignores the dissociation of aggregates and assumes equal forward rate constants for all aggregation reactions of the type

$$X_i + X_j \Rightarrow X_{i+j} \qquad \text{(A10)}$$

in which X_i denotes the concentration of aggregates of order i. We have recently (R. Peled and S. Nir, unpublished results (1991)) extended the programs described in Parente *et al.*

(1990) to allow for the reversible surface aggregation of peptides, but despite an added parameter, we could not obtain a significantly better fit to the data than that in Parente *et al.* (1990b). Hence, here we will employ the Smoluchowski (1917) equation, according to which the number of aggregates consisting of j particles is given by

$$n_j(t) = n_0\tau^{j-1}/(1+\tau)^{j+1} \tag{A11}$$

in which n_0 is the total number of primary particles and τ is a dimensionless time.

Since the total number of aggregates, n_T, is reduced with time according to $n_T(\tau) = n_0/(1+\tau)$, it follows that the fraction of aggregates of order j out of the total number of particles is $\tau^{j-1}/(1+\tau)^j$. Thus,

$$Q_{iM} = \sum_{j=M}^{\infty} \tau^{j-1}/(1+\tau)^j = (\tau/(1+\tau))^{M-1} \tag{A12}$$

However, we would use Q_{iM} directly only if the rate constant of flip-flop is assumed to be independent of j as $j \geq M$.

Here we will employ eq A1. As in Parente *et al.* (1990), τ in eq A11 is given by

$$\tau = cit \tag{A13}$$

in which t is the time, i is proportional to the surface concentration of peptides (units = 1/area), and c is the forward rate of surface aggregation and has a unit of area/time, such that τ is a dimensionless quantity. As in Parente *et al.* (1990b), i was chosen as a number, so that c has a unit of s^{-1}. The parameter c is determined independently from studies on peptide-induced leakage. If we employ eq A1, then instead of using eq A12, we have

$$k_{21}Q_{iM}(t) = \sum_{j=M}^{N} jk_{2i}(\tau)^{j-1}/(1+\tau)^j \tag{A14}$$

which, by utilizing the result in Nir *et al.* (1983), yields for large N

$$k_{2i}Q_{iM}(t) = k_{2i}(M+\tau)(\tau/(1+\tau))^{M-1} \tag{A15}$$

Thus

$$(M+\tau)(\tau/(1+\tau))^{M-1} \tag{A16}$$

When the aggregation of peptides is very slow in comparison with the flip-flop process, we can assume the approximation $\tau \ll 1$, i.e.,

$$dn_{2i}/dt = -k_{2i}(t)t^{M-1} + k_{1i}(1-n_{2i}(t))M\tau^{M-1} \tag{A17}$$

$$= -kn_{2i}M(Ci)^{(M-1)i^{M-1}} + k_{1i}M(Ci)^{(M-1)i^{M-1}} \tag{A18}$$

By defining

$$K(i) \equiv K = k(Ci)^{M-1}$$

$$K_1 = k_{1i}(Ci)^{M-1} \tag{A19}$$

$$K_2 = K - K_1$$

The solution is

$$n_{2i}(t) = (K_1/K)(1-\exp(-Kt))$$

$$= \bar{n}_2(1-\exp(-Kt^M)) \tag{A20}$$

Substitution of eq A20 into eq A8 gives

$$FL_1(t) = \sum_{i=M}^{N} \bar{n}_2(1-\exp(-Kt^M))A_i \tag{A21}$$

At short times, such that $Kt^M \ll 1$, eq A21 indicates that $n_2(t)$ initially increases according to t^M. In contrast, in the perturbation-mediated mode, $n_2(t)$ yields, initially, a linear increase with time.

Pore-Mediated and Perturbation-Mediated Modes of Flip-Flop. We will add the index p to the rate constants describing the perturbation mode. By utilizing eq A1, only two rate constants are required:

$$k_p = k_{1p} + k_{2p}$$

$$k_p\bar{n}_2 = k_{1p} \tag{A22}$$

$$K\bar{n}_2 = K_1$$

where $\bar{n}_2$ will usually equal 0.5 in the case of large vesicles.

The differential equation for $n_{2i}(t)$ is

$$dn_{2i}/dt = -ik_{2p}n_{2i} + ik_{1p}(1-n_{2i}) + [-kn_{2i}(M+\tau)(\tau/(1+\tau))^{M-1} + k_{1i}(M+\tau)(\tau/(1+\tau))^{M-1}] \tag{A23}$$

The first two terms in the equation pertain to the perturbation mode ($i \geq 1$) and use the same definitions as in eqs 6 and A1, whereas the terms within the brackets pertain to the pore-mediated mode ($i \geq M$) and use the same definitions as in eqs A9–A22.

Equation A23 can be written as

$$dn_{2i}/dt = G_i(t)(\bar{n}_2 - n_{2i}(t)) \tag{A24}$$

where

$$G_i(t) = ik_p + k(M+\tau)(\tau/(1+\tau))^{M-1} \tag{A25}$$

The general solution of eq A24 is

$$n_{2i}(t) = \bar{n}_2(1-\exp(\textstyle\int_0^t G_i(s)\,ds)) \tag{A26}$$

For $i \geq M$, we will denote the integral in eq A26 by $g_i(t)$:

$$g_i(t) = ik_pt + \frac{k}{(ci)}\left\{\tau^2/2 + \tau - m(m+1)\ln(1+\tau)/2 + \sum_{j=2}^{m}(-1)^j[1-(1+\tau)^{-j+1}]\left[m\binom{m}{m-j} - \binom{m}{m-(j+1)}\right]/(j-1)\right\} \tag{A27}$$

in which $m = M-1$.

$$\binom{m}{m-j} = m!/(j!(m-j)!) \tag{A28}$$

and the term $\binom{m}{m-(j+1)}$ is applicable for $j = 2$ to $m-1$. At

sufficiently long times, these equations reduce to the final extent expressions given in eqs 9, 13, and 14.

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