An Antimicrobial Peptide, Magainin 2, Induced Rapid Flip-Flop of Phospholipids Coupled with Pore Formation and Peptide Translocation†

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ABSTRACT: The effect of an antimicrobial peptide, magainin 2, on the flip-flop rates of phospholipids was investigated by use of fluorescent lipids, i.e., anionic *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine (NBD-PE), 1-oleoyl-2-[12-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-dodecanoyl]-L-α-phosphatidic acid (C_{12}-NBD-PA), 1-oleoyl-2-[12-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino)dodecanoyl]-L-α-phosphatidyl-L-serine (C_{12}-NBD-PS), and zwitterionic 1-palmitoyl-2-[6-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)caproyl]-L-α-phosphatidylcholine (C_6-NBD-PC). Their intrinsic flip-flop half-lives at 30 °C in the absence of the peptide were 1.1 h, ca. 7 h, ca. 8 days, and >2 days, respectively. The peptide accelerated the flip-flop half-lives of the fluorescent lipids to an order of minutes. Furthermore, the flip-flop was coupled with the membrane permeabilization and the peptide translocation [Matsuzaki, K., Murase, O., Fujii, N., & Miyajima, K. (1995) *Biochemistry 34*, 6521−6526], suggesting pore-mediated flip-flop. The flip-flop rate was independent of the initial labeling conditions (outer leaflet label or inner leaflet label). From these results, a model was proposed, in which the lipids translocate across the membrane by lateral diffusion along the wall of the pores composed of the peptides and the lipids. A simple theoretical calculation could explain the coupling of the flip-flop with the permeabilization.

The interactions of pore-forming peptides with lipid bilayers have been extensively studied for the last 2 decades, for example, to elucidate the action mechanisms of toxins (venoms) and antimicrobial peptides, to understand the functions of ion channel proteins, and to clarify the roles of signal peptides in protein translocation (Dempsey, 1990; Epand, 1993; Saberwal & Nagaraj, 1994; Sansom, 1991; Tamm, 1991). One of the recent most intriguing findings is that the peptide−lipid interactions are much more dynamic than had been thought, although Hall already suggested the possibility 15 years ago (Hall, 1981). Highly charged, relatively large (MW ca. 3000) peptides, such as a mitochondrial presequence (Maduke & Roise, 1993) and an antimicrobial peptide, magainin 2 (Matsuzaki et al., 1995a,b), can permeate through lipid bilayer membranes. Lipid molecules, on the other hand, also rapidly move across the membrane in the presence of a pore-forming synthetic peptide, GALA, and a bee venom, melittin (Fattal et al., 1994). It is important in revealing the underlying mechanisms and principles to elucidate the relationships between the pore formation, peptide translocation, and lipid flip-flop.

The pore formation can be conveniently estimated by the leakage of solutes entrapped within lipid vesicles, such as small ions (Matsuzaki et al., 1989; Venema et al., 1993) and fluorescent dyes (Matsuzaki et al., 1989; Parente et al., 1990; Weinstein et al., 1977). Methods of detecting the peptide translocation have been recently developed (Maduke & Roise, 1993; Matsuzaki et al., 1995a). However, appropriate techniques to evaluate the lipid transbilayer movement in the presence of the peptide are still to be devised. The flip-flop of membrane lipids has been widely investigated by use of endogenous phospholipids (Chandra et al., 1987; Hope & Cullis, 1987) or phospholipid analogs labeled with nitroxide radicals (Bitbol & Devaux, 1988; Kornberg & McConnell, 1971; Schrier et al., 1992; Williamson et al., 1992), fluorescent probes (Connor et al., 1992; Williamson et al., 1992), or radioisotopes (Connor et al., 1992; Anzai et al., 1993). The discrimination between the outer and inner leaflets, which is necessary for the estimation of flip-flop, is based on membrane-impermeable reagents reactive only with lipids on the reagent-added side (Chandra et al., 1987; Hope & Cullis, 1987; Kornberg & McConnell, 1971; McIntyre & Sleight, 1991) or the extraction of lipid analogs on the outer surface with bovine serum albumin (Anzai et al., 1993; Bitbol & Devaux, 1988; Connor et al., 1992; Schrier et al., 1992; Williamson et al., 1992). The former technique will not work in the presence of the membrane-permeabilizing peptides. The latter extraction method will not be suitable for detecting fast (seconds or minutes) flip-flop, because the extraction and following separation procedures take several minutes. Furthermore, this method was reported to suffer from several shortcomings (McIntyre & Sleight, 1991). Other methods to evaluate the flip-flop rate utilize the intervesicular transfer of fluorescent lipids (Fattal et al., 1994; Williamson et al., 1992), which occurs on a time scale of minutes to hours, depending on the type of the probe and the physical properties of the lipsomes. They would not give a direct measure of much faster flip-flop induced by the peptides.

In this paper, we studied the flip-flop of membrane lipids triggered by a pore-forming peptide, magainin 2, H_2N-Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Asn-Ser-COOH. The amphiphilic

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antimicrobial peptide was isolated from the skin of the African frog, *Xenopus laevis* (Zasloff, 1987). It rather specifically recognizes acidic phospholipids, such as phosphatidylglycerol and phosphatidylserine, forming an amphiphilic helix (Matsuzaki et al., 1991, 1994, 1995c). The helix first lies parallel to the membrane surface and then spontaneously forms a transient pentameric pore, followed by translocation across the bilayer (Matsuzaki et al., 1995a,b). In the first part of this paper, we will describe a new technique to measure the rapid flip-flop in the presence of the peptide by use of the reaction between dithionite and 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)[1] groups (McIntyre & Sleight, 1991) combined with the enzymatic degradation of the peptide. We found that the flip-flop is coupled with the pore formation and peptide translocation. In the second part, we will discuss the underlying machinery and propose "the lateral diffusion mechanism".

MATERIALS AND METHODS

Materials. A magainin 2 mutant (12-Trp magainin 2), in which a Phe residue at the 12th position was substituted by Trp, was synthesized by a standard fluoren-9-ylmethoxycarbonyl (Fmoc)-based solid-phase method. The crude peptide was purified by high-performance liquid chromatography and gel filtration (Sephadex G-15, 2.5 × 35 cm column, 0.02 N HCl being used as an eluent). The purity of the synthesized peptide was ascertained by quantitative amino acid analysis. The concentration of the stock solution was routinely determined on the basis of Trp absorbance at 280 nm. 12-Trp magainin 2 has been confirmed to be equipotent to the parent peptide (Matsuzaki et al., 1994). Egg yolk L-α-phosphatidylcholine (egg PC) was purchased from Sigma. L-α-Phosphatidyl-DL-glycerol enzymatically converted from egg PC (egg PG) was a kind gift of Nippon Fine Chemical Co. (Takasago, Japan). 1-Oleoyl-2-[12-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl]-L-α-phosphatidic acid sodium salt (C_{12}-NBD-PA), 1-oleoyl-2-[12-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl]-L-α-phosphatidyl-L-serine sodium salt (C_{12}-NBD-PS), and 1-palmitoyl-2-[6-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-caproyl]-L-α-phosphatidylcholine (C_6-NBD-PC) were obtained from Avanti Polar Lipids (Alabaster, AL). *N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine (NBD-PE) was a product of Molecular Probes (Eugene, OR). Calcein and spectrograde organic solvents were supplied by Dojindo (Kumamoto, Japan). All other chemicals from Wako (Tokyo, Japan) were of special grade. A Tris-HCl buffer (10 mM Tris/150 mM NaCl/1 mM EDTA, pH 7.0) was prepared from water twice distilled in a glass still.

Vesicle Preparation. Large unilamellar vesicles (LUVs), a model membrane system, were prepared by an extrusion method and characterized by dynamic light scattering as described elsewhere (Matsuzaki et al., 1994). Briefly, a lipid film, after being dried under vacuum overnight, was hydrated with the Tris buffer for the flip-flop experiments or a 70 mM calcein solution for the dye release assay (the pH was adjusted to 7.0 with NaOH) and vortex mixed. The suspension was freeze−thawed for five cycles and extruded through polycarbonate filter (a 0.6 μm pore size filter, 5 times). The vesicles were further extruded through two stacked filters (10 times) whose pore size was 0.1 or 0.2 μm (LUVs100 and LUVs200, respectively). The particle size was determined with a Photal laser particle analyzer, LPA-3100, connected to a photon correlator, LPA-3000. The weight averaged radii of LUVs100 and LUVs200 were 43−47 and 62−86 nm, respectively, depending on the hydration medium used. The lipid concentration was determined in triplicate by phosphorus analysis (Bartlett, 1959).

Inner Leaflet Labeling of Vesicles. NBD-labeled LUVs were generated from an equimolar mixture of egg PC and egg PG containing 0.5 mol % NBD-lipid (except for NBD-PE, 0.25 mol %) as described above. The symmetrically labeled vesicles were mixed with 1 M sodium dithionite/1 M Tris ([lipid] = 8 mM, [dithionite] = 60 mM) and incubated for 15 min at 30 °C. Only the NBD groups in the outer leaflets of the bilayers were chemically quenched by dithionite reduction (McIntyre & Sleight, 1991; see also Figure 1). Because of the high lipid concentration, severer reaction conditions than those in the following detection experiment were used. The vesicles were immediately separated from dithionite by gel filtration (Bio-Gel A1.5m, 1.5 × 18 cm column) at 4 °C.

Outer Leaflet Labeling of Vesicles. LUVs composed of egg PC/egg PG (1/1) were prepared as described above (4.25 mM × 3 mL). C_6-NBD-PC dissolved in ethanol (30 μL of a 1.06 mM solution, corresponding to 0.5% of the outer-monolayer lipids of the LUVs) was added to the LUVs with stirring. The vesicles were incubated for 1 h for equilibration.

Detection of Flip-Flop. The fraction of the NBD-lipids which had flipped or flopped during the incubation in the absence or presence of magainin was measured on the basis of fluorescence quenching by sodium dithionite. The asymmetrically NBD-labeled LUVs (2.0 mL) were incubated with or without magainin for various times at 30 °C. In the case of the peptide-containing samples, 20 μL of a trypsin solution (5 mg/mL) was added, and the solution was reacted for 2 min in order to hydrolyze the peptide. After 20 μL of 1 M sodium dithionite/1 M Tris had been added to 2.02 mL of the sample, NBD fluorescence was monitored on a Shimadzu RF-5000 spectrofluorometer whose cuvette holder was thermostated at 30 ± 0.5 °C. The excitation and emission wavelengths were 450 and 530 nm, respectively. Fluorescence intensity, F, was normalized to the intensity prior to dithionite addition, F_0.

Dye Release. Dye-entrapped LUVs were prepared by hydrating an egg PC/egg PG (1/1) mixture with the 70 mM calcein solution. Calcein-entrapped vesicles were separated from free calcein on a Bio-Gel A1.5m column. The release of calcein from the LUVs was fluorometrically monitored at an excitation wavelength of 490 nm and an emission wavelength of 520 nm at 30 °C. The maximum fluorescence intensity corresponding to 100% leakage was determined by

[1] Abbreviations: NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; Fmoc, fluoren-9-ylmethoxycarbonyl; egg PC, egg yolk L-α-phosphatidylcholine; egg PG, L-α-phosphatidyl-DL-glycerol enzymatically converted from egg PC; C_6-NBD-PC, 1-palmitoyl-2-[6-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)caproyl]-L-α-phosphatidylcholine; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine; C_{12}-NBD-PA, 1-oleoyl-2-[12-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl]-L-α-phosphatidic acid (sodium salt); C_{12}-NBD-PS, 1-oleoyl-2-[12-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl]-L-α-phosphatidyl-L-serine (sodium salt); LUVs, large unilamellar vesicles; LUVs100, LUVs prepared with a 0.1 μm pore size filter; LUVs200, LUVs prepared with a 0.2 μm pore size filter.

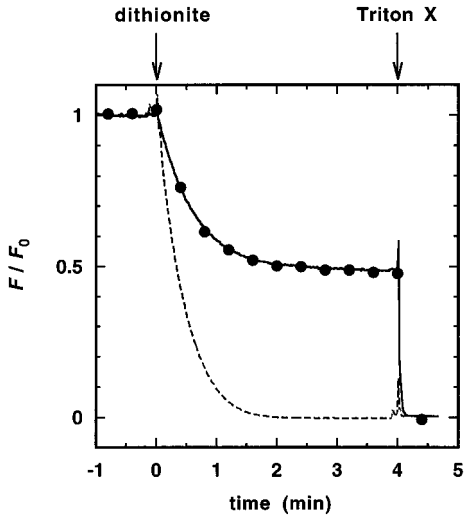

FIGURE 1: Kinetics of reduction of symmetrically distributed NBD-PE by dithionite. LUVs100 (170 μM) symmetrically labeled with 0.25 mol % NBD-PE were incubated with 12-Trp magainin 2 (3 μM) with (circles) and without (broken line) trypsin treatment (25 μg/mL, 2 min, 30 °C). Dithionite was added at time 0 (final concentration 9.8 mM). The addition of 10% Triton X (20 μL) made all NBD lipids accessible to dithionite. The upper trace (solid line) shows the data without the peptide. See text for details. The circles and the solid line are the average of duplicated samples (standard deviations are less than 0.03).

the addition of 10% w/v Triton X-100 (20 μL) to 2 mL of the sample.

RESULTS

Characterization of Asymmetrically Labeled Vesicles. The lamellarity was checked by the dithionite assay (McIntyre & Sleight, 1991). The LUVs symmetrically labeled with NBD-PE were incubated at 30 °C in a cuvette while fluorescence intensity at 530 nm was monitored. The solid line in Figure 1 shows that the addition of dithionite at time 0 selectively reduced the NBD groups exposed to the external aqueous phase. The fluorescence intensity was reduced to 50% of the initial value in 3 min, guaranteeing the unilamellarity. The half-life of the reduction was estimated to be 0.41 min by fitting the decay curve to a single-exponential function with a limiting value. The value is longer than the reported value (ca. 0.1 min) at the identical dithionite concentration (McIntyre & Sleight, 1991). This discrepancy can be attributed to the presence of the acidic phospholipid, which decreases the effective concentration of the $S_2O_4^{2-}$ ion on the membrane surface. To estimate the amount of the exposed lipids in the following experiments, we used the fluorescent intensity at 3 min, where more than 99% of the reaction occurred. Longer reaction times would suffer from errors due to the slow permeation of the reducing ion through the membrane (McIntyre & Sleight, 1991). Addition of Triton X-100 decreased the NBD fluorescence to zero in a biphasic fashion (Figure 1). In the first phase, the NBD fluorescence was decreased to half because of a smaller quantum yield in lipid−detergent micelles than in the bilayers. In the second phase, dithionite reduced the chromophore faster than in the bilayers due to a higher accessibility of the ion resulting from enhanced curvature and decreased charge density.

The asymmetrically labeled vesicles were also characterized by the dithionite reaction. The addition of the reagent

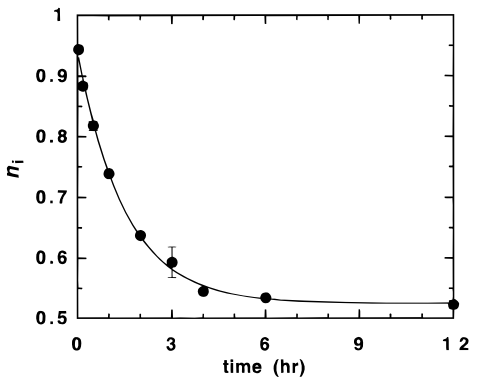

FIGURE 2: Flip-flop of NBD-PE in the absence of the peptide. Inner labeled LUVs100 were incubated at 30 °C. After a period, t, aliquots were sampled to measure the fraction of the probe remaining in the inner monolayer, $n_i(t)$, by dithionite assay. The circles show the observed values (average of duplicated samples). The solid curve is calculated by a least-squares curve fitting (see text).

to the four kinds of inner-leaflet labeled vesicles quenched less than 8% of the NBD fluorescence, indicating almost complete asymmetric labeling. In the case of the outer-leaflet labeled LUVs, it should be confirmed that C_6-NBD-PC molecules were not present (in part) as micelles but incorporated in the membranes. Aliquots of the C_6-NBD-PC ethanol solution were injected into the buffer in the absence of the LUVs. The fluorescence intensity was only 6% of the value in the presence of the vesicles, indicating a significant self-quenching in the micellar form (Chattopadhyay, 1990). The fluorescence of a certain amount of C_6-NBD-PC in the outer-leaflet labeled LUVs was 93% of that in the symmetrically labeled liposome, guaranteeing more than 90% of the fluorescent lipid was incorporated into the membrane. Furthermore, the addition of sodium dithionite quenched >90% of the NBD fluorescence.

Flip-Flop in the Absense of the Peptide. The intrinsic flip-flop rate was determined by use of the inner-leaflet labeled LUVs. The vesicles were incubated for various periods in the absence of the peptide at 30 °C. The amount of the NBD lipids which had flopped during the incubation was evaluated by the dithionite assay and plotted in Figure 2 against the incubation time. The time course was analyzed according to eq 1by use of a least-squares curve-fitting program

$$n_i(t) = k_2/(k_1 + k_2) + [n_i(0) - k_2/(k_1 + k_2)] \exp[-(k_1 + k_2)t] \quad (1)$$

(KaleidaGraph, Synergy Software, Reading, PA). The fraction of the fluorescent lipid in the inner monolayer at time t is denoted by $n_i(t)$. The first-order flop (in → out) and flip (out → in) rate constants are represented by k_1 and k_2, respectively. The obtained parameters for the acidic probe, NBD-PE, are as follows: $k_1 = 0.313$ min^{-1} and $k_2 = 0.345$ min^{-1} ($R = 0.998$). The flip-flop of C_{12}-NBD-PA and C_{12}-NBD-PS was slower. The percent flip-flop values after 24 h of incubation were 89.3% and 8.1%, suggesting half-lives of ca. 7 h and 8 days, respectively. The flip-flop rate of the zwitterionic probe, C_6-NBD-PC, was extremely small. Any measurable flop was not obtained even after 48 h of incubation.

Flip-Flop in the Presence of the Peptide. The broken line in Figure 1 indicates that, in the presence of magainin, the

outer and inner leaflets of the vesicles were indistinguishable because of the peptide-induced membrane permeabilization. The symmetrically labeled LUVs (170 μM) were preincubated with 3 μM 12-Trp magainin 2 for 10 min. Sodium dithionite was injected at time 0. The reducing ion completely quenched NBD fluorescence within 2 min. Therefore, an enzyme digestion method was adopted to inactivate the peptide. Magainin 2 contains four Lys residues at the 4th, 10th, 11th, and 14th positions. We have already shown that trypsin effectively hydrolyzes the peptide (Matsuzaki et al., 1995a). The validity of this technique was ascertained by use of the symmetrically labeled LUVs. The circles in Figure 1 clearly demonstrate that trypsin does stop the membrane permeabilization. The peptide−lipid mixture after 10 min incubation was treated with 25 μg/mL trypsin for 2 min. Sodium dithionite was added at time 0. The kinetics of the NBD reduction was superimposable on that in the absence of the peptide (solid line). These experiments were carried out under conditions corresponding to the highest peptide concentration in the following flip-flop measurements. Our previous paper (Matsuzaki et al., 1995a) reported that 20−30% of the peptide translocated into the inner leaflet under these experimental conditions. Figure 1 shows that the small translocated fraction cannot re-form a $S_2O_4^{2-}$ permeable pore because of the highly cooperative nature of the pore formation (Matsuzaki et al., 1995b).

Addition of magainin 2 to the inner-leaflet labeled LUVs resulted in a rapid flip-flop of the fluorescent lipids. The circles in Figure 3 show the time dependence of the peptide-induced flip-flop. The percent flip-flop value is defined by the equation

$$\% \text{ flip-flop} = 100 \times (n_0 - n)/(n_0 - n_\infty) \qquad (2)$$

where n_0, n, and n_∞ represent the fractions of inner-located lipids in asymmetric LUVs without the peptide, asymmetric LUVs with the peptide, and symmetric LUVs without the peptide, respectively. Considerable amounts of NBD-PE translocated from the inner to the outer leaflets on a time scale of minutes in the presence of the peptide, while the fraction of the flopped NBD-PE in the absence of the peptide was much smaller (5.6%, at 10 min). The solid line in Figure 3 illustrates the peptide-induced dye leakage from the vesicles under the identical conditions. The time course of the pore formation was very similar to that of the lipid movement.

Figure 4A depicts the dose dependences of the dye efflux and the lipid flip-flop. The percent leakage and flip-flop values after 10 min incubation were plotted as a function of the peptide-to-lipid molar ratio. The flip-flop of the four tested NBD-lipids with different polar head groups and acyl chain lengths (NBD-PE, closed circles; C_{12}-NBD-PA, open circles; C_{12}-NBD-PS, open squares; C_6-NBD-PC, closed triangles) initially labeled in the inner leaflets was again almost coupled with the pore formation (solid line). The results did not depend on the initial labeling condition. The magainin-induced lipid movement in the outer-leaflet labeled LUVs (closed squares) was identical to those in the inner-leaflet labeled vesicles (Figure 4A). Both the flip (out → in) and the flop (in → out) rates were equally accelerated by magainin while the peptide itself unidirectionally moved from the outer to the inner side of the membranes.[2]

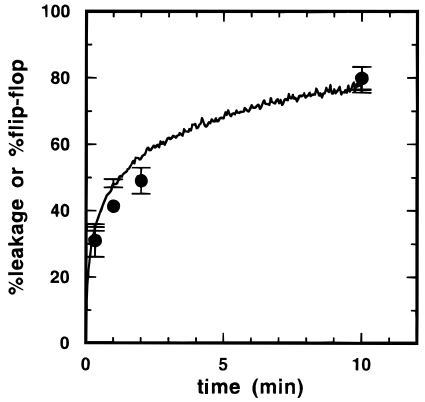

FIGURE 3: Time dependence of magainin-induced NBD-PE flip-flop. LUVs100 (180 μM) asymmetrically labeled with 0.25 mol % NBD-PE in the inner monolayers were mixed with 12-Trp magainin 2 (2 μM) and incubated at 30 °C. The fraction of NBD-PE flopped to the outer monolayer during the incubation was determined by the dithionite assay following the trypsin treatment. Percent flip-flop values were calculated according to eq 2 and plotted as a function of incubation period (circles). The time course of calcein leakage from LUVs100 under the same conditions was also measured (solid line). Data presented are the average of duplicated samples (standard deviations as absolute percentage were less than 6% for flip-flop and 3% for leakage).

The effects of vesicle size on the flip-flop and leakage were examined in Figure 4B. In the case of larger LUVs200, the lipid movement was significantly faster than the dye release. The flip rate (squares) was again identical to the flop rate (triangles).

DISCUSSION

Flip-Flop Measurements. We measured the intrinsic flip-flop rates of the four NBD-labeled phospholipids by use of the dithionite assay.[3] The half-lives of NBD-PE, C_{12}-NBD-PA, C_{12}-NBD-PS, and C_6-NBD-PC were 1.1 h, ca. 7 h, ca. 8 days, and >2 days, respectively. It is reported that the intrinsic flip-flop rates of phospholipids are highly dependent on their head groups (Homan & Pownall, 1988). The half-lives of phosphatidylcholines (∼100 h) are 1 order of magnitude longer than those of phosphatidylethanolamines (∼10 h). The fast flip-flop rate of NBD-PE can be ascribed to the hydrophobicity of its head group labeled with the NBD moiety.

We have developed a new technique to evaluate the flip-flop rate in the presence of the pore-forming peptide by use of a protease, trypsin. This method will be widely applicable if a suitable enzyme for the peptide is employed. The presence of magainin 2 induced the rapid flip-flop of the fluorescent lipids (Figure 3 and 4). We reject the possibility that peptide−NBD-lipid complexes move across the bilayer, because the directions of the peptide movement (out → in) and of the NBD lipids (in → out) are opposite to each other (Figures 3 and 4). Therefore, magainin 2 is considered to scramble all lipid molecules in a pore-mediated way. This

[2] The flux of the "back-translocation" of the peptide (in → out) is negligible under our experimental conditions (Matsuzaki et al., 1995b).

[3] We also tried to examine the flip-flop of C_6-NBD-PG, C_{12}-NBD-PG, and C_{12}-NBD-PE. However, these probes were found to be preferentially located in the outer leaflets under our conditions; addition of dithionite to symmetrically labeled vesicles in the absence of the peptide quenched about two-thirds of the NBD fluorescence. This result did not depend on the percent labeling (0.1−0.5 mol %). The reason is not clear at present.

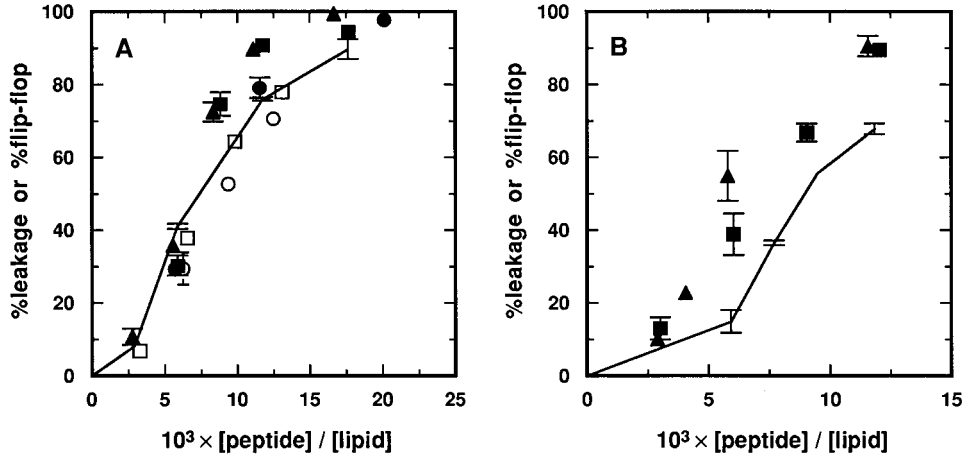

FIGURE 4: Dose−response curves of magainin-induced ion permeation and lipid flip-flop. Leakage of vesicle-entrapped calcein and flip-flop of NBD-labeled lipids during 10 min were investigated for LUVs100 (panel A) and LUVs200 (panel B). Percent leakage of calcein is plotted as solid lines. Percent flip-flop: ●, NBD-PE (in → out); ○, C_{12}-NBD-PA (in → out); □, C_{12}-NBD-PS (in → out); ▲, C_6-NBD-PC (in → out); ■, C_6-NBD-PC (out → in). Plotted data are the average of duplicated samples. Standard deviations are also presented as error bars. Peptide and lipid concentrations are as follows: (A) [peptide] = 0.5−3 μM and [lipid] = 149−180 μM; (B) [peptide] = 0.5−2 μM and [lipid] = 166−173 μM.

conclusion is supported by the results that (1) the peptide induced the randomization of both the anionic and zwitterionic probes irrespective of the initial labeling condition and (2) the time and dose dependences of the flip-flop coincided with those of the pore formation (Figures 3 and 4).

Mechanism of Pore-Mediated Flip-Flop. First of all, it should be noted that vesicular fusion could cause the leakage and the transbilayer transport of both the peptides and lipids. The absence of any fusion has been already confirmed elsewhere (Matsuzaki et al., 1995a).

Enhancement of the flip-flop rate is reported to result from the perturbation of the bilayer, such as the incorporation of guest molecules or the coexistence of solid and fluid domains around the gel to liquid-crystalline phase transition temperature (De Kruijff & Van Zoelen, 1978). The former case includes (1) membrane proteins, e.g., glycophorin, band 3, and cytochrome b_5 (De Kruijff et al., 1978; Gerritsen et al., 1980; Greenhut & Roseman, 1985), and (2) channel (pore) formers, e.g., gramicidin, amphotericin B, and bacterial cytotoxins (Classen et al., 1987; Schneider et al., 1986). In some cases, enhanced membrane permeability was also observed at the same time (Bramhall et al., 1987; Classen et al., 1987; Schneider et al., 1986; Van Hoogevest et al., 1984). However, the solute permeation is usually much faster (seconds to minutes) than the enhanced lipid movement (hours). In contrast, the magainin-mediated flip-flop proceeds at a velocity similar to the leakage rate (Figure 3). Therefore, it is unlikely that magainin 2 merely accelerates the intrinsic slow flip-flop by disturbing the membrane organization. This conclusion is further supported by the observation (Figure 4A) that the peptide induces the concerted flip-flop of the four NBD-lipids, whose intrinsic flip-flop rates are significantly different. Magainin 2 appears to provide a new pathway for the lipid transmembrane traffic.

Recently, the original work by the group of Nir and Szoka (Fattal et al., 1994) reported that pore-forming peptides composed of ca. 30 amino acid residues, i.e., GALA and melittin, trigger the rapid flip-flop of membrane lipids by use of the intervesicular transfer of a NBD-phospholipid, although the limited time resolution of this method did not allow a direct comparison between the rate of leakage and

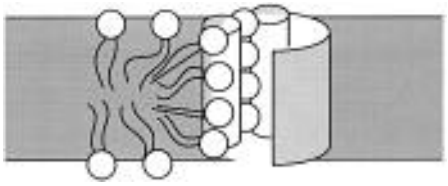

FIGURE 5: Schematic drawing of a magainin pore. The helical peptide molecules are illustrated as smaller cylinders. Spheres aligned between the helices represent the lipid head groups. Shaded and open zones correspond to hydrophobic and hydrophilic regions, respectively.

that of the flip-flop. The data were analyzed by a priori assuming the existence of the two processes, i.e., the pore-mediated one and the membrane perturbation-induced one. The former predominant mechanism assumed that the translocating phospholipids can be accommodated by a small fluctuation in the packing of the pore-forming helices, permitting both the head group and the acyl chains to be in a favorable environment. The flip-flop rate constants of an NBD-phosphatidylcholine accelerated by GALA and melittin were reported to be ≥ 1 and 1 s^{-1}, respectively. It is important to exactly evaluate the flip-flop rate and compare it with the lateral diffusion rate for discriminating between the following two cases. (1) The pore is essentially composed of the peptides. The flip-flop only occasionally occurs through the fluctuating bundle of helices [see Figure 5 of Fattal et al. (1994)]. (2) A supramolecular peptide−lipid dynamic complex constitutes the pore. The lipid molecules almost freely diffuse between the two leaflets through the perimeter of the pore (see Figures 5 and 6). As will be described below, we conclude that the latter case is more probable.

By extending the previous scheme (Fattal et al., 1994), we propose a tentative model where the pores are constructed by both the helix bundles oriented perpendicular to the membrane surface and the rows of lipids interposing the helices (Figure 5). The involvement of acidic phospholipids in the otherwise positively charged pore was proposed by Cruciani et al. (1992) to account for the cation selectivity of the magainin channel. In this situation, the inner and outer monolayers are continuous via the pore-lining lipids. Therefore, the lipid molecules can laterally diffuse between the

leaflets as in a continuum. The diffusion rate is so rapid that a few transmembrane defects would suffice to allow the flip-flop of a larger fraction of the phospholipids (Devaux, 1991).

A simple calculation supports our hypothesis. Assume one open pore (radius, r) in a vesicle (radius, R). The ratio of the diffusion rate constant of the solute through the water-filled pore, k_S, to that of the lipid along the pore wall, k_L, is expressed by the equation (see Appendix)

$$k_S/k_L = 3rD_S/4RD_L \quad (3)$$

where the diffusion coefficients of the solute and the lipid are denoted by D_S and D_L, respectively. The r value was estimated to be ~1 nm because (1) calcein (radius, ca. 0.5 nm) can pass through the pore, whereas trypsin (radius, ca. 2 nm) cannot and (2) the inner radius of a pore composed of five helices and several lipids falls within this range (eq A1). The LUVs100 radius, R, was ~50 nm, as determined by dynamic light scattering. The D_S value for calcein (MW 623) was evaluated to be 2×10^{-10} m^2/s from the value of 2.17×10^{-10} m^2/s for carboxyfluorescein (MW 376) at 23 °C (Blonk et al., 1993). The D_L value of NBD-PE in fluid bilayers around 30 °C is $(3-5) \times 10^{-12}$ m^2/s (Edidin, 1981). Substitution of these parameters into eq 3 gives the ratio (k_S/k_L) of approximately 0.6−1, which is in good agreement with the experimental results in which the rates of the permeability enhancement and the flip-flop are in the same order (Figures 3 and 4). Furthermore, the observation that an increase in R resulted in a decrease in k_S/k_L, i.e., a faster flip-flop relative to dye release, as predicted by eq 3, confirms the validity of our model (see Figure 4B). The k_S/k_L ratios of LUVs100 and LUVs200 were calculated to be 0.81 ± 0.32 and 0.44 ± 0.17, respectively, by use of eq A14. The relative decrease is in keeping with the increase in R from 43−47 to 62−86 nm.

What is the driving force of the pore formation? Magainin can take two orientations, i.e., parallel and perpendicular to the membrane surface (Ludtke et al., 1994), to stably accommodate its amphiphilic helix into the amphiphilic lipid matrix, avoiding the unfavorable exposure of the polar residues to the hydrophobic core of the bilayer. In the perpendicular orientation, i.e., the pore state, several helices should be aggregated. Therefore, an increase in peptide density favors the membrane-spanning state. At the same time, the augmented expansion and curvature modulation of the membrane tend to exclude the surface-lying peptide, facilitating the pore formation. However, the pore is a rather unstable structure, as judged from the short lifetime and the small formation constant of the pore (Matsuzaki et al., 1994, 1995b), indicating that a rapid translocation is possible upon its disintegration. The involvement of negatively charged lipids in the pore structure will electrostatically stabilize the otherwise highly positively charged pore (Cruciani et al., 1992). The appearance of the pore is transient in that it is mainly observable during the early stage of the dynamic peptide−lipid interactions (Matsuzaki et al., 1995a,b), although a trace fraction of the peptide molecules still forms the pore even in the equilibrium state where both bilayers are symmetrically expanded (Matsuzaki et al., 1995b). Therefore, the process appears to involve a quintuplet of surface-lying peptide molecules in the outer leaflet "diving into" the inner leaflet with surrounding lipids to reach an equilibrium distribution. However, the peptide−lipid interaction is not so strong that the lipid molecules in the inner leaflets can also flow into the outer monolayers.

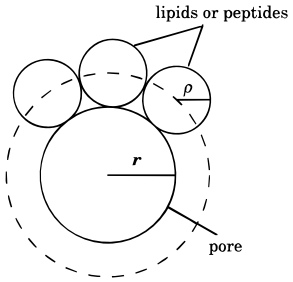

FIGURE 6: Cross-sectional illustration of the pore described in Figure 5. The pore wall is constructed from peptide helices and lipid head groups whose centers are located on the broken circle. Their cross sections are approximated to have an equal size and illustrated as smaller solid circles (radius, ρ). The void surrounded by peptides and lipids corresponding to the ion permeable part of the pore is described by a larger solid circle (radius, r).

In conclusion, we have demonstrated that magainin 2 induces the coupled transbilayer transport of ions and lipids by forming a short-lived pore during the peptide translocation. Our results suggest a new principle: the translocation of an amphiphilic peptide quantitatively (proportionally to the amount of the translocated peptide) generates two coupled signals of short duration, i.e., "an ion pulse" and "a lipid pulse", which short circuit the otherwise insulated two aqueous phases and two lipid monolayers, respectively. In other words, the amphiphilic peptide−lipid system constitutes a signal transduction system. A systematic study using other peptides would reveal that the principles presented here are universal ones. Indeed, our preliminary experiments showed that melittin and mastoparan X also translocate across the bilayer (Matsuzaki et al., 1996a,b).

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APPENDIX

Assume a pore model (Figure 6) in which n_p peptide molecules and n_l lipid molecules constitute a pore of radius r. The radii, ρ, of both the helix and the lipid head group are approximated to be 0.5 nm. Equation A1 gives the

$$r = \rho[1/\sin(\pi/(n_p + n_1)) - 1] \quad (A1)$$

relationship between r and ρ (Sansom, 1991). Because n_p was estimated to be 5 (Matsuzaki et al., 1995b) and n_l is expected to be of the same order, we can approximate

$$r/\rho \approx (n_p + n_1)/\pi - 1 \quad (A2)$$

Dye Diffusion. The dye molecule three dimensionally diffuses from the intravesicular space (volume $\cong 4\pi R^3/3$) to the external aqueous phase through a cross-sectional area of πr^2. The Fick first law can be expressed by the equation [see also Schwarz and Robert (1990, 1992)]

$$-\frac{1}{\pi r^2}\frac{dn_s}{dt} = D_s\frac{C_i - C_o}{d} \quad (3)$$

The number of the solute molecules per vesicle is denoted

by n_S. The solute concentrations in the inside and the outside are represented by C_i and C_o, respectively. The solute diffusion coefficient and bilayer thickness are expressed by D_S and d. Because $n_S \cong 4\pi R^3 C_i/3$ and C_i (mM) $\gg C_o$ (μM), eq A3 can be simplified to

$$\frac{dC_i}{dt} = -\frac{3r^2}{4R^3}\frac{D_s}{d}C_i \quad \text{(A4)}$$

The differential equation can be easily solved.

$$C_i/C_t = \exp(-k_S t) \quad \text{(A5)}$$

$$k_S = 3r^2 D_S/4R^3 d \quad \text{(A6)}$$

The initial dye concentration (70 mM) was set to be C_t.

Lipid Diffusion. The NBD-lipid two dimensionally diffuses from the inner to the outer leaflets through the perimeter of the pore, i.e., the broken line in Figure 6. The effective length of the path, l_{eff}, is described by the equation

$$l_{eff} = 2\pi(r + \rho)n_l/(n_p + n_l) \quad \text{(A7)}$$

By substituting eq A2 into eq A7

$$l_{eff} = 2\pi r n_l/(n_p + n_l - \pi) \quad \text{(A8)}$$

To the first approximation, $n_p - \pi$ (=1.86) may be neglected compared to n_l, which is expected to be close to n_p. This approximation compensates that used in eq A2. That is, the lipid molecules diffuse through the circumference of the pore ($l_{eff} \cong 2\pi r$). The Fick first equation is expressed by eq A9.

$$-\frac{1}{2\pi r}\frac{dn_i}{dt} = \frac{D_L}{4\pi R^2 d}(n_i - n_o) \quad \text{(A9)}$$

The n_i and n_o values represent the numbers of the NBD-lipids in the inner and outer monolayers, respectively. The lipid diffusion coefficient is denoted by D_L. The solution of eq A9 is eq A10. For the inner-leaflet labeling, $\Delta n = n_i$

$$\Delta n/(n_i + n_o) = \exp(-k_L t) \quad \text{(A10)}$$

$$k_L = rD_L/R^2 d \quad \text{(A11)}$$

$- n_o$, and for the outer-leaflet labeling, $\Delta n = n_o - n_i$. Equation 3 can be obtained by eqs A6 and A11.

General Case. In the above treatment, we employed the assumption that only a single pore is open per vesicle. However, in an actual situation, the number of pores is a decreasing function of time (Matsuzaki et al., 1995a,b). According to Schwarz and Robert (1992), the number of the pores, p, which had been opened from $t = 0$ to t, can be estimated by eq A12 in the case of the short-lived pore

$$-\ln\frac{C_i(t)}{C_i(0)} = k_s p\tau \quad \text{(A12)}$$

(Matsuzaki et al., 1994, 1995b). The lifetime of the pore was denoted by τ. Similarly

$$-\ln\frac{\Delta n(t)}{\Delta n(0)} = k_L p\tau \quad \text{(A13)}$$

Therefore, we can estimate the k_S/k_L ratio at time t from the experimental leakage and flip-flop curves according to the equation

$$\frac{\ln[C_i(t)/C_i(0)]}{\ln[\Delta n(t)/\Delta n(0)]} = \frac{k_S}{k_L} \quad \text{(A14)}$$

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