Role of Lipids in the Permeabilization of Membranes by Class L Amphipathic Helical Peptides†

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Received January 8, 1997; Revised Manuscript Received April 22, 1997⊗

ABSTRACT: We studied the mechanism of membrane permeabilization by the 18L model peptide (GIKKFLGSIWKFIKAFVG), which features the consensus class L sequence averaged from the number of naturally occurring lytic peptides. Two aspects of membrane lipid composition significantly affected peptide-membrane interactions: the presence of acidic lipids and, in zwitterionic membranes, and the presence of nonbilayer forming lipids. In zwitterionic membranes, 18L peptide destabilizes the membrane, leading to a transient formation of large defects in the membrane which result generally in contents leakage, but in the presence of bilayer−bilayer contact can alternatively lead to vesicle fusion. In membranes containing acidic lipids (DOPC:DOPG, DOPG), 18L caused leakage but not fusion, probably due to mutual repulsion of acidic vesicles. While the extent of contents leakage was approximately the same as for zwitterionic membranes, the kinetics of leakage could be resolved only by using stopped-flow, leakage being essentially complete within the first minute. Previously, we reported that apolipoprotein (class A) and lytic (class L) peptide analogs have opposing effects on some properties of biological membranes. This reciprocal effect of 18L and Ac-18A-NH_2, class A model peptide, is restricted to membranes with a high propensity for nonbilayer phase formation (DOPE, Me-DOPE, DOPC:DOPE, DOPC:Me-DOPE). The decrease in the content of nonbilayer phase forming lipid or the addition of acidic lipids reduces or eliminates the reciprocal effects. This suggests the importance of nonbilayer phase propensity for certain functions of biological membranes.

Many of the naturally occurring biologically active lytic peptides (insect toxins, vertebrate or mammalian peptide antibiotics) share an important structural feature. Their sequences are such that they can potentially form an amphipathic α-helix, that is an α-helix with opposing hydrophilic and hydrophobic faces oriented along the long axis of the helix (Epand, 1993). Naturally occurring amphipathic helixes have been classified into seven major types on the basis of the size and charge distribution of their hydrophilic domain (Segrest et al., 1990). Sequences of such peptides as magainins, which are antibiotics from frog skin (Zasloff, 1987; Zasloff et al., 1988), or mastoparans (Hirai et al., 1979) and bombolitins (Argiolas & Pisano, 1985), which are components of wasp and bumblebee venoms, belong to the class L helixes according to this classification. This class of amphipathic helix is characterized by a wide and bulky hydrophobic face and a narrow hydrophilic area, containing a number of cationic residues, preferentially lysines. The characteristic distribution of charged and hydrophilic/hydrophobic residues in a series of lytic peptides leads to an idea of defining the common properties inherent to class L helical peptides. Minimalistic approach was used in the design and synthesis of peptides of Lys and Ala by Cornut et al. (1994). Jones et al. (1992) suggested an algorithm for averaging a series of superimposed aminoacid sequences. From a number of naturally occurring class L sequences, the 18L peptide GIKKFLGSIWKFIKAFVG[1] (Tytler et al., 1993) has been derived using this algorithm. It was found that this peptide can decrease the temperature of the phase transition from bilayer to hexagonal phase (T_H). Thus, 18L was denoted as a bilayer destabilizer with regard to its effect on this transition (Tytler et al., 1993). The lytic activity of 18L and other synthetic analogues was shown to correlate with their effects on T_H. The importance of membrane destabilization properties for the 18L peptide lytic activity was also supported by the finding that membrane stabilizers, like class A amphipathic peptides, inhibited several activities of L class peptides (Tytler et al., 1993).

† This work was supported by the Medical Research Council of Canada, Grant MT-7654 and in part by NIH 90734343.

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⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1997.

[1] Abbreviations: 18L, GIKKFLGSIWKFIKAFVG; Ac-18A-NH_2, *N*-acetyl-DWLKAFYDKVAEKLKEAF-amide; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DOPG, dioleoylphosphatidylglycerol; Me-DOPE, *N*-methyldioleoylphosphatidylethanolamine; diPhyPC, diphytanoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; APC, 1-acyl-2-[*trans*-12-(9-anthryl)-11-dodecenoyl]-*sn*-3-glycerophosphocholine; PPC, 1-acyl-2-[9-(3-perylenoyl)nonanoyl]-*sn*-3-glycerophosphocholine; ANTS, aminonaphthalene-3,6,8-trisulfonic acid; DPX, *p*-xylenebis(pyridinium bromide); $diSC_2(5)$, 3−3′-diethylthiacarbocyanine iodide; Tb, terbium III chloride; DPA, dipicolinic acid; FITC-dextran, fluorescein isothiocyanate-dextran (MW 20 000 and 10 000); LUV, large unilamellar vesicles; MLV, multilamellar lipid vesicles; T_H, bilayer to hexagonal phase transition temperature; IMC, intrinsic monolayer curvature.

S0006-2960(97)00045-7 CCC: $14.00

This observation is referred to below as a reciprocal effect of class L and class A helixes.

Despite the data pointing to importance of membrane destabilizing properties for peptide lytic activities, the prevalent model of peptide membrane permeabilization is the formation of transmembrane α-helical bundles. This proposal of peptide oligomerization as the main mechanism of membrane permeabilization by amphipathic peptides is mostly based on conductance measurements of lipid membranes (Sansom, 1991). However, there is some controversy about such an arrangement of cationic class L peptides in the membrane. For example, mastoparans, being only 14 amino acids long, are likely to be too short to form transmembrane α-helixes. Channels formed by mastoparan were found to be weakly cation selective, which was surprising for a helix bundle with a cationic interior (Mellor & Sansom, 1990). Similar findings were observed with other peptides [for a review, see Duclohier (1994)]. In the case of magainin 2, Cruciani et al. (1992) speculate about various alternative discrete arrangements, including lipid headgroups in the channel lining. Studies of the class L peptides magainin 1 and 2 indicated that the α-helixes are oriented parallel to the surface of the membrane, even at leakage-inducing peptide/lipid ratios (Matsuzaki et al., 1994, Bechinger et al., 1993). Matsuzaki et al. (1995) put forward the idea of the action of magainin as a result of a transient pentamer pore formation in the relaxation process of the redistribution of peptide molecules between leaflets of lipid bilayer. Recently, Matsuzaki et al. (1996) proposed that lipid molecules are incorporated in the peptidic pore. Support for inclusion of lipid molecules in the pore formed by magainin also came from recent neutron scattering experiments (Ludtke et al., 1996). This data suggest the importance of the lipid component for membrane permeabilization. However, there have been relatively few systematic studies of lipid effects on the lytic activities of peptides. In this paper, we studied the mode of 18L-induced membrane permeabilization in various lipid systems and found it to be strongly lipid dependent. We found that 18L-lytic activity is modulated by the membrane nonbilayer propensity and by the presence of anionic lipids. We found 18L to simultaneously cause both vesicle contents leakage and fusion of zwitterionic membranes. The relationship between 18L-induced fusion and leakage in zwitterionic systems is also investigated in this work. An additional insight into the mechanism of 18L membrane permeabilization comes from the studies of the reciprocal effects of class L and A amphipathic helixes. The class A amphipathic α-helix, common for apolipoproteins (Segrest et al., 1990), has a relatively narrow hydrophobic area and a broad hydrophilic part with negatively charged residues in the center and sided by positively charged amino acids. As mentioned above, class A helixes were denoted as bilayer stabilizers, because contrary to class L helixes, they increase T_H. Previously, the archetype class A peptide Ac-18A-NH_2 was shown to inhibit 18L-induced erythrocyte lysis and neutrophil activation as well as contents leakage of Me-DOPE liposomes (Tytler et al., 1993). Ac-18A-NH_2, the acetylamide derivative of 18A, was found to be a more potent bilayer stabilizer than unblocked 18A (Venekatachalapathi et al., 1993). Here, we studied the apparent reciprocal effect of 18L and Ac-18A-NH_2 on vesicle contents leakage and vesicle fusion in a series of lipid systems.

MATERIALS AND METHODS

Materials. Details of the synthesis and characterization of the peptides have been described elsewhere (Tytler et al., 1993; Venkatachalapathi et al., 1993). Peptides were synthesized by the solid phase method using t-BOC chemistry. Peptides were cleaved from the resin using anhydrous HF and purified by reverse phase HPLC (Anantharamaiah, 1986). The following peptides were used in the work: 18L, GIKKFLGSIWKFIKAFVG (Tytler et al. 1993); Ac-18A-NH_2, *N*-acetyl-DWLKAFYDKVAEKLKEAF-amide [see Venkatachalapathi et al. (1993)].

DOPC, DOPE, DOPG, Me-DOPE, and diPhyPC were purchased from Avanti Polar Lipids (Alabaster, AL) and were used without further purification. 1-Acyl-2-[*trans*-12-(9-anthryl)-11-dodecenoyl]-*sn*-3-glycerophosphocholine (APC) and 1-acyl-2-[9-(3-perylenoyl)nonanoyl]-*sn*-3-glycerophosphocholine (PPC) were synthesized as previously reported (Molotkovsky et al., 1979) and were kindly provided by Dr. Jul. Molotkovsky. Aminonaphthalene-3,6,8-trisulfonic acid (ANTS), *p*-xylenebis(pyridinium bromide) (DPX), 3−3′-diethylthiacarbocyanine iodide [diSC_2(5)], terbium III chloride (Tb), and dipicolinic acid (DPA) were obtained from Molecular Probes (Junction City, OR). Fluorescein isothiocyanate-dextran (FITC-dextran), MW 20 000, 10 000, and 3000, and valinomycin were purchased from Sigma Chemical Co (St. Louis, MO). Lubrol and Triton X-100 were purchased from Calbiochem (San Diego, CA). All other reagents were of analytical grade. Buffers were prepared in double-distilled deionized water.

Liposome Preparation. Multilamellar vesicles (MLV) were made from vacuum-dried lipid films by suspending them in an appropriate buffer (20 mM Tris-HCl, 1 mM EDTA, and 0.02% NaN_3, pH 7.4, unless otherwise stated) followed by shaking and less than 20 s of low-power sonication. Large unilamellar vesicles were made by multiple extrusion of MLV through two stacked 100 nm pore polycarbonate filters (Nucleopore Corp., Pleasanton, CA). Lipid concentration of the vesicles was determined using a phosphate assay (Ames, 1966).

Fluorescent Measurements. Fluorescence experiments were done on an SLM AB-2 fluorometer (Urbana, IL). Unless otherwise stated, measurements were done in 3 mL quartz cuvettes with stirring and thermostating at 25 °C.

Leakage of Aqueous Content: ANTS/DPX Assay. The ANTS/DPX assay (Ellens et al., 1984), monitors the dequenching of ANTS released into the medium. Multilamellar vesicles (MLV) were made in buffer (20 mM Tris-HCl, 1 mM EDTA, and 0.02% NaN_3, pH 7.4) containing 12.5 mM ANTS and 45 mM DPX. The pH of the buffer was adjusted after dissolution of ANTS and DPX. LUV were prepared by extrusion as described above. Vesicles with encapsulated contents were separated from the media on a Sephadex G-75 column equilibrated with buffer containing 25 mM NaCl for osmotic strength compensation. Vesicles were used within 1−2 days; however, no spontaneous leakage was observed within at least 1 week of storage of the vesicles at 4 °C. Leakage was monitored by following the increase of fluorescence intensity at 530 nm using 360 nm excitation and band widths of 2 and 16 nm for excitation and emission, respectively. A UV band pass filter on the excitation side and a 390 nm cut-off filter on the emission side were used to diminish scattered light. Leakage was initiated by injection of 100−200 μL of vesicle suspension of the desired

lipid concentration into a cuvette containing 2 mL of a dilute peptide solution. Injection of peptide into vesicles was found to be less reproducible because of fast peptide binding to the membrane and transient high local peptide concentrations (Polozov et al., 1994b). The 100% leakage level was determined by vesicle disruption after the addition of 50 μL of 10% Lubrol. Fluorescence increase from background to 100% leakage was more than 10-fold.

Dissipation of Membrane Electrical Potential. An assay (Loew et al., 1985) for the presence of an electrical membrane potential was used in order to determine if there is any loss of potential at peptide lipid ratios below those at which ANTS leakage occurs. The typical experiment was performed as follows: LUV were prepared by extrusion of MLV made in buffer containing 100 mM KCl, 20 mM Tris-HCl, pH 7.4. Of vesicle suspension, 3 μL was diluted into 2.50 mL of isosmotic, K^+-free buffer containing 3 mM of carbocyanine dye $diSC_2(5)$ at a total lipid concentration of 20 μM. A membrane potential was established by the addition of 1 nM valinomycin. Fluorescence was monitored with excitation at 620 nm and emission at 670 nm. Addition of valinomycin results in 98% fluorescence quenching in about 3 min, which is stable for about 2 h and which can be reversed by the disruption of vesicles with detergent. Potential release was initiated by injection of a constant volume (500 μL) of dilute peptide solutions in K^+-free buffer. This mode of mixing was found important for reproducibility of the assay. The percent of fluorescence restored, at the probe and lipid concentrations used, corresponds to the percent of vesicles with completely released potential.

Leakage of Aqueous Content: FITC-Dextran Assay. FITC-Dextran, of molecular weight 3000, 10 000, and 20 000 (Sigma, MO), was encapsulated into vesicles at self-quenching concentrations: 4 mM for MW 20 000, 8 mM for MW 10 000, and 25 mM for MW 3000. Vesicles were separated from unencapsulated material by passing vesicles through a Sephacryl S-300 HR column equilibrated with buffer containing 40 mM NaCl. Internal and external media were isosmotic. Increase of fluorescence upon release of FITC-dextran into the media was used for an estimate of the size of the defects formed by peptides. In order to compare results with the potential release assay, the mode of peptide vesicle mixing was the same as that used in the potential release assay. Initial vesicle fluorescence intensity, corrected for dilution, was taken as zero leakage. Leakage of 100% was obtained by disruption of vesicles with detergent, 30 μL of 10% (w/v) Lubrol. The increase of fluorescence intensity from 0 to 100% leakage was approximately 3-fold. According to the supplier's data, the average Stokes radius of the FITC-Dextrans is 3.3, 2.3, and 1.4 nm for molecular weights 20 000, 10 000, and 3000, respectively.

Lipid Mixing Fusion Assay. The APC/PPC donor−acceptor pair was used for monitoring lipid mixing. Vesicles were labeled by APC and PPC by mixing stock solutions of lipid in chloroform. APC and PPC do not transfer spontaneously between vesicles; they do not segregate in separate phases in fluid lipid bilayers, and they do not have a charged fluorophor which can affect peptide−membrane interactions (Bergelson et al., 1985; Polozov et al., 1994a). All of the above makes this pair convenient for monitoring membrane fusion. Vesicles labeled with 2% APC and 1% PPC were mixed with unlabeled vesicles at a ratio of 1:10. Fusion was initiated by injection of a vesicle suspension into the peptide solution in the fluorescence cell, with stirring. Lipid mixing results in dequenching of APC fluorescence. Fusion was monitored by the increase of fluorescence emission at 434 nm using an excitation wavelength of 370 nm (slits 8 nm and 4 nm, respectively). To reduce scattering effects, an ultraviolet band-pass filter was used in the excitation beam and a yellow cut-off filter (390 nm) was used on emission. Complete dequenching of APC fluorescence was obtained by the addition of excess detergent (50 μL of 10% Triton X-100). Control experiments showed that disruption of APC-labeled vesicles did not affect APC fluorescence.

Aqueous Content Mixing Fusion Assays. Both the ANTS/DPX quenching assay (Ellens et al., 1985) and the Tb/DPA complex formation assay (Wilschut et al., 1980) have been used to measure membrane fusion by the mixing of aqueous contents. We employed the Tb/DPA assay to avoid artifacts caused by turbidity resulting from vesicle aggregation. LUV were prepared by the extrusion procedure. The vesicles were made either in 2.5 mM $TbCl_3$ and 50 mM sodium citrate or in 50 mM DPA (sodium salt) and 20 mM NaCl. In addition, the media contained 2 mM L-histidine and 2 mM TES adjusted to pH 7.4. Vesicles were separated from unencapsulated material by gel filtration on a Sephadex G-75 column eluted with 100 mM NaCl, and 2 mM L-histidine, 2 mM TES, pH 7.4. Fusion was initiated by injection of LUV into the fluorescent cell containing the peptide solution. The sample was excited at 276 nm, and the fluorescence emission was measured at 545 nm. Tb^{3+} itself does not fluoresce significantly. An increase in fluorescence occurs upon Tb/DPA complex formation due to intermolecular energy transfer. Vesicle aqueous contents mixing was followed as an increase in fluorescence.

Electron Microscopy. Vesicles and peptide−lipid complexes were visualized using the electron microscope. For freeze−fracture electron microscopy, 20% glycerol had been added to vesicle suspensions to ensure amorphous freezing. The lipid concentration was 15 mM. Samples (4 μL) were quenched on gold alloy planchets in a slurry of ethane cooled with liquid nitrogen. Freeze−fracture replicas were prepared by platinum shadowing in a Balzers BAF 301 apparatus equipped with electron beam guns. Replicas with traces of lipids removed were picked up on the grids and viewed in a JEOL electron microscope.

Bilayer Conductance Measurements. Planar lipid bilayers were formed across a hole in a Teflon film sandwiched between Teflon blocks containing 2 mL reservoirs, according to Mueller and Rudin (1968). Lipid bilayers were formed by painting a solution of lipid in hexadecane onto a 0.1 mm diameter hole, pretreated with hexadecane, and waiting for the barrier to thin as was determined electrically by an increase in capacitance. The lipid used for the formation of bilayers was diphytanoylphosphatidylcholine (diPhyPC), which is known for its ability to form stable BLMs (Redwood et al., 1971) free of channel-like artifacts. The buffer employed was 2 M KCl and 10 mM Tes, pH 7.4. Peptide solution in distilled water (0.1 mg/mL) was added to one side only (designated as *cis*). Currents were detected and amplified with an Axopatch amplifier and were recorded on a computer. Current records were filtered at 2 kHz.

RESULTS

Since peptide binding to the membrane is the first step in membrane permeabilization, studies of the membrane binding

behavior of 18L and Ac-18A-NH_2 have been performed (Polozov et al., 1995, 1997). From binding studies we conclude that Ac-18A-NH_2 and 18L binding to membranes is of a dynamic character and is lipid dependent. High values of binding constants (for 18L in the range 10^6-10^7 M^{-1}) make possible the choice of conditions for membrane permeabilization studies, so that the peptide is essentially all bound to the membrane, i.e., the total peptide/lipid ratio is the same as the bound peptide/lipid ratio.

18L-Induced Vesicle Contents Leakage. An insight into the mechanism of action of lytic peptide can be derived from the analysis of peptide-induced vesicle content leakage (Parente et al.,1990). The most important feature is the range of leakage-inducing peptide/lipid ratios, as well as if leakage is gradual or follows an all-or-none mechanism. Also characteristic is the dependence of leakage on vesicles size and on lipid concentration.

We studied 18L-induced leakage for vesicles of different lipid composition. As a typical zwitterionic lipid system, where one can also observe a reciprocal effect of 18L and Ac-18A-NH_2, we have chosen DOPC:DOPE 1:1 LUV for detailed studies. We found that high (∼1:20) peptide/lipid molar ratios are required to observe a fast rate and a considerable extent of 18L-induced ANTS/DPX leakage from the DOPC:DOPE 1:1 LUV (Figure 1). At lower bound peptide/lipid ratios, leakage proceeds slowly, on the scale of thousands of seconds. Leakage curves were found to be only modestly sensitive to the size dependence of the vesicles at a constant peptide/lipid ratio and lipid concentration (not shown).

To discriminate between a gradual or an all-or-none vesicle leakage mechanism, we needed to stop leakage and separate partially leaked vesicles from the leaked content. We used peptide digestion by trypsin as a method to stop leakage and to seal vesicles. Trypsin was added to vesicles at various times after initiation of leakage. Vesicles were then separated from the media by gel filtration and analyzed for the degree of ANTS quenching (Table 1). An all-or-none mechanism of leakage would result in the no change of quenching, unlike the case of the gradual leakage from all of the vesicles (Table 1). Several factors contribute to the deviation of the experimental values from Stern−Volmer theory and increase the uncertainty in the determination of quenching. However, they are insignificant for the validity of a general conclusion that the mode of 18L-induced leakage is gradual.

A peculiar dependence on total lipid concentration was found for 18L-induced leakage (Figure 1C). At constant peptide/lipid ratios, the extent and the rates of leakage at low lipid concentrations (Figure 1A) were higher than those at high lipid concentration (Figure 1B). In addition, the extent of leakage at a fixed long time point at 2000 s was found to be nonmonotonic over the peptide/lipid ratios used (Figure 1C). Such behavior can be due to interference with other membrane effects.

18L-Induced Fusion. Using the lipid mixing assay, we found that, indeed, 18L causes membrane fusion at approximately the same peptide/lipid ratios as used for contents leakage. At constant peptide/lipid ratios, the extent and the rate of fusion increased with an increase in lipid concentration. At 200 μM DOPC:DOPE, 1:1, the LUV fusion rate was comparable and somewhat faster than the rate of aqueous contents leakage (Figures 2A and 1B).

To confirm the existence of fusion, we conducted electron microscopy studies. Micrographs of DOPC:DOPE LUV

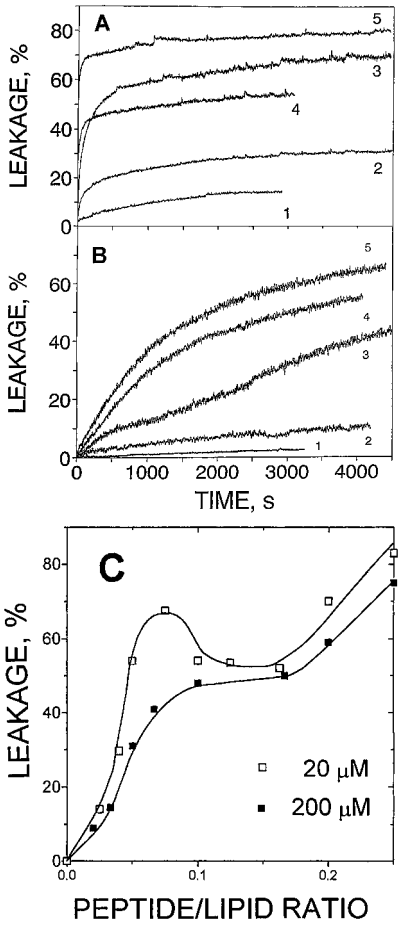

FIGURE 1: ANTS/DPX assay for 18L-induced aqueous contents leakage of DOPC:DOPE (1:1) large unilamellar vesicles for various peptide/lipid ratios. (A) Lipid concentration 20 μM. Typical leakage time traces for various peptide concentrations. Peptide/lipid molar ratios were 0.025 for the curve 1, 0.04 for the curve 2, 0.075 for the curve 3, 0.125 for the curve 4, and 0.25 for the curve 5. (B) Lipid concentration 200 μM. Typical leakage time traces for various peptide concentrations. Peptide/lipid molar ratios were 0.01 for the curve 1, 0.02 for the curve 2, 0.05 for the curve 3, 0.1 for the curve 4, and 0.2 for the curve 5. (C) Dependence of percent of 18L-induced ANTS/DPX leakage observed at 3000 s on peptide/lipid ratios. Lipid concentration 20 μM. (□), 200 μM (■). 18L is more than 95% bound under the experimental conditions. Thus, the total peptide/lipid ratio coincides with bound peptide/lipid ratio.

clearly show the fusion of vesicles in the presence of 18L (Figure 3). It is remarkable that even at very high peptide to lipid molar ratios of up to 1:3 (which is close to 1:1 on a weight basis), there is an apparent retention of a vesicular morphology. No micelles or inverted phase formation were observed. Though, due to the time scale of sample preparation, we were unable to capture intermediate structures and show only final equilibrium structures.

We also studied if 18L induces mixing of aqueous contents of fusing vesicles. The ANTS/DPX assay, based on the quenching ANTS fluorescence by DPX upon vesicle fusion, showed 5−10% fusion (not shown), despite the fact that 18L also causes vesicle leakage. To confirm that this decrease was not an artefact due to changes in light scattering upon vesicle aggregation and/or fusion, we employed the Tb/DPA aqueous contents mixing assay. Contrary to the ANTS/DPX assay, this assay is based on a fluorescence increase upon mixing of Tb^{3+} and DPA, initially encapsulated in separate vesicles. Formation of a Tb/DPA complex can occur both as a result of membrane fusion as well as a result of leakage, albeit with a much lower efficiency in the latter case. At

Table 1. Mechanism of 18L-Induced Leakage. Predicted ANTS Fluorescence Quenching in Vesicles Leaking Gradually or According to an All-Or-None Mode of Leakage and Comparison with Experiment

	degree of fluorescence quenching[a]		
percent of leakage (%)	gradual mode[b]	all-or-none mode[c]	experimental observation[d]
0	1:9.5	1:9.5	1:10−1:8
30	1:7.6	1:9.5	1:5−1:6
50	1:5.75	1:9.5	1:3−1:4
70	1:3.85	1:9.5	1:2−1:3
90	1:1.95	1:9.5	1:1.5−1:2

[a] Presented as the ratio of fluorescence of vesicle contents before (quenched state) and after solubilization of the vesicles by Triton X-100 (dequenched state). [b] Extent of quenching for the gradual mode of vesicle leakage was calculated using the Stern−Volmer equation, assuming dynamic quenching of ANTS by DPX, as determined by Smolarsky et al. (1977). [c] No change is expected in the ANTS quenching, since vesicles are either intact or leaked completely. [d] Measurements were made after termination of leakage by addition of trypsin and removal of extravesicular probes by gel filtration. Leakage was initiated by mixing 18L peptide solution with 1 mM DOPC:DOPE, 1:1 LUV to final peptide/lipid ratio 1:10. After leakage proceeded to certain extents, 100 μL aliquotes were taken and mixed with trypsin solution to final trypsin concentration of 50 μg/mL. Apparent leakage stops within 2−3 min. This partially leaked vesicles were separated from the media by gel filtration and used for determination of the extent of ANTS quenching. Increase in the uncertainty of quenching arises from the additional background fluorescence from trypsin and from sample dilution on passage through the column. Range of the observed values shown.

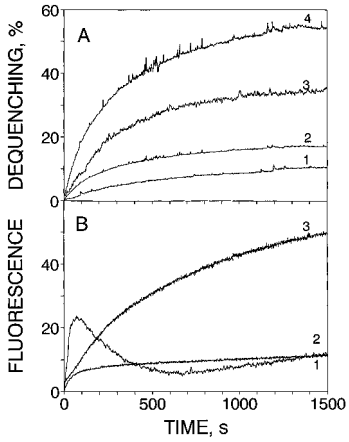

FIGURE 2: (A) DOPC:DOPE (1:1) LUV fusion induced by 18L monitored by a lipid-mixing assay, based on dequenching of anthrylvinyl fluorescence from anthrylvinylphosphatidylcholine (APC)-labeled liposomes upon fusion with unlabeled liposomes. Lipid concentration 200 μM, Peptide/lipid ratios were 0.004 for curve 1, 0.01 for curve 2, 0.04 for curve 3 and 0.09 for curve 4 (18L is more than 95% bound under the experimental conditions). (B) 18L-induced vesicles aqueous contents mixing monitored by the Tb/DPA fusion assay. Lipid concentration 200 μM. Peptide/lipid ratios: curve 1 = 0.01; curve 2 = 0.05; curve 3 = 0.075 (see text).

constant lipid concentration, the rate of fluorescence increase depended on the amount of peptide added (Figure 2B). At low ratios, a slow increase in fluorescence was observed. At ratios approximately corresponding to the plateau on the profile of the ANTS/leakage assay, we observed a fast increase in fluorescence followed by a subsequent decay. At higher ratios, the decay was followed by a slow increase. At still higher ratios, the biphasic behavior changed to a fast increase without decay. While we can not distinguish between a monotonous increase due to leakage or due to fusion, we can definitely assign biphasic behavior to fast

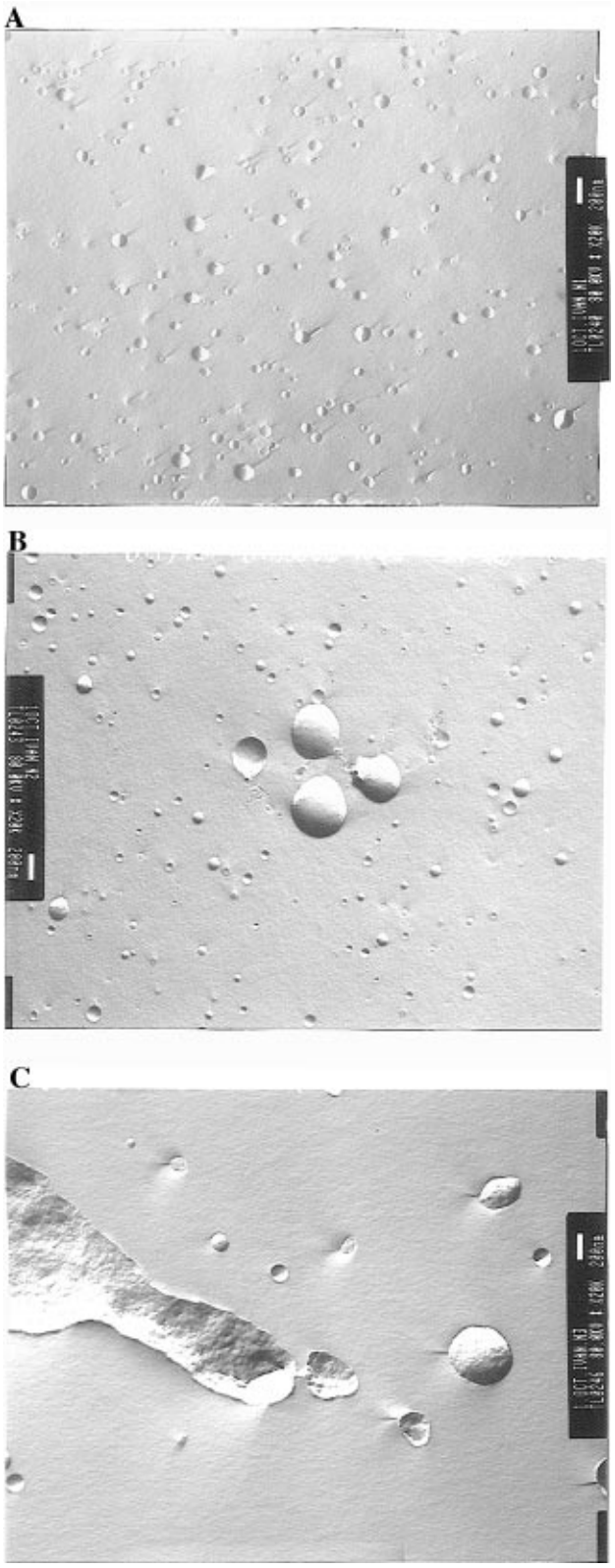

FIGURE 3: Freeze−fracture electron microscopy photographs of DOPC:DOPE (1:1) vesicles (100 nm LUV) with and without 18L addition. (A) No 18L; (B) 18L:lipid = 1:10; (C) 18L:lipid = 1:3. The scale bar corresponds to 200 nm.

fusion followed by leakage. While it is difficult to quantitate the percent of fusion in this assay, it can be estimated as being close to 5−10%, similar to that reported by the ANTS/DPX assay.

Size of 18L-Induced Membrane Defects. To estimate the size of 18L-induced membrane pores or bilayer defects, we employed two additional assays: a potential release assay

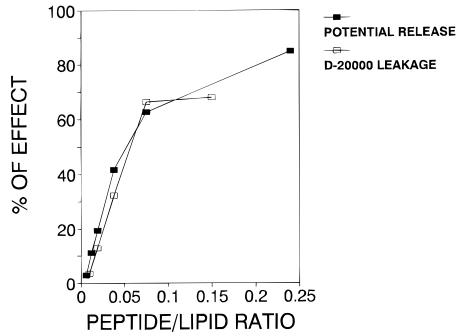

FIGURE 4: Percentage of FITC-dextran (MW, 20 000) leakage and of membrane potential release at 2000 s after 18L addition for various peptide/lipid ratios. Time traces are not shown. Lipid concentration (20 μM) and the method for peptide/lipid mixing was the same in both assays (see Materials and Methods).

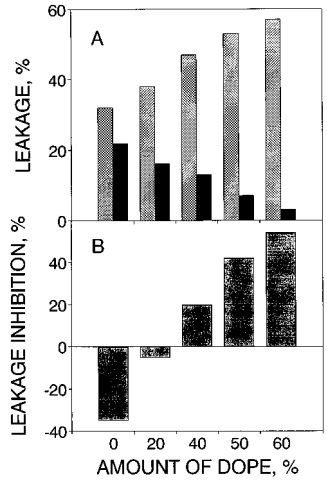

FIGURE 5: (A) Vesicle leakage induced by 18L or Ac-18A-NH_2 from LUV composed of varying DOPC:DOPE mixtures as estimated by the extent of ANTS/DPX leakage. Leakage was initiated by injection of 20 μM DOPC:DOPE LUV of varied composition into the peptide solution. Percent leakage at 2000 s was taken as a measure of leakage extent. At least three consecutive experiments had been averaged for each lipid composition. Average experimental deviation is within 3%. Hatched bars, leakage induced by 1 μM 18L peptide. Solid bars, leakage induced by 2.5 μM Ac-18A-NH_2 peptide. (B) Inhibition by Ac-18A-NH_2 of 18L-induced leakage depending on the ratio of DOPC:DOPE. Lipid concentration was 20 μM, 18L, 1 μM, Ac-18A-NH_2, 1 μM. Leakage inhibition was calculated as a percent of the decrease of leakage caused by addition of Ac-18A-NH_2 to 18L compared to 18L alone. In both cases, the extent of leakage was determined at 2000 s. At least three consecutive experiments had been averaged for each lipid composition. Average experimental deviation is within 3%.

and the leakage of fluorescent dextrans. The potential release assay was used to see if there was any increase in ion permeability at peptide/lipid ratios less than those required for ANTS/DPX leakage. This assay requires the presence of a membrane potential in the vesicles prior to the addition of peptide. To diminish mixing artifacts, peptide was injected in a large volume of buffer (0.5 mL). The extent of potential release as a function of peptide/lipid ratio did not show any significant ionophoretic activity of 18L at peptide/lipid ratios below those required for ANTS/DPX leakage (Figure 4). To estimate the upper boundary of the size of the 18L-induced defects, we studied release of fluorescently modified dextrans (FITC-dextrans) of molecular weights of 3000, 10 000, and 20 000. 18L was able to cause leakage of all of these molecules, which suggests that the size of the defects formed is sufficient for the release of at least 20 000 MW Dextrans. To ensure comparison with the potential release assay, we performed this assay at the same lipid concentrations and with the same peptide injection mode. The extent of dextran leakage (MW 20 000) as a function of peptide/lipid ratio essentially coincides with the plot for the potential release assay (Figure 4). This coincidence, taken together with the similarity of leakage plots for dextrans of various sizes, suggests that 18L does not form small membrane pores, capable of releasing ions but not dextrans.

Lipid Dependence of 18L Membrane Effects and Reciprocal Effects of 18L and Ac-18A-NH_2. We studied 18L lytic activity in several binary lipid mixtures. We also paralleled this with studies of the lytic activity of Ac-18A-NH_2 and reciprocal effects of 18L and Ac-18A-NH_2. We restricted leakage studies to lipids in the fluid state due to the previous observation that 18L as well as Ac-18A-NH_2 peptides are unable to penetrate directly into the gel phase (Polozov et al., 1995, 1997) and partially due to technical reasons, i.e., the difficulty of obtaining stable, nonleaky ANTS/DPX-loaded vesicles from lipids in the gel phase or undergoing the main phase transition.

As we mentioned above, at the peptide and lipid concentrations used, the 18L peptide is essentially all membrane bound. However, incomplete binding of Ac-18A-NH_2 must be accounted for at low (20 μM) lipid concentrations. According to our binding studies (Polozov et al., 1995, 1997), the dissociation constant for Ac-18A-NH2 and DOPC:DOPE, 1:1, is 3.2×10^{-4} M^{-1}. This corresponds to only about 40% of Ac-18A-NH_2 being actually membrane bound at these lipid concentrations. This correction for the efficiency of binding is insignificant for Ac-18A-NH_2 at 200 μM lipid concentration, when ~87% of peptide is in the membrane-bound state.

With zwitterionic lipids, DOPC:DOPE (1:0, 4:1, 3:2, 1:1, 2:3, Figure 5A) and DOPC:Me-DOPE (1:0, 2:1, 1:1, 1:2, 0:1, not shown), the 18L lytic activity increased with increasing content of nonbilayer forming lipid while the opposite trend was observed for Ac-18A-NH_2. Addition of cholesterol (DOPC:Chl, 10:0, 9:1, 4:1) did not significantly affect the lytic activity of 18L or Ac-18A-NH_2 (not shown). Both in DOPC:DOPE and DOPC:Me-DOPE binary mixtures, 18L fusogenic activity increased with the increasing content of nonbilayer forming lipid (not shown). In the case of membranes with a high content of nonbilayer forming lipid [DOPC:DOPE, 2:3, 1:1, 3:2 (Figure 5B); DOPC:Me-DOPE, 1:1, 1:2, 0:1 (not shown)], simultaneous addition of 18L and Ac-18A-NH_2 resulted in less leakage than caused by the addition of 18L alone. In pure DOPC as well as in DOPC:DOPE, 4:1 (Figure 5B), and DOPC:Me-DOPE, 2:1 (not shown), the reciprocal effect was not observed. On the contrary, the simultaneous addition of Ac-18A-NH_2 and 18L to DOPC caused leakage approximately equal to the sum of that observed with the independent addition of these peptides.

Addition of Ac-18A-NH_2 had a similar inhibitory effect regardless if it was added before, simultaneously, or after 18L addition, except that a higher extent of 18L-induced leakage was observed in the case of the delayed addition of Ac-18A-NH_2 due to the high initial leakage rate before Ac-18A-NH_2 addition. A decrease in the inhibition of 18L-induced leakage by Ac-18A-NH_2 with increasing content of DOPC was parallel with an increase in the lytic activity of Ac-18A-NH_2.

We studied how inhibition of 18L-induced leakage depended on Ac-18A-NH_2 concentration (Figure 6A). For 20

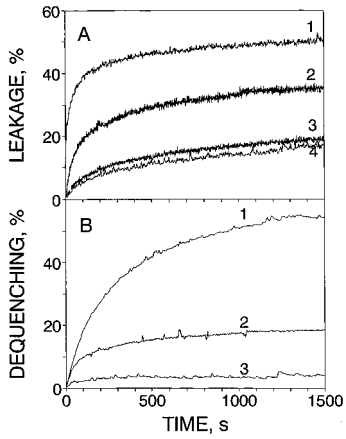

FIGURE 6: (A) Inhibition by various amounts of Ac-18A-NH_2 of 18L-induced leakage of DOPC:DOPE (1:1) LUV. Lipid concentration 20 μM. 18L concentration 1 μM. Concentration of Ac-18A-NH_2: curve 1 = 0; curve 2 = 0.5 μM; curve 3 = 1 μM; curve 4 = 8 μM. Maximum inhibition was observed at 4−8 μM of Ac-18A-NH_2. (B) DOPC:DOPE liposome fusion induced by 18L and its inhibition by Ac-18A-NH_2. Fusion was monitored by a lipid-mixing assay, based on dequenching of anthrylvinyl fluorescence from anthrylvinylphosphatidylcholine (APC)-labeled liposomes upon fusion with unlabeled liposomes. Lipid concentration 200 μM. Curve 1, 18L = 20 μM. Curve 2, 18L = 20 μM; Ac-18A-NH_2 = 5 μM. Curve 3, 18L = 20 μM, Ac-18A-NH_2 = 20 μM.

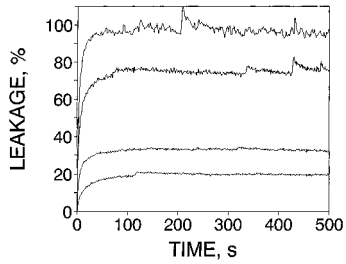

FIGURE 7: 18L-induced DOPG LUV aqueous contents leakage. Stopped-flow experiments. From top to the bottom, the peptide/lipid ratios were 0.2, 0.16, 0.08, and 0.04, respectively. Lipid concentration was 130 μM.

μM DOPC:DOPE, 1:1, and 1 μM 18L, the maximum inhibition was observed at 4−8 μM of Ac-18A-NH_2. Further increase of Ac-18A-NH_2 concentration resulted in an increase of leakage.

The reciprocal effect on peptide-induced leakage also depended on the total lipid concentration. For example, for DOPC:DOPE, 1:1, the reciprocal effect on vesicle leakage was pronounced at low (20 μM) lipid concentrations, but was not observed at high (200 μM) concentrations. Indeed, at 200 μM lipid, the simultaneous addition of Ac-18A-NH_2 and 18L increased the extent of leakage above the sum of the independent additions of each peptide. This lipid concentration dependence is additional evidence of the complex interplay between leakage and fusion effects of 18L and Ac-18A-NH_2. We found that Ac-18A-NH_2 was able to inhibit 18L-induced fusion (Figure 6B). Contrary to leakage activity, a sufficient amount of Ac-18A-NH_2 was able to inhibit fusion completely. It is possible that, at 200 μM, inhibition of 18L-induced fusion directed the peptide activity toward increased content leakage.

18L caused much faster (1−100 s) leakage from liposomes containing acidic lipids [DOPG (Figure 7), DOPC:DOPG, 1:1 (not shown)], although at peptide/lipid ratios comparable to that required for zwitterionic lipids. Resolution of initial leakage rates was possible only by stopped-flow. Leakage

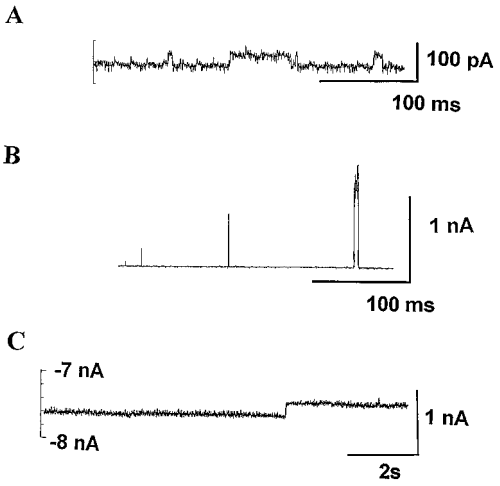

FIGURE 8: Types of bilayer conductance activities induced by 18L peptide. (A) Channel-like activity. Single conductance level of 250 pS is seen. Peptide concentration 1μM. Membrane potential 45 mV. (B) Transient spikes in the conductance with conductance levels in the range of several nS. Peptide concentration 2μM. Membrane potential 145 mV. (C) Stable leaky state with conductances about 50−200 nS. Peptide concentration 3 μM. Membrane potential 45 mV.

was not dependent on the total lipid concentration. 18L did not cause fusion of DOPG vesicles at the leakage-inducing concentrations. Ac-18A-NH_2 induced leakage of DOPG and DOPC:DOPG vesicles with kinetics very similar to that of 18L and also did not promote fusion. With acidic lipids, we did not observe a reciprocal effect between 18L and Ac-18A-NH_2. On the contrary, leakage was close to the sum of that contributed by each of the peptides individually, irrespective of lipid concentration or order of peptide addition.

Bilayer Conductance Measurements are thought to be useful for the characterization of channel forming properties of membrane active peptides. We performed experiments to directly determine the channel forming properties of 18L using black lipid membranes. Injection of peptide into the reservoir, contrary to control buffer injections, often resulted in the collapse of the membrane. We also observed several types of conductance activities induced by 18L (Figure 8). At the lowest peptide concentrations, conductance patterns similar to those of an ion channel were found in strongly polarized membranes (±145 mV). A single conductance level of about 250 pS was detected (Figure 8A). With an increase in peptide concentration, depending on the history of the sample, transient increases in bilayer conductance ranged from 0.5 to 2 nS. Lower voltages (±45 mV) were required for observation of this type of conductance. Alternatively, at the same peptide concentrations, we occasionally also observed transient spikes in conductance (Figure 8B), typical of those usually attributed to membrane defects. Further increase in peptide concentration resulted in the formation of a stable leaky state of the membrane with conductances in the range 50−200 nS (Figure 8C). Further increase in peptide concentration usually resulted in the collapse of the membrane. We also failed to detect channel forming properties of Ac-18A-NH_2 or the existence of reciprocal effects of Ac-18A-NH_2 and 18L in conductance activities.

DISCUSSION

High peptide/lipid ratios required for the lytic activity of 18L suggest that the mechanism of peptide-induced mem-

brane permeabilization involves membrane destabilization. This is supported by the fact that 18L caused leakage simultaneously with fusion. At the same peptide/lipid ratios, 18L measurably affected lipid phase behavior. The size of the 18L-induced membrane defects is sufficient for the release of 20 000 Da fluorescent dextrans, which have average Stokes radii of 3.3 nm. Since 18L-induced membrane potential release is observed at the same peptide/lipid ratios and lipid concentrations at which dextran leakage occurs, we conclude that no small channels are formed. As the mode of ANTS/DPX leakage was found to be gradual rather than all-or-none release, we can conclude that the lifetime of the defect is very short. It is insufficient for the release of the entire vesicle contents, which in our case can be estimated as ~3 ms (pore area ≈ 9 nm^2, vesicle radius ≈ 50 nm) according to Schwarz and Robert (1990, 1992). Such a short lifetime of the leaky state further substantiates the idea that leakage is due to transient membrane defects. Theoretically, it is possible that despite the high peptide/lipid ratios, peptide membrane permeabilization activity is mediated by a small percent of helical bundles formed by associated peptides (Rapaport et al., 1996; Matsuzaki et al., 1995). However, this description seems unlikely for the formation of large, short-lived pores. The distinction between the helical bundle model and membrane destabilization concept is in the pore size, the lining of the pore, it's lifetime, and also the forces or the mechanisms of the formation of the pores. Membrane destabilization suggests inclusion of lipid molecules in the pore lining. Peptide-induced increase in membrane permeability can be viewed as a result of a decreased activation energy for defect formation. This must be the result of peptide incorporation into the pore lining, reducing water exposure of the fatty acid chains. Leakage occuring through the flickering pore is likely to depend on the size of the reporter. Absence of such a pronounced dependence suggests, that the pore formation has a high activation energy, but once formed the pore rapidly grows in size and then collapses back. While there exists certain reservations about the relationship between conductance and leakage activity (Kerr et al.,1995), it is probable that these normally transient defects can be stabilized by an electric potential applied across the membrane (Figure 8C). Conductance levels observed in the planar bilayer membrane experiments correspond to the surface of the aqueous pore in the range from 0.4 (Figure 8A) to ~100 nm^2 (Figure 8C). Transient spikes in the conductance corespond to pore surfaces in the range 1−10 nm^2 (Figure 8B).

From geometric considerations and previous DSC studies (Tytler et al., 1993), Ac-18A-NH_2 and 18L affect the membrane intrinsic monolayer curvature (IMC) in opposite directions. Compensation of changes in intrinsic monolayer curvature leads to cancelling of membrane effects when both peptides are added together, i.e., to the reciprocal effect of 18L and Ac-18A-NH_2. However, the present study shows that this reciprocal effect is pronounced only for systems with high nonbilayer phase propensity. The reason for this is that effects on IMC as the main mechanism responsible for leakage occur only when the membrane already has a negative IMC. Existence of negative IMC in bilayers had been shown to result in an instability (Epand & Epand, 1994). For the unstrained bilayer, i.e., a bilayer with a very low IMC, addition of either peptide cannot easily produce a state with high IMC strain and thus other mechanisms will be prevalent.

Induction of LUV fusion by 18L was pronounced in bilayers with a propensity for nonbilayer phase formation. This can be expected as structures with overall negative curvatures are thought to be structural intermediates in the process of fusion (Markin et al., 1984; Chernomordik et al., 1985; Siegel, 1993). Observation of 18L-induced fusion in parallel with leakage suggests that both processes are alternative ways of reducing strain created by peptide insertion. At high lipid concentration, when there are more frequent bilayer−bilayer contacts, membrane defects are more likely to result in fusion rather than leakage. Support for this explanation comes from the dependence of leakage on lipid concentration (Figure 1C). At higher lipid concentrations (favorable for fusion), we saw less leakage at the same peptide to lipid ratios (in both cases the peptide was essentially totally bound).

Fusion and leakage caused by peptides is initiated by the incorporation of peptides into the outer bilayer leaflet. This insertion modulates the overall bilayer curvature stress (different from IMC) by creating excessive surface in the outer leaflet. This bilayer curvature stress has long been known to be responsible for changes in the shape of lipid vesicles or cells (Sheetz et al., 1976; Sackmann, 1994, 1995). We think it is natural to apply this concept for the understanding of peptide-induced leakage and fusion. This mass imbalance concept is related to the view of Matsuzaki et al. (1995, 1996) of peptide-induced permeabilization as a relaxation process of peptide equilibration between two bilayer leaflets. Alternatively tension can involve redistribution of both peptides and lipids between leaflets (flip-flop). Our data (Polozov, I. V., et al., manuscript in preparation) suggest that transbilayer peptide and lipid redistribution occur transiently after peptide insertion as a relaxation process to reduce bilayer curvature strain. The initially high leakage rate in the system may be caused by such transient redistribution. An input form bilayer curvature stress to peptide-induced leakage is supported by observation of only limited inhibition of 18L-induced leakage by Ac-18A-NH_2 (Figure 6A). Contrary to that, the fact that there is complete inhibition of 18L-induced fusion by Ac-18A-NH_2 (Figure 6B) suggests that bilayer curvature stress by itself is not sufficient to promote vesicle fusion.

Effects of Acidic Lipids. For such lytic peptides as magainins, lytic activity toward acidic membranes is much higher than against zwitterionic ones (Matsuzaki, 1995). Contrary to that, while mellittin binds acidic membranes with higher affinity, its lytic activity is reduced (Benachir & Lafleur, 1995). 18L binding constants for acidic membranes are about 2 orders of magnitude higher than for zwitterionic membranes (Polozov et al., 1997). However, the 18L peptide is only 2-3-fold more disruptive to vesicles of acidic lipids than to those with zwitterionic lipids (Figures 1 and Figure 7). Leakage proceeded much faster and could be resolved only by using a stopped-flow apparatus. A reason for this altered behavior may be the inability to cause membrane fusion, due to electrostatic repulsion between charged vesicles. Also, high peptide binding affinity for acidic membranes correlated with longer times of peptide residence in the membrane (Polozov et al., 1997), which might also correlate with the longer lifetime of a permeable state of the membrane. It is interesting that the Ac-18A-NH_2 peptide was also considerably more lytic in the presence of acidic

lipids and the simultaneous addition of Ac-18A-NH_2 and 18L results in an additive increase in leakage. The description in terms of curvature modulations proposed above for zwitterionic membranes neglects possible specific effects. For example, we have previously shown the preferential association of both 18L and Ac-18A-NH_2 peptides with PG in PC:PG membranes (Polozov et al., 1995, 1997). In contrast, there was no preferential association of these peptides with either PC or PE in DOPC:DOPE mixtures. Thus, a description of peptide effects in terms of curvature may be valid for the PC:PE system, but not for PC:PG.

Peptide Effects and Biological Membranes. The propensity for nonbilayer phase formation and the presence of anionic lipids were the characteristics of the lipid bilayer which modulated peptide−lipid interactions. These two aspects of membrane composition may play an important role in the regulation of lipid−protein interactions and membrane properties *in vivo*. High peptide/lipid ratios, required for permeabilization of zwitterionic membranes, make possible the application of lytic peptides in host defense systems as natural antibiotics. Strong modulation of the binding characteristics by membrane charge, coupled with the dynamic nature of the membrane partitioning, may be sufficient for targeting of peptides to acidic lipid containing membranes.

Previously, reciprocal effects of 18L and Ac-18A-NH_2 peptides in some biological activity assays have been reported (Tytler et al., 1993). In our studies of model membranes, we found that this reciprocal effect of 18L and Ac-18A-NH_2 is pronounced only in lipid systems with a propensity for nonbilayer phase formation. This finding suggests the importance of nonbilayer phase propensity for certain functions of biological membranes.

ACKNOWLEDGMENT

We would like to thank Dr. Yury Tarahovsky for the EM photographs, Dr. Julian G. Molotkovsky for providing the fluorescent lipid probes, and Dr. Shlomo Nir for fruitful discussions.

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BI970045L