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Osmotically Induced Membrane Tension Modulates Membrane Permeabilization by Class L Amphipathic Helical Peptides: Nucleation Model of Defect Formation

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ABSTRACT The mechanism of action of lytic peptides on membranes is widely studied and is important in view of potential medical applications. Previously (I. V. Polozov, A. I. Polozova, E. M. Tytler, G. M. Anantharamaiah, J. P. Segrest, G. A. Woolley, and R. M. Epan, 1997, *Biochemistry*, 36:9237–9245) we analyzed the mechanism of membrane permeabilization by 18L, the archetype lytic peptide featuring the class L amphipathic α -helix, according to the classification of Segrest et al. (J. P. Segrest, G. de Loof, J. G. Dohlman, C. G. Brouillette, and G. M. Anantharamaiah, 1990, *Proteins*, 8:103–117). We concluded that the 18L peptide destabilizes membranes, leading to a transient formation of large defects that result in contents leakage and, in the presence of bilayer-bilayer contact, could lead to vesicle fusion. Here we report that this defect formation is strongly enhanced by the membrane tension induced by osmotic swelling of vesicles. Even below standard leakage-inducing peptide/lipid ratios, membrane resistance to osmotic tension drops from hundreds to tens of milliosmoles. The actual decrease is dependent on the peptide/lipid ratio and on the type of lipid. We propose that under membrane tension a peptidic pore serves as a nucleation site for the transient formation of a lipidic pore. The tension is released upon pore expansion with inclusion of more peptides and lipids into the pore lining. This tension modulation of leakage was observed for other class L peptides (mastoparan, K18L) and thus may be of general applicability for the action of membrane active lytic peptides.

INTRODUCTION

The lipid bilayer of a cell membrane constitutes a continuous selective barrier to polar solutes. However, it has a relatively high permeability to water (Reeves and Dowben, 1969; Deamer, 1996; Huster et al., 1997). Differences in permeation rates between water and other polar solutes promote the well-known phenomena of cell swelling and shrinkage as a response to an osmotic imbalance between the interior and the exterior of the cell. Generally, cells have multiple mechanisms of dealing with osmotic stress by carefully adjusting solute concentrations to avoid osmotic lysis. Osmotic effects are also part of the more general area of lateral tension effects on the functions of biological membranes. A postulated molecular basis for phenomena such as cellular osmoregulation, blood pressure homeostasis, and fluid translocation through higher plants invoke membrane stretch receptors or sensors. Mechanosensitive ion channels have been revealed through the application of patch-clamping techniques to animal, plant, and bacterial cells (Morris, 1990), and biochemical measurements have indicated that turgor-sensitive regulatory proteins and enzymes may be present in bacterial cytoplasmic membranes

(Csonka and Hanson, 1991). The mechanism of these putative mechano-sensors is largely unknown, and more generally, it is largely unexplored how membrane tension affects the functions of membrane proteins. Recent studies with model systems suggest that membrane tension can modulate the activities of membrane-active peptides. For example, it has been shown that a change in the tension of a bilayer lipid membrane modulates the conductance of the alamethicin (Opsahl and Webb, 1994) and the gramicidin (Goulian et al., 1997) ion channels. The lytic activity of melittin was also shown to be modulated by an osmotic gradient (Benachir and Lafleur, 1996).

Effects of osmotic stress on the properties of lipid membranes have been studied in more detail, with most attention devoted to the elucidation of membrane elastic constants from osmotic swelling and osmotically induced membrane lysis (Hallett et al., 1993; Ertel et al., 1993). Recently, attention was brought to another aspect of osmotic effects on vesicle structure. It was shown that relatively small changes in osmotic strength can strongly affect the structure of the membrane-water interface, changing the hydration state of the lipid polar headgroup (Disalvo et al., 1996; White et al., 1996).

In this paper we report how modulation of membrane tension by an osmotic gradient affects the lytic activity of class L amphipathic helical peptides. An amphipathic α -helix, that is, an α -helix with opposing hydrophilic and hydrophobic faces oriented along the long axis, is an often encountered secondary structural motif in biologically active peptides and proteins (Segrest et al., 1990; Epan, 1993). Many naturally occurring lytic peptides such as

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mastoparans (Hirai et al., 1979) and bombolitins (Argiolas and Pisano, 1985), components of wasp and bumblebee venoms, are cationic and possess a highly amphipathic structure, defined as class L (lytic peptides), in the classification by Segrest et al. (1990). A family of structurally similar peptides, the magainins, have been described in the skin of frogs from several species (Zasloff, 1987). The antibiotic activity of magainins is thought to be exerted by permeabilization of the membranes of prokaryotes (Zasloff et al., 1988). However, magainins differ from many of class L peptides by having a broader angle subtended by the hydrophilic face and a different distribution of acidic amino acids (Tytler et al., 1995). All of the peptides of the class L consist entirely of an amphipathic helix. The class L helices have high hydrophobic moments and are highly positively charged. There are two clusters of positively charged amino acid residues in the polar face that is narrower ($<100^\circ$) than in most of the other classes (Segrest et al., 1990). The charged residues are mostly lysines. The classification of amphipathic helices by Segrest et al. (1990) provided a framework for model studies. Jones et al. (1992) suggested an algorithm for averaging a series of superimposed amino acid sequences. Based on this analysis, an archetypical peptide analog of a class L amphipathic helix, 18L, GIKKFLGSIWKFIKAFVG, has been designed de novo (Tytler et al., 1993). The motif of the class L sequence was also modified by designing an amphipathic helical peptide, K18L, in which the cluster of glycine residues was removed from the polar face of the amphipathic helix (Epand et al., 1993). The shape of the axial cross section of the class L helix is an inverted wedge, with the hydrophilic face forming the apex of the wedge. Membrane insertion of a class L peptide increases negative intrinsic monolayer curvature and locally destabilizes a bilayer toward formation of inverted nonbilayer phases (Tytler et al., 1993; Polozov et al., 1997a).

There is much current interest in the mechanism of membrane permeabilization by lytic peptides, and there are potential medical applications of such peptides (Maloy and Kari, 1995). In our recent studies of the mechanism of membrane permeabilization by 18L (Polozov et al., 1997a), we found that in zwitterionic membranes the 18L peptide destabilizes the membrane, leading to a transient formation of large defects in the membrane (pores) that generally result in contents leakage but in the presence of bilayer-bilayer contact can alternatively lead to vesicle fusion. These pores are likely to be dynamic structures with a transient diameter, and they include both peptide and lipid molecules. This defect formation can be accompanied by phospholipid flip-flop and peptide translocation (Matsuzaki, 1998). This description of the pore is somewhat similar to the consensus picture of osmotic lysis of a membrane, that is, that vesicles respond elastically to changes in osmotic pressure, but when the elastic limit is reached, pore formation occurs. As pores grow in size they release excess

pressure with partial release of vesicle content followed by resealing (Hallett et al., 1993; Ertel et al., 1993; Mui et al., 1993). This similarity led us to study the probable synergy between two effects, i.e., osmotic stress and peptide-induced lysis. We found that the presence of small, physiologically relevant osmotic gradients significantly increases peptide lytic activity, resulting in a selective permeabilization of tensed membranes. For the description of the effect we propose the nucleation model for defect formation. This effect was observed for other class L peptides (mastoparan, K18L), and thus the model may be of general applicability for the action of lytic peptides. Another consequence of this observation is the necessity for the control of the tension state of the membranes in a number of in vitro assays, such as vesicle aqueous content leakage or the release of the electrical potential of the membrane.

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 this work: 18L, GIKKFLGSIWKFIKAFVG (Tytler et al., 1993); K18L, KWLLKFYKLVAKLLKAF (Epand et al., 1993); mastoparan X, INWKGAAMAKKLL and Ac-18A-NH₂, N-acetyl-DWLKAFYDKVAEKLKEAF-amide (Venkatachalapathi et al., 1993). Peptides were stored as a powder at -20°C . All these peptides are soluble in water. Stock solutions of the peptides in buffer were prepared before experiments and stored at 4°C . Peptide concentration was determined by weight and controlled by tryptophan fluorescence.

DOPC, DOPE, DOPG, and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL) and were used without further purification. Aminonaphthalene-3,6,8-trisulfonic acid (ANTS) and *p*-xylenebis-(pyridinium bromide) (DPX) were purchased from Molecular Probes (Eugene, OR). Triton X-100 was purchased from Calbiochem (San Diego, CA). All other reagents were of analytical grade. Buffers were prepared in double-distilled, deionized water.

ANTS/DPX assay on leakage of vesicles aqueous content

The ANTS/DPX assay (Ellens et al., 1984; Smolarsky et al., 1977), based on the dequenching of the fluorescence of ANTS released into the medium, was used to monitor leakage of vesicles' aqueous contents. The dye and quencher approach makes possible a study of the effects of smaller osmotic gradients compared with the use of self-quenching dyes (e.g., calcein and fluorescein).

Multilamellar vesicles (MLVs) were made from vacuum-dried lipid films by suspending them in a buffer (pH 7.4, 10 mM Tris-HCl, 1 mM EDTA, 0.02% NaN₃, 12.5 mM ANTS, 45 mM DPX, with a specified concentration of NaCl) followed by shaking and less than 20 s of low-power sonication. The pH of the buffer was adjusted after dissolution of ANTS and DPX. MLVs were subjected to 10 freeze-thaw cycles before preparation of large unilamellar vesicles (LUVs) by multiple consecutive extrusion of MLVs through two stacked polycarbonate filters with pores sizes of 100 or 200 nm (Nucleopore Corp., Pleasanton, CA) using a hand-held extruder (Avestin, Ottawa, Canada). Vesicles with encapsulated

contents were separated from the media on a Sephadex G-75 column equilibrated with isotonic buffer. Lipid concentration of the vesicles was determined using a phosphate assay (Ames, 1966). Vesicles were used within 1 week, although for the compositions used in this work no spontaneous leakage was observed within at least 1 month of storage at 4°C.

Fluorescence measurements were done on an SLM AB-2 fluorometer (Urbana, IL) using 3-ml capacity, 1-cm path-length, quartz cuvettes with stirring and thermostating at 25°C, unless otherwise stated. The temperature of the sample was monitored with a thermistor probe inserted in the cuvette. Leakage was measured by following the increase of fluorescence intensity at 530 nm using 360-nm excitation and bandwidths of 2 and 16 nm for excitation and emission, respectively. A UV band pass filter on the excitation side, and a 390-nm cutoff filter on the emission side were used to diminish scattered light. Use of these filters was essential for samples having low fluorescence intensity.

Leakage was induced by adding a peptide solution to a vesicle suspension. Fast efficient mixing was necessary to avoid artifacts caused by transient high local peptide concentrations (Polozov et al., 1994). The 100% leakage level was determined by vesicle disruption after the addition of 20 μ l of 10% (v/v) Triton X-100. The increase in fluorescence intensity from background to 100% leakage was 5–20-fold, depending on the NaCl concentration. Leakage runs were repeated at least in duplicates and were found to be very reproducible within a single batch of vesicles. Graphs of leakage runs presented in this manuscript are the actual unaveraged time traces. The difference from one leakage run to another was within the size of the symbols used in the figures.

Vesicle characterization

Change in the osmotic strength of the media is expected to affect the shape of vesicles causing swelling or shrinking. Change in the internal volume of vesicles results in a change of the concentration of quencher (DPX) and thus affects the fluorescence of the encapsulated ANTNS. This sensitivity of ANTNS fluorescence intensity was used for the determination of the osmotic strength of the vesicle contents. The dependence of ANTNS fluorescence on the osmotic strength of vesicle exterior for DOPC:DOPG (1:1) and for DOPC:Chol vesicles were determined by either diluting the external media with water or by increasing the osmotic strength with high salt (2 M NaCl). Both types of titration gave similar results for the same osmotic pressure. This approach was less successful with DOPC:DOPE (1:1) vesicles, which were found to leak slowly after being subjected to osmotic swelling. Light scattering also unavoidably contributed to the signal for PC:PE LUVs, which are visibly more turbid than those of only PC, PC:Chol, or PC:PG. These experimental difficulties made correction for dilution less accurate, and thus the relative fluorescence was measured directly upon PC:PE vesicle injection into buffers of defined osmotic strength.

Membrane permeability assay: NBD-PC/DT

McIntyre and Sleight (1991) used dithionite quenching of the fluorescence of *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD)-labeled lipids to study membrane asymmetry. We modified this assay to study membrane permeability. The advantage of this assay is that membrane permeability can be assessed at any moment before or after the addition of the peptide. LUVs labeled inside only with NBD-PC (~0.5%) were prepared from symmetrically labeled LUVs (1% NBD-PC) by short-time (2 min) incubation of LUVs with 10 mM sodium dithionite (DT) followed by vesicle separation from the media by gel filtration or by 200 times dilution with subsequent overnight storage at 4°C for total dithionite deactivation. Freshly prepared solutions of dithionite were used and were stored on ice before use. The dithionite solution was adjusted with NaOH to pH 7.4. For analysis of membrane permeability, vesicles were mixed with peptide at a defined peptide/lipid ratio. After preincubation with peptide for a fixed time, 10 mM DT is added (~40 mOsm). Membrane permeability to dithionite was

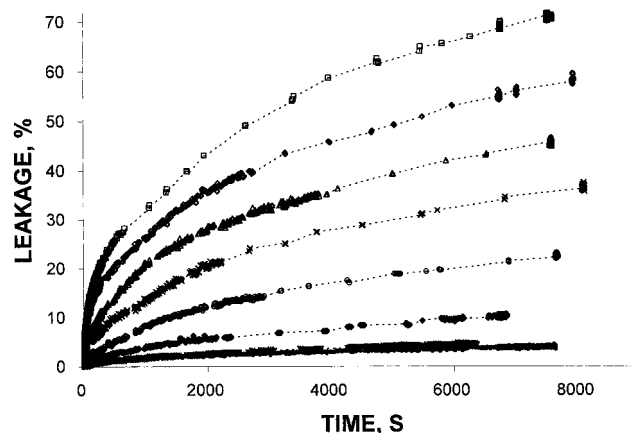


FIGURE 1 Time dependence of aqueous contents leakage of 75 μ M DOPC:DOPE (1:1) LUVs loaded with 12.5 μ M ANTNS and 45 μ M DPX and NaCl to the total osmotic strength of 370 mOsm/kg and diluted into media of varied osmolarity. From top to bottom the osmotic strength of the buffer was 20, 70, 120, 170, 220, 315, 400, and 600 mOsm. Leakage was induced by addition of 1.25 μ M 18L.

monitored by reduction of NBD fluorescence measured with excitation at 468 nm and emission at 535 nm using 4-nm monochromator slits. The percent reduction of NBD fluorescence was determined at 10 min after dithionite addition. Permeability values have been corrected for lipid (NBD-PC) translocation to the outside of the vesicle. Values for lipid translocation had been obtained by determination of the percentage of reduction of NBD-PC in vesicles that were preincubated with trypsin before DT addition. The reduction of NBD fluorescence depends on the percentage of permeable vesicles. Both ANTNS/DPX and NBD-PC/DT assays would report similarly for an all-or-none mechanism of leakage, but in the case of gradual leakage, the slow leakage of all of the vesicles would not result in complete dequenching of ANTNS. In contrast, it would still result in the complete reduction of NBD-PC.

Osmotic strength measurements

The osmotic strength of buffers was measured using a freezing point depression micro-osmometer, Advanced Micro-Osmometer 3MO Plus (Advanced Instruments, Norwood, MA). Relatively low osmotic strengths have been used in this work; thus, the osmotic strength was almost linearly dependent on solute concentrations. Empirical calibration curves have been used for conversion of concentration to osmotic strength in titration experiments.

RESULTS

Effect of osmotic gradient on 18L peptide lytic activity

We found that relatively low, physiologically relevant, osmotic gradients can affect peptide-induced vesicle leakage. Fig. 1 shows leakage induced by the addition of 1.25 μ M 18L to 75 μ M DOPC:DOPE 1:1 LUVs (ANTNS/DPX loaded) suspended in media of varying osmolarity. At this peptide/lipid ratio and this particular lipid concentration, mixing of peptide and vesicles in buffer that was isotonic to media used for vesicle preparation (370 mOsm) resulted in

slow leakage, below 5% in 2 h. The time trace for this low extent of measured leakage can be approximated as a straight line. Considering this as an initial part of the mono-exponential leakage kinetics we can estimate the lifetime of the encapsulated state as 39 h. Application of small osmotic gradients (below 80–100 mOsm) increased the leakage rate without significantly changing this mono-exponential behavior. Larger osmotic gradients further increased the percentage of leakage, and the leakage time traces were no longer mono-exponential. In addition to the change of the slope of the slow leakage, there also appears a faster leakage component with apparent saturation under 2000–3000 s. This empirically defined fast leakage component was also not mono-exponential. At higher gradients, a significant extent of leakage was observed within the first 200–300 s, followed by a marked slowdown of leakage. There was no significant immediate effect of the moderate osmotic gradients applied in the other direction, i.e., the media more concentrated than the contents of the vesicles. However, over a longer time period (on the scale of several hours), a reverse osmotic gradient also results in ANTS dequenching. It should be noted that the high inward osmotic gradient also increases the background bilayer permeability to ANTS/DPX (in the absence of peptide).

We used several controls to test that the observed modulation of peptide lytic activity is, indeed, related to osmotically induced membrane tension. From the previous studies of the membrane-binding properties of the 18L peptide (Polozov et al., 1998), we know that essentially all of the peptide is membrane bound at the experimental conditions used in this work. Thus, the difference among the leakage time traces cannot be attributed to a change in membrane partitioning of the peptide. We also found that leakage can be inhibited by a compensation of the osmotic gradient with sucrose (but not glycerol) as efficiently as with the addition of NaCl (Fig. 2 A). This is direct evidence that it is the osmotic strength, rather than the ionic strength, that modulates the peptide lytic activity. This was also supported by comparison of peptide lytic activity at constant peptide/lipid ratio in a buffer of constant ionic (and osmotic) strength but with the different osmotic strengths within the vesicle (Fig. 3), i.e., when the osmotic gradient was varied without changing the ionic strength of the media outside of the LUVs. Consistently, leakage was higher for vesicles with a higher osmotic gradient.

Osmotic equilibration of LUVs occurs in seconds. Accordingly, prolonged preincubation of vesicles under osmotic stress did not increase peptide lytic activity. Fast peptide-induced leakage from the osmotically stressed vesicles can be immediately inhibited by osmotic compensation (Fig. 2 B).

Dependence of peptide-induced leakage on the osmotic gradient was studied for several peptide/lipid ratios. Fig. 1 shows the situation when the peptide/lipid ratio is not sufficient to cause significant leakage in the absence of osmotic

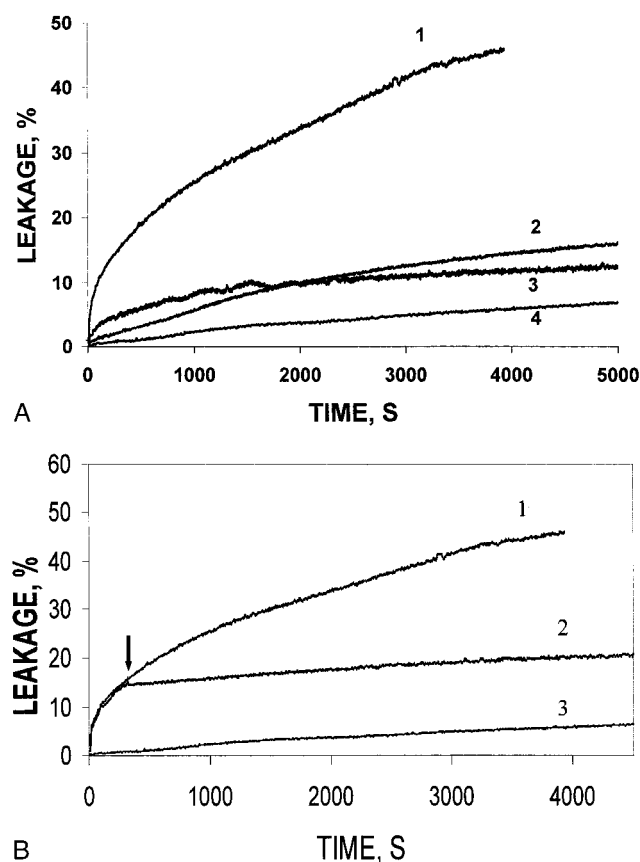


FIGURE 2 (A) Leakage inhibition by sucrose addition; 50 μ M DOPC:DOPE (1:1) LUVs loaded with ANTS/DPX/NaCl to 260 mOsm were added to peptide-containing media (1 μ M 18L) of varied osmotic strength, adjusted by NaCl (line 1, 40 mOsm; line 2, 250 mOsm; line 4, 350 mOsm) or by sucrose (line 3, 250 mOsm). (B) Osmotic compensation can immediately stop the peptide-induced leakage. Line 1, 50 μ M DOPC:DOPE (1:1) LUVs loaded with ANTS/DPX/NaCl to 260 mOsm were added to buffer (40 mOsm) containing 1 μ M 18L peptide; line 2, leakage initiated as in line 1 was immediately inhibited by addition 100 mM NaCl (at the arrow); line 3, control shows only low leakage when media osmotic strength is 350 mOsm (50 μ M DOPC:DOPE (1:1) LUVs loaded with ANTS/DPX/NaCl to 260 mOsm were added to buffer (350 mOsm) containing 1 μ M 18L peptide).

tension. Introduction of an osmotic gradient significantly increased the rate of leakage at higher peptide/lipid ratios, when there was significant leakage independent of membrane tension. However, in this case, complete leakage was eventually reached independent of membrane tension.

Previously we found that the 18L peptide can cause vesicle fusion at the same range of peptide/lipid ratios that it can cause contents leakage. In that study we used electron microscopy and dynamic light scattering as well as fluorescence assays for lipid and vesicle aqueous content mixing (Polozov et al., 1997a). In the present work, we studied whether osmotically induced membrane tension also affects peptide-induced membrane fusion. Using a lipid-mixing assay we found that osmotic swelling had no significant

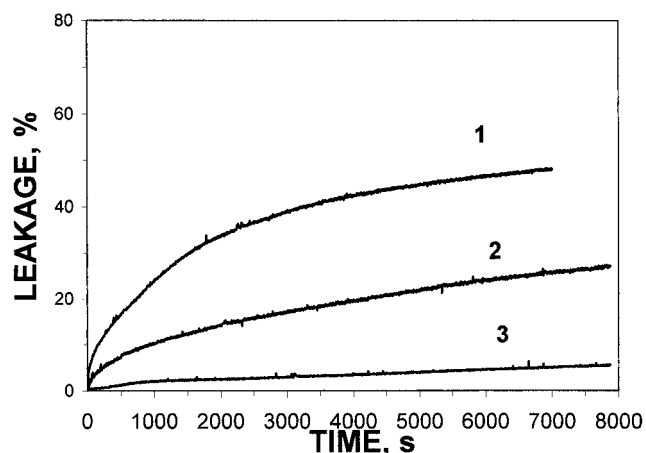


FIGURE 3 18L-induced aqueous contents leakage for vesicles with varied osmotic strength of encapsulated media. Leakage was initiated by addition of $1.25\ \mu\text{M}$ 18L to $75\ \mu\text{M}$ DOPC:Chol (30%) LUVs suspended in 150 mOsm buffer (pH 7.4, 10 mM Tris-HCl, 1 mM EDTA, 0.02% NaN_3 , titrated with NaCl). LUVs were prepared in buffer (pH 7.4, 10 mM Tris-HCl, 1 mM EDTA, 0.02% NaN_3 , 12.5 mM ANTS, 45 mM DPX) containing varied concentration of NaCl so that osmotic strength of vesicle enclosure was 400 mOsm (line 1), 280 mOsm (line 2), or 100 mOsm (line 3).

effect on membrane fusion. At peptide/lipid ratios causing less than 10% of contents leakage of nonstrained vesicles, there was no significant fusion either in the absence or in the presence of osmotic swelling of the vesicles. For the higher peptide/lipid ratios, the extent of peptide-induced lipid mixing was essentially unaffected by the relatively small osmotic gradients used for leakage studies.

Osmotic effects for other lipid and peptide systems

Modulation of peptide activity by osmotic pressure was not restricted to 200-nm DOPC:DOPE liposomes. It was also pronounced for LUVs containing cholesterol (DOPC:Chol) or acidic lipids (DOPC:DOPG). For these lipid systems, the effect was pronounced for vesicles of both 100 and 200 nm, whereas very limited effects were observed in 100-nm DOPC:DOPE vesicles, suggesting that they are almost inactive osmotically.

We tested whether osmotic modulation of peptide lytic activity is pronounced for other membrane-active peptides. We found that similar modulation takes place for other L class peptides such as mastoparan and K18L. Besides, this modulation was not restricted exclusively to class L peptides but was pronounced for other membrane-active peptides, such as the class A model peptide Ac-18A-NH₂.

Osmotic modulation of peptide lytic activity may be used for selective action against osmotically tensed membranes. Leakage time traces from osmotically strained vesicles were essentially not affected by the presence of an equimolar amount of non-strained vesicles (Fig. 4).

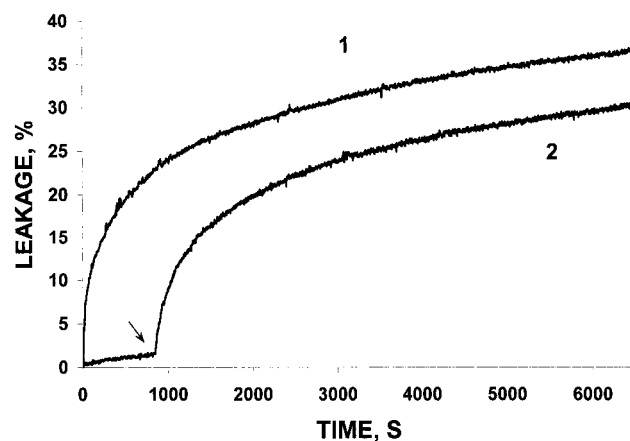


FIGURE 4 Peptide ability to cause selective leakage of osmotically strained vesicles. Line 1, leakage of osmotically strained vesicles; $75\ \mu\text{M}$ DOPC:Chol LUVs loaded to 400 mOsm (pH 7.4, 10 mM Tris-HCl, 1 mM EDTA, 0.02% NaN_3 , 12.5 mM ANTS, 45 mM DPX, NaCl adjusted) were added to 150 mOsm media (pH 7.4, 10 mM Tris-HCl, 1 mM EDTA, 0.02% NaN_3 , NaCl adjusted) containing $1.25\ \mu\text{M}$ 18L. Line 2, selective leakage of osmotically strained vesicles; $1.25\ \mu\text{M}$ 18L causes only low rate of leakage of non-strained vesicles ($75\ \mu\text{M}$ DOPC:Chol LUVs loaded to 100 mOsm), and subsequent addition (at the arrow) of osmotically strained vesicles ($75\ \mu\text{M}$ DOPC:Chol LUVs loaded to 400 mOsm) results in significant leakage.

Osmotic characterization of vesicles

Comprehensive characterization of osmotically induced vesicle swelling and leakage has been well described (e.g., Ertel et al., 1993, Mui et al., 1993, Taupin et al., 1975) and is not the focus of the present manuscript. It is known that the vesicle's elastic response to membrane tension is limited to several percent of volume increase before the membrane ruptures. By contrast, in the deflation mode, large volume changes can be accommodated without vesicle rupture by changes in vesicle shape. Thus, for flexible vesicles, membrane tension created by an osmotic gradient can be determined by the initial shape of the vesicles. There are data indicating that extruded vesicles are not spherical when prepared in a buffer (Mui et al., 1993; Clerc and Thompson, 1994, Jin et al., 1999). Thus, a vesicle may accommodate a small decrease of the external osmotic pressure, without creating any membrane tension, by changing the vesicle shape, rather than by elastic expansion. Presumably, the change in vesicle shape does not cause much stress on the membrane because of the comparatively large dimensions involved. Thus, it is desirable to distinguish vesicle swelling while maintaining a residual osmotic gradient from a complete compensation of an osmotic gradient by vesicle flexibility.

We used the residual ANTS fluorescence for characterizing the swelling of ANTS/DPX-loaded vesicles used for peptide-lipid studies. Depending on the osmotic strength of the encapsulated media, ANTS fluorescence of vesicles

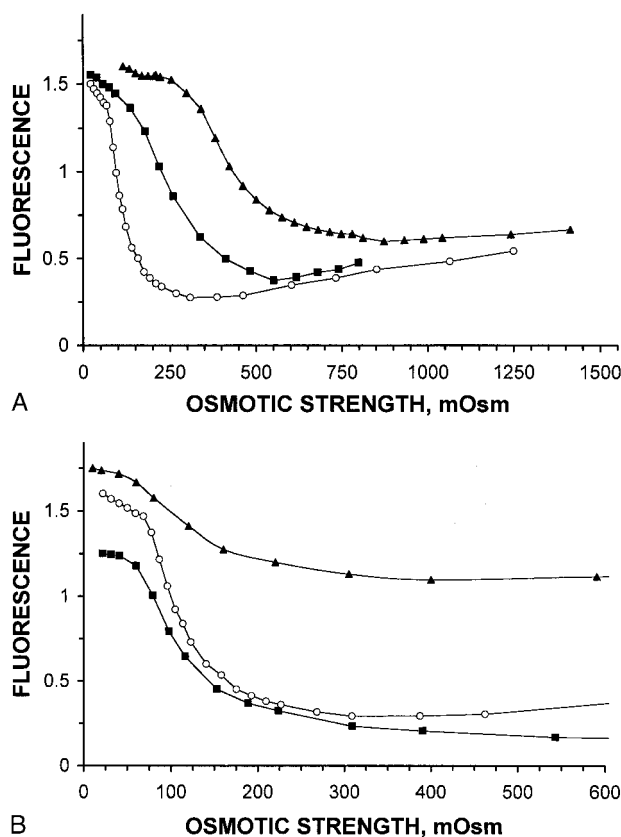


FIGURE 5 Osmotic characterization of vesicles. Dependence of intensity of fluorescence of encapsulated partially quenched ANTS on the osmotic strength of the vesicle environment. (A) DOPC:DOPG (1:1) LUV (100 nm) loaded with ANTS/DPX/NaCl at different osmotic strengths: \blacktriangle , 550 mOsm; \blacksquare , 260 mOsm; \circ , 100 mOsm. (B) LUVs from different types of lipids loaded with ANTS/DPX/NaCl to 100 mOsm: \blacktriangle , DOPC:DOPE (1:1); \blacksquare , DOPC:DOPG (1:1); \circ , DOPC:Chol (7:3).

used for leakage assays is quenched 5–20 times, which still leaves a measurable fluorescence signal. We found that the residual ANTS fluorescence is also sensitive to the osmotic strength of the outside media (Fig. 5). A change in the internal volume of the vesicles results in a change in the concentration of quencher (DPX) and thus affects the fluorescence of the encapsulated ANTS.

Osmotic gradients used in peptide lipid studies were small enough not to induce immediate osmotic lysis of vesicles in the absence of peptides. For all lipid systems used, at high inside-to-outside osmotic gradients there was a region where fluorescence was only modestly sensitive to osmotic strength. Presumably, this region corresponds to the elastic expansion of spherical vesicles. With a further increase in osmotic strength, the fluorescence intensity declines more abruptly, which may be ascribed to vesicle deflation. Eventually the fluorescence becomes insensitive to a further increase of osmotic strength until the onset of a slow increase of fluorescence at an osmotic gradient of several hundred milliosmoles. This increase can be due to a

slow leakage at high osmotic gradients as well as a direct effect of high osmotic strength on ANTS fluorescence.

Micron-sized “giant” unilamellar vesicles are very flexible, in contrast to small unilamellar vesicles that are completely osmotically insensitive (Johnson and Buttres, 1973). Extruded LUVs are somewhere between these extremes. Bending resistance to osmotic squeezing becomes noticeable when local bilayer curvature approaches that of a small unilamellar vesicle, and it will limit the attainable volume reduction. The response of PC:Chol and PC:PG vesicles was much more pronounced than that of PC:PE vesicles. This can be explained by the higher bending modulus of PE-versus PC-containing bilayers (Chen and Rand, 1997). This also correlates with our findings of the size of small unilamellar vesicles of varied lipid composition prepared by fast detergent dilution (I. V. Polozov, A. I. Polozova, and R. M. Epand, manuscript in preparation). Thus, the higher bending rigidity may explain our observation that 100-nm extruded vesicles of DOPC:DOPE were almost osmotically insensitive.

If we consider that the onset of a marked decrease of fluorescence closely corresponds to a state of non-tensed spherical vesicles, we can then conclude that after extrusion vesicles were deflated to $\sim 70\%$ of their initial volume. This correlates well with the more direct measurements of Mui et al. (1993). In the course of LUV preparation, before extrusion, we subjected vesicles to a freeze-thaw procedure. It was previously reported that this produces osmotically swollen vesicles (Chapman et al., 1991). It can be concluded that this swollen state does not survive the extrusion procedure.

Determination of residual osmotic gradients.

With knowledge of the initial osmotic state of vesicles, it is possible to calculate the osmotic gradient at different points of the leakage time trace. It is reasonable to assume that in the initial period there is only outflow of vesicle contents. The residual osmotic gradient $\Delta P(t)$ would then change as:

$$\Delta P(t) = P_{in,o} \times (1 - L(t) - P_{out})$$

$$\text{when } L \leq 1 - P_{out}/P_{in,o} \quad (1)$$

$$\Delta P(t) = 0 \quad \text{when } L \leq 1 - P_{out}/P_{in,o},$$

where $P_{in,o}$ is the initial osmotic strength of the contents of rounded vesicles, P_{out} is the osmotic strength of the outside media, and $L(t)$ is the extent of leakage at the moment t . Fig. 6 shows the time dependence of the residual osmotic gradient for several initial osmotic gradients, as calculated from the data of Fig. 1. The cases with high initial gradient asymptotically coincide at 100 ± 20 mOsm at longer times, whereas the case with an initial gradient below this critical value decrease only slightly over the same period of time. Apparently this asymptotic value corresponds to the critical

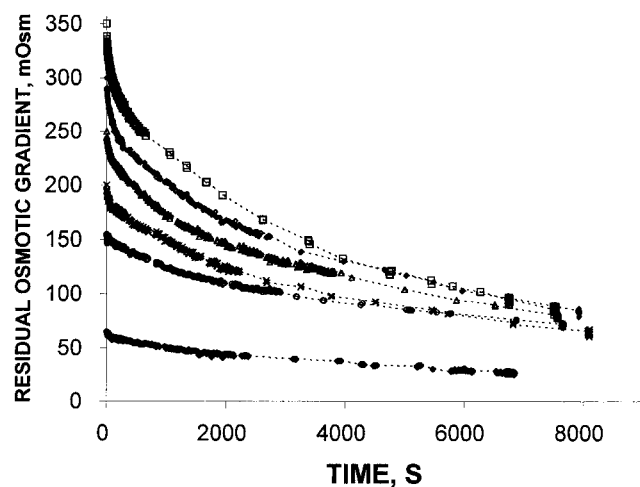


FIGURE 6 Calculations from data shown in Fig. 1, showing the time dependencies of changes of residual osmotic gradients for various initial osmotic gradients. From top to bottom, the initial applied osmotic differential was 350, 300, 250, 200, 155, and 65 mOsm.

membrane tension (σ_c) that vesicles can hold without rapid leakage. According to Laplace's law, for a thin spherical vesicle, membrane tension (σ) depends on osmotic pressure ΔP as $\sigma = \Delta P \times R/2$, where R is the radius of vesicle.

The critical tension estimated from Fig. 6 is only as good as the validity of the description of leakage as a unidirectional outflow of vesicle contents. Although this description is plausible, one cannot exclude the possibility of preferential leakage of either dye or quencher, especially in the mode of slow leakage. Both of these effects will result in an overestimate of the actual critical pressure. Another possibility is that leakage is accompanied with media exchange, rather than outflow only. Although this is unlikely because of hydrodynamic considerations, it may contribute to an underestimate of membrane stability.

Using the approach illustrated in Fig. 6, we determined critical residual gradients for several different peptide/lipid ratios and for DOPC:DOPE 1:1 and DOPC:Chol 7:3 LUVs (Fig. 7). Critical membrane tension decreases with an increase of peptide/lipid ratio. A significant loss of membrane stability occurs at relatively low peptide/lipid ratios that do not cause significant leakage from non-strained vesicles. At higher peptide/lipid ratios the residual osmotic gradients fall to below 10 mOsmol. At these ratios the osmotic effects are pronounced, resulting in the acceleration of leakage kinetics with the application of membrane tension.

Osmotic compression and membrane permeabilization

The ANTS/DPX assay is not sensitive for the detection of inflow into the vesicle under conditions of osmotic compression. To address this question we used an assay based

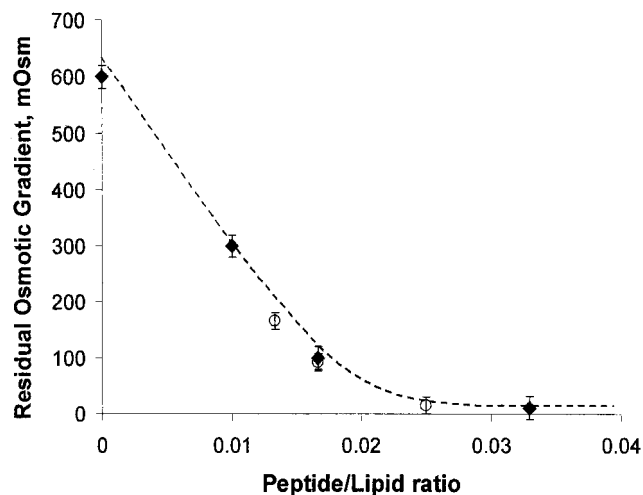


FIGURE 7 Following approach illustrated in Fig. 6 we estimated critical membrane tensions for different peptide/lipid ratios for LUVs of DOPC:DOPE 1:1 (\blacklozenge) and DOPC:Chol 7:3 (\circ). Dashed line illustrates the loss of vesicles resistance to osmotic swelling in the presence 18L peptide.

on dithionite reduction of NBD fluorescence. LUVs were labeled inside only with NBD-PC and mixed with the 18L peptide at a specific peptide/lipid ratio. After preincubation with the peptide for a fixed time, sodium dithionite is added. Dithionite penetration into the vesicles results in a reduction of NBD-fluorescence. Results were corrected for the lipid translocation to the outer leaflet. Typical results are shown in Fig. 8. We see that there also exist characteristic peptide/lipid ratios when essentially all the NBD-fluorescence is quenched. This value also depended on the size of the applied osmotic gradient (not shown).

An additional advantage of this assay is the possibility to measure membrane permeability at any time after peptide-

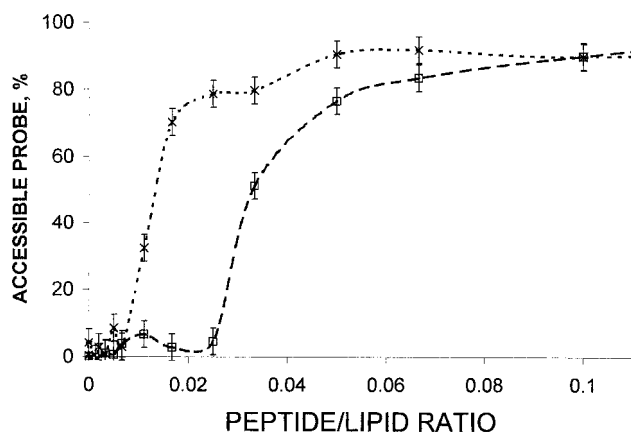


FIGURE 8 Percentage of vesicles permeable to sodium dithionite after prolonged incubation of DOPC:DOPE (1:1) LUVs with 18L at various peptide/lipid ratios. \square , incubation for 2 days (50 h); \times , incubation for 2 h. Lipid concentration was 20 μ M; osmotic strength was 20 mOsm inside LUVs, 60 mOsm outside.

LUV mixing. This is contrary to the ANTS/DPX leakage, which reports on membrane permeability immediately after peptide-LUV mixing. Once the vesicle contents leak out, one cannot easily judge the permeability of the membrane. With the NBD-PC/DT assay we see that there occurs some deactivation of the peptide membrane permeabilizing activity of the peptide with time, especially at low peptide/lipid ratios. However, with each osmotic gradient, even after very prolonged peptide/lipid preincubation, there were always some peptide/lipid ratios that resulted in complete fluorescence quenching.

The osmotic gradient is one reason the NBD-PC/DT assay reports higher permeability values than the ANTS/DPX assay (Polozov et al., 1997a). In addition, the extent of reduction of NBD-PC fluorescence intensity depends on the fraction of vesicles permeable to dithionite, whereas for the ANTS/DPX assay, leakage corresponds to the percentage of leaked DPX. Both the ANTS/DPX and the NBD-PC/DT assays would report similarly for the all-or-none mechanism of leakage, but the NBD-PC/DT assay is expected to give higher values in the case of gradual leakage.

DISCUSSION

Nucleation mechanism of pore formation

Leakage from bilayer vesicles can result from the spontaneous formation of short-lived membrane pores. To a first approximation (Taupin et al., 1975; Lister, 1975; Sung and Park, 1997), the energy (E) of pore formation in a tensed membrane depends on the balance between a reduction of membrane tension (proportional to the surface of the pore) and the hydrophobic exposure of lipids that results in the appearance of line tension on the edges of the pore:

$$E = 2\pi\gamma r - \pi\sigma r^2,$$

where r is the radius of the pore, γ is the line tension of the pore, and σ is the membrane tension. Two types of leakage may occur. The first type, occurring at lower tensions ($dE/dr > 0$) corresponds to alternating opening and closing of smaller pores. The size of a molecule passing through the membrane is important in this case, because if it is too large no leakage will occur. For the second type of leakage ($dE/dr < 0$) occurring at high tensions, the pores are unstable and their radii grow spontaneously. This type of leakage is insensitive to the size of the label. The pore expansion, accompanied by the partial release of vesicle contents, leads to a reduction of the membrane tension σ and subsequent pore closure by the line tension. The boundary condition between these modes: $dE/dr = 2\pi\gamma - 2\pi\sigma \times r = 0$ means that for a given membrane tension σ there exists a critical radius of the pore $r_c = \gamma/\sigma$ such that below r_c pores reseal spontaneously, whereas for pore radii above r_c the pore spontaneously grows. Conversely, the existence of a critical membrane tension σ_c that the membrane cannot maintain

without leakage is an indication of the existence in the membrane of spontaneously forming defects of the size of r_c . Such a dependence $E(r; \sigma)$ means that in the tensed membrane a pore can be nucleated by creating a sufficiently large membrane defect (of the size of r_c).

Creation of membrane defects by electroporation of giant vesicles under controlled tension made possible a study of the kinetics of pore formation (Zhelev and Needham, 1993; Sandre et al., 1999; Brochard-Wyart et al., 2000). It has been shown that the rate of pore closure and the extent of leakage out of the vesicle depend on the size of vesicle and on the viscosity of the encapsulated contents as well as bulk viscosity of the membrane. If the outward leakage of the internal contents is entirely suppressed, membrane tension will be released by reaching some maximum diameter of the pore. This can be simulated by using a viscous ambient liquid (Sandre et al., 1999). Such experiments made it possible to separate contributions to the tension release by contents leakage from that due to pore expansion. It can be concluded that with regard to 100–200-nm osmotically strained unilamellar vesicles, tension release, pore size, and closure rate are dominated by contents leakage. Because the elastic extension of the bilayer is limited to several percent of the initial surface, for large osmotic gradients it may take several cycles of vesicle swelling and membrane pore formation to reach the final residual osmotic gradient. Alternatively, to release the osmotic gradient within one cycle of pore formation and closure, the rate of leakage must be slower than the rate of vesicle osmotic swelling, which is limited by the rate of water permeability across the lipid bilayer. The average time of residence of a water molecule inside a 100-nm vesicle is in the range of milliseconds (Huster et al., 1997). Leakage induced by osmotic shock of vesicles reaches a maximum in the range of seconds. This is consistent with short lifetime of a pore and the overall leakage rate being limited by the necessity of multiple cycles of nucleation followed by pore opening and closing.

An estimate of the size of defects leading to pore nucleation can be obtained from the known lipid-specific values of γ and of the critical tension σ . Experiments with stable pores made by electroporation in giant vesicles (Zhelev and Needham, 1993) give values of γ for SOPC of 0.92×10^{-6} dyn and for SOPC:Chol. (1:1) of 3.05×10^{-6} dyn or 2.6×10^{-6} dyn as recalculated by Moroz and Nelson (1997). We found that 200-nm LUVs of DOPC:DOPE 1:1 can tolerate osmotic gradients ΔP up to 600 mOsm, which corresponds to $\sigma = \Delta P \times R/2 = 149$ dyn cm $^{-1}$. This correlates with findings of Mui et al. (1993) that 100-nm LUVs of egg PC:Chol can tolerate osmotic gradients up to 680 mOsm, which, with the correction for initial nonspherical shape of the vesicle, corresponded to $\sigma = 40$ dyn cm $^{-1}$. Therefore, the size of r_c for the pure lipid systems can be estimated to be in the range $r_c \approx 0.07$ – 0.75 nm (from $1 \times 10^{-6}/149$ to $3 \times 10^{-6}/40$ cm). Although the continuum approximation can serve only as a rough guide for the description of such

molecular size defects, it can be concluded that lipid vesicles do not leak until membrane tension is so high that a defect of the size of less than a lipid molecule is formed, resulting in pore nucleation.

From our data we determined that peptide insertion decreases the critical tension, σ_c , sustained by the membrane. This decrease may be achieved either by increasing the size of intrinsic membrane defects (r_c) or by lowering membrane line tension (γ). Both aspects may be important for pore nucleation. By their nature, peptide molecules and transient peptide aggregates, when inserted into a membrane, can be viewed as irregularities or defects in the structure of the bilayer that potentially can serve as sites for osmotically induced pore nucleation, followed by pore expansion. Also, it is likely that repulsion of cationic peptides and a decrease in the water exposure of the membrane hydrophobic interior will result in lowering the line tension (γ) of the transient peptide/lipid pore, compared with the pure lipid membrane pore. Considering the low concentration of peptides in the membrane, upon expansion the pore will change its properties from those of a peptidic to those of a lipidic pore. If there exists some specific stoichiometrically defined peptide/lipid complex, one should expect a stepwise dependence of the critical tension on the bound peptide/lipid ratio. The approximately linear character of this dependence (Fig. 7) suggests that such a specific arrangement is unlikely and both line tension and defect size may be modulated by peptide insertion.

From the small osmotic gradient necessary for the observation of tension effects, it may appear that it takes a small number of particles to equilibrate the vesicle interior and exterior. This, however, is misleading. In the absence of an electromotive force there is no selective movement of ions across the pore, and membrane tension is released via the outflow of the total content of the vesicle. After tension release and pore resealing, membrane tension is restored by the influx of water through the membrane. The process is repeated until the membrane tension is insufficient for the pore nucleation. The number of pore nucleation cycles therefore depends not on the absolute value of the gradient but on the ratio of the initial and final osmotic strength of vesicle interior. For example, a 300 mOsm gradient resulting from the dilution of 370 mOsm into 70 mOsm buffer results in vesicles with an interior osmotic strength of $\sim 70 + 110 = 180$ mOsm. This requires an estimated $370/180 = 2.06$ times dilution of the contents. Considering a loss of 5% of vesicle content with each pore formation, complete equilibration should take $\sim \ln(1/2.06)/\ln(1 - 0.05) \approx 14$ pore nucleations (total leak-out volume $14 \times 0.05 = 0.7$ of vesicle volume).

Fluorescence assays of vesicle contents leakage can be used to study a very wide range of membrane permeability rates. The lifetime of the encapsulated state can be measured in the range from a fraction of a second to several days. This is an advantage over such assays as a fluorescence assay of

membrane potential release (Loew et al., 1985; Polozov et al., 1997a), in which the effect must be faster than the spontaneous dissipation of membrane potential, which occurs within an hour. In the absence of osmotic stress, there was no detectable leakage, at least within 1 week. On a logarithmic kinetic time scale, subjecting a vesicle to even a modest osmotic gradient significantly enhances its permeability. Ultimately, this is related to the liposome being in a meta-stable, non-equilibrium state. However, in the first approximation, osmotically induced leakage of vesicles reaches a steady state in seconds (under 1 min). If this is the time necessary to get the required size defects in the pure lipid membrane, it is not surprising that peptide-facilitated, tension-induced leakage takes longer, as apparently this requires a transient association of more than one peptide molecule and thus involves additional restriction via lateral diffusion.

Membrane stability and peptide-membrane permeabilization

Two major modes have been discussed regarding membrane permeabilization by L class amphipathic peptides: pore formation of helical clusters and generally defined membrane destabilization (Epand et al., 1995). The concept of peptide oligomerization resulting in pore formation is useful to explain membrane permeabilization observed at extremely low bound peptide/lipid ratios, in the range of several peptide molecules per vesicle (Parente et al., 1990) or single channels in planar bilayer lipid membranes (Sansom, 1991). Contrary to that, membrane destabilization can refer to a case in which a peptide induces membrane permeabilization in the same range of peptide/lipid ratios as it affects the lipid phase behavior. The difference between these cases can also be viewed as the difference in the affinity of self-association of membrane-bound peptides. Permeabilization induced by membrane destabilization ultimately will also occur through the aqueous pores across the membrane. However, in this later case the structure of the pore is less defined and is likely to include lipids as well as peptides. Generally, the mechanism of pore formation is complicated and is influenced by membrane properties such as intrinsic monolayer curvature, membrane lipid composition, charge, potential, and membrane tension.

As regards the membrane permeabilization by class L lytic peptides, the model of helical bundle formation was put forward previously, based mostly on conductance measurements of lipid membranes (Sansom, 1991; Mellor and Sansom, 1990; Duclohier, 1994). However, inclusion of both peptides and lipids in the structure of the pore have been proposed for a number of reasons, such as to explain the anomalous ion selectivity of magainin 2 channels (Cruciani et al., 1992), to explain acceleration of lipid flip-flop by lytic peptides (Matsuzaki et al., 1996), or to explain the size of the water-filled channel as detected with neutron-

scattering experiments (Ludtke et al., 1996). Our recent studies (Polozov et al., 1997a) of the mechanism of membrane permeabilization by 18L peptide concluded that the pores are likely to be transient structures with varying diameter and inclusion of lipid molecules. Leakage occurring through the flickering pore is likely to depend on the size of the reporter. The absence of such a pronounced dependence suggests that pore formation has a high activation energy, but once formed the pore rapidly grows in size and then collapses back. Formation of such spontaneous pores can be viewed as an increase in the size of fluctuations (defects) in the membrane induced by the high peptide/lipid ratios. At high peptide/lipid ratios, mass imbalance results from peptide insertion only into the outer monolayer of the vesicles. Previously (Polozov et al., 1997b) we have used fluorescence resonance energy transfer as an approach to estimate the increase of the membrane surface upon insertion of the 18L peptide. In DOPC:DOPG 1:1 membranes, the inserted peptide had a surface area of 1 nm² or 1.4 times the surface of one lipid molecule. A relatively small increase in membrane surface upon peptide insertion correlates with the observation of a decreased polarity of the bilayer interior and an increase in the density of the membrane (Polozov et al., 1997b).

Our study of the dependency of peptide lytic activity on membrane tension provides another perspective on the process of membrane permeabilization. From the point of membrane stability, the size of spontaneous fluctuations is the limiting factor in the mechanical stability against applied membrane tension. Thus the mechanism of peptide-membrane pore formation can be reformulated as helping to form a lipidic pore. Low membrane permeability is achieved by the high activation energy of lipidic pore formation (and thus the rare occurrence of such events). Peptide insertion decreases the activation energy of pore formation both by increasing the scale of fluctuations (a peptide aggregate serving as the nucleation site for a lipidic pore) and by lowering the line tension of the lipidic pore. Longo et al. (1998) have shown that melittin decreased the lysis tension of SOPC giant vesicles. The peptide can be viewed as a line-active compound, its incorporation into the pore lining will reduce water exposure of hydrophobic acyl chains. In addition, charge-charge repulsion of cationic peptides will favor subsequent pore expansion. This nucleation model may be generally applicable to the action of amphipathic α -helical peptides and other membrane-active agents.

We have seen that the osmotic effects for several class L peptides (18L, K18L, and mastoparan) and for the class A amphipathic peptide, Ac-18A-NH₂, which models the characteristic structure of helical segments of exchangeable apolipoproteins, can be accounted for by the proposed mechanism. This is also in accordance with the finding by Mui et al. (1994) that apolipoproteins can increase the osmotic sensitivity of LUVs. However, this described mode of action alone is insufficient to explain the observed sensitivity

to osmotic stress of some other types of leakage, such as the ion-specific conductance activity of gramicidin A (Goulian et al., 1997) or of the polyene macrolide antibiotic amphotericin B (Wolf and Hartsel, 1995; Hartsel et al., 1998).

In vivo membrane tension can have a variety of origins, including osmotic swelling and hydrodynamic shear stress. The tensed state of a bacterial membrane resulting from turgor maintenance is likely to increase peptide lytic activity and thus may contribute to the specificity of action of peptide antibiotics. On the other hand, the enhancement of lytic activity by lowering membrane tension is likely to also lead to their increased nephrotoxicity.

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