# Capacities of Pardaxin Analogues To Induce Fusion and Leakage of Negatively Charged Phospholipid Vesicles Are Not Necessarily Correlated†

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ABSTRACT: Peptide-induced vesicle fusion is frequently accompanied by leakage of vesicle contents. To determine the correlation between these two processes, we studied the interaction of the amphiphilic peptide pardaxin and two of its analogues with large unilamellar vesicles composed of phosphatidylserine. A pardaxin analogue with a positive charge at both its C- and N-termini induced significantly more fusion but less leakage than the parent peptide. Fusion and leakage were studied with large unilamellar vesicles of two sizes. Aggregation of vesicles was found to be the rate-limiting step in the overall fusion process induced by the peptides. The rates and extents of fusion, determined by membrane mixing, increase in vesicle size, and mixing of aqueous contents, were significantly enhanced in the presence of 2.5–5 mM $Mg^{2+}$ which promoted vesicle aggregation. Model calculations showed that increasing the peptide to lipid ratio resulted in a parallel increase in the fusion rate constants. As the average vesicle diameter was increased, the extent of leakage was enhanced, as more peptide molecules were bound to each vesicle. The mode of leakage induced by the peptides was also investigated. Our results suggest that the potency of a peptide to induce vesicle fusion is not necessarily associated with its capacity to induce leakage, and we further elucidate how these capacities depend on the structures of the peptides.

Membrane fusion is an essential process in both intracellular events and cell–cell interactions. It is also a fundamental step during infection of mammalian cells by enveloped viruses. The fusion process is viewed as consisting of several stages: aggregation and close apposition of the particles, destabilization, and finally, merging of the bilayers (Nir et al., 1980). Although little is known about the molecular mechanisms underlying fusion of biological membranes, it is generally assumed that proteins are involved. The proteins generate the perturbations required to induce local changes in otherwise stable membrane bilayers. Among the few well-characterized fusion proteins to date are viral spike glycoproteins responsible for penetration of enveloped viruses into their host cells (Wiley & Skehel, 1987; Stegmann et al., 1989). A key feature of most viral fusion proteins is a "fusion peptide", a stretch of relatively hydrophobic amino acids, which is believed to destabilize the target membrane and to trigger the fusion process (White, 1990). Thus, many studies on peptide-induced vesicle fusion provide information not only on the possible mode of action of these segments of viral envelope proteins but also on the minimal molecular requirements for protein-mediated membrane fusion (Düzgünes, 1993). Moreover, several studies have demonstrated that amino acid substitution within fusion peptides modifies their fusion-inducing activity to be similar to that of the corresponding site-directed mutants of the viral envelope proteins (Wharton et al., 1988; Rafalski et al., 1991; Rapaport & Shai, 1994).

Studies have employed synthetic peptides that correspond to the putative fusion peptide regions of fusogenic viral proteins (Lear & DeGrado, 1987; Murata et al., 1987a; Rafalski et al., 1990, 1991; Düzgünes & Shavnin, 1992; Yeagle et al., 1991; Martin et al., 1991; Crane et al., 1991; Glushakova et al., 1992; Nieva et al., 1994; Rapaport & Shai, 1994) and model peptides designed *de novo* (Parente et al., 1988; Murata et al., 1991, 1992; Yoshimura et al., 1992). Naturally occurring amphiphilic peptides provide another good model for protein-mediated membrane fusion. Several fusogenic peptides, such as an albumin fragment (Garcia et al., 1984), melittin (Morgan et al., 1984; Murata et al., 1987b), and gramicidin S (Eytan et al., 1988; Tournois et al., 1990) have been identified.

The fusogenicity of peptides is frequently correlated with their ability to induce leakage from vesicles. These properties show similar dependency on (i) the pH of the medium (Subbarao et al., 1987; Kono et al., 1990, 1993; Murata et al., 1992) and (ii) the lipid composition of the target liposomes (Martin et al., 1993). Studies on families of peptides have shown that within each family the relative ability of a peptide to induce fusion correlates with its ability to induce leakage (Wharton et al., 1988; Suenaga et al., 1989; Rafalski et al., 1991; Lee et al., 1992; Fujii et al., 1992). The purpose of the present study was to test whether this parallel behavior should be taken as a general rule.

Our model system is the neurotoxic peptide pardaxin, an amphiphilic 33-residue peptide secreted from certain species of soles within the genus Pardachirus (Primor & Zlotkin, 1975). Pardaxin is postulated to be part of a defence secretion since it has shark-repelling properties and is toxic to various organisms (Primor et al., 1978). It has various physiological and pharmacological effects [reviewed in Shai (1994)], such as it interfers with ion transport in epithelium (Primor, 1983) and nerve cells (Renner et al., 1987). Furthermore, it was found that pardaxin induces morphological changes of synaptic vesicles to membrane structures with large cisternae inside

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(Arribas et al., 1993). These biological activities of pardaxin are probably due to its surface-active properties, as it was shown that it interacts with biological and artificial lipid membranes (Moran et al., 1984; Lazarovici et al., 1986; Shai et al., 1988). These properties of pardaxin validate it as an ideal model in which to study various aspects of peptide–membrane interactions such as pore formation and fusion induction.

Several of its analogues, have been studied extensively, with respect to their interaction with lipid bilayers (Shai et al., 1990, 1991; Rapaport & Shai, 1991, 1992; Pouny & Shai, 1992; Barrow et al., 1992). These studies provided information on the structure, orientation and aggregation state of pardaxin and its analogues when bound to phospholipid vesicles. Moreover, the capacity of pardaxin and several of its analogues to induce fusion of SUV,[1] composed of PC/PS, was also investigated (Rapaport et al., 1993).

In order to gain more understanding of the separate stages of the fusion process, we investigated the correlation between the ability of the amphiphilic peptide pardaxin, and two of its analogues, to induce fusion of and leakage from PS LUV of two sizes. The ability of the peptides to induce fusion of vesicles was studied both with dispersed vesicles and with vesicles preaggregated with $Mg^{2+}$, which by itself does not induce fusion of PS LUV (Wilschut et al., 1981). The latter resembles the fusion process in viral systems where membrane attachment is first established. With the use of $Mg^{2+}$, it is possible to test to what extent the lack of fusion products results from the inability of the peptides to aggregate the LUV or from the intrinsic stability of the large liposomes. The role of peptide secondary structure, charge, orientation, and tendency toward self-aggregation within the membrane, in promoting the fusogenic ability of the peptides, will be discussed.

## EXPERIMENTAL PROCEDURES

*Materials.* Phosphatidylserine (PS) from bovine spinal cord (sodium salt grade I) was purchased from Lipid Products (South-Nutfield, U.K). *N*-(Lissamine Rhodamine B sulfonyl)phosphatidylethanolamine (Rh-PE), *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (NBD-PE), 8-aminonaphthalene-1,3,6–trisulfonic acid, disodium salt (ANTS), and *p*-xylene-bispyridinium bromide (DPX) were purchased from Molecular Probes (Junction City, OR). All other reagents were of analytical grade.

*Synthesis and Purification of Peptides.* The peptides were synthesized by a solid-phase method on PAM-amino acid resin (Merrifield et al., 1982), as previously described (Shai et al., 1990; Pouny & Shai, 1992). The synthetic peptides were purified (>95% homogenicity) by reverse-phase HPLC on a $C_{18}$ column using a linear gradient that increases from 25% to 80% acetonitrile in 0.1% TFA, in 40 min. The peptides were subjected to amino acid analysis in order to confirm their composition.

*Preparation of Lipid Vesicles.* LUV were prepared from phospholipids by extrusion (Hope et al., 1985). Dry lipids were hydrated in buffer and dispersed by vortexing to produce MLV. The lipid suspension was frozen and thawed five times, extruded 10 times through polycarbonate membranes with either 0.1- or 0.4-$\mu$m pore diameter (Poretics Corp., Livermore, CA), and then centrifugated for 5 min at 4000*g* to remove residual MLV. The size distribution of the vesicles was determined by dynamic light scattering in a Malvern 4700 submicron particle analyzer. The mean diameter was found to be 113 or 220 nm for the vesicles that were extruded through the 100- or 400-nm pores, respectively. The lipid concentrations of the liposome suspensions were determined by phosphorus analysis (Rouser et al., 1966).

*Peptide-Induced Lipid Mixing.* Lipid mixing of LUV was measured using a fluorescence probe dilution assay, based on resonance energy transfer measurements (Struck et al., 1981). Lipid vesicles containing 0.6 mol % each of NBD-PE (energy donor) and Rh-PE (energy acceptor) were prepared in 100 mM NaCl and 25 mM Hepes, at pH 7.3, and a 1:4 mixture of labeled and unlabeled vesicles was suspended in 400 $\mu$L of the buffer at room temperature. The increase in NBD fluorescence at 530 nm, with the excitation wavelength set at 460 nm, was monitored using a Perkin Elmer LS-50B spectrofluorometer. The contribution of light scattering to the spectrum was minimized by exciting the sample with vertically polarized light and measuring horizontally polarized emitted light, and by placing a cutoff filter of 515 nm between the sample and the emission detector. The fluorescence intensity before the addition of the peptide, and the fluorescence intensity of a freshly prepared suspension of lipid vesicles containing 0.12 mol % of both probes were referred to as 0 and 100% lipid mixing, respectively.

*Analysis of Membrane Mixing Kinetics.* On the basis of previous work (Wilschut et al., 1981; Nir et al., 1981; Düzgünes et al., 1987) it was assumed that the vesicles were essentially preaggregated after they were preincubated for 5 min in a medium containing several mM $Mg^{2+}$, and that the binding of the peptides that initiate the fusion is a fast process. The fusion rate constant, $f$ ($s^{-1}$), was determined from a simulation of the initial stages of NBD-PE fluorescence increase according to

$$I = 100[1 - \exp(-ft)] \quad (1)$$

in which $t$ is the time in seconds and $I$ is the percent of fluorescence increase relative to 100%. The factor 100 is a product of (i) 80% which is the probability of fusion leading to probe dilution for a 1:4 ratio between labeled and unlabeled vesicles, with (ii) 10/8, which stems from the fact that the 100% level of fluorescence increase corresponds to 80% of probe dilution relative to complete or infinite surface dilution. The final extent of fluorescence increase provides information on the fraction of vesicles capable of fusing, and the number of fusion rounds. We have utilized an equation (Nir et al., 1987) for the final level of fluorescence increase relative to the maximal level at infinite dilution:

$$I = 100[k/(k + L)](n - 1)/n \quad (2)$$

in which $n$ is the number of vesicles in a fusion product and $L/k$ is the ratio between labeled to unlabeled vesicles. In our case complete fusion of vesicles to doublets ($n = 2$) yields $I = 100(10/8)(4/5)(1/2) = 50\%$. Thus, if the final level is $I = 10\%$, only 20% of the vesicles are capable of fusing. We also analyzed the kinetics of vesicle fusion by considering aggregation, dissociation, and fusion processes, when the peptide was added alone without $Mg^{2+}$ (Nir et al., 1983; Bentz et al., 1983). The calculations were performed by introducing certain modifications into the program described by Nir et al. (1986).

[1] Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt; DPX, *p*-xylene-bispyridinium bromide; HIV, human immunodeficiency virus; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phosphatidylethanolamine; PAM, phenylacetamidomethyl; PC, phosphatidylcholine; PS, phosphatidylserine; Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; SUV, small unilamellar vesicles; TFA, trifluoroacetic acid.

*Turbidity Measurements.* Changes in the size distribution and state of aggregation of the vesicles were monitored by absorbance measurements. Aliquots of peptide stock solutions were added to 1-mL suspensions of 91 $\mu$M PS LUV in buffer composed of 100 mM NaCl and 25 mM Hepes, pH 7.3. The absorbance at 405 nm was monitored continuously with a Milton Roy spectrometer.

*Leakage Assay.* The ANTS/DPX assay was used to monitor vesicle leakage (Ellens et al., 1984). ANTS and DPX were coencapsulated in liposomes and thus the ANTS fluorescence was initially quenched. Leakage of lipid vesicles was monitored by measuring the dequenching of ANTS released into solution. PS LUV were prepared as described above in 12.5 mM ANTS, 45 mM DPX, 65 mM NaCl, and 10 mM Tris-HCl, pH 7.4. To remove unencapsulated material, the vesicles were passed through a Sephadex G-75 column and eluted with 150 mM NaCl and 10 mM Tris-HCl, pH 7.4. The fluorescence of the liposomes alone or after the addition of Triton X-100 (0.5% v/v final concentration) corresponds to 0 or 100% leakage, respectively. The increase in ANTS fluorescence at 520 nm, with the excitation wavelength set at 360 nm, was monitored at room temperature using a Perkin Elmer LS-50B spectrofluorometer. The contribution of light scattering to the spectrum was minimized by exciting the sample with vertically polarized light and measuring horizontally polarized emitted light.

*Aqueous Contents Mixing Assay.* ANTS is encapsulated in the lumen of one population of liposomes and DPX in the other (Ellens et al., 1984). Mixing of the aqueous contents of vesicles results in quenching of ANTS fluorescence, whereas leakage does not result in quenching because the probes are too dilute. PS LUV were prepared as described above in 25 mM ANTS and 89 mM NaCl or in 90 mM DPX and 39 mM NaCl, both with 10 mM Tris-HCl, pH 7.4. Unencapsulated material was removed by passing the vesicles through a Sephadex G-75 column and eluting with 150 mM NaCl and 10 mM Tris-HCl, pH 7.4. The fluorescence of a 1:1 mixture of ANTS and DPX containing liposomes and that of liposomes containing both 12.5 mM ANTS and 45 mM DPX correspond to 0 and 100% quenching, respectively. Emission intensity of ANTS fluorescence was monitored using a Perkin Elmer LS-50B spectrofluorometer at 520 nm, with the excitation wavelength set at 360 nm.

## RESULTS

In order to gain more information on the separate stages involved in the membrane fusion process, leakage from and fusion of negatively charged LUV induced by pardaxin, and two of its analogues, were investigated. The native molecule is a 33-mer peptide with the sequence G-F-F-A-L-I-P-K-I-I-S-S-P-L-F-K-T-L-L-S-A-V-G-S-A-L-S-S-S-G-G-Q-E. In one analogue the two carboxylates at the C-terminus were modified to produce two positively charged groups (C-diamino-par), while in the other two L-Leu amino acids located at positions 5 and 19 were replaced with their D-enantiomers (D-LL-par). Fusion was demonstrated by membrane lipid mixing, by contents mixing, and by an increase in vesicle sizes as verified by dynamic light scattering and changes in absorbance.

*Fusion Induced by Peptides Alone.* The induction of intervesicular lipid mixing by the peptides, as a measure of their fusogenic activity, was tested with PS LUV utilizing the probe dilution assay (Struck et al., 1981). The dependence of both the final extent and kinetics of the lipid mixing process on peptide concentration was examined. In separate experiments, increasing amounts of each peptide were added to a fixed amount of PS LUV (91 $\mu$M, extruded through 100-nm pores), and the increase in fluorescence intensity was monitored versus time. Figure 1 shows the extent (panel A) and the initial kinetics (panel B) of the membrane mixing process for the various peptides. It is evident that all the peptides are able to induce lipid mixing. However, while in the low concentration range all the peptides have an apparently similar low activity, at higher peptide concentrations the C-diamino-par peptide can induce higher extents of membrane mixing than the others. The peptides' capacities to cause changes in the absorbance of liposome suspensions were also compared. Peptides (1.25 or 2.5 $\mu$M) were added to 91 $\mu$M PS LUV (extruded through 400-nm pores), and the increase in absorbance at 405 nm was monitored. Under these conditions only C-diamino-par was able to cause significant changes in the absorbance of the vesicles (data not shown), reflecting its higher potential to aggregate vesicles.

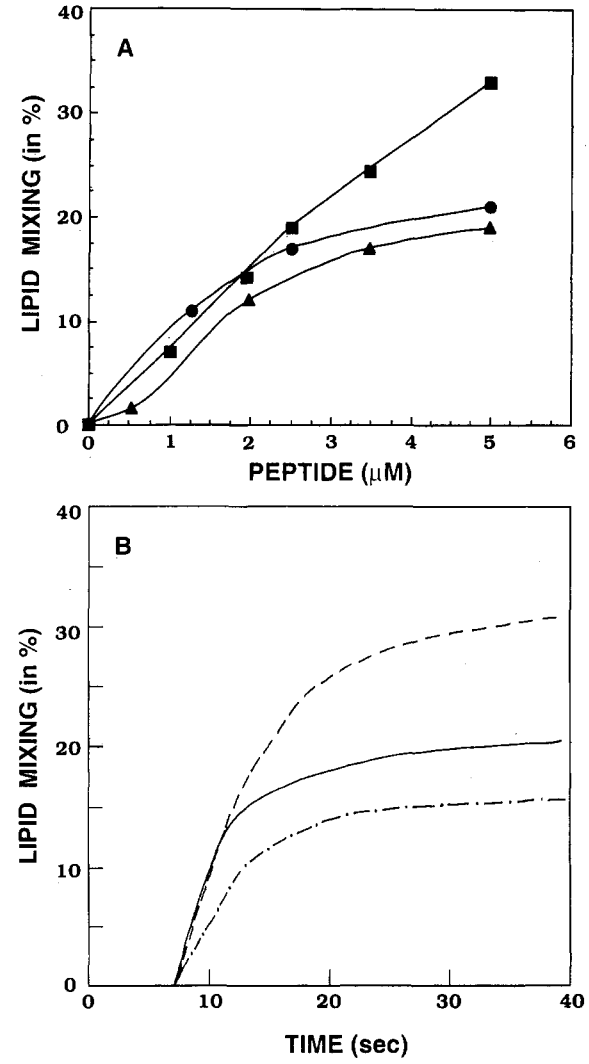


FIGURE 1: (A) Dose dependence of lipid mixing induced by the peptides. Each peptide was added to the liposome suspension (91 $\mu$M) in buffer composed of 100 mM NaCl and 25 mM Hepes, pH 7.3. The increase of the fluorescence intensity of NBD-PE was measured at 10 min after the addition of the peptide, and the percentage of maximal lipid mixing is plotted versus the peptide concentration. Pardaxin, circles; D-LL-par, triangles; C-diamino-par, squares. (B) Initial time course of lipid mixing induced by 5 $\mu$M peptides. The increase in the percentage of maximal lipid mixing is plotted versus time. Pardaxin, continuous line; D-LL-par, dashed-dotted line; C-diamino-par, dashed line.

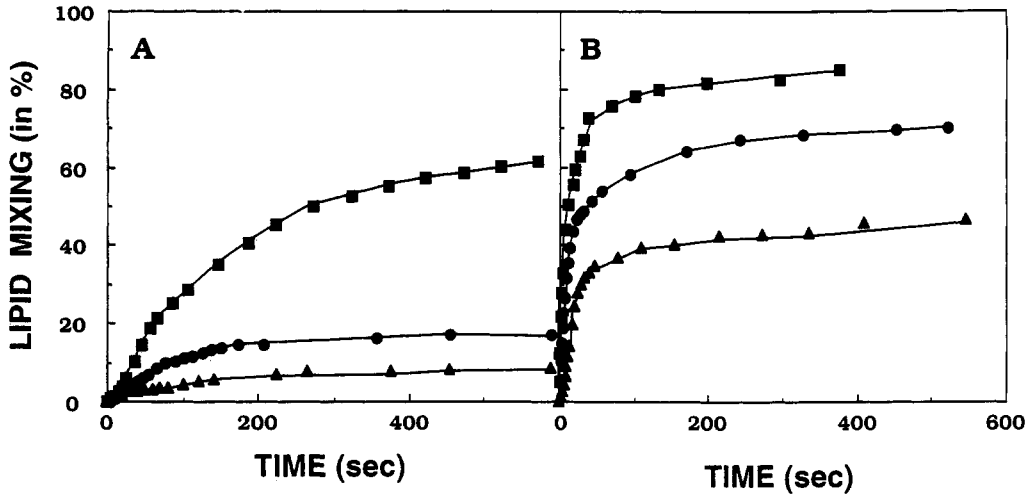


FIGURE 2: Time course of lipid mixing of vesicles extruded through pores of 100 (panel A) or 400 nm (Panel B) in the presence of 2.5 (panel A) or 3.75 (panel B) mM $Mg^{2+}$. Vesicle suspension (91 $\mu$M) in buffer composed of 100 mM NaCl and 25 mM Hepes, pH 7.3, was incubated with $Mg^{2+}$ for 5 min. Peptides (0.55 $\mu$M) were then added at time zero. The increase in NBD-PE fluorescence at room temperature due to the addition of the peptides was monitored, and the percentage of maximum is plotted versus time. Pardaxin, circles; D-LL-par, triangles; C-diamino-par, squares.

*Fusion of Vesicles in the Presence of $Mg^{2+}$.* The next stage in our study was to examine the potential of the peptides to induce fusion of preaggregated vesicles. Figure 2 describes the results when vesicles that were extruded through 100- (panel A) or 400-nm pores (panel B) were preincubated for 5 min in a medium containing 2.5 or 3.75 mM $Mg^{2+}$, respectively, before the addition of the peptides. $Mg^{2+}$ at these concentrations is known to cause aggregation but not fusion of PS LUV (Wilschut et al., 1981; Nir et al., 1983; Düzgünes et al., 1987). A comparison of the results in Figure 2 to those in Figure 1 shows that vesicle preaggregation enhances the extent of fusion in all cases, particularly in the case of the C-diamino-par peptide. Note that in the peptide/lipid ratio used in Figure 2 none of the peptides were significantly fusogenic toward dispersed vesicles. Addition of the peptides to the smaller vesicles preaggregated with 3.75 mM $Mg^{2+}$, or to the larger vesicles pre-aggregated with 5 mM $Mg^{2+}$, resulted in all cases in similar final high levels of lipid mixing (data not shown), but the differences in the kinetics still remain (Table 1). When $Mg^{2+}$ was added to the vesicles after the addition of the peptides, the increase in the extent of fusion with pardaxin (Figure 3) or D-LL-par (data not shown) was reduced as compared to the reverse order of addition. In contrast, addition of $Mg^{2+}$ to vesicles incubated with C-diamino-par yielded a sharp increase in the fluorescence, similar to that observed when the ion was added before the peptide (Figure 3).

The fusion of the preaggregated vesicles was further confirmed by changes in the size distribution of PS vesicles, assessed by dynamic light scattering and absorbance. The size distribution of the vesicles was monitored by a laser particle analyzer before and after the addition of 2.5 mM $Mg^{2+}$, C-diamino-par, and finally EDTA, which disperses $Mg^{2+}$-induced aggregates. The results presented in Figure 4 demonstrate that while the mean diameter of the initial vesicle population was 113 (SD 36) nm, it increased to 647 (SD 232) nm when the vesicles were treated as above. The mean diameter was not changed when $Mg^{2+}$ was added alone for 5 min, followed by addition of EDTA (data not shown).

Changes in vesicle size distribution due to aggregation and/or fusion can also be monitored by following the absorbance of the liposome suspension at 405 nm. $Mg^{2+}$ alone at a concentration of 10 mM can cause massive aggregation of the vesicles; however, no fusion is observed as the absorbance increase is totally reversed by the addition of EDTA (data not shown). Figure 5 shows changes in the absorbance at 405 nm as a function of time after addition of the peptides to PS LUV partially preaggregated with 2.5 mM $Mg^{2+}$. When C-diamino-par was added, a sharp increase in the absorbance intensity could be observed. EDTA could only partially reverse the absorbance increase, reflecting the fact that fusion took place. In contrast, the other two peptides caused only a minor increase in the absorbance, which was almost completely abolished when EDTA was added. When the order of addition was investigated, a large increase in absorbance was observed when $Mg^{2+}$ was added to vesicles preincubated with C-diamino-par. The addition of $Mg^{2+}$ to vesicles preincubated with the other two peptides did not cause any significant changes in the very low absorbance levels (data not shown).

Fusion was also assayed by the mixing of the internal contents of ANTS- and DPX-containing LUV. The fast and extensive leakage of vesicle contents induced by the peptides

Table 1: Rate Constants of Fusion ($f$) for the Different Peptides under Various Conditions

| peptide | extruded size (nm) | lipid conc. ($\mu$M) | $Mg^{2+}$ conc. (mM) | lipid/peptide (molar ratio) | $f$ ($s^{-1}$)[a] |
|---|---|---|---|---|---|
| pardaxin | 100 | 85.5 | 2.50 | 155 | 0.013 |
| | 100 | 91.0 | 2.50 | 18 | 0.043 |
| | 100 | 85.5 | 3.75 | 155 | 0.045 |
| | 400 | 91.0 | 3.75 | 165 | 0.055 |
| | 400 | 91.0 | 5.00 | 165 | 0.045 |
| C-diamino-par | 100 | 34.4 | 2.50 | 160 | 0.010 |
| | 100 | 85.5 | 2.50 | 155 | 0.018 |
| | 100 | 91.0 | 2.50 | 83 | 0.040 |
| | 100 | 34.4 | 2.50 | 43 | 0.050 |
| | 100 | 80.0 | 0.00 | 16 | 0.070 |
| | 100 | 85.5 | 3.75 | 155 | 0.060 |
| | 400 | 91.0 | 3.75 | 165 | 0.070 |
| | 400 | 91.0 | 5.00 | 165 | 0.085 |
| D-LL-par | 100 | 91.0 | 2.50 | 165 | 0.005 |
| | 100 | 91.0 | 2.50 | 83 | 0.012 |
| | 100 | 85.5 | 3.75 | 155 | 0.017 |
| | 400 | 91.0 | 3.75 | 165 | 0.011 |
| | 400 | 91.0 | 5.00 | 165 | 0.030 |

[a] Calculated as described under Experimental Procedures. The estimated uncertainty in f is 40% without $Mg^{2+}$ or in the presence of 2.5 mM $Mg^{2+}$. In the other cases the uncertainty is 20–30%.

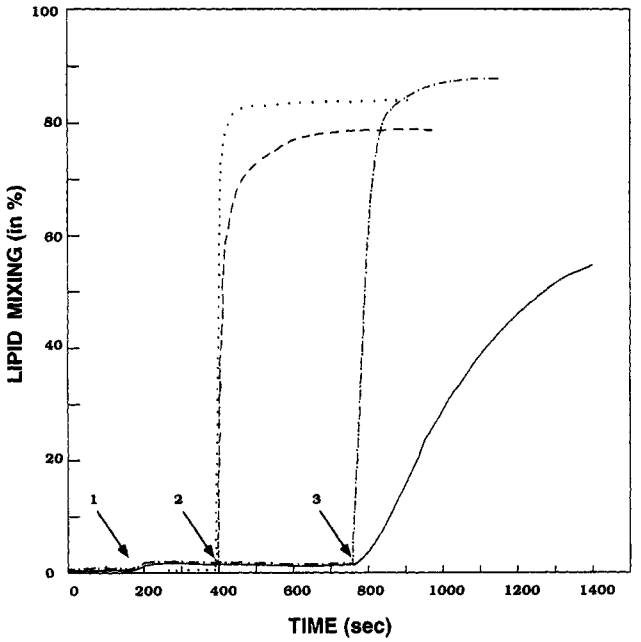


FIGURE 3: Effect of the order of addition of $Mg^{2+}$ and peptides on fusion. Vesicles were extruded through 100-nm pores. The increase in NBD-PE fluorescence was monitored, and the percentage of maximum is plotted versus time. Continuous line: pardaxin (0.55 $\mu$M) was added at point no. 1, followed by $Mg^{2+}$ (3.75 mM) which was added at point no. 3. Dashed line: $Mg^{2+}$ (3.75 mM) was added at point no. 1, followed by Pardaxin (0.55 $\mu$M) which was added at point no. 2. Dashed-dotted line: C-diamino-par (0.55 $\mu$M) was added at point no. 1, followed by $Mg^{2+}$ (3.75 mM) which was added at point no. 3. Dotted line: $Mg^{2+}$ (3.75 mM) was added at point no. 1, followed by C-diamino-par (0.55 $\mu$M) which was added at point no. 2.

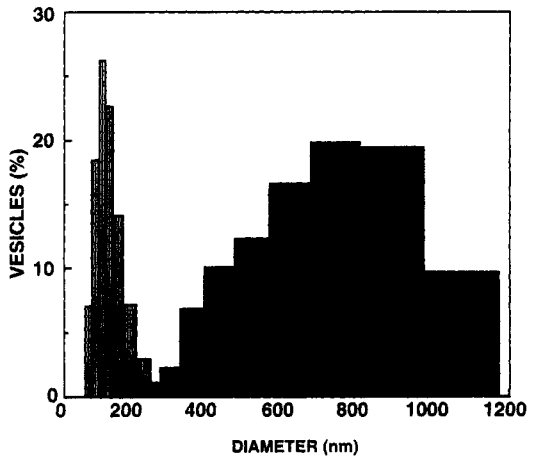


FIGURE 4: Size increase of vesicles as observed by dynamic light scattering. Vertical lines: size distribution of mass of vesicles extruded through 100-nm pores. Horizontal lines: C-diamino-par (0.55 $\mu$M) was added to vesicles (91 $\mu$M) preincubated with $Mg^{2+}$ (2.5 mM). After 10 min of incubation with the peptide EDTA (5 mM) was added, and the size distribution of mass was measured.

(Figures 6 and 7) makes it impossible to estimate fusion ability of the peptides from the contents mixing results. The extents of fusion determined by this method would be much lower than those observed by using the lipid mixing assay. Nevertheless, except for the case of pardaxin, which induced a large extent of vesicle leakage, contents mixing of 22% and 12% was observed when 0.55 $\mu$M C-diamino-par or D-LL-par, respectively, was added to 91 $\mu$M PS LUV (extruded through 100-nm pores) in the presence of $Mg^{2+}$ in the medium (data not shown). In an equimolar mixture of ANTS- and DPX-containing vesicles only half of the contents mixing events can be observed by the assay. Hence, a 22% value of ANTS

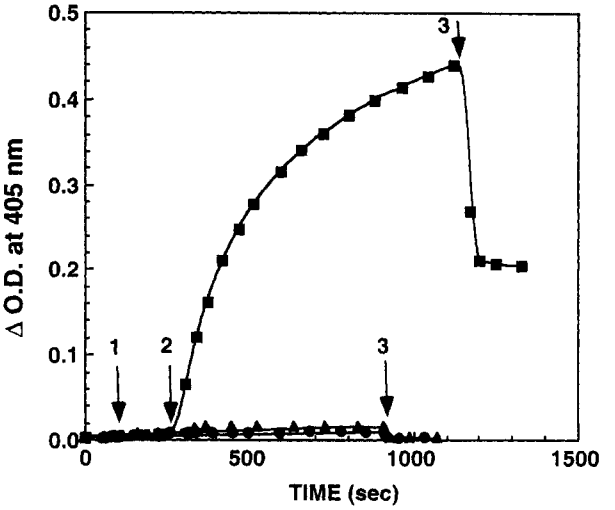


FIGURE 5: Changes in turbidity due to addition of $Mg^{2+}$ and peptides to vesicles. $Mg^{2+}$ (2.5 mM) was added, at the first time point, to PS LUV suspension (91 $\mu$M, at nominal size of 100 nm) in buffer composed of 100 mM NaCl and 25 mM Hepes, pH 7.3. Peptides (0.55 $\mu$M) were then added, at the second time point, and finally 5.0 mM EDTA was added at the third time point. The increase in absorbance at room temperature due to the addition of the peptides is plotted versus time. Some of the data points were removed for the sake of clarity. Pardaxin, circles; D-LL-par, triangles; C-diamino-par, squares.

quenching arises from 44% contents mixing. Taking into account the correction for leakage, the contents mixing results indicate that with C-diamino-par the vesicles undergo at least one round of fusion. The peptide C-diamino-par was the most potent inducer of contents mixing, in accordance with the results of the lipid mixing and absorbance assays and in line with the fact that it was the less potent leakage inducing peptide.

*Analysis of Fusion Kinetics.* In the presence of 3.75 or 5 mM $Mg^{2+}$ a large extent of vesicle aggregation was reached within 5 min; hence the employment of eq 1 could directly yield the fusion rate constants. As pointed out (Figure 2A), a significant enhancement in the rate and extent of membrane mixing was also achieved in the presence of 2.5 mM $Mg^{2+}$. However, eq 1 could not be employed for the analysis of membrane mixing in the presence of 2.5 mM $Mg^{2+}$ since under these conditions the vesicles were only partly aggregated. The increase in turbidity after 5 min of preincubating the vesicles with this concentration of $Mg^{2+}$ was 20-fold below that with 3.75 mM $Mg^{2+}$ (data not shown). In these cases we instead performed an analysis ignoring preaggregation of the vesicles. Hence, $f$ values deduced in the presence of 2.5 mM $Mg^{2+}$ are in a sense upper bounds, since a small degree of vesicle preaggregation did exist. For each peptide the fusion rate constant increases with a decrease in the lipid/peptide ratio (Table 1). For similar lipid/peptide ratios, $f$ values differ by 1.5–3-fold between consecutive members in the sequence C-diamino-par > pardaxin > D-LL-par.

We also analyzed the kinetics of fusion in the absence of $Mg^{2+}$, for the most fusogenic peptide, C-diamino-par, at a lipid/peptide ratio of 16. The $f$ value obtained is apparently within the extrapolated value of that in the presence of $Mg^{2+}$ (Table 1), although $Mg^{2+}$ itself also promotes the fusion induced by the peptides. In all other cases an analysis of kinetics of fusion without $Mg^{2+}$ could not be performed, since an examination of the final extents according to eq 2 indicated incomplete fusion.

The analysis (Table 1) also indicates similarity in $f$ values deduced for the vesicles extruded through different pore sizes.

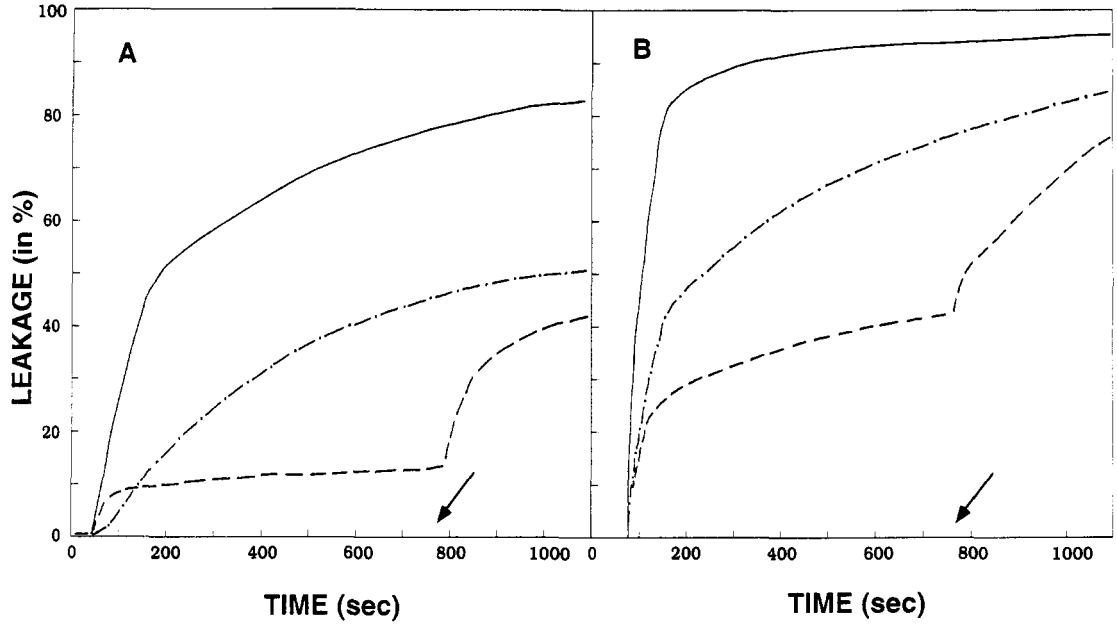


FIGURE 6: Kinetics of leakage induced by peptides. Peptides (0.55 $\mu$M) were added to 91 $\mu$M vesicles at nominal size of 100 (panel A) or 400 nm (panel B) with ANTS/DPX coencapsulated. The percentage of release is plotted versus time. At the time point indicated with arrows $Mg^{2+}$ (2.5 mM) was added. Pardaxin, continuous line; D-LL-par, dashed-dotted line; C-diamino-par, dashed line.

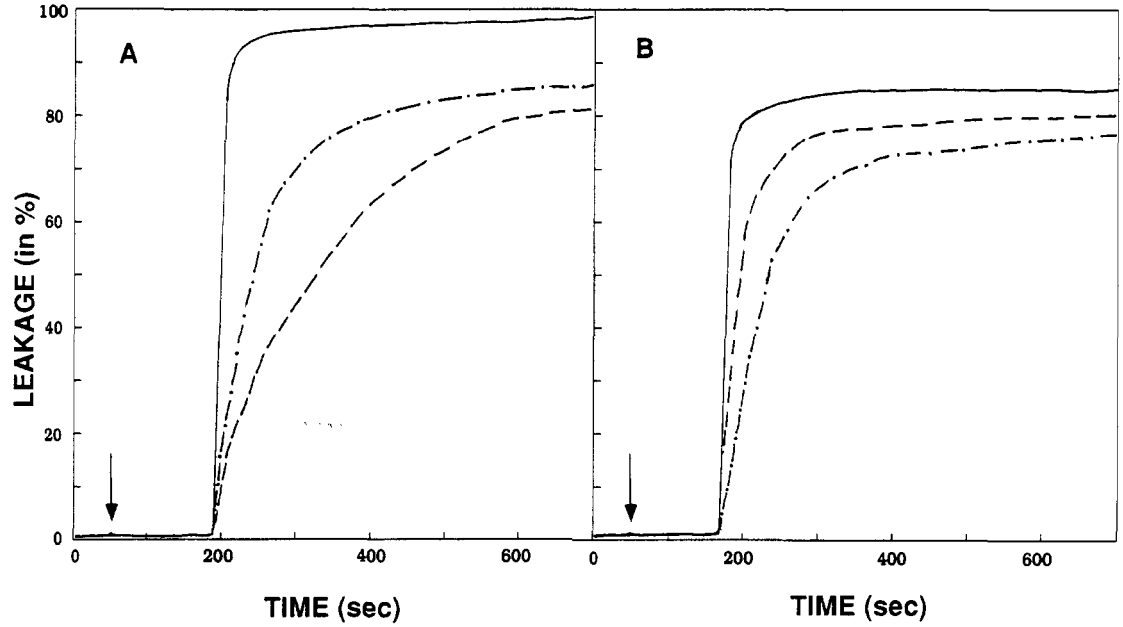


FIGURE 7: Kinetics of leakage induced by peptides in the presence of $Mg^{2+}$. $Mg^{2+}$ at concentrations of 2.5 mM (panel A) or 3.75 mM (panel B) was added at the point indicated with arrow to 91 $\mu$M vesicles at nominal size of 400 nm with ANTS/DPX coencapsulated. Two minutes later peptides (0.55 $\mu$M) were added, and the percentage of release is plotted versus time. Pardaxin, continuous line; D-LL-par, dashed-dotted line; C-diamino-par, dashed line.

However, considering that the number of bound peptides per vesicle is larger for the bigger vesicles, it follows that the larger vesicles are less susceptible to fusion induced by pardaxin and its analogues.

*Peptide-Induced Dye Leakage.* The interaction of the amphipathic peptides with phospholipid vesicles was further investigated by measuring the ability of the peptides to induce leakage of ANTS/DPX from PS vesicles extruded through 100- (Figure A) or 400-nm (Figure 6B) pores. As demonstrated in Figure 6, the relative efficiency of these peptides in inducing leakage does not correlate with their fusogenic activity; C-diamino-par, the most potent peptide in the group in inducing fusion, exhibited the lowest efficiency in inducing dye leakage. Pardaxin was significantly the most effective inducer of leakage, followed by D-LL-par. Interestingly, the addition of $Mg^{2+}$ during the leakage process induced by pardaxin and D-LL-par did not influence the rate of fluorescence increase; however, it caused significant enhancement in the rate of leakage induced by C-diamino-par.

The next step was to test the leakage levels observed when the peptides were added to vesicles preaggregated with $Mg^{2+}$. As demonstrated in Figure 7, the native peptide was the most potent peptide also in this case. Note that the C-diamino-par peptide induced significantly higher leakage levels in preaggregated vesicles than in dispersed vesicles, and in the presence of 3.75 mM $Mg^{2+}$ (panel B) it induced higher levels of leakage than the D-LL-par analogue.

To determine whether the leakage is an all or none event where part of the vesicles release all of their contents, or a graded event, i.e., all of the vesicles release some of their

Table 2: Comparison of the Experimental Leakage Results to Those Expected According to All-or-None Mechanism[a]

| | pardaxin (0.14 μM) | C-diamino-par (1.1 μM) | D-LL-par (0.55 μM) | 3.75 mM $Mg^{2+}$ + C-diamino-par (0.55 μM) | $Ca^{2+}$ (2.9 mM) |
|---|---|---|---|---|---|
| % dye released[b] | 62 | 72 | 67 | 70 | 64 |
| observed[c] % quenching | 92 ± 3 | 88 ± 5 | 76 ± 5 | 73 ± 5 | 70 ± 3 |

[a] From the fluorescence quenching of untreated vesicles the predicted percent quenching according to all-or-none release would be 89 ± 2%. [b] Percentage of leakage after addition of peptides and prior to separation of vesicles from unentrapped dye. [c] The vesicles were collected after the separation from the unentrapped dye and lysed with Triton. The percentage of quenching of the dye within the vesicles was calculated.

contents, we employed the method described in Weinstein et al. (1981) and modified by Parente et al. (1990). After incubation with the peptides, PS vesicles were rapidly separated from unentrapped dyes by means of a Sephadex G-75 column. The collected vesicles were lysed with Triton X-100, and the degree of quenching in the vesicles was calculated from the fluorescence before and after the addition of the detergent. According to the all or none mechanism, the degree of quenching in the collected vesicles should be similar to that in the original population. The data presented in Table 2 indicate that, within the experimental uncertainty, the vesicles treated with the pardaxin and C-diamino-par peptides were quenched to the same extent as those in the original suspension. With D-LL-par the leakage results are less conclusive. We chose cases where the leakage reached 60–70%. In these cases graded release would result in a significantly lower degree of quenching in the collected vesicles. Examples of cases of mainly graded release can be seen when 2.9 mM $Ca^{2+}$ or 3.75 mM $Mg^{2+}$+ C-diamino-par are added to PS LUV. These two cases correspond to a situation where fused vesicles lose part of their contents during the fusion process.

## DISCUSSION

Peptide-induced vesicle fusion is frequently accompanied by leakage of vesicle contents. To determine the correlation between these two processes, we studied the interaction of the amphiphilic peptide pardaxin, and two of its analogues, with large unilamellar vesicles composed of phosphatidylserine. A current model views the overall fusion reaction as a sequence of a second-order process of membrane adhesion, followed by the first-order fusion itself (Nir et al., 1982; Bentz et al., 1983, 1988). In order to test whether the limited fusion frequently observed with fusogenic peptides is the result of an aggregation limited process, and to gain insight into the fusogenic properties *per se*, we compared the fusogenic properties of the peptides toward dispersed PS LUV to those toward aggregated vesicles. Evidently, in a suspension of dispersed vesicles, the peptides carry out both processes, the bringing of the vesicles to close apposition (aggregation) and the fusion itself, each of which is normally carried out by distinct proteins or regions in the viral protein (Bentz et al., 1990).

The peptide C-diamino-par had a much higher ability as compared to the other two peptides in inducing aggregation of the negatively charged vesicles (data not shown). Nevertheless, fusion of dispersed vesicles, although at a relatively high peptide to lipid ratios, was observed with the three peptides tested (Figure 1). It is interesting to note that, even in the highest peptides concentration tested, fusion does not go to completion. According to our calculations, at these elevated peptide to lipid ratios there are about 3000–4000 peptide molecules bound to each vesicle. Therefore, we believe that the explanation for this incomplete fusion is not lack of enough bound peptide molecules, but rather that the peptides are incapable of aggregating large vesicles. In the initial stage of the process the peptides aggregate and fuse the smaller vesicles in the population but after this first round the resultant vesicles are already large and the peptides are not able to aggregate them. At the lower concentrations all the peptides had similar low activity probably because at these peptide to lipid ratios none of them was able to cause massive aggregation of vesicles. C-diamino-par was able to do so at higher concentrations, and indeed at higher peptide to lipid ratios this peptide is much more fusogenic than the other two peptides.

The unique fusion inducing ability of C-diamino-par was demonstrated in particular in the presence of 2.5 mM $Mg^{2+}$. At a 1:165 peptide to lipid ratio, where all the peptides were practically not fusogenic toward dispersed vesicles (Figure 1), C-diamino-par could induce levels of aggregation and/or fusion several-fold higher than those induced by the other peptides (Figures 2A and 5). The fusion levels observed when vesicle aggregation was promoted by 3.75 or even 2.5 mM $Mg^{2+}$ were significantly higher than with dispersed vesicles (Figures 1 and 2). These results confirm the importance, in peptide-induced vesicle fusion, of bringing the vesicles to close apposition, which means that the aggregation step is the rate-limiting one. Once a close apposition of vesicles occurs, fusion is rapidly induced. The results with preaggregated vesicles (Figures 2 and 5 and Table 1) indicate that C-diamino-par is also more effective in inducing fusion of aggregated vesicles. It is possible, however, that the larger rate constants of fusion observed for C-diamino-par (Table 1) are partly due to its higher affinity toward the membrane. Nevertheless, the results with dispersed and preaggregated vesicles indicate that C-diamino-par is the more effective peptide in inducing both vesicle aggregation and the fusion step itself, i.e., the merging of the bilayers.

*Leakage Induced by the Peptides.* To investigate the potential of the peptides to perturb membranes, we monitored their ability to induce release of encapsulated dyes from vesicles. Interestingly, the addition of $Mg^{2+}$ during the leakage process induced by pardaxin or D-LL-par did not have any effect on the rate of leakage. However, when $Mg^{2+}$ was added to vesicles with C-diamino-par, a sharp increase in the leakage rate was observed (Figure 6). This also correlates with the result that the addition of $Mg^{2+}$ after C-diamino-par has a dramatic effect on the rate of fusion, while the addition of $Mg^{2+}$ after the addition of pardaxin or D-LL-par has a much smaller influence (Figure 3). The native peptide also caused the highest levels of leakage when $Mg^{2+}$ was added before the peptides. However, as opposed to the situation with dispersed vesicles, the level of leakage observed with C-diamino-par and vesicles preaggregated with 3.75 mM $Mg^{2+}$ (Figure 7B) was higher than that observed in these conditions for the D-LL-par peptide. We interpret this difference to leakage during the fusion process induced by C-diamino-par. Leakage during the fusion process itself due to leakiness of the fusion intermediate was previously recorded from PS vesicles (Wilschut et al., 1980; Nir et al., 1980, 1982, 1983; Bentz et al., 1983). Therefore, we interpret the influence of $Mg^{2+}$ on the

Table 3: Summary of the Characteristics of the Peptides Studied

| | pardaxin | C-diamino-par | D-LL-par |
|---|---|---|---|
| net charge | +1 | +5 | +1 |
| percent of $\alpha$-helix in membranes | 49[a] | 31[a] | 6[b] |
| induction of fusion | ++ | +++ | + |
| induction of aggregation | + | +++ | + |
| induction of leakage | +++ | + | ++ |

[a] Values are taken from Shai et al. (1991). [b] Value is taken from Pouny and Shai (1992).

leakage rate observed with C-diamino-par to be the result of initiation of fusion and not due to a modified ability of the peptide to perturb the membrane.

The mechanism of peptide-induced leakage for pardaxin and C-diamino-par was found to fit an all or none model (Table 2). This could mean that in these cases leakage from a vesicle occurs only when it binds a minimal number of peptides that form a pore or a defect in the vesicle membrane. This is supported by the observation that, for the same peptide to lipid ratio, the extents of leakage induced by these peptides are larger from the bigger vesicles (Figure 6). This behavior can be anticipated according to the above model since the number of bound peptide molecules per vesicle is significantly higher with the larger vesicles. The leakage induced by other peptides such as GALA (Parente et al., 1990), Magainin (Grant et al., 1992), and the fusion peptide of HIV (Nieva et al., 1994) has also been described as an all or none mechanism. In the present study the high degree of leakage induced by the peptides was accompanied by lower levels of fusion than those in the lipid mixing assay, as determined by contents mixing. Low levels of contents mixing due to fast leakage have also been observed with other fusogenic peptides (Parente et al., 1988; Suenaga et al., 1989; Düzgünes & Shavnin, 1992; Lee et al., 1992). However, for C-diamino-par there is a significant level of contents mixing within the first 2 min, indicating that at that stage the vesicular structure of fused PS LUV is still retained.

*Structural Aspects of Peptide–Lipid Interactions.* The structural and functional differences between the peptides studied are summarized in Table 3. First, the role of peptide self-aggregation in inducing fusion and leakage will be diccussed. Using fluorescently labeled peptides, we have demonstrated that pardaxin molecules tend to self-aggregate efficiently within lipid vesicles (Rapaport & Shai, 1991, 1992). It could be postulated that this peptide forms pores in the membrane, due to its high tendency to self-associate (Rapaport & Shai, 1992) and based on its inducing relatively high levels of leakage and the all or none mechanism found in the present study for pardaxin-induced leakage of vesicles. On the basis of the low tendency of C-diamino-par to cause leakage, which also occurs by an all or none mechanism, we propose that this peptide mostly forms small oligomers in PS vesicles. D-LL-par is able to induce relatively high levels of vesicle leakage. However, the fact that it lacks cooperativity in binding (Pouny & Shai, 1992), together with the partly graded nature of leakage that it induces, may suggest that this peptide does not oligomerize to a large extent within the membrane.

The correlation between the fusogenic ability of a peptide and its oligomerization state appears to be complicated. Current models describe a fusion pore composed of a few protein molecules as an intermediate step in the fusion mechanism (Bentz et al., 1990; Zimmerberg et al., 1993). This model is supported by studies in which oligomerization of a mutant of HIV-1 protein (Freed et al., 1992) and of viral fusion peptides (Wharton et al., 1988; Glushakova et al., 1992) was implicated in their mediation of fusion. Recently, we have demonstrated that the fusion peptide of Sendai virus tends to self-associate in its membrane bound state (Rapaport & Shai, 1994). However, peptide aggregation resulting in membrane destabilization is not sufficient to induce fusion. Native pardaxin, which is capable of forming large intramembrane aggregates and of mediating high levels of leakage, induces less fusion then C-diamino-par which induces less leakage. It appears that a moderate rather than large degree of intramembrane peptide aggregation, with the correct spatial arrangement of charges and hydrophobic residues, is more optimal for induction of fusion.

The secondary structure of peptides is another factor regulating their interaction with lipid membranes. In previous studies we have measured the secondary structure of the peptides in membranes (Shai et al., 1991; Pouny & Shai, 1992). In contrast to previous studies where a correlation between $\alpha$-helical secondary structure and the ability to cause vesicles to leak was suggested (Parente et al., 1990; Pouny & Shai, 1992; Nieva et al., 1994), such clear correlation was not found in the present study, since C-diamino-par with higher $\alpha$-helical content than D-LL-par was found to be less active in induction of leakage.

The correlation between the $\alpha$-helical content of a peptide and its fusogenic properties is also not clear. Such correlation was noted with the model peptide GALA (Parente et al., 1988) and segments of the influenza virus HA-2 protein (Lear & DeGrado, 1987; Wharton et al., 1988; Rafalski et al., 1991), as well as model amphipathic peptides (Lee et al., 1992). The D-LL-par analogue was found to have a low level of $\alpha$-helical structure (Pouny & Shai, 1992), and its lower fusogenic activity toward PC/PS SUV (Rapaport et al., 1993) and PS LUV as was demonstrated in the present study tends to be in line with these cases. However, the observation that C-diamino-par is more fusogenic, while it is less helical than pardaxin, is in line with previous reports showing either that helix formation is not a sufficient condition to trigger membrane fusion (Murata et al., 1991; Burger et al. 1991, Rapaport et al., 1993) or that fusion can be induced by peptides with $\beta$-sheet structure (Fujii et al., 1993; Nieva et al., 1994).

The orientation of a peptide with respect to the membrane is another parameter regulating its capacities. Recent studies on pardaxin and a series of its synthetic analogues (Shai et al., 1990, 1991; Rapaport & Shai, 1991,1992; Zagorski et al., 1991; Barrow et al., 1992; Pouny & Shai, 1992; Lazarovici et al., 1992) have led to the proposal that in its membrane bound state pardaxin consists of two $\alpha$-helices with a proline hinge between them. The hydrophobic N-terminal helix (residues 2–10) is believed to be inserted into the lipid bilayer, thus anchoring the peptide to the membrane, while the C-terminal segment (residues 13–27) lies on the surface.

When the order of addition of $Mg^{2+}$ and the peptides was examined, it was found that in the case of the native and D-LL-par peptides the addition of $Mg^{2+}$ after the peptides reduced the fusogenic activity as compared to a reversed order of addition (Figure 3). When $Mg^{2+}$ is added first, the bound pardaxin and D-LL-par peptides have a better chance of cross-linking and fusing the apposed vesicles before their structure and/or state of aggregation becomes incompatible with fusion. However, when pardaxin or D-LL-par is added to dispersed vesicles, the C-termini may become embedded deeper in the core of the membrane; thus the peptide would be unable to cross-link apposed vesicles, which would explain the observed reduction of fusogenicity. The positive C-terminus of C-diamino-par could be electrostatically attracted to negatively

charged headgroups of the PS and therefore may be located on the interface of the bilayer, which would enable it to cross-link apposed vesicles. A dependence of the fusogenic activity on the order of addition of peptide and divalent ions has been observed for the HIV fusion peptide (Nieva et al., 1994).

We found that the presence of positive charges at the C-terminus of C-diamino-par dramatically increases the overall fusogenic activity of the peptide. These positive charges could cause a reduced tendency of its C-termini to self-aggregate within the vesicular membrane, which would result in a reduced tendency to form pores and a larger potential to cross-link apposed vesicles. A positively charged C-terminus may be attracted by mere electrostatic forces to negatively charged phospholipids located on other liposomes, which could facilitate close apposition of membranes, followed by fusion. The observation that the order of addition of peptide and $Mg^{2+}$ does not influence fusion induced by C-diamino-par, in contrast to the situation with the other two peptides (Figure 3), suggests that the positively charged C-terminus of C-diamino-par is exposed on the surface of the membrane.

In summary, unlike many reported cases where the abilities of a peptide to induce leakage and fusion were suggested to be correlated, the results of the present study suggest that at the peptide–membrane interaction site there are different structural requirements for vesicle fusion and leakage, and therefore modulations of the peptide structure can have opposite effects on the two processes.

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## REFERENCES

Arribas, M., Blasi, J., Lazarovici, P., & Marsal, J. (1993) *J. Neurochem. 60*, 552–558.

Barrow, C. J., Nakanishi, K., & Tachibana, K. (1992) *Biochim. Biophys. Acta 1112*, 235–240.

Bentz, J., Nir, S., & Wilschut, J. (1983) *Colloids Surf. 6*, 333–363.

Bentz, J., Nir, S., & Covell, D. G. (1988) *Biophys. J. 54*, 449–462.

Bentz, J., Ellens, H., & Alford, D. (1990) *FEBS Lett. 276*, 1–5.

Burger, K. N. J., Wharton, S. A., Demel, R. A., & Verkleij, A. J. (1991) *Biochim. Biophys. Acta 1065*, 121–129.

Crane, S. E., Kanada, P., & Clements, J. E. (1991) *Virology 185*, 488–492.

Düzgünes, N. (1993) *Meth. Enzymol. 221*, 82–95.

Düzgünes, N., & Shavnin, S. A. (1992) J. Membr. Biol. 128, 71–80.

Düzgünes, N., Allen, T. M., Fedor, J., & Papahadjopoulos, D. (1987) *Biochemistry 26*, 8435–8442.

Ellens, H., Bentz, J., & Szoka, F. C. (1984) *Biochemistry 23*, 1532–1538.

Eytan, G. D., Broza, R., & Shalitin, Y. (1988) *Biochim. Biophys. Acta 937*, 387–397.

Freed, E. O., Delwart, E. L., Buchschacher, G. L., Jr., & Panganiban, A. T. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 70–74.

Fujii, G., Horvath, S., Woodward, S., Eiserling, F., & Eisenberg, D. (1992) *Protein Sci. 1*, 1454–1464.

Fujii, G., Selsted, M. E., & Eisenberg, D. (1993) *Protein Sci. 2*, 1301–1312.

Garcia, L. A. M., Aranjo, P. S., & Chaimovich, H. (1984) *Biochim. Biophys. Acta 772*, 231–234.

Glushakova, S. E., Omelyanenko, V. G., Lukashevitch, I. S., Bogdanov, A. A., Jr., Moshnikova, A. B., Kozytch, A. T., & Torchilin, V. P. (1992) *Biochim. Biophys. Acta 1110*, 202–208.

Grant, E., Jr., Beelar, T. J., Taylor K. M. P., Gable, K., & Roseman, M. A. (1992) *Biochemistry 31*, 9912–9918.

Hope, M. J., Bally, M. B., Webb, G., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55–65.

Kono, K., Kimura, S., & Imanishi, Y. (1990) *Biochemistry 29*, 3631–3637.

Kono, K., Nishii, H., & Takagishi, T. (1993) *Biochim. Biophys. Acta 1164*, 81–90.

Lazarovici, P., Primor, N., & Loew, L. M. (1986) *J. Biol. Chem. 261*, 16704–16713.

Lazarovici, P., Edwards, C., Raghunathan, G., & Guy, H. R. (1992) *J. Nat. Toxins 1*, 1–15.

Lear, J. D., & DeGrado, W. F. (1987) *J. Biol. Chem. 262*, 6500–6505.

Lee, S., Aoki, R., Oishi, O., Aoyagi, H., & Yamasaki, N. (1992) *Biochim. Biophys. Acta 1103*, 157–162.

Martin, I., Defrise-Quertain, F., Mandieau, V., Nielsen, N. M., Saermark, T., Burny, A., Brasseur, R., Ruysschaert, J.-M., & Vanderbranden, M. (1991) *Biochem. Biophys. Res. Commun. 175*, 872–879.

Martin, I., Defrise-Quertain, F., Decroly, E., Vanderbranden, M., Brasseur, R., & Ruysschaert, J.-M. (1993) *Biochim. Biophys. Acta 1145*, 124–133.

Merrifield, R. B., Vizioli, L. D., & Boman, H. G. (1982) *Biochemistry 21*, 5020–5031.

Moran, A., Korchak, Z., Moran, N., & Primor, N. (1984) in *Toxins, Drugs and Pollutants in Marine Animals* (Bolis, L., Zadunaisky, J., & Gilles, R., Eds.) pp 13–25, Springer Verlag, Berlin.

Morgan, D. G., Williamson, H., Fuller, S., & Hudson, B. (1984) *Biochim. Biophys. Acta 732*, 668–674.

Murata, M., Sugahara, Y., Takahashi, S., & Ohnishi, S. (1987a) *J. Biochem. (Tokyo) 102*, 957–962.

Murata, M., Nagayama, K., & Ohnishi, S. (1987b) *Biochemistry 26*, 4056–4062.

Murata, M., Kagiwada, S., Takahashi, S., & Ohnishi, S. (1991) *J. Biol. Chem. 266*, 14353–14358.

Murata, M., Takahashi, S., Kagiwada, S., Suzuki, A., & Ohnishi, S. (1992) *Biochemistry 31*, 1986–1992.

Nieva, J. L., Nir, S., Muga, A., Goni, F. M., & Wilschut, J. (1994) *Biochemistry 33*, 3201–3209.

Nir, S., Bentz, J., & Wilschut, J. (1980) *Biochemistry 19*, 6030–6036.

Nir, S., Bentz, J., & Düzgünes, N. (1981) *J. Colloid Interface Sci. 84*, 266–269.

Nir, S., Wilschut, J., & Bentz, J. (1982) *Biochim. Biophys. Acta 688*, 275–278.

Nir, S., Bentz, J., Wilschut, J., & Düzgünes, N. (1983) *Prog. Surf. Sci. 13*, 1–124.

Nir, S., Stegmann, T., & Wilschut, J. (1986) *Biochemistry 25*, 257–266.

Nir, S., Stutzin, A., & Pollard, H. B. (1987) *Biochim. Biophys. Acta 903*, 309–318.

Parente, R. A., Nir, S., & Szoka, F. C., Jr. (1988) *J. Biol. Chem. 263*, 4724–4730.

Parente, R. A., Nir, S., & Szoka, F. C., Jr. (1990) *Biochemistry 29*, 8720–8728.

Pouny, Y., & Shai, Y. (1992) *Biochemistry 31*, 9482–9490.

Primor, N. (1983) *J. Exp. Biol. 105*, 83–94.

Primor, N., & Zlotkin, E. (1975) *Toxicon 13*, 227–231.

Primor, N., Parness, J., & Zlotkin, E. (1978) in *Toxin: Animal, Plant and Microbial* (Rosenberg, P., Ed.) pp 539–547, Pergamon Press, New York.

Rafalski, M., Lear, J. D., & DeGrado, W. F. (1990) *Biochemistry 29*, 7917–7922.

Rafalski, M., Ortiz, A., Rockwell, A., van Ginkel, L. C., Lear, J. D., DeGrado, W. F., & Wilschut, J. (1991) *Biochemistry 30*, 10211–10220.

Rapaport, D., & Shai, Y. (1991) *J. Biol. Chem. 266*, 23769–23775.

Rapaport, D., & Shai, Y. (1992) *J. Biol. Chem. 267*, 6502–6509.

Rapaport, D., & Shai, Y. (1994) *J. Biol. Chem. 269*, 15124–15131.
Rapaport, D., Hague, G. R., Pouny, Y., & Shai, Y. (1993) *Biochemistry 32*, 3291–3297.
Renner, P., Caratsch, C. G., Waser, P. G., Lazarovici, P., & Primor, N. (1987) *Neuroscience 23*, 319–325.
Rouser, B., Siakotos, A., & Fleischer, S. (1966) *Lipids 1*, 85–86.
Shai, Y. (1994) *Toxicology 87*, 109–129.
Shai, Y., Fox, J., Caratsch, C., Shih, Y. L., Edwards, C., & Lazarovici, P. (1988) *FEBS Lett. 242*, 161–166.
Shai, Y., Bach, D., & Yanovsky, A. (1990) *J. Biol. Chem. 265*, 20202–20209.
Shai, Y., Hadari, Y. R., & Finkels, A. (1991) *J. Biol. Chem. 266*, 22346–22354.
Stegmann, T., Doms, R. W., & Helenius, A. (1989) *Annu. Rev. Biophys. Biophys. Chem. 18*, 187–211.
Struck, D., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093–4099.
Subbarao, N. K., Darente, R. A., Szoka, F. C., Jr., Nadasdi, L., & Pongracz, K. (1987) *Biochemistry 26*, 2964–2972.
Suenaga, M., Lee, S., Park, N. G., Aoyagi, H., Kato, T., Umeda, A., & Amako, K. (1989) *Biochim. Biophys. Acta 981*, 143–150.
Tournois, H., Fabrie, C. H. J. P., Burger, K. N. J., Mandersloot, J., Hilgers, P., van Dalen, H., de Gier, J., & de Kruijff, B. (1990) *Biochemistry 29*, 8297–8307.
Weinstein, J. N., Klausner, R. D., Innerarity, T., Ralston, E., & Blumenthal, R. (1981) *Biochim. Biophys. Acta 647*, 270–274.
Wharton, S. A., Martin, S. R., Ruigrok, R. W. H., Skehel, J. J., & Wiley, D. C. (1988) *J. Gen. Virol. 69*, 1847–1857.
White, J. M. (1990) *Annu. Rev. Physiol.* 52, 675–697.
Wiley, D. C., & Skehel, J. J. (1987) *Annu. Rev. Biochem. 56*, 365–394.
Wilschut, J., Düzgünes, N., Fraley, R., & Papahadjopoulos, D. (1980) *Biochemistry 19*, 6011–6021.
Wilschut, J., Düzgünes, N., & Papahadjopoulos, D. (1981) *Biochemistry 20*, 3126–3133.
Yeagle, P. L., Epand, R. M., Richardson, C. D., & Flanagan, T. D. (1991) *Biochim. Biophys. Acta 1065*, 49–53.
Yoshimura, T., Goto, Y., & Aimoto, S. (1992) *Biochemistry 31*, 6119–6126.
Zagorski, M. G., Norman, D. G., Barrow, C. J., Iwashita, T., Tachibana, K., & Patel, D. J. (1991) *Biochemistry 30*, 8009–8017.
Zimmerberg, J., Vogel, S. S., & Chernomordik, L. V. (1993) *Annu. Rev. Biophys. Biomol. Struct. 22*, 433–466.