Release of Lipid Vesicle Contents by an Antibacterial Cecropin A−Melittin Hybrid Peptide[†]

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ABSTRACT: A synthetic cecropin A(1−8)−melittin(1−18) hybrid peptide, with antimalarial and antibacterial properties, promotes leakage of aqueous contents of phospholipid vesicles, as determined by measuring the induced release of vesicle-entrapped fluorescence probes. The release of vesicle contents corresponds to an all-or-none mechanism. High molecular weight entrapped solutes (fluorescence-labeled dextrans, 20 and 4 kDa molecular mass) are also released by the peptide. This fact and the high peptide stoichiometry required for the release of vesicle contents suggest a detergent-like disruption of the bilayer. The leakage process is not related to any membrane event requiring lipid-mixing between bilayers. The peptide destabilizes both negatively and neutrally charged phospholipid vesicles. The thermal variation of the fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene-labeled vesicles is modified by the peptide. Circular dichroism and tryptophan fluorescence emission spectra reveal conformational changes in the peptide molecule upon interaction with the lipid vesicles. These changes are consistent with an increased α-helical content and a less polar environment for the single tryptophan residue of the peptide. The leakage induced in phosphatidylserine vesicles is a faster process than in phosphatidylcholine vesicles, while the peptide is more effective in releasing the contents of the latter type of vesicles. This suggests that acidic phospholipids may modulate the effect of the peptide on membranes.

Antimicrobial peptides constitute an important component of the non-adaptative immunity in pluricellular eukaryotes (Janeway, 1994; Boman, 1995; Hoffman, 1995; Maloy & Kari, 1995; White et al., 1995). Other peptides with strong antimicrobial effect are some insect toxins, such as melittin, mastoparan, and bombolitin [reviewed by Saberwal and Nagaraj (1994)], but this activity is associated with high toxicity against eukaryotic cells which prevents their putative therapeutic use in their natural form.

Despite large structural differences among groups of antibiotic peptides, a significative number of them induce membrane permeabilization to ions and other solutes (Saberwal & Nagaraj, 1994; Boman, 1995) which results in disruption of ionic gradients and bioenergetic collapse of the organism. Peptides such as defensins, cecropins, or magainins proceed by inducing leakage of water-soluble contents from liposomes (Steiner et al., 1988; Gazit et al., 1994, 1995; Wimley et al., 1994; Tytler et al., 1995), planar lipid bilayers (Christensen et al., 1987; Ohki et al., 1994), bacteria (Okada & Natori, 1984; Lehrer et al., 1989; Cociancich et al., 1993), other microorganisms (Selsted et al., 1985; Wade et al., 1990; Zasloff, 1994; Díaz-Achirica et al., 1995), as well as some transformed cells (Jaynes et al., 1989; Lichtenstein, 1991). Since all-D analogs of cecropin A and melittin (Wade et al., 1990), as well as magainins (Besalle et al., 1990), are at least as active as their all-L natural enantiomers, the requirement for a chiral receptor has been discarded in favor of a direct peptide−lipid bilayer interaction as the primary cause for killing.

In spite of the consistently demonstrated permeabilization effect of these peptides on membranes, how this is achieved remains an open question. Disagreement among authors is especially evident in the case of linear peptides, such as melittin, cecropins, or magainins, where the structural plasticity of the peptide allows for different interpretations depending on environmental conditions. Two main theories have been proposed to explain the nature of peptide−membrane interaction: (i) a detergent effect, due to the amphiphilic structure of these peptides that interact with the lipid bilayers, disrupting their organization at least temporarily and allowing leakage of the internal components; (ii) a channel formation by aggregation of several peptide molecules in the bilayer plane. Support for a detergent activity arises from permeabilization in absence of membrane potential (Steiner et al., 1988; Pouny et al., 1992), the high peptide stoichiometry required to obtain membrane permeabilization in contrast with the scarce number of molecules involved in the assembly of channels, and from structural studies where the peptide mainly adopts a parallel position in relation to the plane of the membrane (Bechinger et al., 1993; Cross & Opella, 1994; Gazit et al., 1994; Ohki et al.,

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1994). The "pore formation" mechanism is backed up by the discrete conductance obtained in planar lipid bilayers, cooperative effects among the peptides (Vaz-Gomes et al., 1993), and perpendicular positioning of the peptides in relation to the membrane (Ludtke et al., 1994; Mchaourab et al., 1994; Smith et al., 1994). More pragmatic interpretations deal with either the existence of two populations of peptides where the perpendicular one is enriched only at high peptide concentration (Ludtke et al., 1994) and only after a "carpet-like" membrane covering is achieved by horizontally disposed peptides (Pouny et al., 1992), or the formation of a transitory pore, dependent on the translocation of peptides from the outer into the inner leaflet of the vesicle (Matsuzaki et al., 1995).

Another controversial point is how the leakage is produced, either by a graded mechanism as happens with several toxins, where it takes place partially in most of the vesicle population (Grant et al., 1992; Matsuzaki et al., 1995), or in an all-or-none mechanism (Parente et al., 1990; Schwarz et al., 1992; Ostolaza et al., 1993; Wimley et al., 1994), where leakage is complete for some vesicles whereas the rest remain intact.

The availability of melittin, magainins, and cecropin by chemical synthesis has allowed extensive use of synthetic analogs to define structure−activity relationships in order to design more effective antibiotic structures (Merrifield et al., 1994; Maloy & Kari, 1995). Cecropins are basic peptides with 35−39 residues and active against both $Gram^+$ and $Gram^-$ bacteria [see Boman et al., (1991) for a review]. All of them display a strong cationic N-terminal region and a C-terminal portion enriched in hydrophobic amino acids. Melittin, another basic peptide, is a major component of the honey bee venom [see Dempsey (1990) and Sansom (1991) for reviews]. Compared to cecropin, melittin has an opposite sequence polarity with a hydrophobic N-terminal region and a basic carboxyl end, responsible for the hemolytic activity of the molecule. Hybrid peptides formed by the N-terminal region of cecropin followed by the hydrophobic N-region from melittin display a higher antibiotic activity and a broader range of antibacterial action than the parental cecropin A, and they were devoid of hemolytic activity (Boman et al., 1989); peptides with only 15 residues retain a substantial antibiotic effect (Andreu et al., 1992). The CA(1−8)−M(1−18) peptide formed by the first eight residues of cecropin A followed by the first eighteen residues from melittin induced uncoupling and permeabilization of rat liver mitochondria for both charged and neutral solutes, either in the presence or absence of membrane potential, suggesting a detergent-like effect (Díaz-Achirica et al., 1994). The association of CA(1−8)−M(1−18) peptide with mitochondria is very fast and does not allow a good equilibration in the entire mitochondrial population. This peptide forms channels in planar lipid bilayers (Wade et al., 1990), kills the protozoal parasite *Leishmania* (Díaz-Achirica et al., 1995), shows antimalarial action (Wade et al., 1990), and permeabilizes both outer and cytoplasmic membranes in $Gram^-$ bacteria (Piers & Hancock, 1994; Piers et al., 1994). The CA(1−8)−M(1−18) peptide is more effective than the shortened analogs on a molar basis against *Leishmania* (Díaz-Achirica et al., 1995). In the present work, we have studied the effect of this peptide on lipid vesicles, as a simple model of biological membranes, in order to explain its action at the molecular level.

EXPERIMENTAL PROCEDURES

Materials. Bovine brain phosphatidylserine (PS),[1] egg phosphatidylcholine (PC), *N*-(7-nitro-2-(1,3-benzoxadiazol)-4-yl)dimyristoyl phosphatidylethanolamine (NBD-PE), and *N*-(lissamine rhodamine B sulphonyl)diacyl phosphatidylethanolamine (Rh-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). 8-Aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and *N*,*N*′-*p*-xylenebispyridinium bromide (DPX) were purchased from Molecular Probes (Eugene, OR). Fluorescein isothiocyanate (FITC)-derivatized dextrans FD-20 and FD-4 were from Sigma (St. Louis, MO). All other reagents were of analytical grade. Buffers were prepared in Milli-Q water (Millipore, Millford, MA).

Peptide Synthesis. The CA(1−8)−M(1−18) peptide was synthesized by the solid-phase method (Merrifield, 1986) as previously described (Andreu et al., 1992) using BOC-chemistry protocols on *p*-methylbenzhydrylamine resin. The peptide was deprotected and cleaved from the resin by HF acidolysis and purified by reverse-phase chromatography on a C_{18} column, as described. The amino acid sequence of the peptide is KWKLFKKIGIGAVLKVLTTGLPALIS-NH_2.

Circular Dichroism and Fluorescence Spectroscopy. Circular dichroism (CD) spectra were obtained on a Jobin Yvon Mark III dichrograph (Longjumeau, France) fitted with a 250 W xenon lamp. The spectra were recorded at 0.2 nm/s scanning speed. Samples were analyzed in either 0.10 or 0.01 cm optical path cells. CD results were expressed in units of degree $\times$ cm^2 $\times$ (dmol of amino acid residue).$^{-1}$ These values were calculated on the basis of 107 as the mean residue weight for this peptide. Fluorescence emission spectra of the peptide were recorded at 1 nm/s scanning rate on a SLM Aminco 8000 spectrofluorimeter (Urbana, IL), for excitation at 275 nm wavelength. The optical path of the cells was 0.2 cm. The slit widths for both excitation and emission beams were 4 nm. Glan−Thompson (90°/0°) polarizers were used to avoid any potential light-scattering interference on the measurements. Fluorescence emission was expressed in arbitrary units. The peptide concentration was determined by absorbance measurements at 280 nm based on the extinction coefficient at this wavelength of the single tryptophan residue of the molecule.

Leakage Measurements. Leakage of vesicle aqueous contents was measured by using the ANTS/DPX assay as previously described by Ellens et al. (1985). Vesicles containing 12.5 mM ANTS, 45 mM DPX, 20 mM NaCl, 10 mM Tris, pH 7.4, were prepared by five cycles of freezing−thawing and further extrusion through two stacked 0.1 μm or 0.4 μm (pore diameter) polycarbonate membranes (Nucleopore, Costar, Cambridge, MA) in an Extruder (Lipex Biomembranes Inc., Vancouver, Canada). Vesicles were separated from unencapsulated materials on Sephadex G-75 (Pharmacia, Uppsala, Sweden) by using Tris buffer (10 mM Tris, 0.1 M NaCl, 1 mM EDTA, pH 7.4) as elution buffer. In a typical assay a small volume of peptide in the Tris buffer was added to the vesicles at zero time. The variation of fluorescence intensity was measured through a 3−68 Corning

[1] Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; CD, circular dichroism; DMPC, dimyristoyl phosphatidylcholine; DMPS, dimyristoyl phosphatidylserine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPX, *N*,*N*′-*p*-xylenebispyridinium bromide; FITC, fluorescein isothiocyanate; PC, egg phosphatidylcholine; PS, bovine brain phosphatidylserine.

cutoff filter (>530 nm) upon excitation at 386 nm on a SLM Aminco 8000 spectrofluorimeter. The 0% and 100% leakages were taken as the fluorescence intensity of the corresponding vesicle suspension before and after addition of Triton X-100 at a final concentration of 0.5%, respectively. It has been described that the mode of mixing vesicle and peptide solutions in the leakage assays can affect the kinetics of the process (Polozov et al., 1994). Thus, the same amount of peptide can produce different leakage curves depending on the peptide concentration of the initial solution. In the present case, a single peptide stock solution was used and neither the stirring conditions nor the sample volume were altered along the study.

The release of FITC-dextrans was analyzed essentially as described (Ostolaza et al., 1993). FD-20 (MW_{av} = 19 600) and FD-4 (MW_{av} = 4300) were encapsulated in PC or PS vesicles at self-quenching concentrations (4 and 20 mM, respectively) in 5 mM Mops buffer, pH 7.5, containing 40 mM NaCl. After five cycles of freezing−thawing and five additional cycles of extrusion through two polycarbonate filters (0.4 μm pore diameter), unencapsulated material was separated from the vesicles by gel filtration on a Sephacryl S-300 HR column equilibrated in 5 mM Mops buffer, pH 7.5, containing 65 mM NaCl. The fluoresence emission of fluorescein was measured through a 3-68 Corning cutoff filter (>530 nm) for excitation at 465 nm. Percentages of leakage were calculated as above.

Other Analytical Procedures. Other vesicles were formed by hydrating a dry lipid film with Mops buffer (50 mM Mops, pH 7.0, containing 0.1 M NaCl and 1 mM EDTA) for 60 min at 37 °C (Mancheño et al., 1995). The lipid suspension was subjected to five cycles of extrusion through two stacked 0.1 μm (pore diameter) polycarbonate membranes. Phospholipid concentration was determined as described (Barlett, 1959). Intermixing of membrane lipids was analyzed by considering fluorescence energy transfer assays (Struck et al., 1981). A vesicle population of egg PC or brain PS containing 1% NBD-PE (donor) and 0.6% Rh-PE (acceptor) was mixed with unlabeled vesicles at 1:9 molar ratio (75 μM final lipid concentration) in the Mops buffer. At zero time, a small volume of peptide in the same buffer was added. A decrease in the donor-to-acceptor fluorescence energy transfer indicates lipid-mixing between membranes. Other experimental details were previously described (Mancheño et al., 1994).

Measurements of the fluorescence depolarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) (Aldrich, Milwaukee, WI) were made on an SLM Aminco 8000 spectrofluorimeter equipped with 10 mm Glan−Thompson polarizers. Labeling of the vesicles with DPH was carried out as previously described (Gavilanes et al., 1985). Peptide−vesicle mixtures were incubated for 1 h above the transition temperature of the phospholipid and later cooled down. The fluorescence emission was measured at 425 nm for excitation at 365 nm, after equilibration of the samples at each required temperature.

The binding of the peptide to the vesicles has been analyzed by measuring the tryptophan fluorescence emission of the free peptide in the supernatant obtained by centrifugation (160 000*g*/20 min, Airfuge Beckman) of peptide−vesicle mixtures at different peptide/lipid ratios. A calibration plot, fluorescence emission *versus* peptide concentration (determined by amino acid analysis of acid-hydrolyzed peptide samples), was used for the above calculations. The binding of the peptide satisfied a partition relation. For 100 μM lipid concentration the constant fraction of peptide bound is 0.9 and 0.6 for PS and PC vesicles, respectively, along the range of concentrations used in this study. The binding data have been analyzed as partition equilibrium (Schwarz et al., 1986; Beschiaschvili & Seelig, 1990; Rapaport & Shai, 1991), and the calculated surface partition coefficients are 1.5×10^5 and 2.5×10^4 M^{-1} for PS and PC vesicles, respectively.

RESULTS

The CA(1−8)−M(1−18) peptide promotes permeabilization of both negatively and neutrally charged vesicles. Negative vesicles composed of bovine brain phosphatidylserine (PS), an acidic phospholipid expected to interact with the cationic peptide, and neutral vesicles of egg phosphatidylcholine (PC), a phospholipid very abundant within membranes, were used as model membrane systems. The release of intravesicular aqueous contents produced by this peptide would not be related to any membrane fusion event, since no lipid-mixing between membranes has been observed for any of these two types of phospholipid vesicles in the corresponding peptide concentration ranges, based on a classical assay of resonance energy transfer between a donor (NBD) and an acceptor (Rh) fluorescence probes (see Experimental Procedures).

Figure 1 shows the representative kinetics of the leakage of the ANTS/DPX system encapsulated in PS and PC vesicles induced by the peptide. DPX quenches the fluorescence emission of the ANTS probe when both are coencapsulated, and dequenching is produced upon dilution of both probe and quencher in the extravesicular medium due to the permeabilization of the membrane. Thus, the leakage of aqueous contents can be analyzed by measuring the fluorescence emission increase of ANTS. The clearly distinct shapes of these two kinetics are not dependent on the peptide concentration employed, although the parameters of the curves obviously varied. The leakage induced in PS vesicles is a fast process, with a maximum leakage value reached within a few seconds (ca. 10 s). In contrast, the leakage induced in PC vesicles exhibits a complex kinetics: a fast (on the order of seconds) is followed by a slow (taking several minutes) process (Figure 1), and the maximum leakage requires up to about 1 h at the lowest concentration of the peptide studied. No significant leakage is observed throughout this period of time in the absence of peptide. A very similar kinetics has been reported for the leakage of carboxyfluorescein induced by a synthetic peptide analog of pardaxin, another antibacterial peptide, on egg PC vesicles (Saberwall & Nagaraj, 1993). Also, the antimicrobial peptide magainin 2a causes the release of carboxyfluorescein from PS vesicles through a process exhibiting a rapid initial phase, about 100 s, followed by a much slower phase (Grant et al., 1992).

The dependence of the extent of leakage on the peptide/phospholipid molar ratio (R_i) is given in Figure 2. The % leakage represents the percentage of the ANTS fluorescence increase related to that produced by the detergent Triton X-100 which produces a complete lysis of the vesicles. It can be observed that the maximum leakage is obtained at about $R_i = 0.025$ for PS vesicles (Figure 2A). In general, the leakage of aqueous contents from lipid vesicles can be

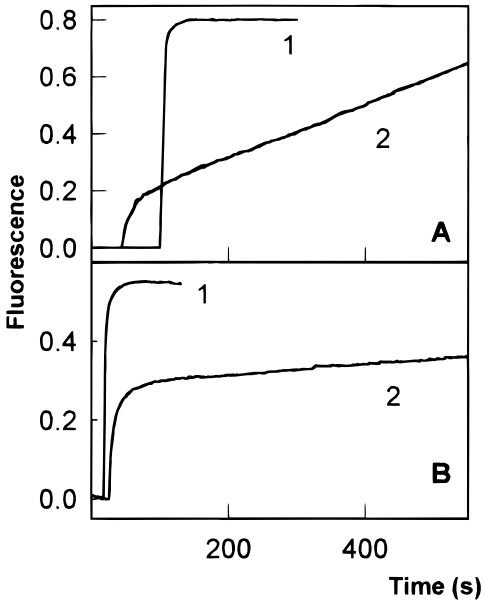

FIGURE 1: Assays for vesicle leakage induced by the CA(1−8)−M(1−18)-NH_2 peptide. (A) Release of aqueous contents (ANTS/DPX) from (1) bovine brain PS and (2) egg PC vesicles (prepared by extrusion through 100 nm pore diameter membranes). The peptide and lipid concentrations were 4.4 and 105 μM, respectively. Fluorescence is expressed in arbitrary units. Fluorescence emission of ANTS at >530 nm cutoff for excitation at 386 nm was measured at 25 °C. The fluorescence unit corresponds to the value obtained after addition of Triton X-100 (0.5% final concentration) that corresponds to the 100% leakage. Both kinetic plots are shifted to facilitate the comparison. (B) Leakage of FD-20 dextran induced by the CA(1−8)−M(1−18)-NH_2 peptide encapsulated in (1) PS and (2) PC vesicles (prepared by extrusion through 400 nm pore diameter membranes). Both kinetic plots correspond to a peptide/lipid molar ratio of 0.025. Fluorescence is expressed in arbitrary units (the unit corresponds to the fluorescence increase promoted by the addition of Triton X-100, 0.5% final concentration).

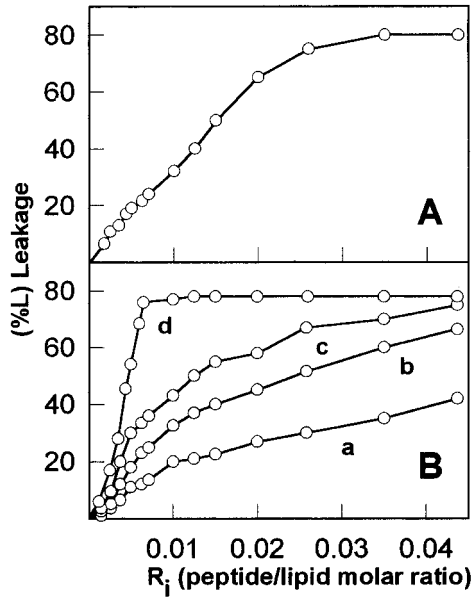

FIGURE 2: Effect of CA(1−8)−M(1−18)-NH_2 peptide on the leakage of aqueous contents (ANTS/DPX) of (A) bovine brain PS and (B) egg PC vesicles. Vesicles have been prepared by extrusion through 100 nm pore diameter membranes. Leakage values (%*L*) are expressed as percentages of the release of aqueous contents promoted by 0.5% Triton X-100 final concentration. Measurements have been performed at a 105 μM PS vesicle concentration for all the assays. Leakage values are referred to the peptide/PS molar ratio (R_i) for each assay. (B) Leakage values obtained at (a) 5 min; (b) 10 min; (c) 15 min; and (d) 50 min.

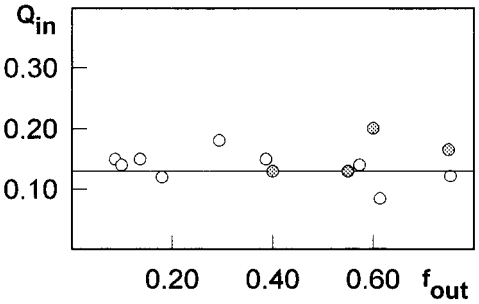

FIGURE 3: Plot of the internal quenching (Q_{in}) of vesicle (ANTS/DPX) contents as a function of the fraction of contents released (f_{out}). The quenching of the ANTS fluorescence emission by DPX inside the vesicles of bovine brain PS (empty circles) and egg PC (shadow circles) after addition of the CA(1−8)−M(1−18)-NH_2 peptide was determined by DPX titration as described. The initial internal quenching of the ANTS fluorescence emission is given by the horizontal line for both PS and PC vesicles.

adjusted to either an all-or-none or a graded mechanism. The first possibility implies that a percentage of vesicles releases all their contents, whereas the second one involves a partial release in the overall vesicle population, that would be explained by the formation of vesicle pores whose lifetime is not long enough to deplete the intravesicular content. The fluorescence of ANTS inside the intact vesicles would be quenched at the same initial extent, according to the first mechanism; but the internal quenching by DPX would be decreased as the leakage occurs by dilution of probe and quencher inside the vesicles if a graded leakage is produced. Both possibilities are compatible with the results in Figure 2A since partial leakage is observed in a wide range of peptide concentrations. To distinguish between both possibilities, we have studied the internal quenching of the ANTS fluorescence as a function of the released ANTS fluorescence, based on the experimental approach described by Wimley et al. (1994). Results are given in Figure 3. It can be observed that the fluorescence of ANTS inside the vesicles, either PS or PC, remains quenched at the same extent regardless of the fraction of fluorescence released. This indicates that the leakage produced by the peptide corresponds to an all-or-none mechanism.

The dependence of the % leakage promoted by the CA(1−8)−M(1−18) peptide on PC vesicles with the peptide/lipid molar ratio has been also studied (Figure 2B). As indicated above, the leakage is a slower process than for PS vesicles. Therefore, the % leakage at different times is also given in this figure. Plot d in Figure 2B reveals that the maximum leakage is obtained for R_i values higher than 0.005. A minimum R_i value is required to detect leakage (about $R_i = 0.0020$) and maximum leakage is observed at lower values of R_i than for PS vesicles.

The effect of the peptide on the leakage of PC and PS vesicles has been also studied by varying the lipid concentration (from 30 to 300 μM) at a constant peptide concentration. The results are coincident with those described above when both are expressed in terms of peptide/lipid molar ratios.

The above results have been obtained for lipid vesicles prepared by extrusion through 100 nm pore diameter membranes. We have also performed the same experiments for larger vesicles, obtained by using 400 nm pore diameter membranes. For unilamellar vesicles, the number of vesicles per mole of lipid depends upon the vesicle diameter (Szoka

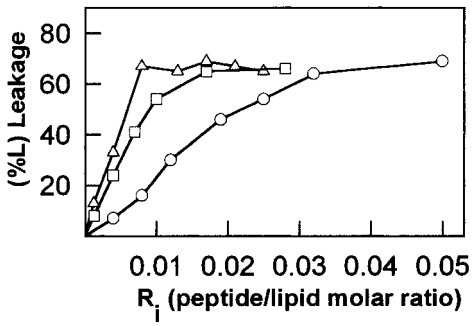

FIGURE 4: Effect of the CA(1−8)−M(1−18)-NH_2 peptide on the leakage of aqueous contents (ANTS/DPX) of (△) egg PC, (○) bovine brain PS, and (□) 80% PC/20% PS vesicles prepared by extrusion through 400 nm pore diameter filters. Leakage values (%*L*) are expressed as percentages of the release of aqueous contents promoted by Triton X-100 (0.5% final concentration). Leakage values are referred to the peptide/PC molar ratio (R_i) for each assay.

& Papahadjopoulos, 1980), and thus the peptide to vesicle ratio can be altered at a constant lipid/peptide ratio by changing the diameter of the vesicles. The results (Figure 4) are highly similar to those observed with smaller vesicles. Thus, the maximum leakage is observed at about the same R_i for both vesicle sizes of either PC or PS. In addition, the dependence of the initial rates (expressed as % leakage per second) on the peptide/lipid ratio is almost coincident for the vesicles of both sizes (data not shown). Considering that the two sets of experiments have been carried out at the same total phospholipid concentration, this coincidence of results indicates that the leakage is produced once a given peptide/lipid ratio, and not a peptide/vesicle ratio, is achieved.

Self-quenched fluorescence-labeled high molecular weight dextrans (FD-20 and FD-4, 19.6 and 4.3 kDa average molecular masses, respectively) have been also encapsulated in PS and PC vesicles in order to analyze the dependence of the leakage promoted by the peptide on the molecular weight of the entrapped solutes. Results in Table 1 demonstrate that these large molecular weight solutes are released from both types of vesicles at % leakage values similar to those obtained for ANTS/DPX-containing vesicles (Figure 4), supporting the notion of a detergent-like mode of action for the peptide. Representative kinetic plots of this release are given in Figure 1B.

We have also studied the effect of the CAM peptide on vesicles composed of 80% PC/20% PS. Results are also given in Figure 4. The R_i values for 50% of the maximum leakage observed are 0.004, 0.006, and 0.015 for PC, 80% PC/20% PS, and PS vesicles, respectively. It can be observed that the presence of PS results in a higher peptide concentration required for maximum leakage as it may be expected from the experiments performed with vesicles of each type of pure phospholipid.

Addition of PS vesicles containing the ANTS/DPX system to empty PS vesicles/peptide mixtures (3:1 empty-to-loaded vesicle ratio; 105 μM in the loaded vesicles), previously incubated for a period of time as that required for completion of leakage, at equilibrium does not result in any leakage of the fluorescence probes. Therefore, the peptide does not equilibrate among these vesicles. However, when the same experiment is performed for PC vesicles, release of ANTS is detected. Successive additions of either PS or PC vesicles to PC or PS vesicles previously incubated with the peptide corroborate the above conclusions. Leakage is observed

Table 1: Release of FITC-Labeled FD-20 and FD-4 Dextrans[a]

	(PS vesicles)			(PC vesicles)		
R_i	FD-20	FD-4	ANTS	FD-20	FD-4	ANTS
0.025	55	60	54	52	53	65
0.004	8	10	7	25	35	33

[a] Values are expressed as percentages of leakage (see Experimental Procedures) determined after equilibration of the fluorescence signal (10 min and 1 h for PS and PC vesicles, respectively). R_i is the peptide/lipid molar ratio. The results correspond to vesicles obtained by extrusion through 400 nm pore diameter membranes (see Figure 4 for ANTS leakage values).

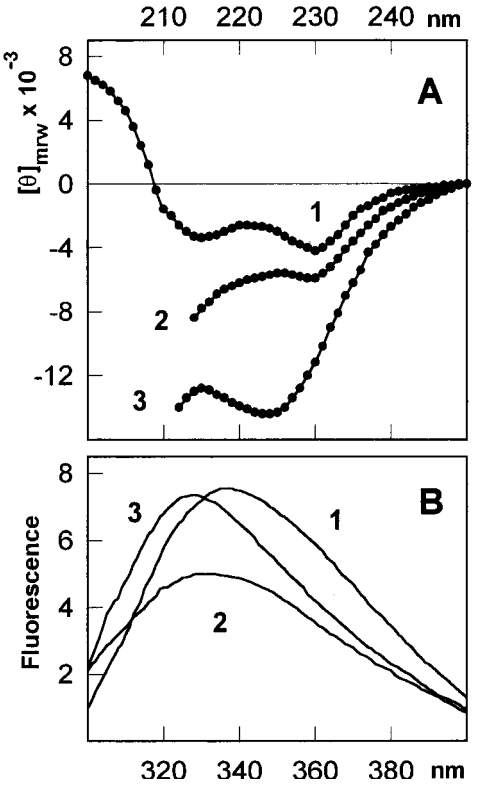

FIGURE 5: Circular dichroism (A) and fluorescence emission (B) spectra of the CA(1−8)−M(1−18)-NH_2 peptide (1) and in the presence of PC (2), and PS (3) vesicles at an R_i (peptide/lipid molar ratio) of 0.006 (the peptide concentration was 20 μM). $|\Theta|_{mrw}$, mean residue weight ellipticities, are expressed in units of degree $\times$ cm^2 $\times$ dmol^{-1}. *F*, fluorescence emission intensity is expressed in arbitrary units and was obtained for excitation at 275 nm. Samples were prepared in 50 mM Mops buffer, pH 7.0, containing 0.1 M NaCl and 1 mM EDTA. Spectra were recorded after incubation of the peptide/vesicle mixtures for 1 h at 25 °C. The constant fraction of peptide bound is 0.9 and 0.6 for PS and PC vesicles, respectively (see Experimental Procedures).

when PS vesicles are added to previously equilibrated PC vesicles/peptide mixtures, while the release of the ANTS/DPX system is not detected when PC vesicles are added to PS vesicles/peptide mixtures at equilibrium.

Although the CA(1−8)−M(1−18) peptide promotes leakage of aqueous contents of PC, PS, and PC−PS vesicles, the differences observed for neutrally and negatively charged vesicles, in terms of both kinetics and dependence on peptide/lipid molar ratio, may suggest a different mode of interaction. This possibility may result in a different structure for the peptide−vesicle complex. Therefore, we have studied the structure of the peptide in each type of peptide/vesicle mixtures by considering the fluorescence emission and circular dichroism properties of the peptide in the presence

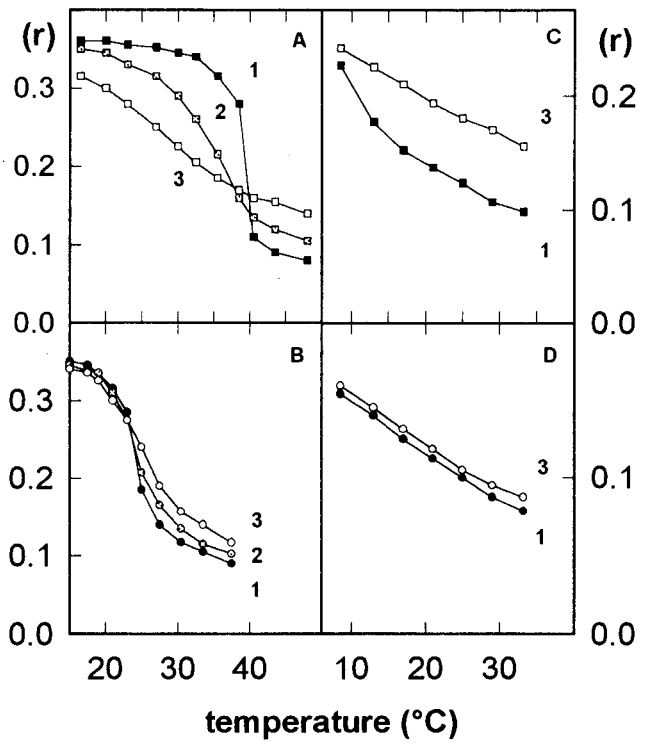

FIGURE 6: Effect of the CA(1−8)−M(1−18)−NH_2 peptide on the thermotropic behavior of DMPS (A), DMPC (B), PS (C), and PC (D) vesicles. Anisotropy of DPH (*r*) was measured at different temperatures. Samples (120 μM phospholipid concentration) were in 50 mM Mops buffer, pH 7.0, containing 0.1 M NaCl and 1 mM EDTA, and different peptide/lipid molar ratios were studied: (1) 0; (2) 1:40; and (3) 1:10.

of PC and PS vesicles. Figure 5 shows the far-UV CD (Figure 5A) and tryptophan fluorescence emission (Figure 5B) spectra of the peptide upon interaction with PC and PS vesicles. These promote drastic changes in the spectral properties of the peptide. The effect of the vesicles would result in an increased α-helical content according to the CD results in the peptide bond region. In addition, the single tryptophan residue of the peptide would be located in a less polar environment upon interaction with the lipids, on the basis of the blue-shift observed in the Trp fluorescence emission maximum (about 10 and 5 nm for PS and PC, respectively). These two changes are more pronounced in the presence of PS vesicles, in terms of absolute value of the ellipticity at 222 nm and blue-shift of the Trp fluorescence emission. The slow kinetics displayed by the leakage of aqueous contents from PC vesicles is not correlated with the time course of the spectral changes observed. In fact, the spectra shown in Figure 5 do not show any modification during incubation of the peptide−vesicle mixtures up to 1 h, the largest period of time required for maximum leakage when PC vesicles are considered.

Finally, the effect of the peptide on the thermotropic behaviour of DMPC and DMPS has been also studied. Results are summarized in Figure 6. The peptide decreases the amplitude of the phospholipid phase transition, the effect on DMPS vesicles also being more pronounced. In this latter case, the change may reflect the abolition of the characteristic phase transition of the bilayers at the peptide/lipid molar ratio at which maximum leakage is detected (Figure 6A). We have also studied the effect of the peptide on the thermal variation of the fluorescence anisotropy (*r*) of DPH-labeled PC and PS vesicles. The results are given in Figure 6C and D, respectively. In both cases the peptide increases the *r* value, and the increase is also higher for PS than PC vesicles.

DISCUSSION

The results we have obtained demonstrate that the CA-(1−8)−M(1−18) peptide promotes the release of lipid vesicle contents, a process not related to any fusion of bilayers since no lipid-mixing has been detected. The studies performed clearly suggest that the peptide acts through an *all-or-none mechanism*, the % leakage being the percentage of destabilized vesicles that have completely released their contents. The similar characteristics observed for the leakage of high molecular weight dextrans and ANTS/DPX suggest that pores are not involved in the process. This fact and the high peptide stoichiometry required for leakage suggest a *detergent-like* effect.

The peptide destabilizes bilayers of both neutrally charged and negatively charged phospholipids. However, the leakage promoted by the peptide on PS and PC vesicles shows very different features: while in PS vesicles the leakage occurs at the order of seconds, it takes minutes in PC vesicles and, in this latter case, an amount of peptide around 5-fold smaller is required for maximum leakage. Thus, the peptide promotes a faster but less efficient leakage in PS than in PC vesicles (considering effectiveness in terms of amount of peptide required to produce maximum leakage; a low amount corresponds to a high effectiveness).

It is conceivable that the effect of the peptide responds to the following scheme: (i) binding to the membrane; (ii) membrane destabilization, and stabilization of the destabilized peptide−lipid complex. This last process may respond to different possibilities, namely, formation of a hole or any other leaky structure. Regarding to the binding of the peptide, it can be described according to a partition equilibrium, the corresponding coefficient being higher for PS than for PC vesicles. This may explain why the leakage was more effective in PC than in PS vesicles, since the smaller partition coefficient would allow equilibration of the bound peptide among the latter type of vesicles. On the contrary, such an equilibration of the peptide among PC vesicles would result in a slower leakage; the slow kinetic component would arise from the destabilization extended to other vesicles during peptide equilibration. This would explain that a further addition of either PS or PC vesicles to PC−peptide mixtures at equilibrium resulted in an additional leakage that did not occur with PS vesicles. Incorporation of PS into PC vesicles reduces the effectiveness of the peptide to produce leakage, which would also support such a notion. In this regard, it has been proposed a different mechanism for the binding of melittin (one of the parent peptides) to negatively and neutrally charged lipid vesicles (Ohki et al., 1994; Monette & Lafleur, 1995). When melittin interacts with negatively charged membranes, it may be electrostatically adsorbed to the membrane, while when interacting with neutrally charged membranes, it may be adsorbed in hydrophobic interaction (Stanislawski & Ruterjans, 1987; Altenbach et al., 1989; Frey & Tamm, 1991; Ohki et al., 1994). Recently, significant differences between a cationic cecropin A−melittin peptide and its anionic per-succinyl derivative in their interaction with neutrally charged PC liposomes have been reported and discussed also on electrostatic terms (Fernández et al., 1994). It must be also

remembered that acidic phospholipids appear to inhibit the lytic activity of melittin (Portlock et al., 1990; Benachir & Lafleur, 1995; Monette & Lafleur, 1995), the ionic properties of which should not be far from those of the CA(1−8)−M(1−18) hybrid, since the four basic residues at the eight amino acid region of C-terminal melittin are comparable to the other four from the cecropin A part in the N-terminal of the hybrid peptide.

The differences observed when comparing PC and PS vesicles may be related to the formation of a different final equilibrium structure. In this regard, the effect of melittin on membranes strongly depends on the nature of the lipid component. Thus, melittin produces micellization of pure saturated phosphatidylcholine bilayers—although the resulting small comicelles refuse into bilayers upon heating above the phospholipid phase transition (Dufourc et al., 1986)—and formation of inverted phases upon interaction with phosphatidylserine (Batenburg et al., 1987). The results from CD and fluorescence emission spectroscopies as well as those from the analysis of the thermal behavior of the lipid may support the above possibility. However, these results can be also explained by the observed different partition coefficient of the peptide in PC and PS vesicles.

Finally, it has been demonstrated that the CA(1−8)−M(1−18) peptide permeabilizes the mitochondrial inner membrane at concentrations already shown to be bactericidal (Díaz-Achirica et al., 1994). At the lower peptide concentrations a limited population of mitochondria is affected, thus indicating that the peptide does not fully equilibrate within the entire system. The negatively charged mitochondrial membranes thus behave as those of PS vesicles. Indeed, the kinetics of mitochondrial permeabilization suggests a fast association of the peptide (Díaz-Achirica et al., 1994), as it also occurs for the PS membranes. The addition of the polyanion heparin resulted in a slower permeabilization process of the mitochondrial membranes but the entire mitochondrial population was affected (Díaz-Achirica et al., 1994). This effect was explained by a modification of the peptide/mitochondrial membrane association. It is remarkable that this is the behavior observed when the peptide acts on PC vesicles. Heparin would decrease the effective association of the peptide to the membrane and would allow a more homogeneous distribution of the peptide in the system, as we have discussed when comparing PS and PC vesicles.

To summarize, the CA(1−8)−M(1−18) peptide permeabilizes membranes. The leakage occurs through an all-or-none mechanism and does not appear to involve the formation of discrete pores. The characteristics of the process (effectiveness and rate) are dependent on the charge properties of the membrane. When the biological targets of this peptide are considered, the leakage rate may be more relevant in terms of its bactericidal action. A slow although efficient (requiring a small amount of peptide) effect may allow some repairing mechanisms to act, which could not act if the process is fast as in negatively charged membranes. In this regard, it is important to note that lipopolysaccharide, a polyanion, is one of the main components of the membrane playing an important role in the interaction of antibiotic peptides with Gram-negative bacteria (Rana et al., 1991).

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