The Concentration-Dependent Membrane Activity of Cecropin A[†]

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ABSTRACT: Cecropin A is a naturally occurring, linear, cationic, 37-residue antimicrobial peptide. The precise mechanism by which it kills bacteria is not known, but its site of action is believed to be the cell membrane. To investigate the nature of its membrane activity, we examined the ability of cecropin A to alter membrane permeability in synthetic lipid vesicles and in Gram-negative bacteria. Cecropin A exerted distinctly different types of membrane activity depending on its concentration. In synthetic lipid vesicles, cecropin A dissipated transmembrane electrochemical ion gradients at relatively low concentrations, but much higher concentrations were required to release an encapsulated fluorescent probe. Cecropin A dissipated ion gradients whether or not the vesicle membranes contained anionic lipid, although the presence of anionic lipid dramatically increased peptide binding, and modestly increased the release of an encapsulated probe. Cholesterol did not prevent the dissipation of ion gradients by low concentrations of peptide, but it did inhibit release of the encapsulated probe by high concentrations of peptide. At the highest concentrations examined, cecropin A remained monomeric in solution, and did not aggregate, lyse, or otherwise alter vesicle size. In Gram-negative bacteria, cecropin A was potently bactericidal at concentrations which dissipated ion gradients in lipid vesicles, but much higher concentrations were required to cause the release of cytoplasmic contents. These findings point to the conclusion that cecropin A kills bacteria by dissipating transmembrane electrochemical ion gradients. They weigh against theories comparing the antimicrobial activity of cecropin A to the release of encapsulated probes from lipid vesicles, and against roles for cholesterol or anionic lipid headgroups in the selectivity of peptide action against bacteria.

Insects produce a wide variety of polypeptides that exhibit potent *in vivo* and *in vitro* antimicrobial activity (Hoffmann, 1995). Among the most extensively studied antimicrobial polypeptides from insects are the "cecropins" from *Hyalophora cecropia* (Hultmark et al., 1980). The larvae of this moth produce at least three different cecropins, designated types A, B, and D; all are linear basic polypeptides composed of 35−37 residues sharing 62−65% primary sequence homology (Boman & Steiner, 1981; Steiner et al., 1981; Hoffmann, 1981). Subsequent investigations have since identified numerous polypeptides in various animals which are now classified as cecropins, as well as many other polypeptides with functional similarities but distinctive structural features (Boman, 1995). All of these polypeptides are composed exclusively of ordinary L-amino acids, and they differ in this respect from antibiotics of microbial origin such as gramicidin and alamethicin.

Despite numerous studies on various antimicrobial peptides, no definitive explanation has emerged for their mechanism of antimicrobial action, or for the basis of their selective action against bacteria. For antimicrobial peptides in general, the preponderance of data suggest that they cause an increase in membrane permeability, in some cases to ions, in other cases to much larger molecules (Duclohier et al., 1989; Lehrer et al., 1989; Diaz-Achirica et al., 1994; Wimley et al., 1994; Mancheño et al., 1996). The available data also suggest that the presence of cholesterol and the relative paucity of anionic lipids in the outer surface of host cell membranes are protective against this permeabilizing activity (Christensen et al., 1988; Mchaourab et al., 1994; Matsuzaki et al., 1995). For the cecropins, the most detailed information available to date has been provided by Christensen et al. (1988) in an electrophysiological study of their action on planar lipid membranes. They showed that various cecropins and cecropin analogues initially formed weakly selective ion channels, and then destabilized the membranes, leading to complete electrical breakdown. The presence of cholesterol in these preparations inhibited both channel formation and subsequent destabilization.

There is no clear consensus on the mechanism by which membrane active peptides bring about these changes. Mancheño et al. (1996) studied a hybrid peptide composed of 8 residues from cecropin A, and 18 residues from melittin, showing that it underwent conformational changes upon binding to lipid vesicles, and caused them to leak moderately-sized fluorescence probes. Matsuzaki et al. (1995) studied erythrocyte hemolysis and the release of a fluorescence probe from vesicles by magainin-2, concluding that erythrocytes were protected from magainin-induced damage by a lack of acidic lipids in the outer leaflet of their membranes, by a relatively low transmembrane potential, and by the presence of cholesterol. Tytler et al. (1995) link the protective effect

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of cholesterol to an interaction between cholesterol and a Glu residue in model amphipathic helical peptides. Gazit et al. (1995) studied a mammalian cecropin, suggesting that it does not aggregate on the membrane surface, but causes permeabilization by forming a carpet-like monolayer of peptide on the membrane surface.

These studies notwithstanding, a definitive mechanistic understanding of the activity and selectivity of antimicrobial peptides still eludes us. This is partly due to the difficulty of comparing different peptides and different lipid mixtures in various studies, and partly due to the limited types of measurement techniques that have been applied. Therefore, we have employed a variety of techniques in combination to study cecropin A under conditions designed to better understand the antimicrobial activity and selectivity of cecropin A.

MATERIALS AND METHODS

Materials. Cecropin A was synthesized by the Peptide Core Facility at the Mayo Clinic using ordinary solid-phase methods, and was verified to have a molecular mass of 3933 daltons by mass spectrometry, appropriate for the sequence:

+KWKLFKKIEK VGQNIRDGII
KAGPAVAVVG GATQIAK -$CONH_2$

Cholesterol and the lipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (POPA) were obtained from Avanti Polar Lipids (Alabaster, AL). Calcein, 3,3′-dipropylthiadicarbocyanine iodide [$DiSC_3(5)$], 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), and *p*-xylenebis(pyridinium bromide) (DPX) were obtained from Molecular Probes (Eugene, OR); octaethylene glycol dodecyl ether ($C_{12}E_8$) was supplied by Calbiochem (San Diego, CA); valinomycin and 3-(*N*-morpholino)propanesulfonic acid (MOPS) were purchased from Fluka (Uppsala, Sweden). Solvents were HPLC grade or better, and all water used was glass-distilled. Peptide concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, IL) with an albumin standard. The concentrations determined in this manner agreed closely with those determined by assuming an extinction coefficient of 5700 M^{-1} at 280 nm for the single tryptophan residue in cecropin A.

Analytical Ultracentifugation. Sedimentation equilibrium analysis was performed using a Beckman XLA analytical ultracentrifuge on samples of cecropin A with an $A_{280} = 0.15$ prepared in a high-salt buffer (150 mM NaCl, 30 mM MOPS, and 2 mM EDTA) and a low-salt buffer (30 mM NaCl, 30 mM MOPS, and 2 mM EDTA). Samples were centrifuged at 42 000 and 48 000 rpm until successive radial scans at the same speed were indistinguishable.

Molecular weights were determined by fitting the equilibrium radial distribution data to the radial absorbance profile, $A(R)$, calculated for an ideal single species at equilibrium:

$$A(R) = A(R_{ref}) \exp[\omega^2 M_w(1 - nr)(R^2 - R_{ref}^2)/2NkT]$$

where M_w is the molecular weight, n is the partial specific volume (cm^3/g) of the solute, r is the density of the solution, and ω is the angular velocity of the cell. R and R_{ref} are distances (cm) measured from the center of rotation, N is Avogadro's number, k is Boltzmann's constant, and T is temperature in degrees kelvin. A partial specific volume of 0.7668 was calculated for cecropin A using the weighted average of the amino acid content (Cohn & Edsall, 1943). Radial absorbance profiles recorded at several different wavelengths yielded statistically indistinguishable results.

Table 1: Vesicle Composition by Mole Percent

vesicle type	POPC	POPA	cholesterol
C	100		
AC	80	20	
ACCh	42	20	38

Vesicle Preparation. Three types of vesicles are used in this work: 100% POPC (type C), 20% POPA with 80% POPC (type AC), and 20% POPA with 42% POPC and 38% cholesterol (type ACCh) (Table 1). Stock solutions of POPC and POPA were prepared in cyclohexane and assayed for phospholipid concentration by wet ashing followed by a colorimetric phosphorus assay (Chen, 1956; Bartlett, 1959). Cholesterol stock solutions were prepared in ethanol. Volumes of these stock solutions appropriate to give the desired compositions and molar ratios for each vesicle type were mixed with 0.05% BHT to retard oxidation, and lyophilized overnight to remove organic solvent. After adding buffer to the dried lipid mixtures, they were vortexed, sonicated in an ultrasonic bath (125 W, 10 min), subjected to three freeze/thaw cycles, and extruded 11 times through a 0.1 μm polycarbonate membrane (McDonald et al., 1991).

Calcein-filled vesicles were prepared by the use of a calcein-containing buffer (30 mM calcein, 30 mM MOPS, 2 mM EDTA, 75 mM NaCl, and 18.6 mM KCl at pH 7) (Goldfine et al., 1995). Calcein-containing vesicles were separated from unencapsulated calcein by gel filtration chromatography with Sephadex G-75 (Pharmacia Biotech) using iso-osmotic buffer (30 mM MOPS, 2 mM EDTA, 150 mM NaCl, and 26 mM KCl at pH 7). ANTS/DPX-filled vesicles were prepared similarly in buffer containing 12.5 mM ANTS, 45 mM DPX, 30 mM MOPS, 1 mM EDTA, and 150 mM NaCl at pH 7.

For experiments involving transmembrane potential differences in a calcein-free system, vesicles were prepared in buffer containing KCl (30 mM MOPS, 2 mM EDTA, 150 mM NaCl, and 10.8 mM KCl at pH 7). For experiments involving transmembrane potential differences in calcein-containing vesicles, it was necessary to compensate for the K^+ counterion of calcein. The K^+ concentration in the batch of calcein used for all of these experiments was determined by inductively coupled plasma analysis to be 7.8 mM for a 30 mM solution of calcein. According to this analytical method, there was no Na^+ in the sample.

Calcein Release. Calcein release from vesicles was measured by illuminating calcein-containing vesicles at 495 ± 1.7 nm and recording changes in fluorescence intensity at 525 ± 1.7 nm. Measurements were made at 7.6 μM cecropin A and 30 μM lipid. The fluorescence intensity of encapsulated calcein increases to a maximum (non-self-quenched) level after vesicles are solubilized with detergent ($C_{12}E_8$) in an excess of calcein-free buffer. Changes in intensity were taken as a percentage of the increase achieved when vesicle contents were completely released by $C_{12}E_8$. By this measure, the fluorescence of encapsulated calcein is 84−88% self-quenched at a concentration of 30 mM.

Measurements of self-quenching were taken immediately after preparation and periodically thereafter to assure vesicle integrity. Individual preparations were stored at 4 °C and proved to be stable (i.e., they exhibited no detectable diminution in the degree of self-quenching) for at least 2 weeks.

ANTS/DPX Release. The mechanism by which cecropin A released the fluorescent probe ANTS and the quencher DPX from type AC vesicles was characterized using a fluorescence requenching assay described by Wimley et al. (1994) and Ladokhin et al. (1995). In brief, ANTS fluorescence was measured at 510 ± 5 nm using excitation at 360 ± 5 nm. Peptide was added to ANTS/DPX-containing type AC vesicles and incubated for 20−30 min to reach a plateau level of probe release. Aliquots of a concentrated DPX solution (0.45 mM DPX, 30 mM MOPS, 1 mM EDTA, and 150 mM NaCl) were then added to determine the degree to which released ANTS could be requenched by DPX. Finally, the fluorescence intensity of fully released ANTS was determined by detergent lysis of vesicles with $C_{12}E_8$.

Quenching is defined as the fractional attenuation of fluorescence. Total quenching of ANTS (Q_{tot}) is given by $Q_{tot} = (Q_{out} - Q_{in})f_{out} + Q_{in}$ where Q_{out} is the degree of quenching of ANTS outside vesicles, Q_{in} is the degree of quenching of ANTS inside vesicles, and f_{out} is the fraction of ANTS outside vesicles. Q_{in} and f_{out} were determined for each trial (as aliquots of DPX were added to the cecropin A-treated vesicles) from the intercept and slope of a plot of Q_{tot} versus Q_{out}. If Q_{in} is constant over a range of f_{out}, this indicates that probe release occurs in an all-or-none manner, while values of Q_{in} which increase with f_{out} indicate that probe release is partial or "graded".

Light Scattering Measurements. Vesicle size distributions were characterized by photon correlation spectrometry (dynamic light scattering) using the 514 nm line of a 4 mW argon ion laser and a Malvern 4700C submicron particle analyzer. Measurements were made at a 90° angle, and autocorrelator settings were adjusted so that it could resolve the presence of two populations in a standard mixture of 100 nm and 400 nm latex particles (Duke Manufacturing, Palo Alto, CA).

Binding Affinity. All fluorescence measurements were made on a SPEX Fluorolog-2 spectrofluorometer (Edison, NJ) in right-angle configuration. Steady-state anisotropy measurements (r_{ss}) were made using Glan-Thompson polarizers (aligned to yield a polarization ratio of 160), a 4 × 10 mm quartz cell, an excitation wavelength of 298 ± 1.7 nm, and an emission wavelength of 350 ± 1.7 nm.

The steady-state anisotropy of the tryptophan residue at position 2 in cecropin A is $r_{free} = 0.058$ in solution, and increases to $r_{bound} = 0.136$ in the presence of a high concentration of vesicles. *G*-factor-corrected anisotropy measurements (r_i) were recorded as a solution of cecropin A was titrated with a vesicle suspension. The fraction of cecropin A bound to a vesicle surface (f_b), adjusted for the increase in quantum yield of the fluorophore that occurs with binding, was obtained by solving the expressions for f_b:

$$r_i = A \cdot r_{bound} + (1 - A) r_{free}$$

$$A = \frac{f_b}{f_b + (1 - f_b)/Q_b}$$

where Q_b is the ratio of quantum yields for the bound versus unbound peptide (1.77 in this system). The anisotropy data were fit to a single site binding equation (Rafalski et al., 1991):

$$[P](f_b - 1) - [L]/N - K_d + [L]/(N \cdot f_b) = 0$$

where [P] and [L] are the total peptide and lipid concentrations, N represents the number of lipid molecules of all species comprising a peptide binding site, and K_d is the dissociation constant. For the determination of K_d and N, the cecropin A concentrations ranged from 16 to 21 μM and the lipid concentrations ranged from 0 to 2 mM. Given values for K_d, N, [P], and [L], this equation permits calculation of f_b and, in turn, the total lipid to surface-bound peptide ratio, L:P = [L]/([P]f_b). The degree of Rayleigh scattering by vesicles using a nonbinding probe (L-tryptophan) was negligible; hence, the data did not require correction.

Membrane Potential Measurements. Transmembrane electrochemical potential gradients were produced by diluting vesicles prepared in a potassium-containing buffer ($[K^+]_i$) into a buffer with various other potassium ion concentrations ($[K^+]_o$) and adding the K^+-selective ionophore valinomycin (1.0 μM in ethanol stock for a final valinomycin to lipid ratio of 1:10 000). The Nernst equation was used to calculate the $[K^+]_o$ needed to provide the desired potential difference. A fluorescent membrane potential sensitive dye [$DiSC_3(5)$, stock 0.2 mM in ethanol] was used to detect and measure changes in potential difference. This dye registers a positive-inside change in the membrane potential as an increase in fluorescence intensity at 668 nm when the dye is excited at 606 nm. The use and mechanism of action of $DiSC_3(5)$ and similar dyes are detailed in Sims et al. (1974). Intensity changes typically stabilized within 1 min of changing the external potassium concentration. The combined level of ethanol from both the valinomycin and the $DiSC_3(5)$ stock solutions in any preparation did not exceed 0.5%.

Bactericidal Activity. A clinical isolate of *Escherichia coli* bacteria with a K1 capsule (Weiser & Gotschlich, 1991) and an unencapsulated laboratory strain, HB101 (K12) (Boyer & Roulland-Dussoix, 1969), were grown to mid-log phase in Luria−Bertani media, centrifuged, and resuspended with buffer (30 mM MOPS, 150 mM NaCl at pH 7). Concentrated bacterial suspensions were incubated for either 10 or 60 min at 25 °C with selected concentrations of cecropin A before placement on ice, dilution, and spotting on Luria−Bertani agar plates. Colony-forming units on the plates were counted visually after an overnight incubation at 37 °C. Data from three separate assays were averaged to determine the concentration which reduces the population surviving treatment by 50% (MBC_{50}).

Release of bacterial cytoplasm induced by cecropin A was assessed using the indicator *o*-nitrophenolic galactoside (ONPG), a strain of *E. coli* (ML35p) which constitutively expresses cytoplasmic β-galactosidase (Lehrer et al., 1988, 1989), and a Molecular Devices Spectra MAX 250 to record absorbance changes at 420 nm. Peptide-induced release of β-galactosidase from the bacterial cytoplasm catalyzes the conversion of ONPG to *o*-nitrophenol (ONP). The production of ONP was followed by measuring its absorbance at 1 min intervals while samples were incubated at 37 °C. As in the bactericidal activity assays, type AC vesicles were

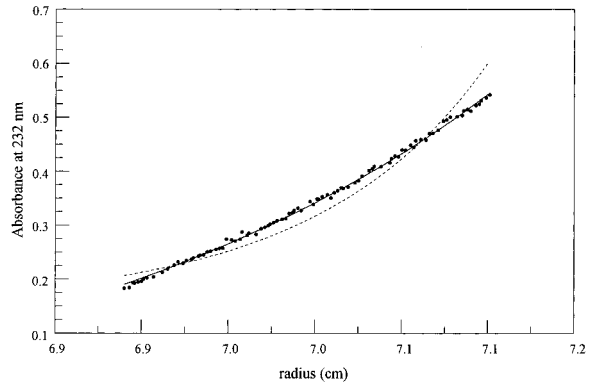

FIGURE 1: Sedimentation equilibrium results for cecropin A in high-salt buffer at 42 000 rpm. The data are represented by points; the solid line shows the expected behavior of a monomer. The dashed line shows the expected behavior for a dimer. The cecropin A concentration was 26 μM in high (150 mM NaCl, 30 mM MOPS, and 2 mM EDTA) and a low (30 mM NaCl, 30 mM MOPS, and 2 mM EDTA) salt buffer. The results for cecropin A in high- and low-salt buffer at 48 000 rpm, and in low-salt buffer at 42 000 rpm, were similar to those shown here.

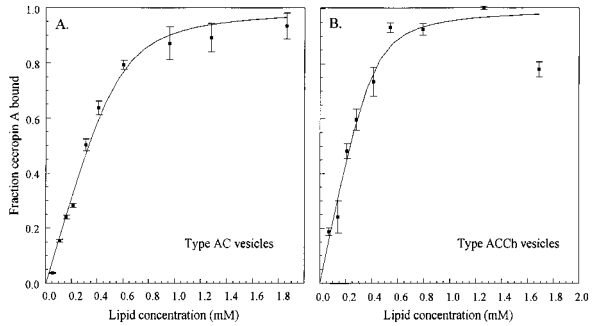

FIGURE 2: Binding isotherm for cecropin A and lipid vesicles. Solutions of cecropin A (21 μM) were titrated with a vesicle suspension. (A) Type AC vesicles. The fitted line represents K_d = 1.83 ± 0.78 μM and N = 27.8 ± 2. (B) Type ACCh vesicles. The fitted line represents K_d = 1.53 ± 1.5 μM and N = 20.1 ± 4.1.

Table 2: Dissociation Constants for Cecropin A with Lipid Vesicles

parameter	type AC	type ACCh
K_d (μM)	1.83 ± 0.78	1.53 ± 1.5
N	27.8 ± 2.7	20.1 ± 4.1
correlation	0.9952	0.9773

added to bacterial suspensions to make the L:P ratio either 10:1 or 1000:1 with respect to vesicle lipid.

RESULTS

Sedimentation equilibrium studies of cecropin A were performed with 26 μM peptide at pH 7.0 in 30 or 150 mM NaCl. Predicted optical density (OD) curves for monomer and dimer species were generated for 42 000 and 48 000 rpm. The results indicate that cecropin A is monomeric under conditions which are similar to those used in the studies below (Figure 1). This agrees with the findings of Mchaourab et al. (1993) for cecropin AD.

Affinity. Cecropin A binding to type AC and ACCh vesicles reached plateau levels at a lipid concentration slightly above 1 mM (Figure 2A,B). The data were fit to the single site binding equation, yielding K_d = 1.83 ± 0.78 μM and N = 27.8 ± 2.7 for type AC vesicles. For type ACCh vesicles, K_d = 1.53 ± 1.5 μM and N = 20.1 ± 4.1 (Table 2). In both cases, N pertains to the number of lipid molecules per site, including both inner and outer layers of the bilayer. At much lower cecropin A concentrations (0.02−20 μM) and a lipid concentration of 80 μM, the f_b did not change significantly. Therefore, the binding curves do not represent saturation of liposomes with cecropin A.

It was not possible to reach a binding plateau with type C vesicles. With the highest concentration of type C vesicles that could be examined (3.5 mM), we obtained f_b = 0.13, suggesting that some cecropin A binds to type C vesicles, but that the dissociation constant is much greater than that of the other types. If a binding site size of 20 is assumed, one obtains $K_d \approx$ 1.1 mM as a rough estimate for the dissociation constant.

Quantity of Cecropin A Bound at Saturation. The quantity of cecropin A bound to vesicles at saturation with cecropin A is defined as the amount of peptide bound to each vesicle at the threshold of the plateau observed in the aforementioned binding studies. This threshold occurred at approximately 1 mM lipid and 18.4 μM cecropin A for both type AC and type ACCh vesicles. Inserting these values into the binding equation with the appropriate dissociation constants yields f_b = 0.91 and 0.96, and concentrations of bound cecropin A of 16.8 and 17.6 μM for types AC and ACCh vesicles, respectively. Assuming the vesicle diameter to be 100 nm, and the surface area of both the outer and inner leaflet lipid headgroups to be 70 Å^2 (i.e., 9 × 10^4 lipids/vesicle), it follows that there are between 1500 and 1600 cecropin A molecules on the surface of each vesicle.

Assuming that 20% of the lipids have a net charge of −1, and that each cecropin A molecule has a net charge of +7, these results indicate that there are about 1.2 positive charges from the peptide for each negative charge from a lipid. This implies either that cecropin A has a low effective positive charge [as found for cecropin AD by Mchaourab et al. (1994)] or that factors in addition to electrostatic charge promote the binding of cecropin A to anionic vesicle surfaces. The latter is consistent with our finding of low, but detectable, levels of cecropin A bound to uncharged type C vesicles. If the peptide assumes a transbilayer configuration [as found for cecropin AD by Mchaourab et al. (1994)], negatively charged lipids on the inner leaflet may compensate for the excess positive charge.

Calcein Release. Due to differences in peptide affinity for the three vesicle types, experimental conditions in this section are related in terms of total lipid to bound peptide (L:P) ratios (calculated as described under Materials and Methods). Under the conditions of these experiments, L:P = 35:1 for type AC vesicles and L:P = 25:1 for type ACCh vesicles. This corresponds to roughly 11−16% of the quantity of cecropin A bound at saturation. We estimate the L:P ratio for type C vesicles to be 3100:1 under these conditions.

Cecropin A released calcein from all three vesicle types in a time- and concentration-dependent manner. It released readily detectable amounts of calcein from type C vesicles at 1 and 5 min (Figure 3A). Despite a manyfold higher surface concentration, cecropin A released only 4 times as much calcein in type AC vesicles at 1 min (Figure 3B). The presence of 38% cholesterol in type ACCh vesicles negated this increase. The diminished effect of cecropin A on calcein released in type ACCh vesicles cannot be explained by a lower surface concentration, since it is slightly higher for these vesicles than for type AC vesicles. Qualitative

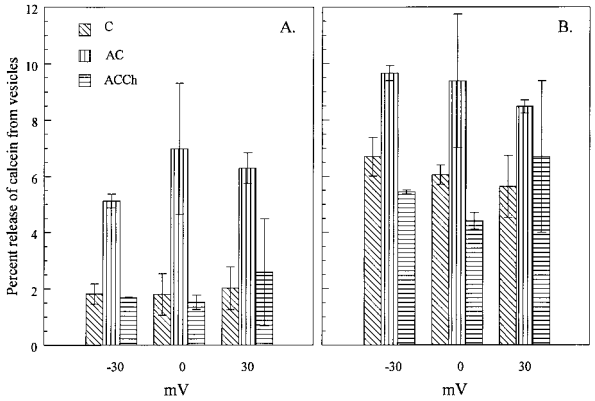

FIGURE 3: Cecropin A induced release of calcein from vesicles with initial membrane potentials of −30 mV, 0 mV, and +30 mV. Results are expressed as a percentage of the total contents released, and each point is an average of three trials. The cecropin A concentration was 7.6 μM, and the lipid concentration was 30 μM. (A) After a 1 min exposure. (B) After a 5 min exposure.

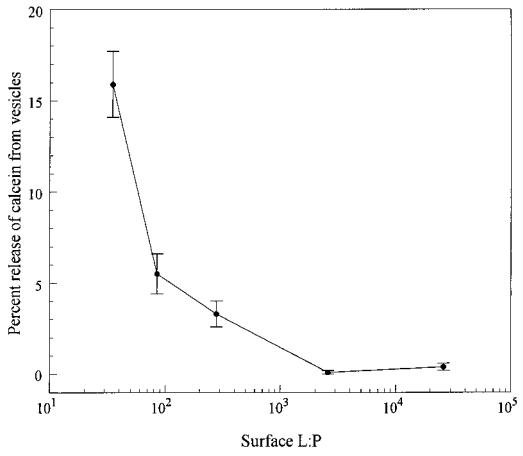

FIGURE 4: Calcein release from type AC vesicles after a 5 min exposure to cecropin A for various surface L:P ratios. The lipid concentration was 30 μM, and the cecropin A concentration ranged from 7.6 μM to 3.1 nM. Results are expressed as a percentage of the total contents released.

differences between vesicle types in the amount of calcein released persist after 5 min, but they are quantitatively less significant. The dependence of calcein release on peptide concentration was examined in type AC vesicles. Calcein release was relatively high after a 5 min exposure to cecropin A at L:P = 35:1 (Figure 4) compared to ratios of 85:1 and 280:1. At higher L:P ratios, calcein release could not be demonstrated.

Because bacterial cell membranes have transmembrane electrochemical potential differences which might influence the membrane activity of cecropin A, we examined the effect of transmembrane potential differences, created by the use of valinomycin and K^+ gradients, on cecropin A-induced calcein release. In all three types of vesicles, calcein release after a 5 min exposure to cecropin A was not measurably affected by potential differences of +30 mV or −30 mV, or by treatment with valinomycin in the absence of a K^+ gradient (i.e., 0 mV) (Figure 3A,B). The pattern observed in the absence of such treatments persisted, namely, that the ability of cecropin A to release calcein from type AC vesicles was somewhat greater than from type C and type ACCh vesicles.

The mechanism by which encapsulated probes are released by cecropin A was examined in type AC vesicles containing

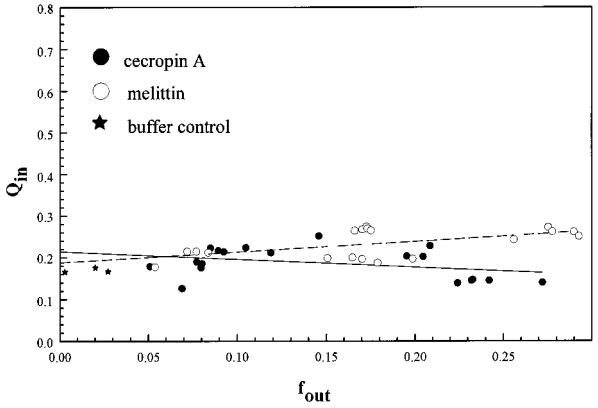

FIGURE 5: Internal quenching (Q_{in}) of ANTS/DPX-containing, type AC vesicles as a function of the contents released (f_{out}) after incubation with either melittin or cecropin A. The lipid concentration was 10 μM, the melittin concentration ranged from 0.11 to 0.78 μM, and the cecropin A concentration ranged from 0.24 to 1.1 μM. ○ = melittin treatment. ● = cecropin A treatment.

the fluorescent probe ANTS, and the quencher DPX. The activity of cecropin A was compared to that of melittin which has previously been shown to release vesicle contents by an all-or-none mechanism. Figure 5 shows that the degree to which encapsulated ANTS was quenched by coencapsulated DPX remained constant regardless of how much ANTS was released by either melittin or cecropin A. These results confirm an all-or-none mechanism for melittin-induced calcein release, and indicate that cecropin A likewise releases vesicle contents in an all-or-none manner.

In summary, the ability of cecropin A to release encapsulated calcein depends on lipid composition and the L:P ratio but not membrane potential. Release appears to proceed by an all-or-none mechanism. Anionic lipid dramatically increases the affinity of vesicles for cecropin A, but it only modestly increases the ability of cecropin A to release encapsulated calcein, and this increase becomes quantitatively less pronounced with time. Cholesterol opposes the increase in cecropin A-induced calcein release due to anionic lipids.

Vesicle Size. Photon correlation spectroscopy revealed a mean diameter of 93.5 ± 18.0 nm and a uniform size distribution for type AC vesicles. After the addition of cecropin A at a concentration calculated to yield an L:P on the vesicle surface of 35:1, the mean vesicle size was unchanged at 96.3 ± 17.1 nm. Hence, the vesicles did not fragment or aggregate in the presence of a cecropin A concentration which causes extensive calcein release from vesicles.

Membrane Potential Changes. We examined the ability of cecropin A to change the transmembrane potential of a vesicle using the potentiometric dye $DiSC_3(5)$. This dye registers a change in the transmembrane potential difference toward more positive values as an increase in the fluorescence intensity. Conversely, a change toward a more negative potential difference is indicated by a decrease in the emission intensity. The response of the dye as measured by the percent change in fluorescence was calibrated to changes in the external potassium concentration. Figure 6 shows that changes in the fluorescence of this dye are not linearly related to $\ln([K]_o/[K]_i)$. Changes toward more positive potential differences are somewhat more readily measured than negative changes because of this nonlinearity.

Cecropin A was added to suspensions of all three vesicle types in which potential differences of −40, 0, and +40 mV

Table 3: Cecropin A-Induced Membrane Potential Changes and L:P Ratios for Vesicles

						apparent change (mV)		
nominal L:P	vesicle type	L (μM)	P (μM)	f_b	surface L:P	−40 mV	0 mV	+40 mV
4:1	AC	30	7.6	0.11	35:1	56 ± 2	14 ± 3	56 ± 4
100:1	C	31	0.31	0.001	74 000:1	7 ± 3	1 ± 0	−11 ± 1
	AC	31	0.31	0.36	280:1	14 ± 3	0 ± 1	−14 ± 1
	ACCh	31	0.31	0.48	210:1	27 ± 9	0 ± 0	−21 ± 5
1000:1	AC	31	0.031	0.38	2600:1	9 ± 5	−5 ± 3	−26 ± 4
	ACCh	31	0.031	0.50	2000:1	14 ± 6	1 ± 0	−13 ± 4

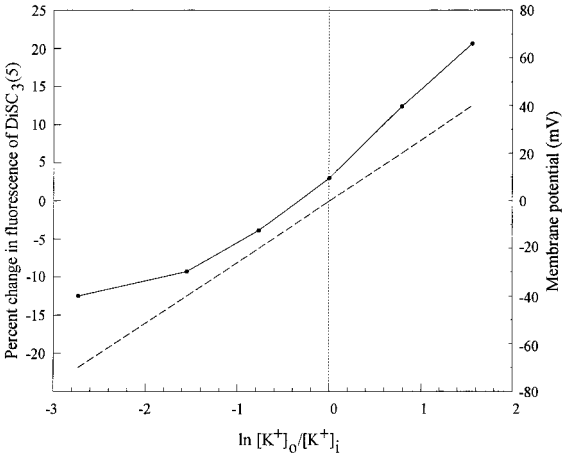

FIGURE 6: Response of a fluorescent probe, $DiSC_3(5)$, to changes in membrane potential in type AC vesicles. The solid line indicates the measured changes in response as expressed as the percent change in fluorescence intensity (left axis). The dashed line represents the potential expected from the Nernst relationship (right axis). The concentration of potassium inside the vesicles was 10.8 mM.

were established; fluorescence intensities were recorded before and after a 5 min exposure to cecropin A. Surface L:P ratios were calculated to enable a direct comparison of results from the different vesicle types (Table 3). The changes in $DiSC_3(5)$ fluorescence indicate that, for all three vesicle types, cecropin A at L:P > 200 causes membranes with potential differences of −40 and +40 mV to depolarize toward 0 mV, whereas there is little change seen in membranes with an initial potential difference of 0 mV (Figure 7). These findings were similar in type AC and ACCh vesicles, indicating that cholesterol did not protect against the cecropin A-induced depolarization of the membrane. Our results were distinctly different with type AC vesicles at L:P = 35:1. In this case, the change in membrane potential for vesicle membranes at each of the starting potentials changed in a markedly positive direction.

Bactericidal Activity. To characterize the antimicrobial activity of the cecropin A used in these experiments, we measured its bactericidal activity against two strains of *E. coli.* K12 is a laboratory-adapted strain which tends to be highly vulnerable to antimicrobial agents. K1 is a recent pathological isolate (from a case of infant meningitis) which has not undergone extensive laboratory adaptation.

Cecropin A quickly killed both strains of *E. coli* at low concentration (Figure 8). The MBC_{50} for the K1 strain after a 10 min incubation with cecropin A was approximately 1.7 μM, and for the K12 strain it was 0.7 μM. After a 60 min incubation, only 40% of K1 bacteria survived at 0.36 μM, while virtually no bacteria survived 60 min incubations with 4−18 μM peptide. Since it is difficult to determine a meaningful L:P ratio with respect to bacterial lipid, we added small numbers of bacteria to vesicle suspensions such that the nominal L:P ratio with respect to the vesicles ranged from 450:1 to 20000:1, and the bacterial lipid represented a negligible contribution to the total. The MBC_{50} for the K1 strain increased to 4.5 μM under these conditions. Survival assays and the MBC_{50} values derived from them are inherently imprecise, however, so we cannot be certain that this represents a significant increase.

The effect of cecropin A on the release of cytoplasmic contents from a β-galactosidase-producing strain of *E. coli* was also examined in vesicle suspensions at L:P ratios of 1000:1 and 10:1 with respect to vesicle lipid (Figure 9). The rate and extent of release of intracellular β-galactosidase at an L:P of 1000:1 are similar to those which occur in the absence of cecropin A, whereas the rate and extent of release at an L:P of 10:1 are much higher. This suggests that cecropin A does not induce the release of β-galactosidase from bacterial cytoplasm at high L:P, consistent with our finding with respect to calcein release from vesicles at this L:P.

DISCUSSION

There are two key findings in these data. The first is that cecropin A exhibits concentration-dependent changes in the nature of its membrane activity. In lipid vesicles, cecropin A dissipated ion gradients at low concentrations of peptide, while substantially higher concentrations were required to release encapsulated calcein. In Gram-negative bacteria, cecropin A was lethal at low concentrations, while substantially higher concentrations were required to release cytoplasmic β-galactosidase. Thus, the concentration-dependent change in membrane activity we observe in synthetic vesicles corresponds to the change in activity we observe in bacteria, and suggests that the bactericidal activity of cecropin A at low concentration is due to the dissipation of transmembrane electrochemical ion gradients.

The ability of cecropin A to dissipate the electrochemical ion gradients established by valinomycin and K^+ appears to be a consequence of passive "ion channel" formation (Durell et al., 1992). This is a common bactericidal mechanism among naturally occurring antibiotics, and an altogether plausible mechanism for cecropin A. At high L:P ratios, the ion channels formed by cecropin A are capable of depolarizing the vesicle membrane (Figure 7), but not of releasing measurable amounts of calcein (Figure 5). At low L:P ratios where it does cause the release of calcein, cecropin A also causes a dramatic increase in the fluorescence intensity of the $DiSC_3(5)$ probe. Since it is unlikely that cecropin A would cause vesicles initially at +40 mV to develop an even more positive potential difference, we must assume that membrane integrity is breached at low L:P, and

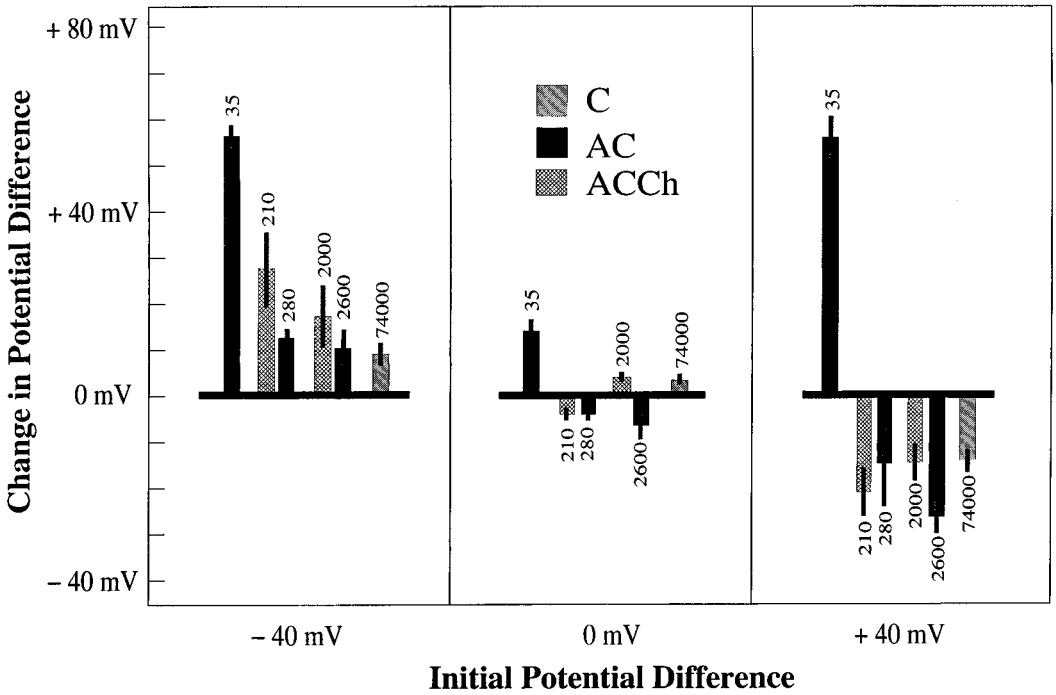

FIGURE 7: Changes in membrane potential after a 5 min exposure to cecropin A for initial membrane potentials of −40, 0, and +40 mV. The vertical bars indicate the direction and magnitude of the potential change for each vesicle type. The number above or below each vertical bar is the surface L:P ratio for that measurement.

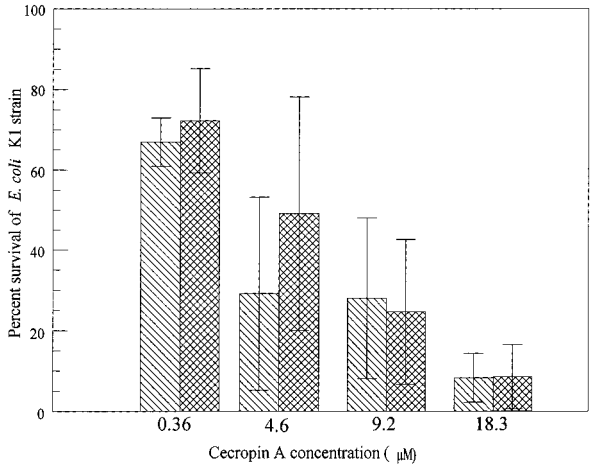

FIGURE 8: Survival of *E. coli* (K1 strain) after a 10 min exposure to cecropin A. The bars on the left of each pair represent survival in the absence of vesicles, and the bars on the right of each pair represent survival in the presence of AC vesicles (the L:P ratio varied from 450:1 to 20 000:1).

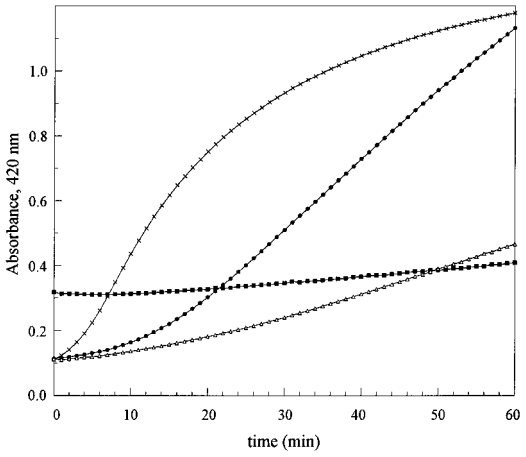

FIGURE 9: Release of cytoplasmic β-galactosidase from *E. coli* strain ML35p upon treatment with cecropin A. Symbols are as follows: × = melittin, 20 μg/mL in buffer suspension; ● = cecropin A, 5 μM, and L:P of 10:1; ■ = cecropin A, 5 μM, and L:P of 1000:1; △ = bacteria in buffer. The initial absorbance for an L:P of 1000:1 was higher than the other three because this concentration of lipid scattered a substantial amount of light.

that this interferes with the relationship between $DiSC_3(5)$ fluorescence and membrane potential. Addition of enough $C_{12}E_8$ detergent to lyse these vesicles resulted in similar increases in the apparent membrane potential (data not shown), substantiating this assumption. This result has a significant corollary, namely, that membrane integrity has not been compromised at the higher L:P where channel formation occurs. The behavior of $DiSC_3(5)$ thus provides further evidence that cecropin A exhibits distinct changes in the nature of its membrane activity as a function of concentration.

The second key finding is that cecropin A can bind, form ion channels, and release calcein in the absence of anionic lipid (i.e., in type C vesicles). Anionic lipid profoundly increases the affinity of cecropin A for membranes, and the amount of cecropin A bound at saturation. Yet, the presence of anionic lipid had no discernible effect on channel formation, and only a modest effect on calcein release. These findings suggest that cecropin A can bind to lipid vesicles by at least two different mechanisms: one which is independent of headgroup charge, and one which depends on the presence of anionic lipid. Charge-independent binding is directly observed only in type C vesicles, but we presume that it is responsible for ion channel formation and calcein release in all three vesicle types. Anionic lipid-dependent binding, on the other hand, increases overall affinity for the peptide, but not necessarily its membrane activity. Membrane activities such as ion channel formation and calcein release imply that the peptide physically penetrates the membrane bilayer, and that there is a direct association between peptide and the hydrophobic region of the vesicle

membrane at some point. Therefore, charge-independent binding may be mediated by interactions between the peptide and the hydrophobic region of the bilayer.

The physiological consequences of cecropin A activity are distinctly different at high and low surface concentrations, but they may arise from similar processes. For example, both types of membrane activity may result from cecropin A monomers combining to form multimeric pores. At low concentrations, relatively few cecropin monomers are available, and may combine to form small pores just large enough to pass ions. Hypothetical models of such channels have been developed for cecropins by Durell et al. (1992). At high concentrations, more monomers are available, and they may combine in greater numbers to form the larger pores which pass calcein ($M_w = 623$) or β-galactosidase. This scenario is entirely consistent with our results, although the data do not address the specific structure or multimeric state of the bound polypeptide.

In electrophysiological studies of various cecropins on planar lipid bilayers, Christensen et al. (1988) showed that cecropins formed weakly selective ion channels, and that channel formation was typically followed by catastrophic electrical breakdown of the membrane. While this sequence of events could be due to time-dependent conformational changes in the membrane-bound cecropin, it seems more likely that these measurements were made while peptide was accumulating on the membrane, with small amounts of bound cecropin causing the formation of ion channels early in the course of the experiment, and larger amounts subsequently leading to electrical breakdown. Therefore, these results are consistent with our conclusions about concentration-dependent differences in the membrane activity of cecropin A.

It is straightforward to reconcile our results and those of Christensen et al. (1988) with respect to the protective effect of cholesterol against ion channel formation. Christensen et al. (1988) found that cholesterol profoundly slowed the *time course* of channel formation, whereas we found that it did not interfere with the depolarization of membranes over a 5 min incubation period. These incubations are severalfold longer than the typical time course of channel formation observed by Christensen et al. (1988). Thus, cholesterol may delay, but it does not appear to prevent, channel formation by cecropins. This is an important result because it casts serious doubt on the ultimate ability of cholesterol to protect cell membranes against the channel-forming effect of cecropin A.

Steiner et al. (1988) found that cholesterol did not protect against the cecropin A-induced release of carboxyfluorescein from vesicles, whereas we found that cholesterol protected against calcein release. Here again, temporal considerations help reconcile our results because Steiner et al. (1988) incubated cecropin A with vesicles for 60 min vs 10 min in our studies. We found that cholesterol protected against calcein release at both 1 and 5 min after exposure to cecropin A, but the protective effect was smaller at 5 min (Figure 3b). Therefore, the presence of cholesterol may offer only a transient protective effect against calcein release.

Our sedimentation studies indicate that cecropin A exists as a monomer in solution; therefore, its binding affinity for the lipid membrane is not affected by self-association in solution. The light-scattering studies show that, once bound, cecropin A causes neither the aggregation nor the dissolution of vesicles. These results simplify the approach required to examine the affinity of peptides for lipid membranes with a quantitative model. The model we have chosen regards a block of N lipids as a single binding site, and fits the binding isotherm with a term that represents N. The dissociation constant in this case pertains to the aggregate dissociation of the peptide from the N-lipid site. The estimates of N we obtained suggest that a single peptide interacts with more than one lipid molecule, as to be expected in the interaction between basic peptides and anionic lipid membranes (Mosior & McLaughlin, 1992).

The dissociation constants provide an important piece of information by showing that cholesterol does not exert its effects by lowering the affinity, the quantity bound at saturation, or the surface concentration of the peptide. Nevertheless, we do not attribute any specific physical significance to these dissociation constants because a truly meaningful interpretation of the binding isotherms would require the examination of additional factors such as ionic strength, the extent to which the peptides insert into the membrane, the amino acid composition of the polypeptides, and conformational changes which occur upon binding. The data fit well to a simple model, so that the use of more sophisticated models (e.g., Kim et al., 1991; Seelig et al., 1993; Montich et al., 1993; White & Wimley, 1994; Mchaourab et al., 1994; Ben-Tal et al., 1996; Ben-Shaul et al., 1996) is not mandated on the basis of goodness-of-fit to the data.

Direct comparisons between the vesicle and bacterial systems used in these experiments are confounded by the complexities of working with a living organism. The cell membranes of a Gram-negative bacterium are composed of a complex lipid mixture. The outer membrane and various components of the cell wall could either hinder access of peptides such as cecropin A to the inner membrane, or bind them via nonproductive associations. Therefore, we cannot accurately determine an L:P ratio at the bacterial cell membrane which is directly comparable to the value obtained for vesicles. In lieu of being able to compare the actual concentrations of cecropin A required to achieve specific effects in vesicles and in bacteria, our evidence that ion channel formation is the chief mechanism by which cecropin A exerts bactericidal activity is chiefly that the membrane activity of cecropin A exhibits the same type of *concentration-dependence* in both vesicles and bacteria.

We have measured the antimicrobial effect of cecropin A using a survival assay, rather than growth inhibition, chiefly so that peptide concentrations to which the bacteria are exposed may be defined unambiguously. This permits us to compare the peptide concentrations required to kill bacteria to the concentrations required to release cytoplasmic β-galactosidase. It is not clear that the minimum inhibitory concentrations (MIC's) derived from growth inhibition assays are suitable for this purpose because they depend on assumptions about peptide diffusion in agar or agarose (Hultmark et al., 1983), and the results vary with the technique and materials used (Boman et al., 1989). Yet, the two types of measurement yield results which are in reasonable relationship to one another, with MBC_{50} results between 2-fold and 9-fold higher than the typical MIC's reported.

Clearly, cecropin A is bactericidal at the relatively high concentrations, where it increases membrane permeability to large molecules, and it is possible that this type of

permeability change is the dominant bactericidal mechanism under these conditions. However, our results show that cecropin A has a more potent bactericidal mechanism, which appears to involve ion channel formation, does not depend on the presence of anionic lipid, and is unaffected by cholesterol. If cecropin A exhibits greater toxic effects on bacterial cells than on host cells at low concentrations, the basis for this selectivity is more likely due to differences in the susceptibility of these cells to ion gradient dissipation than to the presence of anionic lipid in the bacterial membrane, or to cholesterol in the host cell membrane.

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