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Binding and action of cecropin and cecropin analogues: antibacterial peptides from insects

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The mechanism of action of cecropin was studied by using liposomes as a model system. The bilayer was efficiently destroyed if the liposome net charge was zero or negative. Cecropin analogues with an impaired N-terminal helix had reduced membrane disrupting abilities that correlate with their lower antibacterial activity. The reduced bactericidal activity of the analogues was rationalized in terms of reduced binding to bacteria. The stoichiometry of cecropin killing of bacteria suggests that amounts of cecropin sufficient to form a monolayer strongly modify the bacterial membrane. Although some bacteria were resistant to cecropin they did bind large amounts in a non-productive manner. In contrast, mammalian erythrocytes achieve resistance by avoiding the binding of cecropin.

Introduction

Insects possess an efficient inducible humoral immune system. In the saturniid *Hyalophora cecropia* the active agent has been shown to consist of a mixture of lysozyme and two antibacterial protein families, named cecropins and attacins. The latter two possess poorly understood modes of action. Cecropins are small, basic proteins synthesized as a response to a bacterial infection. Their antibacterial spectrum is broad, including both Gram-positive and Gram-negative species (reviewed by Boman and Hultmark [1]). The primary structures of the 35- or 37-cecropins have been determined [2]. From Chou and Fasman [3] calculations it was evident that the N-terminal half has a high potential for an unusually polarized amphipathic helix [4,5], a structural element commonly found in membrane-associated proteins (for a review see Epand [6]). The cecropins were also shown to easily form such helical structures in a slightly hydrophobic environment [4].

Cecropins induce a rapid lysis of bacterial cells, indicating a direct action on their membranes. The possibility that lysis is a secondary effect in a cellular system, however, could not be ruled out [7]. A cecropin-like peptide, sarcotoxin [8], has been shown to destroy the cytoplasmic membrane of bacteria causing a loss of active transport and ATP generation [9]. Synthetic cecropin analogues have been used to study the structural requirements for cecropin action on bacteria [10,11]. The amphipathic helix was shown to be important for

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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lysis of the least susceptible bacterial species whereas no single amino acid replacement was deleterious to the action on the most susceptible species such as *Escherichia coli*

All eucaryotic cell types tested so far are resistant to cecropin action, indicating that the cecropins specifically attack bacteria [7]. In the present study the cecropins are shown to possess membrane-disrupting activity by using unilamellar liposomes as a model system. The lysis potential of the different cecropin analogues is correlated with their membrane affinity.

Materials and Methods

The syntheses of cecropin A and its analogues have been described [10,11]. These peptides were synthesized by solid phase methods and purified by reverse-phase liquid chromatography on C_{18} silica columns. They were homogeneous by HPLC. Natural cecropins were purified as previously described [12]. The cecropin concentration was estimated from the absorbance at 280 nm taking $A_{280}^{0.1\%} = 1.36$ for the cecropins containing one tryptophan residue or by using the fluorescamine method [13]. Phosphatidylcholine, as well as mixtures of dicetyl phosphate, stearylamine, cholesterol and phosphatidylcholine and a synthetic mixture of phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, phosphatidylserine, cardiolipin, phosphatidylinositol (12 : 10 : 12 : 5.2 : 0.4 : 0.4) mimicking the phospholipid composition of human erythrocytes were from Pharmacia PL Biochemicals (Uppsala, Sweden). They were used without further purification. Carboxyfluorescein was from Eastman Kodak and was recrystallized as described [14].

Medium-sized liposomes were prepared by employing a French press method [15]. In brief, a film of phospholipid (10 mg) was dispersed in Hepes (2 mM), histidine (2 mM) buffer, pH 7.4, containing either carboxyfluorescein (100 mM) or sodium chloride (100 mM) by vigorous vortexing. The suspension was then passed four times through a French press. Large aggregates were removed by centrifugation at $10000 \times g$ for 30 min. Large liposomes were prepared by the reversed-phase technique [16]. Typically, phospholipid (9 μmol) was dissolved in 0.9 ml diethyl ether and 0.1 ml chloroform. After addition of buffer the evaporation scheme given by Wilschut et al. [17] was followed, giving a total of 1 ml of vesicles. The non-entrapped material was in all cases removed by one or two passages over a Sephadex G-25 column (10×1.8 cm).

Phospholipid content was determined with ammonium ferrothiocyanate [18].

Electron microscopy

Liposomes were negatively stained with 1% aqueous potassium phosphotungstate on carbon-coated Formvar grids and examined with a Jeol 100 S microscope.

Assay of liposome lysis

The method used by Blumenthal et al. [14] to record carboxyfluorescein release from liposomes was adapted to micro-test plates. The fluorescence was measured with a Leitz Diavert fluorescence microscope interfaced to a Hewlett Packard 85 computer using the Leitz MPV compact MT software. Excitation was through a 450–490 nm band pass filter and emission was recorded after a 510 nm reflection short-pass filter.

Results

Effect of cecropin and cecropin analogues on large liposomes

The hypothesis that the bacterial membranes are the targets for cecropin action was tested by preparing liposomes and measuring the release of entrapped carboxyfluorescein upon incubation with cecropins. This method, which records the decreased self-quenching of the fluorescence when the dye is released from the liposomes, was adapted to microtitre plates by using an inverted fluorescence microscope. The dose-response curves for a set of different cecropin analogues are shown in Fig. 1. The liposomes are readily lysed by cecropin and its analogues. In this experiment the liposomes were composed of phosphatidylcholine, cholesterol and dicetyl phosphate and were prepared by the reversed-phase method. The size of these mostly unilamellar liposomes was rather heterogeneous, ranging between 30 and 200 nm, typically 80 nm, as judged from electron micrographs. The native cecropin was most effective. The ana-

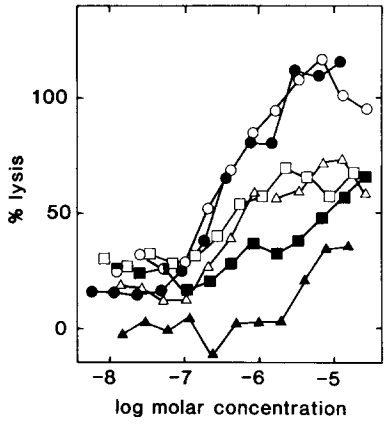

Fig. 1. Lysis of negatively charged liposomes (12 nmol phospholipid/ml) by cecropin A analogues. Liposomes were composed of phosphatidylcholine, dicetyl phosphate and cholesterol (7 : 2 : 1) and were prepared by the reversed-phase method. The liposomes were incubated for 1 h at 25 °C with cecropin A (1–37) (●), (2–37) (○), (3–37) (▲), [Phe^2]-cecropin A (△), [Pro^8]-cecropin A (■), [Pro^4]-cecropin A (□) in 2 mM Hepes, 2 mM histidine, 0.12 M NaCl. Total lysis was obtained by adding 0.2% Triton X-100.

logues with a break in their N-terminal helix obtained by introducing a proline residue at position 4 or 8 (Fig. 2) had significantly reduced membrane-disrupting activity. Shortening of the N-terminal helix by one residue had no effect, whereas removal of two residues lowered the lytic activity drastically. Substituting phenylalanine for tryptophan-2 resulted in an intermediate activity, showing that the tryptophan ring system is not mandatory for activity. The weak activity of cecropin A(3–37) is most likely due to a weakened amphipathic character and a shortened helix.

Influence of liposome composition and charge on cecropin action

The preferential lysis of bacterial membranes was initially believed to be determined by the different phospholipid compositions of bacterial and eucaryotic membranes. Liposomes containing lipopolysaccharide were thus prepared and found to be sensitive (not shown). So also were liposomes containing the negatively charged phospholipid analogue dicetyl phosphate. Incorporation of cholesterol (Fig. 1), which is mainly found in eucaryotic membranes, did not make the liposomes resistant either. In Fig. 3 it is shown that

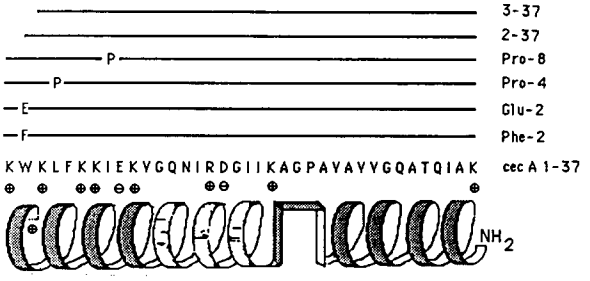

Fig. 2. Structure of cecropin A and cecropin A analogues [10,11]. The secondary structure, consisting of two helices joined by a β-turn, is believed to be induced upon contact with bacterial membranes. It is based on Chou and Fasman predictions and circular dichroism measurements in a slightly hydrophobic solvent [4]. The non-shaded area has a low helical potential but it is assigned to be helical due to the high helical content obtained from circular dichroism measurements.

even liposomes with a lipid composition mimicking the one found in red blood cells were sensitive. However, positively charged liposomes composed of phosphatidylcholine, stearylamine and cholesterol (7 : 2 : 1) were insensitive to cecropin at the highest concentration tested (14 μM). A strict comparison of the absolute sensitivities of differently charged liposomes is difficult as their sensitivity differed between preparations as well as in a particular preparation that had been stored for different periods of time.

Liposomes were prepared with two different

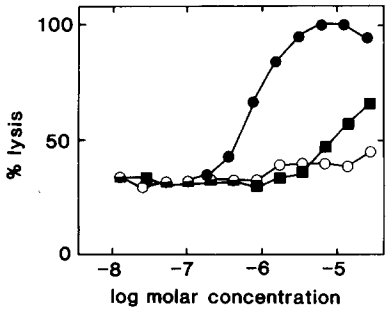

Fig. 3. Lysis of liposomes (6 nmol of phospholipid/ml) composed of a mixture of phospholipids with proportions of phospholipids identical to that found in human erythrocytes. Liposomes were prepared by the French press method and were incubated for 1 h at 25 °C with cecropin A (1–37) (●), cecropin A (2–37) (○); and [Pro^8]-cecropin A (■). Other conditions as in Fig. 1.

methods reported to produce differently sized liposomes: reversed-phase evaporation and French press extrusion. Liposomes prepared with the reversed-phase method were mostly unilamellar with a typical diameter of 80 nm. The French-pressed liposomes had a somewhat broader size distribution and mean values were smaller, typically 50 nm. These medium-sized liposomes as well as larger liposomes prepared by the reversed-phase method were sensitive to cecropins.

Kinetics of cecropin lysis of liposomes

In Fig. 4 is shown the time course of cecropin-induced release of carboxyfluorescein from liposomes prepared from phospholipids of the same composition as in human erythrocytes. The initial rates were approximately linearly dependent on the cecropin concentration. If there is a rapid binding of cecropin followed by a slower step leading to lysis, the cecropin concentrations employed must have been well below the maximum binding capacity of the membranes. The slight decrease in fluorescence observed at longer times with the higher cecropin concentrations might reflect a change in light scattering that was not corrected for.

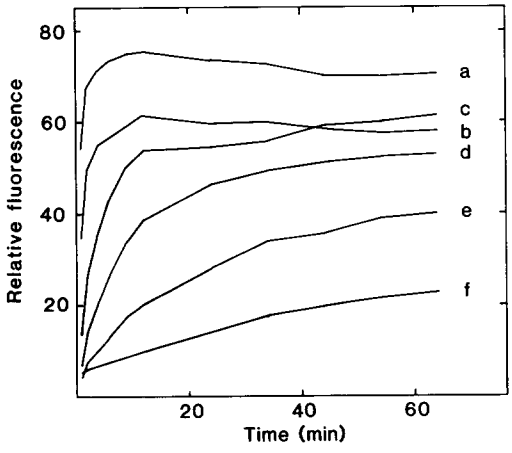

Fig. 4. Kinetics of cecropin-induced lysis of liposomes composed of a mixture of phospholipids with proportions of phospholipids identical to that found in human erythrocytes. Liposomes (6 nmol of phospholipid/ml) were incubated at ambient temperature with cecropin A (a, 48; b, 24; c, 12; d, 6; e, 3; and f, 1.5 μM) in the same buffer as in Fig. 1. Fluorescence was recorded at timed intervals.

Cecropin binding to bacteria

Different bacterial species show different sensitivity to cecropins, ranging from the resistant insect pathogen *Bacillus thuringiensis* to the most susceptible *E. coli*. In an attempt to correlate affinity with killing ability, both binding and bacterial viability were recorded for radioactivity labelled cecropin A acting on *B. thuringiensis, B. subtilis* and *E. coli* (Fig. 5). *B. thuringiensis* is normally totally resistant to the cecropin concentrations employed but in this experiment 50% were killed at the highest cecropin concentration. The binding curves were rather parallel for all three bacterial species, whereas the LD_{50} values differ, giving different amounts of cecropin bound when 50% of the cells are killed. The different bacterial susceptibilities can thus not be explained by differential binding. Rather, the different

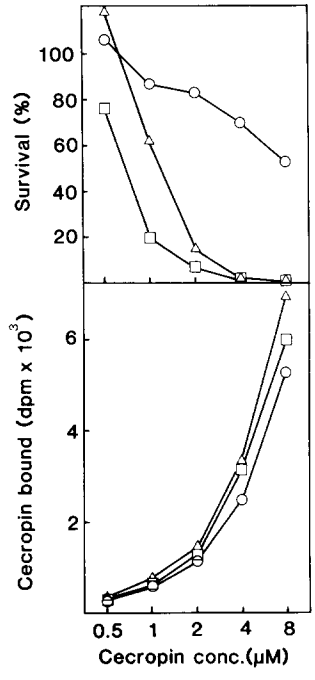

Fig. 5. Comparison of cecropin A-mediated killing and binding to bacteria. *E. coli* D31 ($5.8 \cdot 10^7$ cfu, □), *B. megaterium* Bm11 ($3.3 \cdot 10^6$ cfu, △), and *B. thuringiensis* Bt11 ($4.6 \cdot 10^6$ cfu, ○) were incubated with cecropin A (2200 dpm $^{14}C/\mu g$) for 10 min at ambient temperature in a total volume of 0.1 ml. A sample was withdrawn for colony counting (cfu). The remaining bacteria were centrifuged at $10000 \times g$ for 5 min and the radioactivity in the supernatant was determined by liquid scintillation counting.

TABLE I

BINDING OF CECROPIN ANALOGUES TO BACTERIA AND LETHAL CONCENTRATIONS

Incubation was in 0.1 M sodium phosphate buffer, pH 6.4, at 37 °C for 10 min. The number of cells used give the same Klett value at 350 nm and represent the same bacterial mass. The cells were centrifuged and the remaining cecropin in the supernatant was assayed with the inhibition zone assay [19].

Analogue	Concn. (mg/ml)	Bound cecropin (% of total)			Lethal concentration (μM)		
		P. aeruginosa OT97 ($6 \cdot 10^9$ ml^{-1})	*B. megaterium* Bm11 ($6 \cdot 10^8$ ml^{-1})	*E. coli* D21 ($4 \cdot 10^9$ ml^{-1})	*P. aeruginosa* OT97	*B. megaterium* Bm11	*E. coli* D21
Cecropin A	0.29	87	73	94	1.5 [a]	0.56 [a]	0.28 [a]
[Phe^2]-cecropin A	0.33	82	64	95	3.5 [a]	0.78 [a]	0.34 [a]
[Glu^2]-cecropin A	0.30	37	20	94	170 [b]	39 [b]	3.2 [b]
[Pro^4]-cecropin A	0.43	78	66	79	8.1 [b]	11 [b]	0.36 [b]
[Pro^8]-cecropin A	0.32	78	49	78	15 [b]	31 [b]	0.50 [b]

[a] Values taken from Ref. 10.
[b] Values taken from Ref. 11.

bacterial species can resist different amounts of bound cecropin before they lyse.

The cecropin binding to bacteria is hampered by a gradual membrane disintegration. When radioactively labelled cecropin was used, a decrease in binding with time was observed, indicating that cecropin-containing particles were released that could not be pelleted by low-speed centrifugation. If, however, free cecropin in the supernatant was estimated from measurements of the biological activity with the inhibition zone assay [19], no decrease in binding with time was obtained. The cecropin in the material released was thus biologically inactive. By using this technique the binding of different cecropin analogues to *Pseudomonas aeruginosa* OT97, *Bacillus megaterium* Bm11, and *E. coli* D21 was estimated (Table I). The method can unfortunately measure binding only at a very limited range of cecropin concentrations. Binding has thus been obtained at a single cecropin concentration. The correlation of binding of the different analogues with their respective killing abilities was estimated by the Spearman rank correlation coefficients [20]. Values obtained were 0.98 and 0.90 for OT97 and Bm11, respectively, indicating a highly significant correlation. The strong binding of cecropin to *E. coli* precluded a comparison of binding and lethal concentration. The different susceptibility of different bacteria cannot be explained by differential binding.

TABLE II

CECROPIN INTERACTION WITH ERYTHROCYTES

Washed erythrocytes (RBC) were incubated with cecropin for 1 h at 37 °C. Percent lysis was estimated at 577 nm with 100% taken as the value obtained after ultrasound treatment in distilled water. ^{3}H,^{14}C-labelled cecropin A (1100 cpm/μg) was incubated with the indicated number of erythrocytes in a total volume of 50 μl at 37 °C for 1 h. The samples were centrifuged in an Eppendorf centrifuge and the total counts in the supernatant were quantified in a scintillation counter. –, not determined.

	Concn. (mg/ml)	Percent lysis		Percent bound					
		bovine RBC (nl^{-1})	sheep RBC (nl^{-1})	bovine RBC (nl^{-1})			sheep RBC (nl^{-1})		
		11	11	50	1	0.1	50	1	0.1
Cecropin A	0.58	0	13	–	–	–	–	–	–
^{3}H,^{14}C-Cecropin A	0.06	–	–	−1	3	7	5	4	5

Binding to and lysis of red blood cells

It has been shown that Chang liver cells are resistant to cecropin [7]. Red blood cells from cow and sheep were used in the present study to find out whether such resistance is a common property of eucaryotic cells. The bovine cells were totally resistant and the sheep cells were lysed to a very low extent at a cecropin concentration 700-times higher than the lethal concentration for *E. coli* (Table II). The membrane surface area employed is comparable to the *E. coli* surface area in Fig. 5. In a binding experiment it was also demonstrated that cecropin has no or very low affinity for red blood cells. The lack of lytic activity can thus be explained by a lack of cecropin binding to eucaryotic erythrocytes.

Discussion

It has long been suspected that the cecropins belong to the group of amphipathic peptides with membrane-disrupting ability [21]. It is now clearly demonstrated that artificial membranes are disrupted by cecropins.

The liposome lysis potential of the different cecropin analogues and their bacterial killing ability is strongly correlated. It should be noted that the effective concentration of cecropin as well as the total membrane surface area employed are comparable in the liposome assay (Fig. 1) and the bacterial killing assay (Fig. 5). The lysis of bacteria is thus most likely caused by a primary membrane attack similar to that found in liposomes. The strong correlation between bacterial and liposome lysis for a number of cecropin analogues also suggests that the cecropin target is the phospholipid bilayer. However, the different susceptibilities of different bacterial species cannot be explained from these studies on model membranes but are likely to be caused by membrane components other than phospholipids.

The cecropin analogues used, all of which contain impaired N-terminal amphipathic helical structures, are not as efficient in disrupting membranes as cecropin itself. The weakened helical character of the analogues [11] could either have decreased their intrinsic ability to disrupt membranes or lowered their binding affinities to membranes. The present study indicates that the latter is the case. A strongly amphipathic helix in the N-terminal part of the cecropin is needed for an efficient binding but minor alteration of its precise structure and polarity are not critical for the intrinsic membrane-disrupting activity of cecropin.

One objective of this work was to gain some information about the stoichiometry of the interaction of cecropin with bacteria. Cecropin action is not of a catalytic nature but rather a matter of mass action. This follows from the fact that the final level of lysis is proportional to the amount of cecropin used when lysis is recorded as a function of time (Fig. 4).

The number of cecropin molecules bound per bacterium when 50% of the bacterial cells are dead can be calculated from the binding experiments (Fig. 5). This gives $5.3 \cdot 10^5$, $5.6 \cdot 10^6$ and $2.2 \cdot 10^7$ cecropin molecules per cell for *E. coli* D31, *B. megaterium* Bm11 and *B. thuringiensis* Bt11, respectively. It was estimated that there were 3.6 cells per colony-forming unit of Bm11 and Bt11. Assuming that D31, Bm11 and Bt11 are rod-shaped objects of the dimensions 0.5×2, 1.5×4 and 1.1×4 μm, respectively, the number of cecropin molecules per surface area can be calculated. There are thus $1.5 \cdot 10^5$, $2.5 \cdot 10^5$ and $1.4 \cdot 10^6$ cecropin molecules per μm^2 of bacterial surface for D31, Bm11 and Bt11, respectively. This is equivalent to an available surface area of 660, 400, and 72 $Å^2$ per cecropin molecule. The actual area occupied by one cecropin molecule is dependent on both its folding and orientation in the membrane. Assuming that the predicted N-terminal amphipathic helix is 21 residues long with a pitch of 1.5 Å per residue and a density of 1.37 g/cm^3, this part alone will have a projected surface area of 350 $Å^2$, if its axis is parallel to the membrane surface. Considering the calculated available area, this would imply close packing of cecropin molecules on the bacterial surface. For *B. thuringiensis* even more than one layer of cecropin molecules is indicated. For Gram-negative bacteria one can also take into consideration the fact that there are two membranes. By forming membrane structures with smaller diameters, such as mixed micelles, there would be a somewhat larger space to accommodate the large numbers of cecropin molecules. The release of such structures is indicated from the binding studies.

It can be noted that cecropin has similar affinities to the resistant insect pathogen *B. thuringiensis* and to the very susceptible *E. coli*. The pathogen is thus not able to avoid binding of cecropin but the bacterium is inherently resistant to attack by the bound cecropin. This can be achieved by non-productive binding of cecropin that prevents it from reaching its membrane target.

The precise physical nature of the interaction of cecropin and membrane is not possible to deduce from this work but some general features are suggested. At the cecropin concentrations employed, formation of discrete holes (as reviewed by Bhakdi and Tranum-Jensen [22]) is rather unlikely due to the extensive binding of cecropin. A more general membrane disintegration is more likely as the final state. The initial event could still be the formation of discrete holes through the membrane.

The resistance of erythrocytes to lysis by cecropin was shown to be obtained by a lack of affinity. The rationale for this is not clear. The hypothesis that the phospholipid composition conferred specificity can be rejected from the fact that liposomes with a phospholipid composition similar to that of erythrocytes were sensitive. Alternatively, the lack of a membrane potential could inhibit cecropin binding. This idea would be analogous to what has been suggested to explain melittin interaction with nerve cell membranes [23]. A similar model has also recently been proposed for the specific interaction of amphipathic mitochondrial signal sequences with the energized mitochondrial membrane [24]. Two facts speak against such an explanation for cecropin-mediated lysis. First, the liposomes were readily lysed without the presence of a membrane potential. Second, the bacterial outer membrane that has no membrane potential has to be crossed by cecropin. This molecule of molecular weight 4000 is most probably too large to freely pass across the outer membrane. Thus, it probably has to destroy this membrane that lacks membrane potential, unless the target is at junctions between the outer and inner membranes.

Acknowledgements

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