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# Mechanism of Interaction of Different Classes of Cationic Antimicrobial Peptides with Planar Bilayers and with the Cytoplasmic Membrane of *Escherichia coli*<sup>†</sup>

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**ABSTRACT:** Antimicrobial cationic peptides are prevalent throughout nature as part of the intrinsic defenses of most organisms, and have been proposed as a blueprint for the design of novel antimicrobial agents. They are known to interact with membranes, and it has been frequently proposed that this represents their antibacterial target. To see if this was a general mechanism of action, we studied the interaction, with model membranes and the cytoplasmic membrane of *Escherichia coli*, of 12 peptides representing all 4 structural classes of antimicrobial peptides. Planar lipid bilayer studies indicated that there was considerable variance in the interactions of the peptides with model phospholipid membranes, but generally both high concentrations of peptide and high transmembrane voltages (usually  $-180$  mV) were required to observe conductance events (channels). The channels observed for most peptides varied widely in magnitude and duration. An assay was developed to measure the interaction with the *Escherichia coli* cytoplasmic membrane employing the membrane potential sensitive dye 3,5-dipropylthiobarbituric acid in the outer membrane barrier-defective *E. coli* strain DC2. It was demonstrated that individual peptides varied widely in their ability to depolarize the cytoplasmic membrane potential of *E. coli*, with certain peptides such as the loop peptide battenecin and the  $\alpha$ -helical peptide CP26 being unable to cause depolarization at the minimal inhibitory concentration (MIC), and others like gramicidin S causing maximal depolarization below the MIC. We discuss the mechanism of interaction with the cytoplasmic membrane in terms of the model of Matsuzaki et al. [(1998) *Biochemistry* 37, 15144–15153] and the possibility that the cytoplasmic membrane is not the target for some or even most cationic antimicrobial peptides.

Antibiotic resistance has been a great concern in recent years due to the extensive clinical use of classical antibiotics. The development of a new class of antibiotics has become increasingly important. Among the possible candidates, the antimicrobial cationic peptides have attracted increasing research and clinical interest. Antimicrobial cationic peptides have been found in a variety of sources, such as amphibians, mammals, insects, plants, and bacteria (1, 2), including magainins, defensins, cecropins, melittin, and thionins. These cationic peptides and many others exhibit potent activities against a rather broad spectrum of microbial organisms, including Gram-positive and Gram-negative bacteria, fungi, and enveloped viruses (2, 3). It is now clear that these endogenous peptide antibiotics are key components of the

innate immunity, and constitute a primary defense system of host.

Based on their secondary structure, cationic peptides can be categorized into four major groups, including  $\beta$ -sheet structures stabilized by two to three disulfide bridges,  $\alpha$ -helices, extended structures with a predominance of one or more amino acid, and loop structures containing only one disulfide bridge (3). Despite this diversity, the secondary structure is usually composed both of a hydrophobic surface and of a hydrophilic surface. This amphipathic structural feature is believed to play a key role in the antimicrobial mechanism of action. The hydrophilic (positive charged) property is proposed to initiate peptide interaction with the negatively charged bacterial surface and the negatively charged headgroups of bilayer phospholipids. The hydrophobic property would permit the peptides to enter the membrane interior.

It has been proposed that the antibacterial target of cationic peptides is the cytoplasmic membrane (4). Cationic peptides are generally able to interact electrostatically with the negatively charged headgroups of bacterial phospholipids and then insert into the model membranes of planar bilayers or liposomes, forming transient channels or pores (5–8). Assuming this also happens in bacterial cells, it has been proposed that such channel formation leads to the leakage

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Table 1: Peptides Used in This Study

peptide	class	sequence <sup>a</sup>	size	net charge	<i>E. coli</i> DC2 MIC ( $\mu$ g/mL)	MIC in 0.1 M KCl ( $\mu$ g/mL)
CP26	$\alpha$ -helical	KWK <sup>d</sup> SIKKLTSAAKKVVTTAKPLISS	26	+6	0.5	0.5
CEME	$\alpha$ -helical	KWKLFKKIGIGAVLKVLTGTPALIS	26	+4	2	2
CEMA	$\alpha$ -helical	KWKLFKKIGIGAVLKVLTGTPALTLTK	28	+5	1	1
CP29	$\alpha$ -helical	KWK <sup>d</sup> SIKKLTAVKKVLTGTPALIS	26	+5	1	1
indolicidin	extended	ILPWKWPWWPWRR-NH <sub>2</sub>	13	+3	4	8
CP11-NH <sub>2</sub>	extended	ILKKWPWWPWRR-NH <sub>2</sub>	13	+5	1	4
bactenecin	loop	Cyclized RL(CRIVVIRVC)R	12	+3	2	8
linear Bac	linear $\beta$	RLCRIVVIRVCR	12	+3	2	16
Bac2S	linear $\beta$	RLSRIVVIRVSR	12	+3	2	16
gramicidin S	$\beta$ -structured loop	cyclic (PF <sup>d</sup> LOVPF <sup>d</sup> LOV)	10	+2	2	8
Gram474	$\beta$ -structured loop	cyclic (PVKLKVY <sup>d</sup> PLKVKLY <sup>d</sup> )	14	+4	32	32
Gram4112	$\beta$ -structured loop	cyclic (PVKLKVY <sup>d</sup> PLKVKLY <sup>d</sup> )	14	+4	2	8
polymyxin B	cyclic lipopeptide	cyclized iso-octanoyl BTBB(BF <sup>d</sup> LBBT)	10	+5	0.125	0.125

<sup>a</sup> Amino acids are labeled according to the one-letter code with the following additions: O = ornithine, B = diaminobenzoate, superscript d, e.g., F<sup>d</sup>, indicates the D enantiomer. Bolded residues are basic (i.e., carry a positive charge); amino acids in parentheses are cyclized.

of cell contents and cell death. However, there is very little data for peptides pertaining to measurement of the disruption of the bacterial cytoplasmic membrane permeability barrier, despite ample evidence that membrane disruption can occur in model membrane systems. Shai (9) pointed out that in model membrane systems, very high ratios of peptide to lipid are required and suggested an alternative "carpet" model in which the peptides cooperatively destroy the membrane barrier without channel formation. On the other hand, Matsuzaki et al. (10) indicated that peptides bind to the outer leaflet of model membranes and flip inward, carrying lipids with them and creating brief disruptions in permeability. Although some investigators (e.g., ref 6) have utilized measurements of the accessibility of a normally membrane-impermeable substrate to cytoplasmic  $\beta$ -galactosidase, these assays suffer from using a bulky substrate (*o*-nitrophenyl galactoside), as well as an inability to fully dissociate cytoplasmic from outer membrane permeabilization. To circumvent this, we have adopted here an assay involving the membrane potential sensitive dye diSC3-5<sup>1</sup> to measure the disruption of the electrical potential gradient across the cytoplasmic membrane of intact bacteria using the *E. coli* mutant DC2 to permit us to perform this assay in the absence of EDTA. Bacteria, through either electron transport or ATP hydrolysis, eject protons and maintain a protonmotive force comprising an electrical potential gradient ( $\Delta\psi$ , oriented internal negative) and a pH gradient ( $\Delta$ pH, oriented internal alkaline) (11). At neutral pH in the medium, by far the major contribution to the protonmotive force is provided by the membrane potential gradient of about  $-150$  mV (12), which is maintained by K<sup>+</sup> flux utilizing the large K<sup>+</sup> pool inside cells (approximate concentration 100–150 mM K<sup>+</sup>). Permeabilization of the cytoplasmic membrane would allow ions to equilibrate across the cytoplasmic membrane to reduce or destroy the membrane potential gradient.

We have used the diSC3-5 assay as a tool to study the interaction of cationic peptides of several structural classes with the cytoplasmic membrane and correlated this with the activity of these peptides in planar bilayer membranes. In particular, many previous studies have examined  $\alpha$ -helical peptides 22–26 amino acids in length, which can in principle

span the membrane bilayer (1). We have examined 3 classes of shorter peptides 10–14 amino acids in length.

## EXPERIMENTAL PROCEDURES

**Bacterial Strains and Growth Conditions.** The bacterial strain used was the antibiotic-supersusceptible, outer membrane barrier-altered *E. coli* strain DC2 (13). It was grown in Luria broth (10 g/L tryptone and 5 g/L yeast extract, 5 g/L NaCl).

**Chemicals.** 3,5-Dipropylthiarcocyanine (diSC3-5) was from Molecular Probes (Eugene, OR). Gramicidin S was purchased from Sigma (St. Louis, MO). Diphytanoylphosphatidylcholine and diphytanoylphosphatidylglycerol were purchased from Avanti Lipids.

**Peptide Synthesis.** Peptides were synthesized by Fmoc [N-(9-fluorenyl)methoxycarbonyl] chemistry by the Nucleic Acid/Protein Service unit at the University of British Columbia using an Applied Biosystems, Inc. (Foster City, CA), Model 431 peptide synthesizer. Gramicidin S was purchased from Sigma, and Dr. Bob Hodges (PENCE, Edmonton, Canada) provided Gram474 and Gram4112 (14). All the peptide sequences are listed in Table 1. Bactenecin was obtained in its fully reduced form. Its disulfide bond was formed as described previously (16).

**Peptide Concentration Determination.** Concentrations of bactenecin and its derivatives were determined by amino acid analysis by the NAPS unit at UBC. Concentrations of CP26, CEME, CEMA, CP29, indolicidin, and CP11-NH<sub>2</sub> were estimated by the dinitrophenylation assay, which measures the presence of free amino groups (15). Concentrations of gramicidin S and its analogues were determined by the dried weight of powder.

**Minimal Inhibitory Concentration (MIC) Determination.** The MICs of peptides were determined by a modified 2-fold microtiter broth dilution method (16).

**Planar Bilayer Assays.** The basic methods have been reported previously (17, 18). Membranes were made from 1% lipid (comprising 0.2% diphytanoylphosphatidylglycerol and 0.8% diphytanoylphosphatidylcholine) in *n*-decane. Bilayers were painted across a 2 mm<sup>2</sup> hole in a Teflon divider separating two compartments containing 5–6 mL each of a bathing solution of 1 M KCl. Voltages were applied across this membrane through Calomel electrodes connected by a salt bridge, and the resultant current was boosted 10<sup>9</sup>–10<sup>10</sup>.

<sup>1</sup> Abbreviations: MIC, minimal inhibitory concentration; diSC3-5, 3,5-dipropylthiarcocyanine.

fold by a current amplifier, monitored on a Tektronix Model 7633 oscilloscope and recorded on a Rikadenki R-01 strip chart recorder.

**Cytoplasmic Membrane Permeability Assay.** Cytoplasmic membrane permeabilization was determined by using the membrane potential-sensitive cyanine dye diSC3-5 (19), which distributes between cells and the medium depending on the cytoplasmic membrane potential gradient. Once inside cells, it becomes concentrated and self-quenches its own fluorescence, probably due to the formation of dye aggregates inside cells (19). If peptides form channels or otherwise disrupt the membrane, the membrane potential will be dissipated, and diSC3-5 will be released into the medium causing the fluorescence to increase. The mutant *E. coli* DC2 with increased outer membrane permeability was used so that diSC3-5 could readily reach the cytoplasmic membrane in the absence of EDTA (cf. refs 20, 21). Bacteria were grown at 37 °C with shaking to mid-logarithmic phase ( $OD_{600} = 0.5-0.6$ ). Cells were collected by centrifugation, washed once with buffer (5 mM HEPES, pH 7.2, 5 mM glucose), and resuspended in the same buffer to an  $OD_{600}$  of 0.05. The cell suspension was incubated with 0.4  $\mu$ M diSC3-5 until diSC3-5 uptake was maximal (as indicated by a stable reduction in fluorescence due to fluorescence quenching as the dye became concentrated in the cell by the membrane potential). Then 100 mM KCl was added to equilibrate the cytoplasmic and external  $K^+$  concentrations. A 1 mL cell suspension was placed in a 1 cm cuvette, and the desired concentration of tested peptide was added. The fluorescence reading was monitored by using a Perkin-Elmer Model 650-10S fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, CT), at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. The maximal fluorescence increase due to the disruption of the cytoplasmic membrane was recorded. A blank with only cells and the dye was used to subtract the background.

## RESULTS

**Model Membrane Studies.** To provide a basis for understanding the results of whole cell experiments, each of the peptides tested here was examined for its ability to influence the conductance of planar bilayer membranes. Membranes were constructed from 20% anionic lipid (diphytanoylphosphatidylglycerol, as a surrogate for the *E. coli* lipids phosphatidylglycerol and cardiolipin) and 80% zwitterionic lipid (diphytanoylphosphatidylcholine, as a surrogate for phosphatidylethanolamine). Twelve different peptides were examined including four 26-28 amino acid  $\alpha$ -helical peptides, two 11-mer extended peptides, the 12-mer loop peptide bactenecin and its linear variants, and three 10-14 amino acid cyclic  $\beta$ -structured peptides including gramicidin S and two variants. In Figure 1, some illustrative recordings of the channel-forming behavior of four of these peptides are presented, and a summary of the observations with all of the peptides is provided in Table 2. In general, these peptides required large concentrations (approximately 1  $\mu$ g/mL or more) to permit the observation of reasonable numbers of channels, and most of the studies reported in Figure 1 and Table 2 were performed at such concentrations. Certain generalizations could be made regarding the observed activities. First it required a high voltage across the mem-

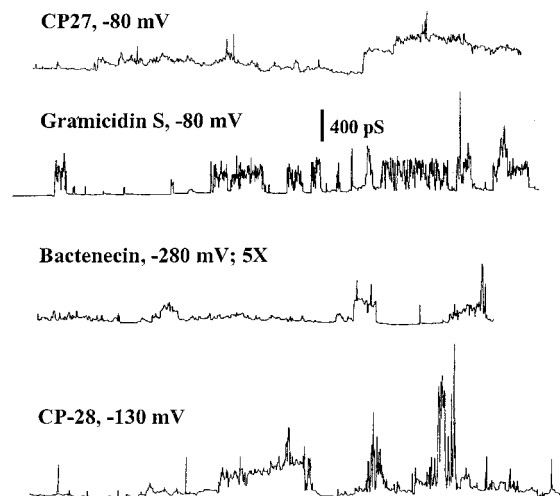


FIGURE 1: Chart recorder tracings of conductance events that occurred upon the addition of 1  $\mu$ g/mL of the indicated peptides to the solution (1 M KCl) bathing a planar lipid bilayer. The indicated voltages were applied. Approximately 2 min of chart recordings is shown. The bar indicates 400 pS ( $1\text{ S} = 1\text{ }\Omega^{-1}$ ).

brane (generally  $-180$  mV) to initiate conductance across the membrane. When the applied voltage was subsequently decreased to  $-80$  mV, conductance events were still observed but often at a lower frequency. Second, these conductance events were not initiated by a positive applied voltage (e.g.,  $+180$  mV), a result that seemed reasonable given the positive charge on the peptides. Indeed, a switch to a positive voltage often led to a complete loss of all trans membrane conductance events. Third, although channel-like conductance events of variable duration were observed (e.g., see Figure 1), many of the events observed involved very rapid conductance alterations which were quite variable in magnitude (see, e.g., the CP-28 CEMA trace in Figure 1). An interesting observation was that there were few situations where conductance events lead to a substantial increase in transmembrane conductance since events tended to be rapid and the net result was a rapid or eventual return to base line conductance. Also none of these peptides caused rapid destabilization of the membrane despite the very high concentrations and voltages utilized, in contrast to the membrane destabilization predicted by the carpet model of action (9).

Two of the peptides showed somewhat different behavior to sequence-related peptides. Peptide CP26 ( $\alpha$ -helical) was relatively inactive, compared to the related peptides CEME, CEMA, and CP29, and tended to require higher voltages to initiate conductance events and stabilized the membrane to  $-280$  mV, despite the observation that conductance events once initiated for all four peptides were of a similar size and lifetime range. The loop peptide bactenecin was similarly poorly active, required higher voltages to initiate conductance events, led to ultrastable membranes, and formed smaller channels compared to its reduced version (Lin Bac) and linearized version, respectively. The other two peptides that had reduced activity were indolicidin and CP11-NH<sub>2</sub> (extended structures), and both also resulted in membranes that were hyperstable to voltages of  $-280$  to  $-330$  mV.

**Relationship between Membrane Potential and diSC3-5 Fluorescence Intensity in *E. coli* DC2 Cells.** Upon addition to mid-log phase *E. coli* DC2 cells ( $OD_{600} = 0.05$ ) at room



Table 2: Black Lipid Bilayer Observations on Antimicrobial Peptides

peptide	activation voltage (mV)	still active at -80 mV	lifetime of channels (ps)	size of channels at -80 mV (pS)	size of channels at -180 mV, pS	polarity: effect of switching voltages	effect on membrane stability	activity
CP26	-180 to -280	yes but less active	10 ms to 3 s	60–180 and up to 420	same	switch to +180 mV turns off channels	stable to -280 mV	not very active at 2 $\mu$ g/mL
CEME	-80 or less	yes	10 ms to >30 s	40–180 and up to 300	same	switch to +80 mV turns off channels	not very stable at -180 mV	very active
CEMA	-180	yes	10 ms to 3 s	30–60 and up to 375	38–154 and up to 769	not done	stable at -180 mV for >5 min	active
CP29	-180	yes but less active	10 ms to 6 s	25–70	18–720	switch to +20 mV turns off channels	not very stable at -180 mV	very active
indolicidin	-80	yes but less active	10 ms to 15 s	2.5–25	5–20	switch to +50 mV turns channels off	membrane very stable at -330 mV	not very active
CP11-NH <sub>2</sub>	-180	just as active	10 ms to 15 s	25–50	15–400	not done	membrane very stable at -230 mV	not very active even at 4 $\mu$ g/mL
Bac	-180 to -280	yes but less active	15 ms to 10 s	4–5	6	not done	stable to -380 mV	not very active even at 3 $\mu$ g/mL
Lin Bac	-180	active	0.1–4 s	13–200 and up to 688	56–222	still active	not very stable at -280 mV	active
Bac2S	-80	yes	15 ms to 4 s	12–25, and up to 188	11–28 and up to 139	not done	very stable at -280 mV	active
gramicidin S	-80	active	0.5–6 s	most 375, some 125	~280	switch to +180 (not to +130) turns off channels	stable to -230, membrane broke at -280 mV	very active
Gram4112	-180 then -80	yes but less active	most <10 ms up to 2 s	3–90, and up to 300	15–60 and up to 200	loss of activity	membrane broke at -180 (1 $\times$ ) and -230 mV (2 $\times$ )	very active
Gram474	-180	yes	10 ms to 15 s	3	25 at -330 mV	no effect	very stable at -330 mV, 5 min and -380 mV, 3 min	active

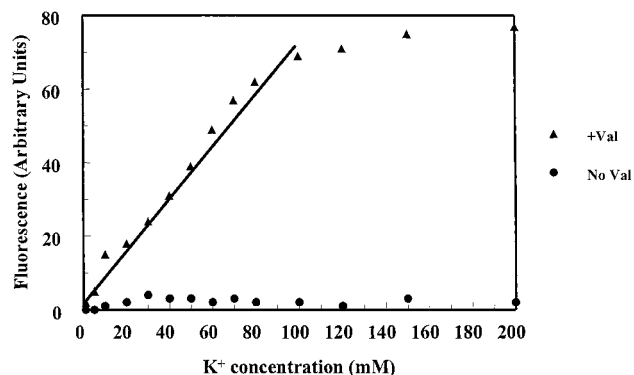


FIGURE 2: Effect of the external KCl concentration on the fluorescence intensity of *E. coli* DC2 cells incubated with 0.4  $\mu$ M diSC3-5 and 1  $\mu$ M valinomycin. log-phase *E. coli* cells ( $OD_{600} = 0.05$ ) were incubated with 0.4  $\mu$ M diSC3-5 until maximal fluorescence quenching was observed, and then 1  $\mu$ M valinomycin was added ( $\blacktriangle$ ) or not added ( $\bullet$ ). Various concentrations of KCl were added to 1 mL aliquots of the mixture to alter the  $K^+$  gradient. Then cell suspensions were incubated at room temperature for 10 min before fluorescence readings were taken.

temperature, the fluorescence of diSC3-5 decreased gradually until it stabilized to a steady level of about 10% of its original level after an hour. The fluorescence quenching was only induced in *E. coli* cells with an intact membrane potential gradient (inside negative). The addition of 0.1 M KCl without the presence of valinomycin after this stabilization had no effect on the fluorescence intensity.

Changing the concentration of potassium outside the cells will change the potassium concentration gradient accordingly. The potassium concentration gradient opposes the electrical gradient and thus maintains the membrane potential gradient  $\Delta\psi$  across the membrane (12). Valinomycin, an ionophore, renders the cytoplasmic membrane permeable to potassium (11). Therefore, varying the  $K^+$  concentration in the presence of valinomycin outside cells will change the equilibrium  $\Delta\psi$  accordingly. This was indicated by plotting the fluorescence quenching of diSC3-5 as a function of  $K^+$  concentration outside the cells (Figure 2). As the concentration of  $K^+$  outside increased, the fluorescence intensity increased proportionally (i.e., fluorescence quenching decreased as diSC3-5 left the cells), indicating a decrease of membrane potential. A linear relationship was observed over the range 10–100 mM external potassium concentrations. At 0.1 M KCl, the increase reached its maximum, due to equilibration of the outside and inside  $K^+$  concentrations. This result demonstrated that the partitioning of diSC3-5 between the suspension medium and cells was membrane potential-dependent and was directly proportional to the size of the membrane potential. To prevent any effects due to the movement of potassium ions (which could oppose the dissipation of the electrical potential gradient), an external concentration of 0.1 M KCl was used in the subsequent cytoplasmic membrane permeability assay. As a control, we demonstrated that 1  $\mu$ M valinomycin alone did not cause a change in fluorescence intensity, while the subsequent addition of 0.1 M KCl resulted in a release of diSC3-5 over the subsequent 10 min.

We confirmed above that most of the cationic peptides with which we worked were able to form conductance increases in model membranes. If this occurred in the bacterial cytoplasmic membrane, it would be predicted to cause the membrane potential to be dissipated resulting in

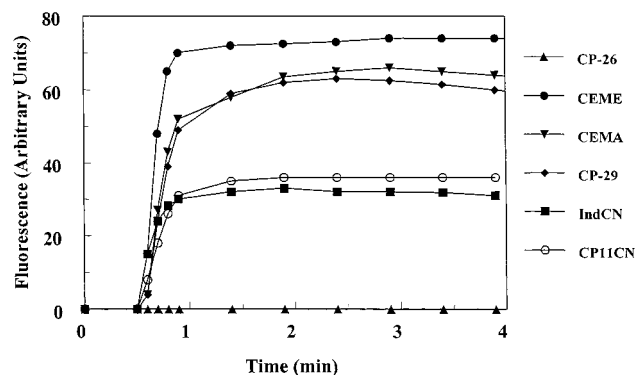


FIGURE 3: Kinetics of fluorescence intensity changes of *E. coli* DC2 in the presence of  $\alpha$ -helical and extended helical cationic peptides. Peptides at their MICs were added separately to respective log-phase *E. coli* DC2 cells preincubated with  $0.4 \mu\text{M}$  diSC3-5 in the presence of  $0.1 \text{ M KCl}$ . CP26,  $0.5 \mu\text{g/mL}$  (▲); CEME,  $2 \mu\text{g/mL}$  (●); CEMA,  $1 \mu\text{g/mL}$  (▼); CP29,  $1 \mu\text{g/mL}$  (◆); indolicidin,  $8 \mu\text{g/mL}$  (■); CP11-CN,  $4 \mu\text{g/mL}$  (○).

redistribution of diSC3-5, causing the fluorescence to increase. A cytoplasmic membrane permeability assay was developed based on this assumption.

**Antimicrobial Activities in the Presence of Potassium Chloride.** Since  $0.1 \text{ M KCl}$  was added to the assay buffer to equilibrate the cytoplasmic and external  $\text{K}^+$  concentrations, the effect of  $0.1 \text{ M KCl}$  on the antimicrobial activity was tested. Table 1 shows the MIC of a variety of cationic peptides in the presence and absence of  $\text{K}^+$ . It seemed that the activity of the  $\alpha$ -helical cationic peptides was not affected by the presence of  $\text{K}^+$ . Among the tested  $\beta$ -structured peptides, the analogue Gram474 was not affected, but the MICs of gramicidin S and Gram4112 were increased by 4- and 8-fold, respectively. MICs of the extended structured peptide, indolicidin, and its variant CP11-NH<sub>2</sub> were increased by 2- and 4-fold. The MIC of polymyxin B did not change, but MICs of the loop peptide cyclic batenecin and of its reduced form and linear variant bac2S were increased by 4–8-fold.

**Interaction of  $\alpha$ -Helical Cationic Peptides with the Cytoplasmic Membrane.** CP26, CEME, CEMA, and CP29 belong to the group of amphipathic  $\alpha$ -helical peptides which are unstructured in free solution but upon interaction with lipid bilayers adopt a conformation that comprises at least 50%  $\alpha$ -helix as judged by circular dichroism (23). CEME is hybrid of moth cecropin (residues 1–8) and bee melittin (residues 1–18) (23–25). CEMA is a derivative of CEME with two extra positive charges at the C-terminal end (23, 25). CP26 and CP29 were CEME variants designed in our laboratory to have improved  $\alpha$ -helicity (22).

Although these four peptides had only 2–4-fold differences in MIC toward *E. coli* and other bacteria, their abilities to dissipate the membrane potential were quite different. CP26 had the lowest MIC, yet it failed to dissipate the membrane potential at its MIC (Figure 3). It started to disrupt the membrane potential at  $2 \mu\text{g/mL}$ , 4-fold higher than its MIC, and achieved maximal membrane potential disruption at  $8 \mu\text{g/mL}$  (Figure 4). CEME, CEMA, and CP29 had similar MICs and showed similar patterns (Figures 3 and 4) in completely dissipating the membrane potential at concentrations within 2-fold of their MICs. The fluorescence increases caused by CEME, CEMA, and CP29 occurred rapidly and reached a maximum equivalent to complete loss of diSC3-5

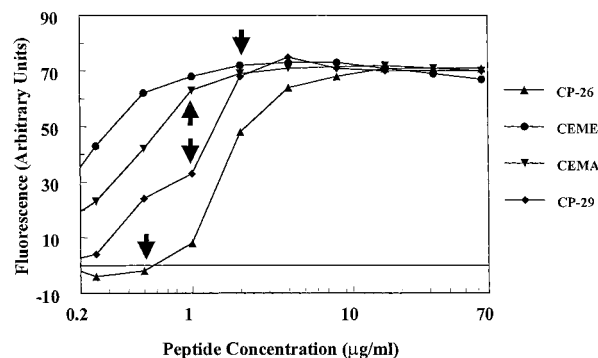


FIGURE 4: Effect of  $\alpha$ -helical cationic peptides on the fluorescence intensity changes of *E. coli* DC2 cells incubated with diSC3-5. Readings were taken when the maximal fluorescence increase was reached, typically within 5 min. CP26 (▲); CEME (●); CEMA (▼); CP29 (◆). Arrows indicate the MIC values.

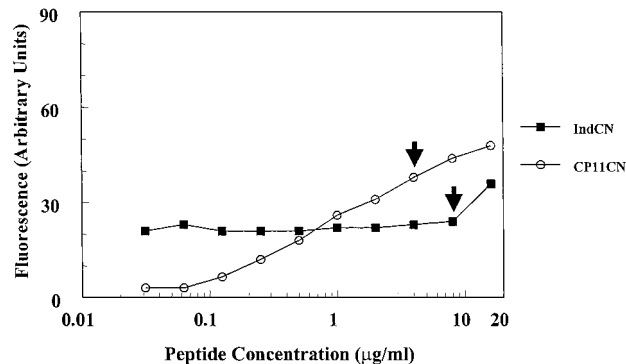


FIGURE 5: Effect of extended structured cationic peptides on the fluorescence intensity of *E. coli* DC2 cells preincubated with diSC3-5. Readings were taken when the maximal fluorescence increase was reached. Indolicidin (■); CP11-CN (○). Arrows indicate the MIC.

within 2 min. CEME had the lowest activity (highest MIC) among these peptides, but it seemed to have the greatest ability to dissipate the membrane potential.

**Interaction of Extended Structured Cationic Peptides with the Cytoplasmic Membrane.** Indolicidin, with its carboxyl terminus amidated, is identical to the 13-mer peptide present in the cytoplasmic granules of bovine neutrophils (26). CP11-NH<sub>2</sub> is a derivative of indolicidin with Lys3 replacing Pro3 and Trp4, and an additional Arg residue at the C-terminus producing a molecule with a greater positive charge (27). At their MICs, both peptides rapidly dissipated membrane permeability (Figure 3). However, the increase was only 30–35 arbitrary units, less than half that of the  $\alpha$ -helical peptide CEME. These peptides failed to cause full dissipation of the cytoplasmic membrane potential even at concentrations 4-fold the MIC (Figure 5), and a very flat concentration dependence was observed for both peptides.

**Interaction of  $\beta$ -Structured Cationic Peptides with the Cytoplasmic Membrane.** Gramicidin S is a naturally occurring 10 amino acid, cyclic peptide antibiotic first isolated from *Bacillus brevis* (28). It has been shown to possess a two-stranded  $\beta$ -sheet structure connected by  $\beta$ -turns (29, 30). Gram4112 and Gram474 are two 14 amino acid analogues of gramicidin S (18, Table 1) that were respectively as active and 4-fold less active than gramicidin S (Table 1). At their MIC, all three gramicidin peptides caused very rapid membrane depolarization (Figure 6), although Gram4112 was less effective than the other two. Both gramicidin S and Gram474 started to cause membrane

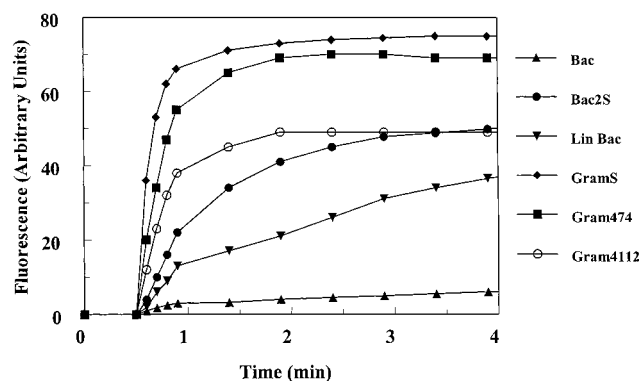


FIGURE 6: Kinetics of the fluorescence intensity changes of *E. coli* DC2 in the presence of  $\beta$ -structured and loop cationic peptides. Peptides at their MICs were added to log-phase *E. coli* DC2 cells that had been preincubated with 0.4  $\mu$ M diSC3-5 in the presence of 0.1 M KCl. Gramicidin S, 8  $\mu$ g/mL (◆); G474, 32  $\mu$ g/mL (■); Gram4112, 8  $\mu$ g/mL (○); battenecin, 8  $\mu$ g/mL (▲); battenecin (reduced), 16  $\mu$ g/mL (▼); Bac2S, 16  $\mu$ g/mL (●).

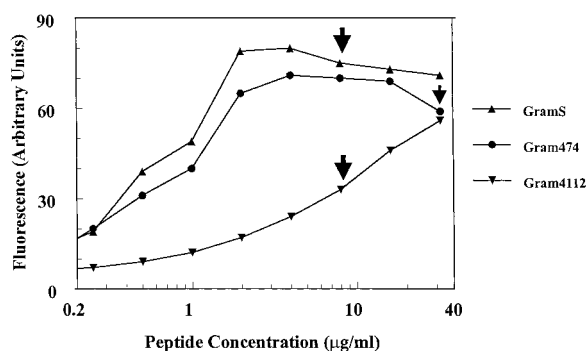


FIGURE 7: Effect of  $\beta$ -structured cationic peptides on the fluorescence intensity of *E. coli* DC2 cells incubated with diSC3-5. Readings were taken when the maximal fluorescence increase was reached. Gramicidin S (◆); Gram474 (●); Gram4112 (▼). Arrows indicate the MIC.

depolarization at very low (sub-MIC) concentrations and achieved maximal dissipation of membrane potential at concentrations well below their MICs, at 2  $\mu$ g/mL (Figure 7). Gram4112, on the other hand, did not cause maximum membrane depolarization at its MIC, and achieved this only at 4-fold MIC.

**Interaction of Loop-Structured Cationic Peptides with the Cytoplasmic Membrane.** Studies on the mechanism of action of the 12 amino acid battenecins using the above-described assay have indicated substantial differences in their ability to permeabilize the membrane at their MICs according to whether they are in their natural cyclic form or linearized (16). This observation was confirmed (Figure 6) and extended (Figure 8) here. Despite the similar MICs for cyclic battenecin, linear battenecin, and Bac2S against *E. coli* DC2, the influence of different concentrations of these peptides on the membrane potential was quite different (Figures 6 and 8). The linear derivatives led to rapid depolarization (<4 min), and caused maximal dissipation of membrane potential at concentrations well below their MIC concentrations (Figure 8). In contrast, native cyclic battenecin caused cytoplasmic membrane depolarization gradually and very slowly (Figure 6; 16). A concentration of 64  $\mu$ g/mL (8-fold the MIC) was required to achieve maximum depolarization, and even at this concentration, it took 90 min to achieve this level of probe release.

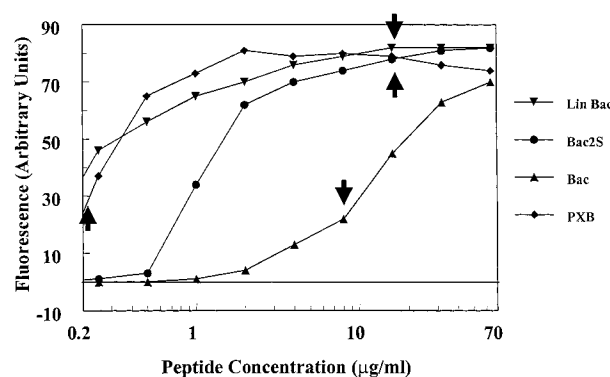


FIGURE 8: Effect of loop-structured cationic peptides on the fluorescence intensity of *E. coli* DC2 cells preincubated with diSC3-5. Readings were taken when the maximal fluorescence increase was achieved: battenecin (▲); battenecin (reduced) (▼); Bac2S (●); polymyxin B (◆). Arrows indicate the MIC.

## DISCUSSION

It has often been proposed that the antibacterial target of cationic peptides is the cytoplasmic membrane (e.g., see ref 4). Cationic peptides are generally able to interact electrostatically with the negatively charged headgroups of bacterial phospholipids and then insert into the cytoplasmic membrane, forming conductance events which are proposed to lead to the leakage of cell contents and cell death (5, 6, 7, 18). The observations presented here (Table 2), regarding the conductances observed as a result of peptide addition to model membranes, are similar to these other studies, although some heterogeneity was observed. There is ample evidence that membrane disruption can occur in model membrane systems (8), although it has been correctly pointed out that this occurs at very high peptide-to-lipid ratios (9). However, there have been very few measurements of the disruption of the cytoplasmic membrane permeability barrier by cationic peptides. In this paper, we have adapted an assay involving the membrane potential sensitive dye diSC3-5 to measure the disruption of the electrical potential gradients across the cytoplasmic membrane in intact bacteria under conditions where the normally large  $K^+$  concentration gradient across the cytoplasmic membrane is equilibrated. It provided a sensitive, rapid assay of cytoplasmic membrane permeabilization. However it must be noted that this assay does not measure membrane destruction or an equivalent lethal event since, e.g., valinomycin can destroy the membrane potential but is not lethal (but rather reversibly bacteriostatic) at the concentrations utilized here.

Different peptides varied substantially in their abilities to depolarize the membrane potential. There was no absolute correlation between the ability to permeabilize the cytoplasmic membrane and the antimicrobial activity. For example, CP26 and battenecin had quite good activities against bacteria like *E. coli* but were unable to depolarize the cytoplasmic membrane at their MIC concentrations. Moreover, there was a correlation between reduced activity in lipid bilayers and reduced ability to disrupt membrane potential (for CP26, battenecin, indolicidin, and CP11-CN). This strongly suggests, at least for these peptides, that the mechanism of action involves some event other than the breakdown of the cytoplasmic membrane permeability. For all peptides, the data from both the model membrane and intact bacteria depolarization experiments were not consistent



with the carpet model of Shai (9). This model suggests that cationic antimicrobial peptides act by coating the membrane until they reach a trigger concentration (supposed to be when the peptides saturate the membrane surface), and at this concentration they collapse inward, destroying the membrane barrier. Instead, there were few instances of membrane breakage even at the high concentrations and large transmembrane voltages used in the planar bilayer experiments presented here. Furthermore, there were also no obvious precipitous events in intact cells, and instead the membrane potential alterations occur over a broad range of concentrations.

Given the above precedents, and a variety of suggestions in the literature that peptides may act in ways other than membrane breakdown (32–35), it is worth asking the question as to whether other peptides kill by breaking down the cytoplasmic membrane barrier. We believe that there is a good possibility that this is not the primary mechanism of action of many antimicrobial peptides. We know already that permeabilization of the cytoplasmic membrane to destroy the membrane potential is not lethal per se (e.g., the effects of uncouplers such as carbonyl cyanide *m*-chlorophenyl hydrazone are reversible). Looking at all of the peptides studied here (representing most structural classes), there was clearly no correlation between the concentrations leading to complete permeabilization and MIC. Some peptides, e.g., gramicidin S, depolarized the membrane partially at concentrations well below the MIC, whereas others, e.g., bactenecin, were effective only at concentrations well above the MIC. Thus, it is possible that the known abilities of peptides to act on lipid membranes reflect their mechanism of passage across the membrane and that their actual targets are in the cytoplasm. In this regard, Matsuzaki et al. (31) have clearly demonstrated that  $\alpha$ -helical magainins can pass across membranes. Furthermore, a variety of other mechanisms of action have been suggested, including stimulation of autolytic enzymes (32), interference with bacterial DNA and/or protein synthesis (33), inhibition of DNA synthesis leading to filamentation (34), or general binding to and inhibition of cellular nucleic acids (35). Our own studies have confirmed that the cationic peptides readily interact with nucleic acids (36), and we would suggest this as a credible alternative mechanism.

Previous researchers have proposed a barrel–stave model (18) to explain similar types of conductance events observed with peptides such as Pep5 and alamethicin. In this model, the peptides are supposed to form the staves of a barrel-like channel in which the number of peptides that come together to form a channel will determine the size (conductance) of the channel, and the stability of the barrel determines the lifetime. However, we learned in this study that similar conductance events occur with peptides of all sizes (10–28 amino acids, including cyclic 10–14-mers), some of which cannot span the bilayer. Thus, we favor the general model of Matsuzaki et al. (10), who proposed that  $\alpha$ -helical antimicrobial peptides bind to the outer leaflet of model membranes and flip inward, carrying lipids with them (37, 38) and creating brief disruptions in permeability. Indeed Matsuzaki et al. suggested that a peptide–lipid supramolecular complex is formed which mediates the mutually coupled transbilayer transport of lipid and peptides. We would add to this by suggesting that these complexes will

contain irregular aggregates of peptide molecules within the membrane (i.e., without any fixed stoichiometry), that will form in a concentration- and voltage-dependent manner. Such aggregates would offer both hydrophobic and hydrophilic surfaces that can contact, respectively, the hydrocarbon chains of the membrane lipids, and the external medium and headgroups of the lipids (which may be within the membrane if hexagonal phase structure develops; 39). It is implicit within this concept that there will exist, within these aggregates, informal aqueous channels that will allow the passage of at least ions and possibly larger molecules. As long as these supramolecular complexes are of variable size and stability, this would explain the observed variations in both the magnitude and duration of conductance events observed in our and other planar bilayer studies. This paper thus extends this model to other structural classes of antimicrobial peptides including quite small peptides, and provides evidence that it is relevant to interaction with the cytoplasmic membrane of bacteria.

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