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Improvement of Outer Membrane-Permeabilizing and Lipopolysaccharide-Binding Activities of an Antimicrobial Cationic Peptide by C-Terminal Modification

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Antimicrobial cationic peptides have been discovered in many different organisms and often possess a broad range of activity. In this study, we investigated the mechanisms of actions of melittin and two synthetic peptides, CEME (a cecropin-melittin hybrid) and CEMA, against gram-negative bacteria. CEMA was produced by recombinant DNA procedures and is an analog of CEME with a modified C terminus resulting in two additional positive charges. All three peptides showed good antimicrobial activity against four different gram-negative bacteria, but only CEMA was able to somewhat augment the activity of some conventional antibiotics in synergy studies. Studies using the bacteria *Pseudomonas aeruginosa* and *Enterobacter cloacae* showed that the peptides all possessed the ability to permeabilize bacterial outer membranes to the hydrophobic fluorophor 1-*N*-phenylnaphthylamine and the protein lysozyme, with CEMA being the most active. CEMA also had the strongest relative binding affinity for bacterial endotoxin (lipopolysaccharide). These data collectively indicated that these peptides all cross the outer membrane by the self-promoted uptake pathway and that CEMA is the peptide most effective at accessing this pathway.

The past few decades have witnessed the emergence of many different peptide antibiotics, including defensins (19), insect cecropins (5), magainins (42), and melittin (12). Cecropins and melittin belong to the group of antimicrobial peptides that exist in a random-coil configuration in aqueous solutions but adopt a helix-turn-helix structure upon interaction with membranes (9, 34). Both of these peptides have one amphipathic α-helix and one hydrophobic α-helix, but the order of these helices in the two peptides is inverted. Many synthetic peptides have been created in attempts to improve antibacterial activity. Included in these are cecropin-melittin hybrid peptides which possess the amphipathic N-terminal α-helix of cecropin A followed by the hydrophobic N-terminal α-helix of melittin (40). These peptides have been shown to have a broad range of antibacterial activity against both gram-negative and gram-positive bacteria (40). Cecropins (6), melittin (36), and the hybrid peptides (41) have all been shown to form ion-permeable channels in lipid membranes.

Gram-negative bacteria pose an additional challenge to these peptides in the form of an outer membrane which constitutes a permeability barrier to antibiotics (14, 24, 37). Very few studies have investigated the interaction of antimicrobial peptides with the outer membranes of gram-negative bacteria. Sawyer et al. (31) demonstrated that β-structured defensins from rabbits (macrophage cationic proteins) could permeabilize the outer membrane of *Pseudomonas aeruginosa*. This was confirmed by studies that showed that human neutrophil peptide 1 (18) and bactenecin (33) could permeabilize the *Escherichia coli* outer membrane. In addition, macrophage cationic proteins (rabbit defensins) were shown to bind with high affinity to lipopolysaccharide (LPS) (31). These data were consistent with the proposal that cationic peptides cross the outer membrane by the self-promoted uptake pathway (13, 31). In this pathway, cationic compounds displace the divalent cations that form stabilizing cross bridges between adjacent LPS molecules (13). This results in a localized outer membrane perturbation through which the cationic compound is taken up (13). However, only limited biochemical and kinetic studies have been made with cationic peptides (5, 9, 19), including our own preliminary study on a cecropin-melittin hybrid named CEME (28a).

To study cationic peptides more thoroughly, we have developed a bacterial expression system and used it to produce recombinant CEME with properties identical to those of chemically synthesized CEME (28). In this study, we have compared CEME with a prototype antibacterial cationic peptide, melittin, and with a variant peptide, CEMA, that was also produced by recombinant DNA technology through modification of the carboxy terminus of CEME.

MATERIALS AND METHODS

Materials. Melittin was purchased from Calbiochem. Polymyxin B, gentamicin, lysozyme, and 1-*N*-phenylnaphthylamine (NPN) were obtained from Sigma. Dansyl polymyxin B was prepared by S. Farmer as previously described (32). *P. aeruginosa* LPS was isolated by N. Karunaratne using the method of Darveau and Hancock (7). The CEME peptide (Fig. 1) was isolated as previously described (28). Oligonucleotides encoding CEMA preceded by a methionine residue were cloned into pRIT5 (25) and electroporated into *Staphylococcus aureus*

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CEME **KWKLFKKIGIGAVLKVLTTGLPALIS**

CEMA **KWKLFKKIGIGAVLKVLTTGLPALKLTK**

Melittin **GIGAVLKVLTTGLPALISWIKRKRQQ**

FIG. 1. Amino acid sequences of the peptides used in this study. Positively charged residues are in bold. Residues 1 to 8 of CEMA and CEME are derived from cecropin (5).

RN4220. The CEMA peptide (Fig. 1) was then isolated as described previously for CEME (28). Strains used in this study are found in Table 1.

MIC assays. The MICs of different antibiotics were determined by the broth dilution method (1). The antibiotics were serially diluted in 100 μl of Luria-Bertani medium (10 g of tryptone and 5 g of yeast extract per liter) in 96-well microtiter plates. Each well was inoculated with 10 μl of 10^4 to 10^5 CFU of the organism per ml. After incubation at 37°C overnight, the MIC was determined as the lowest antibiotic concentration that inhibited growth. Where indicated, 5 mM $MgCl_2$, 80 mM NaCl, or various sublethal concentrations of peptide were included in the Luria-Bertani medium.

Membrane permeabilization studies. Cells were grown to an optical density at 600 nm (OD_{600}) of 0.5 to 0.6, centrifuged, and washed with 1 volume of assay buffer (5 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7.2; 5 mM KCN). The cells were resuspended in assay buffer at an OD_{600} of 0.5. For the lysozyme lysis assays (16), 600 μl of cells was placed in a cuvette and lysozyme was added to a final concentration of 50 μg/ml. A potential permeabilizing agent was added to the cuvette, and the subsequent decrease in OD_{600} was recorded with a dual-beam spectrophotometer. The experiments were repeated in the absence of lysozyme to determine the lytic activities of the test compounds. In some experiments, various concentrations of $MgCl_2$ were added to the assay mixture prior to the addition of the test compound. The NPN uptake assay mixtures consisted of 1 ml of cells and 10 μM NPN. To this, various concentrations of test compound were added, and the increase in fluorescence, as a result of the partitioning of NPN into the membrane interior, was measured in a fluorescence spectrophotometer as previously described (20). The permeabilizing activity of a given compound was designated as the total NPN fluorescence (in arbitrary units) minus the NPN fluorescence in the absence of the compound. Control experiments showed that the fluorescence enhancement was due to the uptake of NPN into the cells.

Dansyl polymyxin B displacement studies. The relative binding affinities of various compounds for LPS were investigated by dansyl polymyxin B displacement assays (23). Briefly, 2.5 μM dansyl polymyxin B and 3 μg of *P. aeruginosa* H103 (15) LPS per ml were mixed together in 1 ml of 5 mM HEPES (pH 7.2), which resulted in 90 to 100% maximum fluorescence as measured by a fluorescence spectrophotometer. Samples (5 μl) of test compounds were added to the cuvette, and the decrease in fluorescence due to the displacement of dansyl polymyxin B from the LPS was measured after each addition. The data were plotted as the fraction of dansyl polymyxin B bound to the LPS as a function of compound concentration. For each compound, the compound concentration which resulted in 50% maximal displacement (I_{50}) was determined directly from these graphs. The assays were repeated with washed *P. aeruginosa* H309 whole cells resuspended in 5 mM HEPES buffer (pH 7.2). The bacterial cells were prepared exactly as described for the membrane permeabilization studies. The assay consisted of 10 μl of cells (10^7 cells per ml), 990 μl of 5 mM HEPES (pH 7.2), 5 mM KCN, and a concentration of dansyl polymyxin B that was previously determined to result in 90 to 100% maximum fluorescence. Compounds were added, fluorescence decreases were recorded, and I_{50}s were determined as above. All experiments were repeated three times.

RESULTS

Antibacterial activity of cationic peptides. The MICs of CEME, CEMA, and melittin were determined for four different gram-negative bacteria (Table 1). Generally, CEME and

TABLE 1. MICs of various antimicrobial agents

Species and strain	Relevant phenotype	MIC (μg/ml)[a]					
		PX	GM	CEME[b]	CEMA	MEL	CTZ
P. aeruginosa							
H309	Wild type	0.5	1	2.4	2.8	8	0.5
K799	Parent of Z61	0.5	1	4.8	2.8	8	0.5
Z61	Antibiotic supersusceptible	0.06	0.25	1.2	1.4	8	0.03
E. coli							
UB1005	Parent of DC2	0.5	1	2.4	2.8	8	0.5
DC2	Polymyxin susceptible	0.06	0.5	0.6	0.7	4	0.5
SC9251	Parent of SC9252	0.06	2	1.2	1.4	8	0.5
SC9252	Polymyxin resistant	4	4	1.2	1.4	8	0.5
S. typhimurium							
14028s	Parent of MS7953s	1	4	2.4	5.6	16	ND[c]
MS7953s	Defensin supersusceptible	0.25	2	0.6	1.4	8	ND
E. cloacae							
218S	Parent of 218R1	0.5	0.5	2.4	2.8	8	0.1
218R1	β-Lactam resistant	0.5	0.5	2.4	1.4	8	20

[a] PX, polymyxin B; GM, gentamicin; MEL, melittin; CTZ, ceftazidime.
[b] CEME data were taken from reference 28a.
[c] ND, not determined.

CEMA had similar MICs, which were consistently lower than those of melittin. The exception to this was with *S. aureus*, which showed similar susceptibilities (MIC = 8 to 10 μg/ml) to all three peptides. This is in agreement with data from the work of Wade et al. (41), who demonstrated that melittin had a lethal concentration which was lower than that of CEME for gram-positive bacteria but higher for gram-negative bacteria. The MICs of the peptides were usually higher on a weight basis than those of gentamicin (an aminoglycoside), but CEME and CEMA were equally or more effective at killing these organisms on a molar basis (MICs of 0.25 to 2.0 μM).

Both outer membrane-altered antibiotic-supersusceptible mutants (*P. aeruginosa* Z61 [3] and *E. coli* DC2) were two- to fourfold more susceptible to the cationic peptides (the exception being melittin against Z61) than the parental strains, indicating that the outer membrane of these bacteria did constitute a significant barrier to peptide effectiveness. SC9252 had a 64-fold-increased resistance to the cationic cyclic peptide polymyxin B, but it showed no increased resistance to any of the cationic peptides. These data indicated that there were differences between polymyxin B and the cationic peptides in their interactions with the outer membrane. MICs of CEME, CEMA, and melittin were increased 16-fold, 8-fold, and ≥8-fold by the addition of 5 mM Mg^{2+} (as $MgCl_2$) to the growth medium. MICs of polymyxin and gentamicin were increased twofold and fourfold, respectively, by 5 mM Mg^{2+}. This was not just an ionic effect, since 80 mM Na^+ (as NaCl) altered MICs by less than twofold.

The *Salmonella typhimurium* defensin-supersusceptible strain (11) was fourfold more susceptible to CEME and CEMA and twofold more susceptible to melittin than the wild-type parent. Against the same strain, the MIC of ceftazidime, which is taken up across the outer membrane by a porin-mediated pathway different from that of the peptides, remained unchanged (data not shown). These results suggested that the mutation was affecting the uptake pathway only for these cationic compounds. Common mechanisms of resistance observed in clinical isolates, including intrinsic resistance due to low outer membrane permeability (cf. *P. aeruginosa* and *E. coli*) and β-lactamase overproduction (cf. *Enterobacter cloacae* 218S and 218R1 [21]), had no impact on susceptibility to these peptides (Table 1).

Synergy studies with cationic peptides and other antibiotics. Previous studies have shown that some cationic-membrane-permeabilizing agents such as polymyxin B nonapeptide and $lysine_{20}$, at sub-MIC levels, were able to increase the susceptibility of bacteria to a number of different antibiotics (38). Therefore, MIC assays with ceftazidime, imipenem, tetracycline, novobiocin, fusidic acid, and polymyxin were performed in the presence of one-half or one-fourth the MIC of each of the cationic peptides to determine whether any synergy existed between them. Generally the peptides had little effect on the MIC of the first five above-named antibiotics, although CEMA at one-half the MIC consistently reduced their MICs twofold. However, the MIC of polymyxin B was decreased 4-, 8-, and 16-fold in the presence of sub-MIC levels of CEME, CEMA, and melittin, respectively.

Cationic peptides as outer membrane-permeabilizing agents. We tested the abilities of the various cationic peptides to permeabilize the outer membranes of *P. aeruginosa* H309 and *E. cloacae* 218R1 to lysozyme and NPN. These probes have previously been used successfully to identify outer membrane-permeabilizing agents (16, 20). Since lysozyme is a 14-kDa basic protein and NPN is a very small hydrophobic probe, one might predict that their accessibilities to permeabilized outer membranes would be different according to the extent of the disruption of the outer membrane permeability barrier. There was little difference in the abilities of individual peptides to promote lysozyme-mediated lysis of *P. aeruginosa* H309 or *E. cloacae* 218R1 (Fig. 2), despite the substantial difference in the intrinsic outer membrane permeabilities of these organisms. Neither organism showed susceptibility to lysozyme in the absence of any peptides. CEMA demonstrated an ability to enhance lysozyme uptake across the outer membrane (leading to peptidoglycan degradation and lysis), especially at lower concentrations, superior to that of polymyxin B for both *P. aeruginosa* and *E. cloacae*. CEME was similar to polymyxin B, except that it did show stronger outer membrane-permeabilizing activity at low concentrations against *P. aeruginosa*. Melittin (Fig. 2A) showed a weaker ability to enhance lysozyme uptake across the outer membrane. If the lytic activity of melittin in the absence of lysozyme is considered, melittin appeared to be quite a weak outer membrane permeabilizer. In all cases, the inclusion of 5 mM $MgCl_2$ inhibited the peptide-mediated uptake of lysozyme (data not shown).

The NPN uptake assays showed somewhat different results (Fig. 3). First, the two organisms appeared to have different sensitivities in this assay, since NPN uptake was enhanced at lower peptide concentrations in *P. aeruginosa* than in *E. cloacae*. Second, in these assays, polymyxin B showed permeabilizing activity equivalent to that of CEMA. Third, melittin, which was a weak permeabilizer in the lysozyme lysis assays, promoted uptake of NPN to an extent that was comparable to that of CEME (Fig. 3A). Analysis of these data indicated that both CEME and CEMA were able to permeabilize outer membranes to NPN at concentrations that were similar to those that permeabilized the outer membranes to lysozyme. In contrast, the lower concentrations of polymyxin B and melittin that enhanced the uptake of NPN (Fig. 3) could not enhance the uptake of lysozyme (Fig. 2).

LPS binding of cationic peptides. Previous studies have shown that the fluorescent probe dansyl polymyxin B can be used to assess the relative binding affinities of various compounds to LPS (23). Dansyl polymyxin B fluoresces strongly when bound to LPS but only weakly when free in an aqueous solution. Therefore, the relative ability of a particular compound to bind to LPS can be measured by its ability to displace dansyl polymyxin B from the LPS, resulting in a decrease in fluorescence. It has previously been shown that defensins (31), magainins (29), and melittin (8) can interact with the LPSs of different organisms. In this study, we tested the ability of various cationic compounds to displace dansyl polymyxin B from *P. aeruginosa* H103 purified LPS and *P. aeruginosa* H309 whole cells. The binding sites of purified LPS and of LPS in whole-cell environments were titrated with dansyl polymyxin B to achieve >90% saturation as indicated by the fluorescence level. Cationic compounds were titrated in, and the resulting decrease in fluorescence was monitored. When the fraction of dansyl polymyxin B bound was plotted as a function of compound concentration, the I_{50}s for each compound (the concentration leading to 50% maximal displacement of dansyl polymyxin) could be read directly from the graphs. The results (Table 2) indicated that CEMA had the highest binding affinity for purified LPS, followed closely by polymyxin B, CEME, and melittin. The cationic trisaccharide antibiotic gentamicin and especially Mg^{2+} showed lower affinities. The results obtained with *P. aeruginosa* whole cells were slightly different. While polymyxin B had I_{50}s for purified LPS and whole cells that were similar, the cationic compounds had two- to threefold-lower I_{50}s for whole cells than for purified LPS. These data indicated that the α-helical peptides interacted with LPS in whole-cell environments better than did polymyxin B.

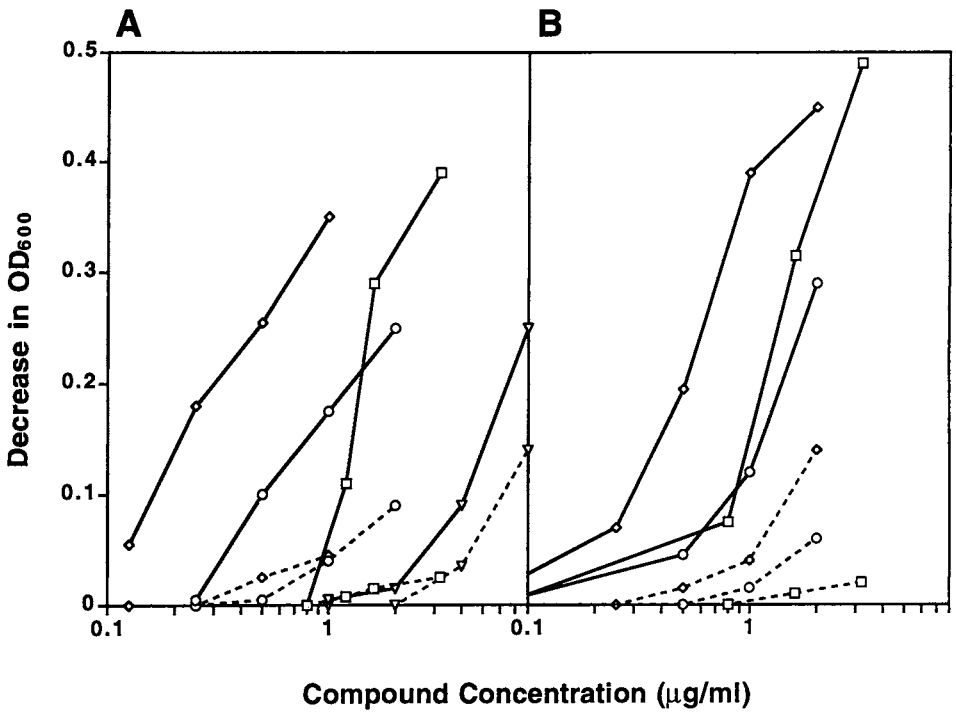

FIG. 2. Peptide-mediated uptake of lysozyme. Various peptides were tested for their ability to enhance the uptake of lysozyme across the outer membranes of *P. aeruginosa* (A) or *E. cloacae* (B). Cells at an OD_{600} of 0.5 were incubated with (solid lines) or without (dashed lines) 50 μg of lysozyme per ml before the addition of different concentrations of polymyxin B (squares), CEME (circles), CEMA (diamonds), or melittin (triangles). The decrease in OD_{600} was measured and plotted as a function of compound concentration. CEME data in panel A were taken from the work of Piers and Hancock (28a).

DISCUSSION

Antimicrobial cationic peptides have been shown to play a significant role in host defenses (5, 19) and are now being considered for use as therapeutic agents. It is therefore necessary to obtain a better understanding of how these peptides kill different microorganisms. Some studies have addressed the ability of these peptides to form channels in lipid bilayer membranes (10, 22, 39). In contrast, very few studies have dealt with the issue of how these peptides interact with and cross the barrier of the outer membrane in gram-negative bacteria. The self-promoted uptake pathway that was originally proposed to be used by the cationic antibiotics polymyxin B and gentamicin was also suggested as the mechanism of uptake for the defensins macrophage cationic proteins 1 and 2 across the outer membrane of *P. aeruginosa* (31). This was followed by a study that suggested that an α-helical cecropin-melittin hybrid peptide (CEME) could also access this pathway (28a). Several peptides in nature, including the cecropins, have an amidated carboxy terminus. This amidation is essential for cecropins to demonstrate a full range of antibacterial activities (2). Therefore, we created here a variant of CEME, termed CEMA, with two extra charges in the carboxy-terminal region to assess the role of charge in the interactions of peptides with the self-promoted uptake pathway. We found that although CEMA had MICs similar to those of CEME, it possessed outer membrane-permeabilizing and LPS-binding activities significantly higher than those of CEME. We also examined the interaction of melittin with outer membranes and extended these studies to bacteria other than *P. aeruginosa*.

The self-promoted uptake hypothesis (16) proposes that compounds taken up by this pathway interact initially with divalent-cation binding sites on surface LPS. Several pieces of evidence indicated that all the cationic peptides tested in this study fulfill this requirement. First, all the peptides were able to displace dansyl polymyxin B from LPS with high efficiency, as indicated by their low I_{50}s. Second, dansyl polymyxin B was also displaced by Mg^{2+}, indicating that these sites are indeed divalent-cation binding sites. Third, Mg^{2+} ions were able to increase the MIC of the cationic peptides against *P. aeruginosa* (data not shown), indicating that binding to the divalent-cation binding sites on the LPS was necessary to initiate killing activity. Fourth, the cationic peptides were more active against the *E. coli* mutant DC2, which has been shown to have an LPS mutation resulting in decreased LPS esterification and consequently an increase in negative charge on the LPS (30). This might provide the peptides with an increased number of sites to interact with, thus increasing the susceptibility of this organism to the peptides.

It should be noted that the binding of cationic peptides to whole cells, while necessary for bactericidal activity, is not sufficient. Polymyxin B nonapeptide, a deacylated analog of polymyxin B, is able to bind to LPS (23) but possesses an MIC of >100 μg/ml (38). As well, some *E. coli* strains that are resistant to cecropin action show no differences in their abilities to bind radioactive cecropin molecules (35).

Compounds proposed to be taken up by the self-promoted uptake pathway are also proposed to be able to destabilize the bacterial outer membrane (16, 17). We have demonstrated that the peptides tested in this study all possess outer membrane-disrupting abilities against both *P. aeruginosa* and *E. cloacae* that result in the enhanced uptake of NPN and lysozyme. Indeed, the studies reported here are the first to demonstrate the existence of the self-promoted uptake pathway in an *Enterobacter* species.

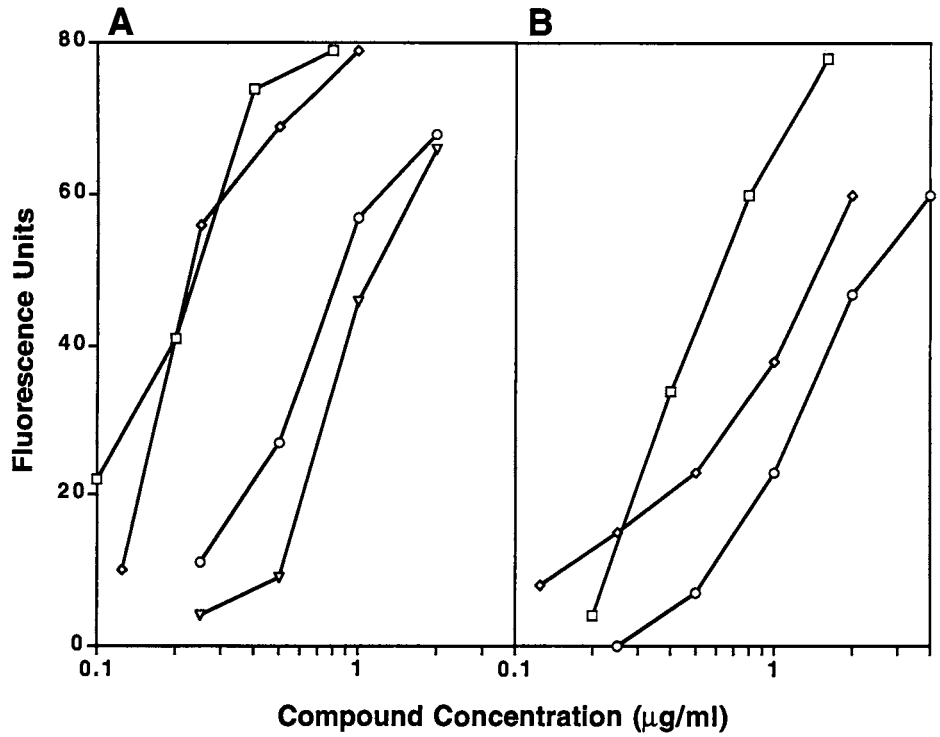

FIG. 3. Peptide-enhanced uptake of NPN. Various peptides were tested for their ability to permeabilize the outer membranes of *P. aeruginosa* (A) or *E. cloacae* (B) to the hydrophobic probe NPN. To cuvettes containing cells and 10 μM NPN, polymyxin B (squares), CEME (circles), CEMA (diamonds), or melittin (triangles) was added to various-final concentrations. The resulting increase in fluorescence due to the partitioning of NPN into the membrane was plotted as a function of compound concentration. CEME data were taken from the work of Piers and Hancock (28a).

The ability of a compound to cross the outer membrane via the self-promoted uptake pathway might be expected to depend on the intrinsic structural properties of the compound itself. Consistent with this, the peptides studied here showed differential activities with respect to the steps involved in self-promoted uptake. While all compounds taken up by this pathway were considered to bind at divalent-cation binding sites on the LPS, the exact sites appeared to be heterogeneous (or differentially accessible) for polymyxin B and the cationic peptides. Thus, differences were observed in the degree to which 5 mM Mg^{2+} influenced MICs, the effect on susceptibility of the LPS mutation (27) in *E. coli* SC9252, and the relative concentrations required to permeabilize bacteria to NPN or lysozyme.

In this report, we have described a new peptide, CEMA, which demonstrated potent LPS-binding and outer membrane-permeabilizing activities and is significantly more active than CEME. CEMA differs from CEME only at the C-terminal end, where it has an additional two amino acid residues and two positive charges. The role of C-terminal basic residues has been studied for certain other peptides. A magainin analog with a $lysine_{10}$ C-terminal chain extension showed 16-fold-lower MICs than those of magainin against *P. aeruginosa* and *S. aureus* (4). A cecropin analog that was shortened to remove the C-terminal lysine residue was completely inactive (40). The basic residues at the C terminus of melittin were found to play a key role in binding to the polar region of membranes but were not needed for ion channel formation (26). This result is in agreement with the data obtained for CEMA, which showed increased LPS-binding affinity and membrane-permeabilizing activity compared with that of CEME but did not demonstrate higher killing activity. This suggested that the extra basic amino acids at the C-terminal end of CEMA enhanced its ability to access the self-promoted uptake pathway.

TABLE 2. I_{50}s for various compounds against *P. aeruginosa* LPS and whole cells

Compound	I_{50} (μM) against *P. aeruginosa*[a]:	
	H103 LPS	H309 whole cells
Polymyxin B	0.93 ± 0.03	0.85 ± 0.13
Gentamicin	12.17 ± 0.58	19.0 ± 5.2
$MgCl_2 \cdot 6H_2O$	850 ± 132	127 ± 31
CEME[b]	1.30 ± 0.37	0.41 ± 0.08
CEMA	0.70 ± 0.10	0.33 ± 0.03
Melittin	1.41 ± 0.05	0.43 ± 0.06

[a] Each value is the average of at least three trials ± the standard deviation.
[b] CEME data were taken from the work of Piers and Hancock (28a).

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