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Antimicrobial Action of Rabbit Leukocyte CAP18$_{106–137}$

DAVID J. MASON,[1] RICHARD DYBOWSKI,[1] JAMES W. LARRICK,[2] AND VANYA A. GANT[1]*

Infection and Immunity Laboratory, Division of Infection, United Medical and Dental Schools of Guy's and St. Thomas's Hospitals, London SE1 7EH, United Kingdom,[1] and Palo Alto Institute of Molecular Medicine, Mountain View, California 94043[2]

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CAP18 is a cationic antimicrobial protein originally isolated from rabbit neutrophils, of which a 32-mer sequence from its C-terminal and (CAP18$_{106–137}$) has been found to be the most active. The bactericidal action of this peptide has been characterized by conventional culture techniques and flow cytometry. Cultures of *Escherichia coli* NCTC10418 were exposed to the MBC (12 μM) of the peptide for up to 60 min and stained with a fluorochrome sensitive to changes in either membrane potential {bis-(1,3-dibutylbarbituric acid)trimethine oxonol [DiBAC$_4$(3)]}, or membrane integrity (propidium iodide [PI]) before flow cytometric analysis. Addition of CAP18$_{106–137}$ to *E. coli* in broth culture resulted in immediate collapse of membrane potential [as determined by uptake of DiBAC$_4$(3)] and loss of membrane integrity (as indicated by uptake of PI), with a corresponding 6- to 8-log decrease in viable counts as determined by colony formation on solid media. In identical experiments, the presence of Mg^{2+} (1 to 10 mM), K^+ (50 to 250 mM), or EDTA (5 mM) or incubation in nutrient-free buffer or at 4°C had no effect on peptide-induced dye uptake. In contrast, addition of Ca^{2+} (1 to 10 mM) or the respiratory chain poison carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (50 μM) inhibited the uptake of both dyes. These findings, however, did not relate to bacterial recovery on solid media, where (unless in the presence of K^+ 150 to 250 mM) CAP18$_{106–137}$ at 12 μM fulfilled the MBC criteria (99.9% killing). We conclude that CAP18$_{106–137}$ exerts a rapid and profound action on *E. coli* cytoplasmic membranes and viability as measured by colony formation. The results suggest, however, that CAP18$_{106–137}$ may exert its action at sites additional to the cell membrane and that its activity profile is unique among cationic antimicrobial proteins.

CAP18 is an 18-kDa cationic protein originally isolated from rabbit neutrophils (15). The isolation of CAP18, a particularly lysine-rich molecule consisting of 142 amino acids, was originally made possible because of its ability to bind to bacterial lipopolysaccharides (LPS) (15). This LPS-binding activity is associated with the C-terminal fragment of the molecule; thus, two synthesized C-terminal peptides consisting of 30 to 40 amino acids and termed CAP18$_{106–142}$ and CAP18$_{106–137}$ not only display the LPS-binding properties of the intact protein but also inhibit LPS-induced cytokine release from human peripheral blood mononuclear cells (21). These peptides also inhibit LPS-induced lethality in galactosamine-sensitized mice, as well as suppress the growth of a wide range of bacteria such as *E. coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* (20). Protein sequences similar to that of rabbit CAP18$_{106–137}$ have been identified in pigs (34), cows (19), and humans (19); where investigated, these share many, if not all, of the functions attributed to the original rabbit protein.

Thus, this protein displays a high degree of conservation across several animal species; has antimicrobial, LPS-binding, and LPS-neutralizing properties; and may play an important role in the innate defense mechanisms of multicellular organisms against bacterial infection.

Other cationic antibacterial proteins have been purified from neutrophils. However, neither bacterial permeability increasing protein (BPI) (9) nor the defensin family of peptides (11) displays sequence homology with the active C-terminal fragments of CAP18. CAP18-derived peptides also differ in other ways: unlike BPI, they are active against both gram-negative and gram-positive organisms, and unlike the defensins (11), they are active in the presence of protein at physiological pH (20).

While the antimicrobial properties of defensins and other peptide families, such as the insect cecropins (3) and amphibian magainins (45), are thought to relate to their ability to form ion channels in artificial membranes (5, 17), less is known about the bactericidal mechanism of CAP18$_{106–137}$. Many antimicrobial peptides (including CAP18$_{106–137}$) are predicted to have an α-helical structure when partitioned in lipid (4) and to induce cytoplasmic membrane permeabilization subsequent to insertion into the membrane. The release of β-galactosidase from CAP18$_{106–137}$-exposed *E. coli* lends support to this hypothesis (36).

Thus, evidence to date on several protein families with disparate sequences and predicted tertiary structures implicates the bacterial membrane as a target. We therefore set out to further characterize CAP18$_{106–137}$ action with particular reference to the known physicochemical activity profiles of other cationic proteins. We used, on the one hand, classical microbiological techniques to detect ability to form colonies on semisolid media and, on the other hand, flow cytometric analysis of bacterial membrane potential and membrane integrity. To achieve this, we used bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC$_4$(3)] and PI to measure membrane potential and membrane integrity, respectively. DiBAC$_4$(3) is an anionic membrane potential-sensitive probe which partitions only into depolarized cell membranes (43), while PI is a small cationic dye excluded from cells with intact membranes (10). If membrane integrity is damaged, however, the latter dye penetrates

* Corresponding author. Mailing address: Infection and Immunity Laboratory, Division of Infection, United Medical and Dental Schools of Guy's and St. Thomas's Hospitals, St. Thomas's Campus, Lambeth Palace Road, London SE1 7EH, U.K. Phone: 44 0171 928 9292, ext. 2861. Fax: 44 0171 928 0730.

the cell and tightly binds to nucleic acids. Both dyes have previously been used in conjunction with flow cytometry to investigate bacterial responses to antibiotics (10, 25, 26). We have applied these techniques in the context of a variety of physicochemical conditions to further determine the membrane-specific and antimicrobial properties of CAP18$_{106–137}$.

MATERIALS AND METHODS

Bacterial strains and media. Strains used for this study included *E. coli* NCTC10418, *S. aureus* BB255 (1), and clinical isolates of *E. coli* and *Proteus mirabilis*. Organisms were cultured in IsoSensitest broth (ISO-B; Oxoid, Basingstoke, United Kingdom) at 37°C on an orbital shaker at 200 rpm.

Protein isolation and purification. While CAP18 was originally purified from rabbit peritoneal exudate cells, the peptide used in this study (CAP18$_{106–137}$) was synthesized in either an Applied Biosystems 430A or 431A peptide synthesizer as previously described (21). The peptide was dissolved in phosphate-buffered saline (PBS; pH 7.4) to give a stock solution of 1 mg/ml.

Bacterial sensitivity to CAP18$_{106–137}$. The MIC and MBC for *E. coli* NCTC10418 and *S. aureus* BB255 were determined by a broth dilution method. Doubling dilutions of CAP18$_{106–137}$ were prepared in ISO-B. Each dilution was inoculated with approximately 10^5 CFU/ml and incubated at 37°C for 18 h. The MIC was defined as the lowest concentration of CAP18$_{106–137}$ that visibly inhibited growth. Subcultures were made on nutrient agar from tubes in which growth had been inhibited. The MBC was defined as the concentration that caused >99.9% killing (33).

Plate assay of bacterial susceptibilities. The susceptibility of two clinical isolates each of *E. coli* and *P. mirabilis* to CAP18$_{106–137}$ at 64 μg/ml (equivalent to 12 μM) was determined by a plate assay. Nutrient agar plates were seeded with the appropriate test organism (10^5 CFU/ml with 20 ml of nutrient agar). A 16-μl aliquot containing 64 μg of CAP18$_{106–137}$ in PBS (pH 7.4) was added to each bacterial lawn together with an equal aliquot of PBS (pH 7.4) as a control. Growth of susceptible bacteria was inhibited where the drop was initially placed. *E. coli* NCTC10418 was used as a sensitive control.

Antibacterial activity of CAP18$_{106–137}$. Cultures of *E. coli* NCTC10418 were grown at 37°C to early log phase. Several 500-μl aliquots were removed and incubated in parallel at 37°C with selected concentrations of CAP18$_{106–137}$ for 60 min, or less for time course experiments. Two aliquots (2 × 200μl) were then removed for staining with DiBAC$_4$(3) or PI before flow cytometric analysis as described below. The remainder of the sample was used for viable count estimation. To investigate the effects of low temperature, the 500-μl aliquots referred to above were spun at 13,000 rpm for 30 s (Eppendorf centrifuge) after a 60-min incubation at 4°C; the resulting pellet was then washed and resuspended in 0.5 ml of fresh broth prior to staining and plating. In other experiments, the medium was modified by addition of either $CaCl_2$ (1 to 10 mM), $MgCl_2$ (1 to 10 mM), or KCl (50 to 250 mM) and the incubation time was limited to 10 min at 37°C. To determine the effect of EDTA or the respiratory poison CCCP (Sigma Chemical Co., Poole, United Kingdom) on the action of CAP18$_{106–137}$, cell suspensions with or without the peptide (12 μM) were coincubated with EDTA (5 mM) or CCCP (50 μM) for 10 min at 37°C. To investigate peptide action in nutrient-free buffer, cells were removed from broth suspension by centrifugation at 13,000 rpm for 30 s and reincubated (after a washing step) for 10 min in PBS (pH 7.0) with or without CAP18$_{106–137}$ at a final concentration of 12 μM.

Experiments were repeated in triplicate, and statistical analysis of the pooled data was carried out where appropriate. Wherever possible, the difference between the control and experimental samples was calculated and plotted. Due to the large values involved in the viable count data, however, actual values were plotted, as calculating the differences led to large variation.

Viable counts. Counts of CFU per milliliter were used as an independent estimate of viability. These were obtained by the method of Miles et al. (29), except that nutrient agar (Oxoid) was used. The limit of detection was 50 CFU/ml, but a zero value was recorded for samples that failed to produce colonies.

Fluorescence staining and flow cytometry. DiBAC$_4$(3) (excitation, 493 nm; emission, 516 nm) and PI (excitation, 536 nm; emission, 617 nm) were supplied by Molecular Probes, Inc., and Sigma Chemical Co., respectively. A stock solution (1 mg/ml) of DiBAC$_4$(3) was prepared in acetone. This solution was then diluted 1:10 in 70% ethanol to give a concentration of 100 μg/ml. PI was dissolved in deionized water to a concentration of 100 μg/ml. DiBAC$_4$(3) or PI was added to 200 μl of cells suspended in broth to give a final concentration of 10 μg/ml.

As a positive control of dye performance, a culture of *E. coli* NCTC10418 in the early logarithmic phase was fixed with 70% ethanol, washed, and resuspended in fresh ISO-B before staining with DiBAC$_4$(3) or PI as described above.

The flow cytometer used was a Bryte HS (Bio-Rad, Hemel Hempstead, United Kingdom) dual-parameter flow cytometer fitted with a combination xenon-mercury arc lamp. The instrument was used as previously described by Mason et al. (26). In brief, fluorescence measurements were made with the standard fluorescein isothiocyanate filter block with the following characteristics: excitation, 470 to 490 nm; emission, 520 to 560 nm (beam splitter at 510 nm). The sheath flow pressure and sample flow rate were set to 0.7 kPa · cm^{-2} and 2μl · min^{-1}, respectively.

Statistical analysis of results. The data obtained was nonparametric; hence, median values are plotted and bars are used to depict the range of counts or changes in fluorescence. Probability (*P*) values were calculated from Spearman's test of rank correlation. Mann-Whitney tests (one sided at the 5% level) were carried out to determine significance.

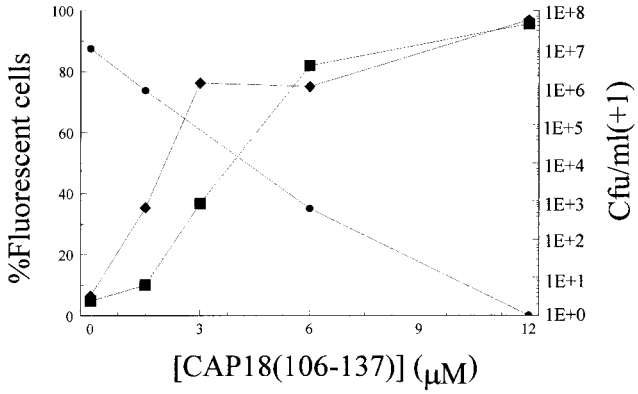

FIG. 1. Dose-response curve of *E. coli* NCTC10418 exposed to CAP18$_{106–137}$ after 60 min of incubation at 37°C. Viable counts (●) and proportions of fluorescing cells in the presence of DiBAC$_4$(3) (◆) and PI (■) are shown.

RESULTS

DiBAC$_4$(3)- and PI-associated fluorescence of fixed bacterial cells. Fixation of *E. coli* NCTC10418 with ethanol during the early log phase caused an approximately 10-fold increase in cell-associated DiBAC$_4$(3) or PI fluorescence intensity, a finding very similar to that previously reported by Mason et al. (26) for the related strain *E. coli* KL16. These results indicate that, as for *E. coli* KL16, DiBAC$_4$(3) and PI are sensitive to destruction of cytoplasmic membrane potential and integrity, respectively, in *E. coli* NCTC10418 (14). In the experiments described here, the proportion of cells in a bacterial culture which exhibited cell-associated fluorescence with DiBAC$_4$(3) or PI, equivalent to that of alcohol-fixed organisms, is expressed as a percentage.

Bacterial sensitivity and dose response to CAP18$_{106–137}$. The MICs of CAP18$_{106–137}$ for *E. coli* NCTC10418 and *S. aureus* BB255 were estimated as 6 and 28 μM, respectively. The MBCs of the peptide were 12 μM for *E. coli* NCTC10418 and 56 μM for *S. aureus* BB255.

Figure 1 shows a typical dose-response curve for *E. coli* NCTC10418 exposed to CAP18$_{106–137}$. Viable counts decreased with increasing concentrations of the peptide, and bacterial growth was below the detection limit (<50 CFU/ml) at a concentration of 12 μM. Figure 1 also shows that the percentage of cells rendered fluorescent by DiBAC$_4$(3) or PI increased with the peptide concentration, reaching a maximum (>90%) in the presence of 12 μM peptide. In contrast, no peptide-induced dye uptake or loss of viability was observed in cultures of *P. mirabilis* following exposure to 12 μM CAP18$_{106–137}$.

In plate susceptibility tests, bacterial susceptibility was determined by the presence or absence of growth within the zone in which 64 μg of CAP18$_{106–137}$ had been delivered. No growth inhibition was observed with the clinical isolate of *P. mirabilis*, demonstrating that this strain was unaffected by 64 μg of the peptide; conversely, the clinical isolate of *E. coli* showed a zone of clearing where CAP18$_{106–137}$ had been added.

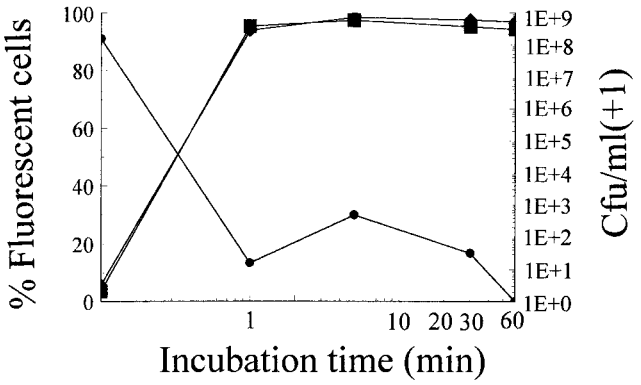

FIG. 2. Time course of action of $CAP18_{106–137}$ (12 μM) against *E. coli* NCTC10418 at 37°C. Viable counts (●) and proportions of fluorescing cells in the presence of $DiBAC_4(3)$ (◆) and PI (■) are shown.

Time course of $CAP18_{106–137}$ action. Figure 2 demonstrates the very rapid action of $CAP18_{106–137}$ on *E. coli*. Thus, exposure to $CAP18_{106–137}$ for 1 min resulted in staining of 92 to 94% of the organisms with $DiBAC_4(3)$ and PI, respectively, and an approximately 7-log decrease in CFU. Longer periods of exposure to $CAP18_{106–137}$ eliminated colony formation with no further increase in dye uptake.

Effect of cation concentration on $CAP18_{106–137}$ action. No inhibition of peptide activity was detected in our ISO-B, which contained concentrations of unbound Ca^{2+} and Mg^{2+} of 0.15 and 2.59 mM, respectively. Figure 3a shows, however, that added Ca^{2+} (1 to 10 mM) marginally inhibited peptide action. We found a correlation between the number of colonies after peptide exposure and increasing Ca^{2+} concentrations ($P < 0.0001$). Addition of Ca^{2+} also significantly decreased the uptake of both dyes; unlike CFU (rescued), however, the effects of added Ca^{2+} only became clearly significant upon addition of 5 mM Ca^{2+} for PI and 10 mM Ca^{2+} for $DiBAC_4(3)$ ($P < 0.0001$ and $P < 0.01$, respectively) (Fig. 3b). In contrast, Mg^{2+} ion (1 to 10 mM) had less effect on peptide-induced changes and only addition of at least 5 mM Mg^{2+} produced a significant 3-log rescue of bacterial growth ($P < 0.0001$; Fig. 4a). Interestingly, this ion had no detectable effect on peptide-induced cell-associated fluorescence after addition of $DiBAC_4(3)$ or PI ($P = 0.54$ or $P = 0.61$, respectively) (Fig. 4b).

Increasing the concentration of K^+ (50 to 250 mM) restored some cell viability post-$CAP18_{106–137}$ treatment (Fig. 5a). This increase in cell viability became significant at concentrations of 150 mM K^+ and above ($P < 0.0001$). The reduced change in $DiBAC_4(3)$ cell-associated fluorescence following peptide exposure (Fig. 5b) is due to the control culture also becoming fluorescent as the electrochemical null point is reached or exceeded. It is assumed that this point reflects the balanced distribution of K^+ across the cell membrane (8, 24, 42). Peptide-induced PI uptake was unaffected by increasing K^+ concentrations.

Effect of CCCP or EDTA on $CAP18_{106–137}$ action. $CAP18_{106–137}$ eradicated colony formation, irrespective of coincubation with EDTA or CCCP (Table 1); these agents, therefore, had no significant effect on peptide action. Both agents increased the percentage of $DiBAC_4(3)$- and PI-fluorescing cells in control cultures; while EDTA failed to prevent further increases in dye uptake consequent upon peptide addition, the presence of CCCP decreased the number of peptide-induced $DiBAC_4(3)$- and PI-fluorescent cells.

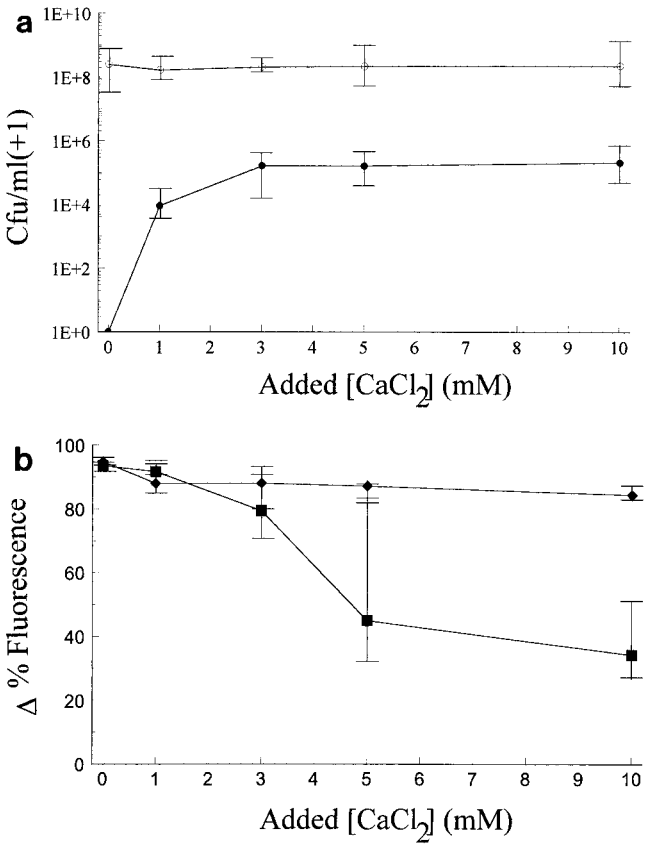

FIG. 3. (a) Viable counts of $CAP18_{106–137}$ (12 μM)-treated and untreated *E. coli* NCTC10418 incubated at 37°C for 10 min in the presence of increasing concentrations of Ca^{2+}. Bars depict the range of counts, and the plot through the points represents the median values. Symbols: ○, control culture; ●, peptide-treated culture. (b) Fluorescence data obtained from *E. coli* NCTC10418 cultures incubated at 37°C for 10 min in the presence of increasing concentrations of Ca^{2+}. The graph represents changes in the proportion of fluorescing cells between $CAP18_{106–137}$ (12 μM)-treated and -untreated cultures stained with $DiBAC_4(3)$ (◆) or PI (■). Bars depict the range of changes observed, and the plot represents the median values.

Temperature effects. Incubation of cells at 4°C resulted in a moderate increase in CFU, as well as an increase in baseline fluorescence with both dyes (Table 1). Interestingly, while the expected increase in peptide-induced fluorescence was also seen at 4°C, we observed a small but significant number of organisms being rescued from the otherwise lethal effects of $CAP18_{106–137}$ (Table 1).

$CAP18_{106–137}$ action in nutrient-free buffer. Preincubation of bacteria in PBS (pH 7.4) did not affect colony formation, despite a moderate increase in baseline fluorescence (Table 1). As was observed for low-temperature incubation, PBS exerted a minor but significant effect on peptide-induced eradication of bacterial colony formation although dye uptake was not affected.

DISCUSSION

$CAP18_{106–137}$ (present at 12 μM in ISO-B) induced a greater-than-6-log decrease in *E. coli* NCTC10418 CFU, with collapse of membrane potential and disruption of membrane permeability in more than 95% of the organisms within 5 min. In addition, $CAP18_{106–137}$ at this concentration fulfilled MBC criteria (>99.9% killing) in the presence of ISO-B, Ca^{2+} (1 to

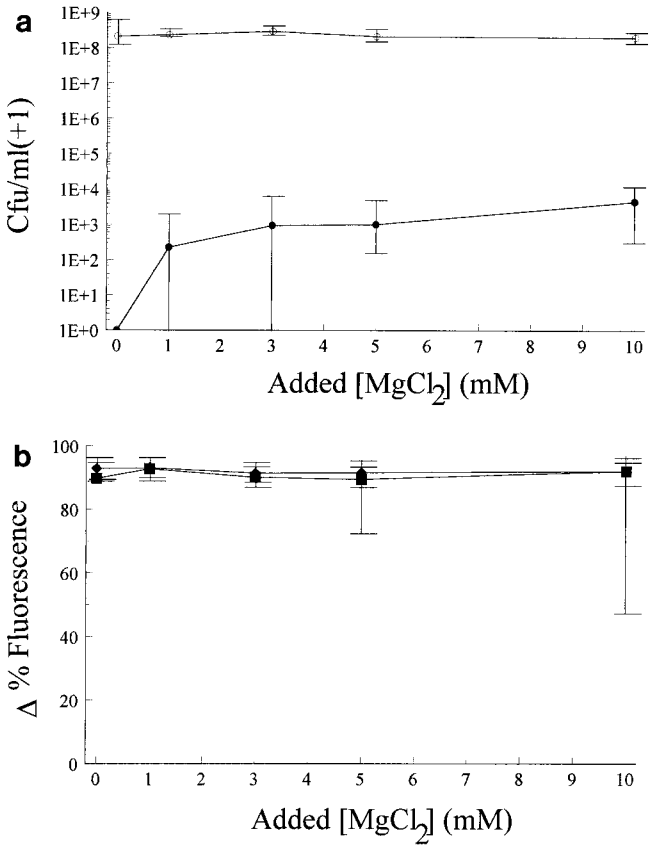

FIG. 4. (a) Viable counts of CAP18$_{106–137}$ (12 μM)-treated and untreated *E. coli* NCTC10418 incubated at 37°C for 10 min in the presence of increasing concentrations of Mg^{2+}. Bars depict the range of counts, and the plot through the points represents the median values. Symbols: ○, control culture; ●, peptide-treated culture. (b) Fluorescence data obtained from *E. coli* NCTC10418 cultures incubated at 37°C for 10 min in the presence of increasing concentrations of Mg^{2+}. The graph represents changes in the proportion of fluorescing cells between CAP18$_{106–137}$ (12 μM)-treated and untreated cultures stained with DiBAC$_4$(3) (◆) or PI (■). Bars depict the range of changes observed, and the plot represents the median values.

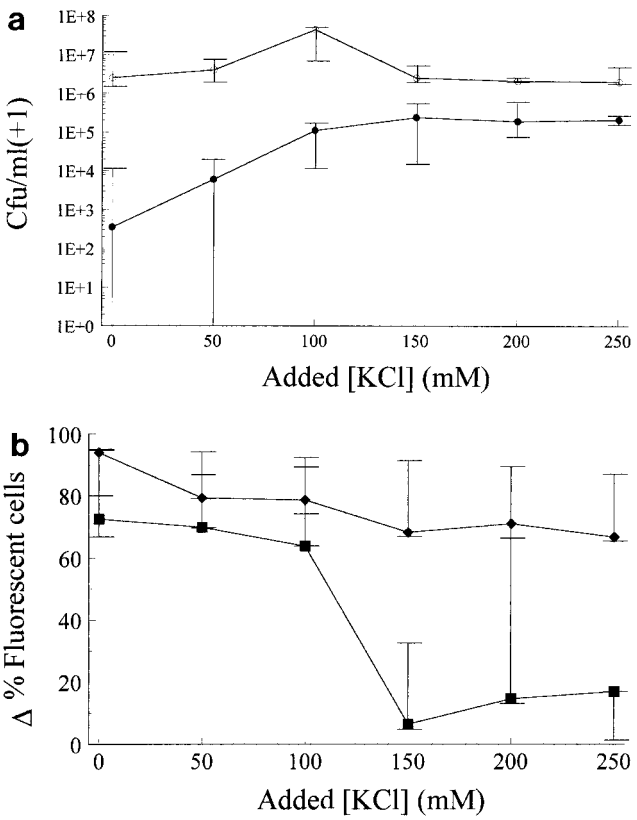

FIG. 5. (a) Viable counts of CAP18$_{106–137}$ (12 μM)-treated and untreated *E. coli* NCTC10418 incubated at 37°C for 10 min in the presence of increasing concentrations of KCl. Bars depict the range of counts, and the plot through the points represents the median values. Symbols: ○, control culture; ●, peptide-treated culture. (b) Fluorescence data obtained from *E. coli* NCTC10418 cultures incubated at 37°C for 10 min in the presence of increasing concentrations of KCl. The graph represents changes in the proportion of fluorescing cells between CAP18$_{106–137}$ (12 μM)-treated and untreated cultures stained with DiBAC$_4$(3) (◆) or PI (■). Bars depict the range of changes observed, and the plot represents the median values.

10 mM), Mg^{2+} (1 to 10 mM), CCCP (50 μM), or EDTA (5 mM) or after incubation in PBS (pH 7.4) or at 4°C.

These findings contrast with the antimicrobial properties of other cationic proteins. Thus, defensin action is completely inhibited by the presence of protein, nutrient-depleted media, and low temperatures (11, 35), and magainin 2 requires 90 min of incubation with *E. coli* to reduce viable numbers by 95% (45). Furthermore, the candidacidal activity of the defensin HNP-1 is almost completely inhibited by Ca^{2+} at a concentration of 0.25 mM (22), and both Ca^{2+} and Mg^{2+} at concentrations ranging from 0.25 to 7 mM also completely inhibit other cationic peptides (2, 16, 35, 44).

The resistance of *P. mirabilis* to CAP18$_{106–137}$ may relate to a reduced outer membrane negative charge (30, 32). These organisms are also resistant to polymyxins and BPI (9, 30, 41), again suggesting that surface charge may determine peptide efficacy. Additionally, the ability of CAP18$_{106–137}$ to bind LPS (and, presumably, concentrate at the cell surface) may account for its differential MBCs for *E. coli* and *S. aureus* (12 μM versus 48 μM, respectively). In conclusion, both access to and concentration at the outer membrane may be essential for antibacterial action.

The reduction in CAP18$_{106–137}$-induced dye uptake subsequent to addition of Ca^{2+} (1 to 10 mM) or CCCP (50 μm) suggests inhibition of peptide-induced membrane damage; such a reduction was not seen with Mg^{2+} (1 to 10 mM), K^+ (50 to 250 mM), or EDTA (5 mM). Peptide-induced dye uptake was also unaffected by incubation in PBS or at 4°C. Interestingly, these findings did not relate to bacterial recovery on solid media. Despite achieving >99.9% killing, we noted that addition of Ca^{2+} (1 to 10 mM) or Mg^{2+} (1 to 10 mM) to *E. coli* exposed to CAP18$_{106–137}$ (12 μM) in ISO-B resulted in a small but significant number of recovered colonies. Similarly, addition of K^+ (150 to 250 mM) reduced CAP18$_{106–137}$-induced killing to 90%. This partial recovery was possibly caused by limiting the available and necessary peptide to below a critical level. Such a reduction in peptide levels would result from either cation-induced changes in the organism or direct peptide-cation interaction. In this context, the finding that monovalent ions such as K^+ and Na^+ (50 to 250 mM) inhibit defensin action by preventing binding to cells may be relevant (23). Ca^{2+} and Mg^{2+} ions can also increase hydrophobic interactions within a peptide, leading to precipitation and loss of active soluble molecules. We observed some precipitate upon incubation of CAP18$_{106–137}$ with 10 mM Ca^{2+} and noted similar observations with some magainin peptides (38). Precipitation of CAP18$_{106–137}$ by Ca^{2+} may explain the equivalence of

TABLE 1. Effects of CCCP, EDTA, temperature, and incubation in nutrient-free buffer on *E. coli* NCTC10418 exposed to $CAP18_{106–138}$[a]

Condition	No. of CFU/ml at $CAP18_{106–138}$ concn of:		% $DiBAC_4(3)$-fluorescent cells at $CAP18_{106–138}$ concn of:		% PI-fluorescent cells at $CAP18_{106–138}$ concn of:	
	0 μm	12 μM	0 μM	12 μM	0 μM	12 μM
CCCP (0 μM)	1.1×10^7	0.0	4.0	92.1	2.5	95
CCCP (50 μM)	1.5×10^7	0.0	57.9	58.3	21.9	60.9
EDTA (0 μM)	5.5×10^6	0.0	4.0	92.1	2.5	97.1
EDTA (5 μM)	3.4×10^7	0.0	24.8	88.3	24.8	95.8
Temp of 4°C	1.2×10^7	3.0×10^4	21.7	94.1	25.3	94.6
Temp of 37°C	8.0×10^7	0.0	3.9	93.3	2.3	93.0
ISO-B	5.5×10^6	0.0	6.5	94.7	2.0	92.5
PBS	1.6×10^7	2.0×10^3	18.3	91.8	28.5	96.9

[a] Median values of combined results of three experiments are shown.

peptide action at 6 and 12 μM when 1 mM Ca^{2+} is added to the latter (Fig. 1 and 3a). Ca^{2+} and Mg^{2+} ions can, additionally, stabilize bacterial LPS; their removal by EDTA, conversely, results in destabilization (31, 32, 39). However, we found no evidence of enhanced antibacterial action in the presence of EDTA. Unlike Ca^{2+}, added Mg^{2+} and K^+ failed to form a precipitate or influence bacterial dye uptake, despite the rescue of significant numbers of bacteria capable of forming colonies. Taken together, these results suggest that (i) $CAP18_{106–137}$ access to its target and action are unrelated to those bacterial properties which are influenced by divalent cation concentration, and (ii) Ca^{2+}, Mg^{2+}, and K^+ interfere with $CAP18_{106–137}$ by distinct mechanisms.

Recovery of a minority of peptide-exposed organisms following incubation at 4°C or in nutrient-free PBS (pH 7.4) at 37°C may be linked to a reduction in bacterial metabolic activity (18), suggesting that actively metabolizing cells are more susceptible. This may relate to energization and membrane potential.

Defensins, magainins, and cecropins form voltage-dependent ion channels in artificial membranes for which an electronegative membrane potential appears essential (5–7, 13, 17, 22). Matsuzaki et al. (27, 28) additionally proposed that magainin-induced membrane permeabilization may also relate to peptide-induced destabilization of the lipid bilayer through the cumulative effect of single monomeric peptides on lipid bilayer integrity. Whether permeabilization occurs by channel formation or lipid destabilization may further depend on membrane site, composition, and potential (13, 37). Our results (Table 1) demonstrating that addition of CCCP has no effect on $CAP18_{106–137}$-induced loss of viability despite considerable depolarization suggest that a pre-existing membrane potential is not a prerequisite for subsequent peptide action. Garcerá et al. (12) have described similar findings relating to the pore-forming activity of the lantibiotic nisin, although the maintenance of the total proton motive force enhances the action of this peptide. Formation of channels in the bacterial membrane by an ionophore-like action may not, however, be critical to the antimicrobial mechanism of these peptides. The dissociation between peptide effects on the membrane and colony formation is supported by our finding that addition of the ionophore valinomycin along with K^+ (200 mM) extensively depolarizes bacteria, whose subsequent growth is unaffected (data not shown). Finally, magainin- and cecropin-induced membrane damage has been compared to detergent-induced damage (13, 40). Detergents induce bacteria to immediately lose $DiBAC_4(3)$-associated fluorescence and to rapidly lyse (data not shown); however, we observed no loss in either $DiBAC_4(3)$-associated bacterial fluorescence or bacterial particle concentration subsequent to peptide exposure, making a powerful detergent-like role for $CAP18_{106–137}$ action unlikely.

$CAP18_{106–137}$-induced membrane permeabilization, also described by Tossi et al. (36), was believed to be the primary killing event. Other events, however, may be involved in peptide-induced cell death in addition to membrane permeabilization. The dissociation between membrane-pertubative events and bacterial recovery on solid media which we describe is in accord with similar findings of Tytler et al. (37) when investigating magainin-induced cell death. The stability of $DiBAC_4(3)$ cell-associated fluorescence from treated organisms suggests that $CAP18_{106–137}$-membrane interaction may occur with minimal perturbation to the lipid bilayer, similar to the model proposed for the magainins by Matsuzaki et al. (27, 28). Such minimal perturbation suggests that other targets are responsible for the action of $CAP18_{106–137}$ on colony formation.

Our results show how flow cytometric analysis can rapidly detect separate perturbative effects of $CAP18_{106–137}$ on membrane potential and barrier function; furthermore, the spectrum of activity of $CAP18_{106–137}$ differs in several important respects from those of other antimicrobial peptides. We are currently seeking to determine the basis for these differences, which may relate to a second site of action unrelated to membrane-perturbing properties.

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