Interaction of CAP18-Derived Peptides with Membranes Made from Endotoxins or Phospholipids

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ABSTRACT Antimicrobial peptides with α-helical structures and positive net charges are in the focus of interest with regard to the development of new antibiotic agents, in particular against Gram-negative bacteria. Interaction between seven polycationic α-helical CAP18-derived peptides and different types of artificial membranes composed of phosphatidylcholine or lipopolysaccharide of the Gram-negative bacterium *Escherichia coli* were investigated using different biophysical techniques. Results obtained from fluorescence energy transfer spectroscopy with liposomes, monolayer measurements on a Langmuir trough, and electrophysiological measurements on planar reconstituted asymmetric bilayer membranes including the lipid matrix of the outer membrane of *E. coli* were correlated, and these data were, furthermore, correlated with structural parameters of the peptides (net charge, α-helical content, hydrophobic moment, and hydrophobicity). All peptides induced current fluctuations in planar membranes due to the formation of transient lesions above a peptide- and lipid-specific minimal clamp voltage. Antibacterial activity was exhibited only by those peptides that induced lesion formation in the reconstituted outer membrane at clamp voltages below the transmembrane potential of the natural membrane. Thus, we propose that the physicochemical properties of both the peptides as well as of the target membranes are important for antibacterial activity.

INTRODUCTION

Antibacterial peptides and proteins are an integral part of the host's defense barrier against invading bacteria. They provide immediate protection by direct physicochemical attack on the surface membrane of the microorganisms (Hancock, 1997; Schröder, 1999). One group of these antibacterial proteins is the cathelicidins, which have been identified in various mammals including humans (Zanetti et al., 1995). They contain a highly conserved N-terminal domain called cathelin and a C-terminal domain that comprises an antimicrobial peptide. Human and rabbit cathelicidins are termed 18-kDa cationic antibacterial protein hCAP18 and rCAP18, respectively. CAP18 is stored in the intracellular granules of neutrophilic granulocytes and is liberated into the phagocytic vacuoles during phagocytosis (Cowland et al., 1995). The C-terminal domain of CAP18 exhibits lipopolysaccharide (LPS)-binding, LPS-neutralizing, antibacterial, and anticoagulant activities (Hirata et al., 1995). A major difference in the interaction of human and rabbit CAP18 with cell membranes is observed in their effect on human red blood cells: the hCAP18-fragment FALL-39 ($hCAP18_{102-140}$) is hemolytic whereas $rCAP18_{106-142}$ is not (Travis et al., 2000).

Gram-negative bacteria (50% inhibitory concentration 0.4–2 μg/ml) such as *Escherichia coli*, *Salmonella minnesota* and *S. typhimurium*, and Gram-positive bacteria (50% inhibitory concentration, 2.3–3.6 μg/ml) such as *Streptococcus pneumoniae* and *Staphylococcus aureus* are known to be sensitive toward the antimicrobial action of $rCAP18_{106-142}$. There is, however, no activity of rCAP18 against the Gram-negative strain of *Proteus mirabilis*, fungi, and multiple-drug-resistant strains of *Mycobacterium avium* and *M. tuberculosis* (Larrick et al., 1993). At low salt concentrations, however, LL-37 ($hCAP18_{104-140}$) is also active against *P. mirabilis* (Turner et al., 1998).

The cell envelope of Gram-negative bacteria consists of the cytoplasmic membrane, the peptidoglycan layer, and an additional barrier, the outer membrane (OM), which is strictly asymmetric with respect to its lipid composition. Whereas the inner leaflet of this membrane contains only phospholipids, the outer leaflet is composed of LPS. This outer membrane is the primary target of CAP18, but the lethal event appears to be depolarization of the inner membrane (IM) (Matsuzaki, 1999).

CAP18 belongs to the group of antimicrobial peptides having a net positive charge, an α-helical conformation most pronounced in the presence of negatively charged lipids, and a high amphipathicity (Chen et al., 1995; Travis et al., 2000; Turner et al., 1998; Oren et al., 1999). These structural features are comparable to those of magainin, cecropin, and KLAL-model peptides (Oren and Shai, 1998; Dathe and Wieprecht, 1999). Different parameters may modulate activity and selectivity of these peptides. Among these are overall charge, distribution of the charges in the peptide, helicity, hydrophobic moment, hydrophobicity, angle subtended by the hydrophilic/hydrophobic surface, and the hydrophobic gradient (Dathe and Wieprecht, 1999; Travis et al., 2000; Pathak et al., 1995).

Recently, we have characterized the interaction between $rCAP18_{106-137}$ and differently composed lipid membranes

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with different biophysical techniques (Gutsmann et al., 1999, 2000). The mechanism of interaction of rCAP18 with these membranes comprises several steps: after the accumulation of the positively charged protein at the membrane, having a negative surface potential, and intercalation into the membrane, the α-helical conformation of the protein is enhanced and the orientation of the protein molecules changes under the influence of a LPS-specific transmembrane potential from a nonconductive to a transmembranous conductive state. CAP18 oligo- or multimers then form transient lesions with heterogeneous characteristics and limited lifetimes. The depth of intercalation of the peptide into the membrane is governed by the type of LPS. Each LPS differs mainly in its chemical structure, which is, therefore, decisive for the resistance/sensitivity of the respective bacterial species toward CAP18. In particular, in the case of *P. mirabilis,* the additional L-Ara*p*4N $\beta(1\rightarrow8)$-linked to the first Kdo is the structural component being responsible for the resistance of these bacteria to CAP18 lethality (Gutsmann et al., 2000). The depth of intercalation of rCAP18 into the membrane is correlated inversely to the amplitude of the clamp voltage required for the induction of membrane lesions.

In this paper, we focus on three major points: 1) comparison of results on the interaction between five 20-amino-acid (aa) and two 32-aa fragments of rCAP18 and hCAP18 and lipid membranes. These results are derived from different biophysical techniques including fluorescence resonance energy transfer (FRET) spectroscopy on liposomes, monolayer measurements with a Langmuir trough, and electrophysiological measurements on planar reconstituted asymmetric bilayer membranes. In addition, we focus on 2) correlation between structural features (net charge Q, α-helical content α, hydrophobic moment μ, and hydrophobicity H) of the peptides and the biophysical data and 3) conclusions from the biophysical data with respect to the antibacterial and cytotoxic activity of the seven peptides.

We verify our observation that the physicochemical properties of the antibacterial peptides, those of the membranes, and in particular the interaction between peptide and membrane are of importance for sensitivity and selectivity of the peptides. The finding that the minimal clamp voltage (and with that the membrane potential of cells) required for the induction of lesions in different membranes discriminates between resistance and sensitivity of Gram-negative bacteria provide important complementary information for the design of improved antibacterial therapeutics based on CAP18.

MATERIALS AND METHODS

Lipids, peptides, and other chemicals

For the formation of planar membranes, monolayers, and liposomes, deep rough mutant LPS from *Escherichia coli* strain F515 (F515 LPS) (chemical structures according to Wiese et al., 1998) was used. LPS was extracted by the phenol/chloroform/petroleum ether method (Galanos et al., 1969), purified, lyophilized, and transformed into the triethylamine salt form.

Phosphatidylethanolamine (PE) from bovine brain (type I), phosphatidylglycerol (PG) and phosphatidylcholine (PC) from egg yolk lecithin (sodium salt), and diphosphatidylglycerol (DPG) from bovine heart (sodium salt) were from Sigma (Deisenhofen, Germany), and diphytanoylphosphatidylcholine (DPhyPC) was from Avanti Polar Lipids (Alabaster, AL). All phospholipids were used without further purification. The fluorescent dyes *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PE (NBD-PE) and *N*-(rhodamine B sulfonyl)-PE (Rh-PE) were purchased from Molecular Probes (Eugene, OR).

Synthetic CAP18-derived peptides were prepared by Merrifield synthesis as previously described (Larrick et al., 1994) and stored in 0.01% acetic acid. Three fragments of rabbit CAP18 (rCAP18), two of human CAP18 (hCAP18), and two hCAP18 with a modified amino acid sequence as compared with the original peptide sequence (mhCAP18) were used. The respective amino acid sequences are summarized in Table 1.

Calculations

The net charge Q was calculated under the assumption that K, R, and the N-terminal NH_2 are positively charged and E, D, and the C-terminal COOH bear a negative charge. The α-helical content of the seven peptides is the average of the α-helical probability of each residue calculated by PSA (http://bmerc-www.bu.edu/psa/) (Stultz et al., 1993; White et al., 1994). H is the mean residue hydrophobicity, calculated on the basis of the Eisenberg consensus scale of hydrophobicity, and μ is the hydrophobic moment calculated as the absolute value of the vector sum of the hydrophobicities of all residues, assuming an ideal α-helix (Eisenberg, 1984). The results are shown in Fig. 1.

Assay of bacterial killing

For determination of the antibacterial activity of the peptides, an in vitro assay was performed as described before (Capodici et al., 1994). Briefly, *E. coli* J5 (10^6/ml) which were grown in triethanolamine-buffered minimal salts medium, were incubated for 30 min at 37°C alone or with different concentrations of the seven CAP18-derived peptides in a reaction mixture containing 0.8% (wt/vol) nutrient broth in physiological saline buffered with 20 mM sodium phosphate, pH 7.4. After termination of the incubation, an aliquot of the reaction mixture was serially diluted in physiological saline and than plated in 1% agar. Bacterial colonies were counted after incubation at 37°C for 24 h and the 50% inhibition concentration (IC_{50}) was determined.

TABLE 1 Amino acid sequences of the seven CAP18-derived peptides used in this study

Name	Sequence
$hCAP18_{98\text{-}117}$	DNKRFALLGD FFRKSKEKIG-NH_2
$mhCAP18_{98\text{-}117}$	DNKRFALLRN KFRKSKEKIG-NH_2
$mhCAP18_{104\text{-}123}$	LLGDFFRKSN EKIGNEFKRI-NH_2
$rCAP18_{98\text{-}117}$	AQESPEPTGL RKRLRKFRNK-NH_2
$rCAP18_{106\text{-}125}$	GLRKRLRKFR NKIKEKLKKI-NH_2
$rCAP18_{106\text{-}137}$	GLRKRLRKFR NKIKEKLKKI GQKIQGLLPK LA-NH_2
$hCAP18_{104\text{-}135}$	LLGDFFRKSK EKIGKEFKRI VQRIKDFLRN LV-NH_2

Amino acids that are modified as compared with the origin sequence are underlined.

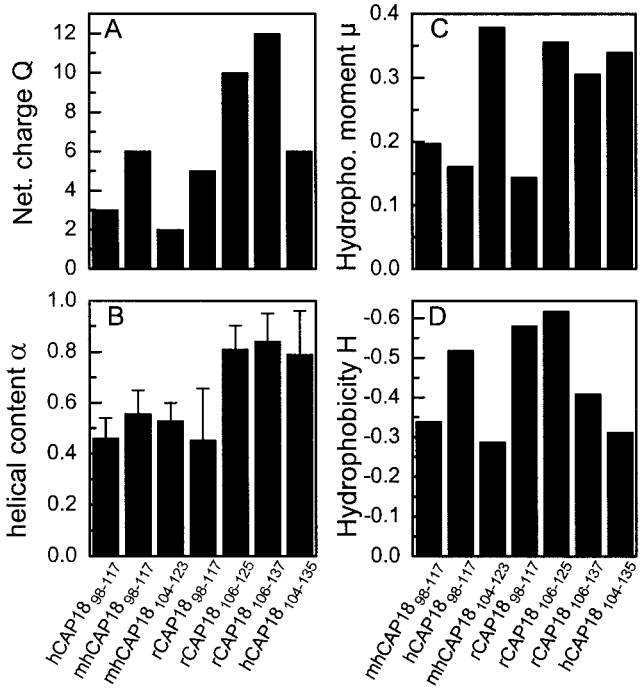

FIGURE 1 Calculated structural parameters of CAP18-derived peptides. (*A*) The net charge Q was calculated under the assumption that K, R, and the N-terminal NH_2 are positively charged and E, D, and the C-terminal COOH carry a negative charge. (*B*) The α-helical content of the seven peptides is the average of the α-helical probability of each residue calculated by PSA. (*C*) μ is the hydrophobic moment calculated as the absolute value of the vector sum of the hydrophobicities of all residues, assuming an ideal α-helix. (*D*) H is the mean residue hydrophobicity, calculated on the basis of the Eisenberg (1984) consensus scale of hydrophobicity.

FRET spectroscopy

The FRET spectroscopy was used as a probe dilution assay (Struck et al., 1981) to obtain information on the intercalation of CAP18 into liposomes made from various phospholipids and LPSs. For the FRET measurements, PC or F515 LPS liposomes were double labeled with NBD-PE and Rh-PE. The fluorescent dyes were dissolved together with PC or F515 LPS in chloroform in molar ratios [lipid]:[NBD-PE]:[Rh-PE] of 100:1:1. The solvent was evaporated under a stream of nitrogen, and the lipids were resuspended in bathing solutions with different salt concentrations at pH 7.0, mixed thoroughly, and sonicated with a Branson sonicator for 1 min (1 ml of solution). Subsequently, the preparation was cooled for 30 min at 4°C, heated for 30 min at 56°C, and recooled to 4°C. Preparations were stored at 4°C overnight before measurement. A preparation of 900 μl of the double-labeled phospholipid or LPS liposomes (0.1 mM) at 37°C was excited at 470 nm (excitation wavelength of NBD-PE), and the intensities of the emission light of the donor NBD-PE (531 nm) and acceptor Rh-PE (593 nm) were measured simultaneously on a fluorescence spectrometer (SPEX F1T11, SPEX Instruments, Edison, NY). The peptides were added after 50 s to a final concentration of 5 μg/ml. Intercalation could be detected as changes in fluorescence intensities as a function of time (increase of donor signal, decrease of acceptor signal). In the following, the quotient of the donor and the acceptor intensities is denoted as the FRET signal. In Fig. 2 *B*, the difference between the FRET signals before and after peptide addition (when no further changes were observed) is plotted. To check whether FRET signals were influenced by fusion or micellization, electron micrographs of freeze-fractured vesicles with and without CAP18 peptide were taken (data not shown), which did not give any evidence for significant peptide-induced changes of the vesicles structure.

Film balance measurements

From pressure/area isotherms of monolayers at the air/water interface of a film balance, the area of the composing molecules, lipids and/or proteins, can be calculated at a given lateral pressure.

The incorporation of the peptides into lipid monolayers with respect to the composition of the aqueous subphase was studied with monolayers spread from 1 mM chloroform solutions of DPhyPC or 1 mM chloroform/methanol (9:1 v/v) solutions of LPS. The experiments were than conducted as described earlier (Gutsmann et al., 2000).

To investigate the capacity of CAP18-derived peptides to displace divalent Ca^{2+} ions from LPS monolayers, a subphase containing 12.5 μM Ca^{2+} doped with radioactive $^{45}Ca^{2+}$ (adjusted to a final activity of 250 Bq/ml) (Amersham Buchler, Braunschweig, Germany) and 5 mM HEPES at pH 7 was used. This Ca^{2+} concentration was sufficient for saturating the LPS monolayer (160 nM F515 LPS). The experiments were performed as described in detail elsewhere (Gutsmann et al., 2000). The peptides were added to the subphase in different concentrations, and the equilibrium β-counting rates were recorded. The relative $^{45}Ca^{2+}$ concentration was calculated from the following equation:

$$I_{rel} = \frac{I_{\text{peptide}} - I_{\text{sub}}}{I_{\text{mono}} - I_{\text{sub}}}, \qquad (1)$$

where I_{rel} is the relative $^{45}Ca^{2+}$ intensity and I_{sub}, I_{mono}, and I_{peptide} are the β-intensities of the pure subphase, after spreading the monolayer, and after peptide addition, respectively. The experiments were performed at a subphase temperature of 20°C to avoid condensation at the β-counter.

Preparation of planar bilayers and electrical measurements

Planar bilayers according to the Montal-Mueller technique (1972) were prepared as described before (Wiese and Seydel, 1999; Gutsmann et al., 1999). Briefly, asymmetric bilayers were formed by opposing two lipid monolayers prepared on aqueous subphases (bathing solutions) from chloroformic solutions of the lipids at a small aperture (typically 150-μm diameter) in a thin Teflon septum (12.5-μm thickness). The essential step in bilayer reconstitution is the formation of lipid monolayers. This step requires a chloroform or chloroform/methanol solubility and a sufficiently high hydrophobicity of the lipids. Because this requirement is not fulfilled by wild-type LPS, we used here F515 LPS instead of J5 LPS.

For the formation of bilayer membranes, natural phospholipids and DPhyPC were dissolved in chloroform (2.5 mg/ml) and LPS in chloroform/methanol (10:1 v/v) (2.5 mg/ml) by heating to 95°C for 2 min. The PL leaflet of asymmetric LPS/PL membranes consisted of a mixture of PE, PG, and DPG in a molar ratio of 81:17:2 resembling the phospholipid composition of the inner leaflet of the outer membrane of *Salmonella typhimurium* (Osborn et al., 1972) being composed of the same constituents as that of *E. coli* (Shaw, 1974). For electrical measurements, planar membranes were voltage clamped and the compartment opposite (trans-compartment) to the one the peptide was added to (cis-compartment) was grounded. Therefore, in comparison with the natural system, a positive clamp voltage represents a membrane that is negative on the inner side. Current is defined as positive when cation flux is directed toward the grounded compartment. For the determination of the single-fluctuation amplitudes, the current traces were filtered at a corner frequency of 2 kHz and digitized with a sampling frequency of 5 kHz.

All measurements were performed with bathing solutions consisting of 100 mM KCl and 5 mM $MgCl_2$ at a temperature of 37°C. To adjust to pH

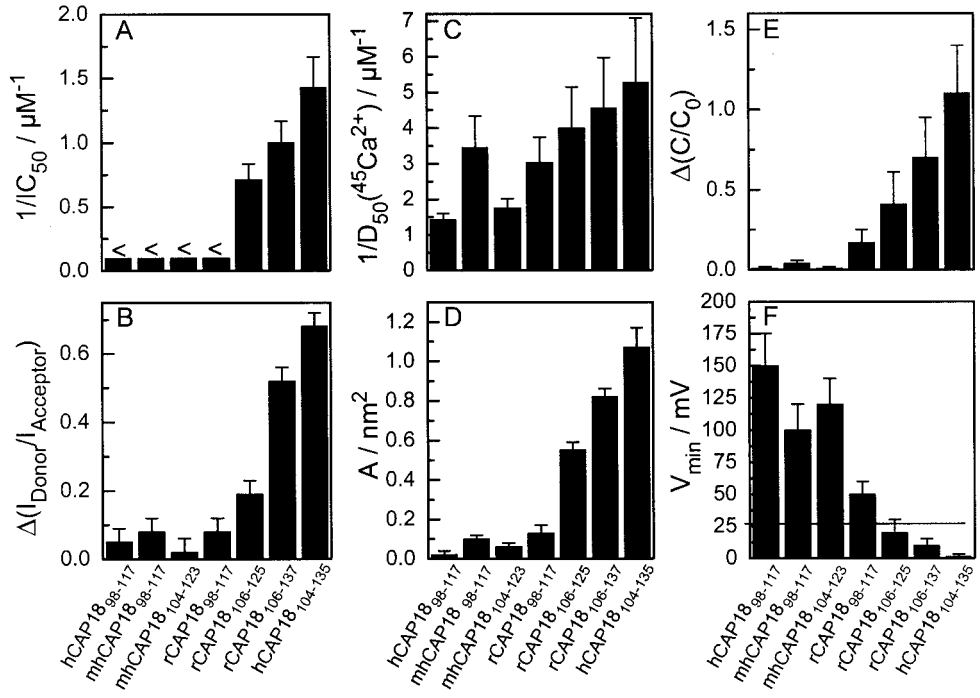

FIGURE 2 Summary of the effects induced by CAP18-derived peptides in different biological and biophysical experiments. (*A*) Inverse of the peptide concentrations necessary to kill 50% of *E. coli* J5 bacteria. (*B*) Changes of the FRET signals (quotient of the intensities of the donor and acceptor dyes) after addition of 5 μg/ml peptide to 100 μM suspensions of double-labeled F515 LPS liposomes. Bathing solution: 100 mM KCl, 5 mM HEPES at pH 7 and 37°C. (*C*) Inverse of the peptide concentrations necessary to displace 50% $^{45}Ca^{2+}$ from F515 LPS monolayers. Bathing solution: 12.5 μM total Ca^{2+}, 10 mM HEPES at pH 7 and 20°C; the F515 LPS concentration was 9.6 nmol (160 nM). (*D*) Areas occupied by the peptides in F515 LPS monolayers at a lateral pressure of 30 mN m^{-1}. Bathing solution: 100 mM KCl, 5 mM HEPES at pH 7 and 37°C. (*E*) Relative changes of the capacitance of F515 LPS/PL planar membranes after addition of 2 μg/ml peptide to the LPS side of the bilayer. Bathing solution: 100 mM KCl, 5 mM $MgCl_2$, 5 mM HEPES at pH 7 and 37°C. (*F*) Minimal clamp voltage necessary to induce lesions after the addition of 2 μg/ml of the peptides to the LPS side of F515 LPS/PL bilayers. Bathing solution: 100 mM KCl, 5 mM $MgCl_2$, 5 mM HEPES at pH 7 and 37°C.

7, the bathing solutions were buffered with 5 mM HEPES. The specific electrical conductivity of the bathing solutions at 37°C was 17.2 mS cm^{-1}. Mg^{2+} was used to improve the stability of the membranes. Peptides (100 μg/ml) were added in aliquots of 30 μl to the cis-side (named first) of the bilayer, e.g., F515 LPS/PL.

The determination of the membrane capacitance yields information on area, thickness, and composition of the bilayer. Membrane capacitance can be determined by applying step-like voltage pulses and measuring the resulting transient charging currents (Alvarez and Latorre, 1978). In our setup the capacitance was determined at a frequency of 1 Hz and a maximum clamp voltage of 1 mV. This way we were able to analyze membrane qualities and membrane protein interactions without influencing the system by other agents such as carriers or by high clamp voltages.

RESULTS

Antibacterial activity

The 20-aa $rCAP18_{106-125}$ and both 32-aa CAP18 fragments $rCAP18_{106-135}$ and $hCAP18_{104-135}$ showed antibacterial activity against *E. coli* J5, and the 50% inhibition concentrations (IC_{50}) were 1.4, 1.0, and 0.7 μM, respectively (Fig. 2 *A*). In contrast, the other four 20-aa peptides did not show any antibacterial activity against *E. coli* J5 up to concentrations of 10 μM.

FRET spectroscopy

It has been described that the human peptide LL-37 is cytotoxic (Johansson et al., 1998) and interacts with vesicles prepared of PC (Oren et al., 1999) and that, in contrast, rabbit $CAP18_{106-137}$ does not interact with PC membranes (Gutsmann et al., 1999, 2000). Therefore, we tested the capability of the seven peptides to intercalate into liposomes prepared of PC in FRET experiments. We observed a significant increase in the FRET signal after addition of the same amount of $hCAP18_{104-135}$ (Fig. 3, *trace a*). However, neither 5 μg/ml $rCAP18_{106-137}$ nor any of the five 20-aa fragments (Fig. 3) caused a change of the FRET signal of double-labeled PC liposomes (10 μM) with respect to the control (0.01% acetic acid) (Fig. 3, *trace b*). The peptide concentrations used in these experiments were significantly below the saturation concentration to guarantee that all peptide molecules could bind to the vesicles.

The results on the intercalation of the peptides into F515 LPS liposomes are summarized in Fig. 2 *B*. From the changes of the FRET signal after addition of 5 μg/ml of each peptide to 10 μM F515 LPS liposome suspensions, the

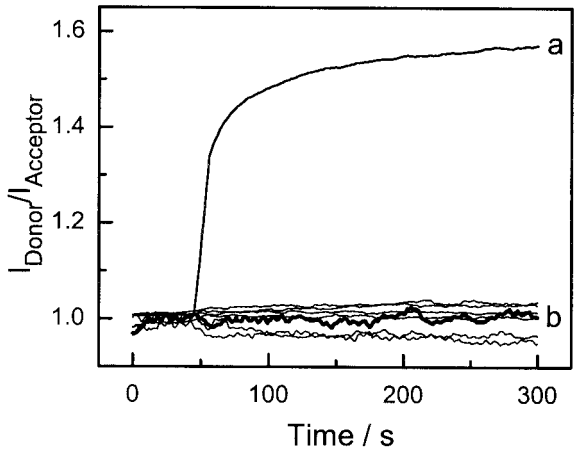

FIGURE 3 Intercalation of CAP18-derived peptides into phosphatidylcholine (PC) liposomes. Changes of the FRET signal versus time after addition of 5 μg/ml peptide to 10 μM suspensions of PC liposomes double labeled with NBD-PE and RH-PE. Trace a, addition of 5 μl (1 mg/ml) of $hCAP18_{104-135}$; Trace b, addition of 5 μl of 0.01% acetic acid as a control; remaining traces, addition of 5 μl (1 mg/ml) of the other peptides. Bathing solution: 100 mM KCl, 5 mM HEPES at pH 7 and 37°C.

capacity to intercalate into the LPS liposomes is in the order $hCAP18_{104-135}$ > $rCAP18_{106-137}$ > $rCAP18_{106-125}$ > $rCAP18_{98-117}$ $mhCAP18_{98-117}$ > $hCAP18_{98-117}$ > $mhCAP18_{104-123}$.

Film balance measurements

The Ca^{2+}-displacement experiments provide important information on the binding of the seven peptides to the membrane surface and a resulting displacement of divalent ions. All peptides have the potency to displace Ca^{2+} from F515 LPS monolayers (Fig. 4), and the respective peptide concentrations necessary to displace 50% of $^{45}Ca^{2+}$ [$D_{50}(^{45}Ca^{2+})$] from the monolayers are shown in Fig. 2 *C*. Five peptides displaced more than 90% $^{45}Ca^{2+}$ each, but $hCAP18_{98-117}$ and $mhCAP18_{104-123}$ displaced only about 60% $^{45}Ca^{2+}$ each (Fig. 4).

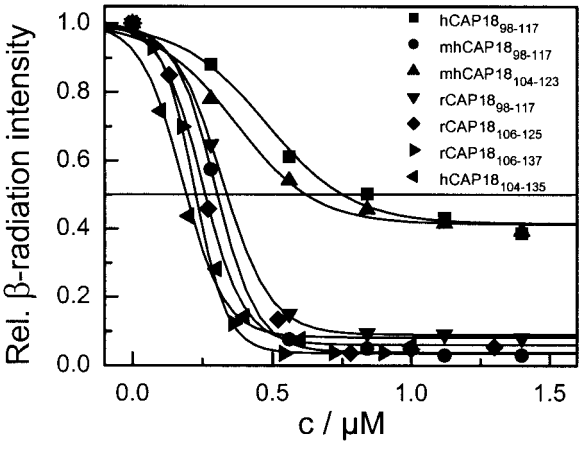

FIGURE 4 Displacement of $^{45}Ca^{2+}$ from F515 LPS monolayers by CAP18-derived peptides, plotted as relative change of β-radiation intensity versus peptide concentration. Bathing solution: 12.5 μM total Ca^{2+}, 10 mM HEPES at pH 7, and 20°C; F515 LPS concentration was 9.6 nmol (160 nM).

We then determined the area of $hCAP18_{104-135}$ molecules in monolayers composed of DPhyPC or F515 LPS in the presence (5 mM) or absence of Mg^{2+} in the subphase and the area of the five fragments in F515 LPS monolayers in the absence of Mg^{2+}. In contrast to $rCAP18_{106-137}$, which has been shown not to intercalate into monolayers composed of the zwitterionic PC (Gutsmann et al., 2000) (Table 2), $hCAP18_{104-135}$ intercalated into these membranes and occupied an area of 1.1 $\pm$ 0.2 nm^2 in the absence of Mg^{2+} and 1.0 $\pm$ 0.1 nm^2 in the presence of 5 mM $MgCl_2$. The difference in the molecular areas observed on the two subphases is not significant. Nearly identical areas were observed for $hCAP18_{104-135}$ and $rCAP18_{106-137}$ molecules intercalated into F515 LPS monolayers in the absence of Mg^{2+} and for $rCAP18_{106-137}$ also in the presence of 5 mM $MgCl_2$. For $hCAP18_{104-135}$, however, twice that area (2.0 $\pm$ 0.1 nm^2) was obtained in the presence of 5 mM $MgCl_2$ (Table 2).

In FRET experiments we did not observe an interaction between the five 20-aa CAP18-derived peptides and zwitterionic PC membranes, and even the interactions with F515 LPS liposomes were reduced as compared with those of $rCAP18_{106-137}$. For this reason, we determined the size of the area occupied by these peptides in F515 LPS monolayers in the absence of Mg^{2+}. The capacity of the peptides to intercalate into F515 LPS monolayers differs significantly and decreases in the following order (Fig. 5): $hCAP18_{104-135}$ (*trace a*) > $rCAP18_{106-137}$ (*trace b*) > $rCAP18_{106-125}$ (*trace c*) > $rCAP18_{98-117}$ (*trace d*) $\geq$ $mhCAP18_{98-117}$ (*trace e*) > $mhCAP18_{104-123}$ (*trace f*) > $hCAP18_{98-117}$ (*trace g*). The respective calculated areas occupied by one peptide molecule are depicted in Fig. 2 *D*.

Electrical measurements using planar bilayers

Membrane capacitance

Changes in membrane capacitance after addition of membrane-active agents are indicative of their accumulation or incorporation into the lipid bilayer. Thus, capacitance changes after the addition of the CAP18-derived peptides provide information on the interaction between peptides and different bilayers.

First, we characterized the influence of $hCAP18_{104-135}$ on the membrane capacitance of differently composed asymmetric planar membranes. The addition of 2 μg/ml (500 nM) $hCAP18_{104-135}$ to the LPS leaflet of asymmetric F515 LPS/PL membranes led to an increase in membrane capacitance to a maximum of 130% of the initial value, followed by a decrease to 95% (Fig. 6 *A*). Nearly identical changes of membrane capacitance were observed after ad-

TABLE 2 Comparison of the sizes of $rCAP18_{106-137}$ and $hCAP18_{104-135}$ in DPhyPC and F515 LPS monolayers and the minimal clamp voltage V_{min} needed to induce lesions in the respective planar lipid/PL bilayers

	$rCAP18_{106-137}$		$hCAP18_{104-135}$	
Lipid/subphase	Area per molecule/nm^2*	V_{min}/mV*	Area per molecule/nm^2	V_{min}/mV
PC/Sub: +0 mM $MgCl_2$	0 ± 0.1	ND	1.1 ± 0.2	ND
PC/Sub: +5 nM $MgCl_2$	0 ± 0.1	60 ± 7	1.0 ± 0.1	40 ± 10
F515 LPS/Sub: +0 mM $MgCl_2$	0.8 ± 0.1	ND	1.1 ± 0.1	ND
F515 LPS/Sub: +5 mM $MgCl_2$	0.8 ± 0.1	10 ± 5	2.0 ± 0.1	1 ± 2

The monolayers were prepared on subphases containing 100 mM KCl, 5 mM HEPES with varying concentrations of $MgCl_2$ (first column) at pH 7 and a temperature of 37°C. The intercalation was determined at a lateral pressure of 30 mN m^{-1}. V_{min} was determined after addition of 2 μg/ml of the respective peptide to the planar lipid/PL bilayers. For the formation of PC/PL bilayers DPhyPC was used instead of natural PC. Bathing solution: 100 mM KCl, 5 mM $MgCl_2$, 5 mM HEPES at pH 7 and 37°C. ND, not determined.
*Data taken from Gutsmann et al. (2000).

dition of the same amount of this peptide to DPhyPC/PL membranes (Fig. 6 *B*). Also, the differences in the kinetics of the capacitance changes between the two membrane types were not significant. The observed changes of the membrane capacitance could result from changes of thickness, size, and/or dielectric coefficient of the membranes and have been described in more detail for $rCAP18_{106-137}$ earlier (Gutsmann et al., 1999).

We than compared the seven CAP18-derived peptides for their influence on membrane capacitance by adding an identical amount (2 μg/ml) of each peptide to the LPS side of F515 LPS/PL membranes. As a parameter we used the sum of the initial increase and the subsequent decrease of the capacitance relative to the value of the untreated membrane. The respective data are shown in Fig. 2 *E*.

Membrane conductance

Membrane conductance is a measure of the integrity of the lipid bilayer. Thus, the formation of transient membrane lesions leads to fluctuations of membrane conductance and current. All investigated peptides induced current fluctuations in F515 LPS/PL membranes at a concentration of 2 μg/ml. The characteristics of the fluctuations induced by the seven peptides are comparable with respect to size and lifetime and have already been described in more detail for the interaction of $rCAP18_{106-137}$ with this type of membrane (Gutsmann et al., 1999). The transient lesions had lifetimes in the range of some milliseconds and some minutes. The calculated diameters of the lesions ranged between 0.2 (detection limit) and 5 nm or, in some cases, even larger. In Fig. 7, an example is given for the $hCAP18_{104-135}$-induced lesions in F515 LPS/PL bilayers. Lesion formation started above a peptide-specific minimal clamp voltage, V_{min} (Fig. 2 *F*).

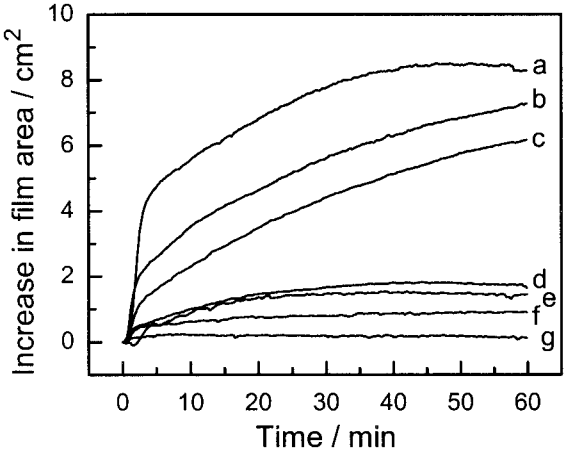

FIGURE 5 Increase in film area of F515 LPS monolayers (concentration of LPS was 3 nmol (75 nM)) versus time at a constant lateral pressure of 30 mN m^{-1} after addition of 125 ng/ml $hCAP18_{104-135}$ (*trace a*), $rCAP18_{106-137}$ (*trace b*), $rCAP18_{106-125}$ (*trace c*), $rCAP18_{98-117}$ (*trace d*), $mhCAP18_{98-117}$ (*trace e*), $mhCAP18_{104-123}$ (*trace f*), and $hCAP18_{98-117}$ (*trace g*) to the subphase (100 mM KCl, 5 mM HEPES at pH 7 and 37°C).

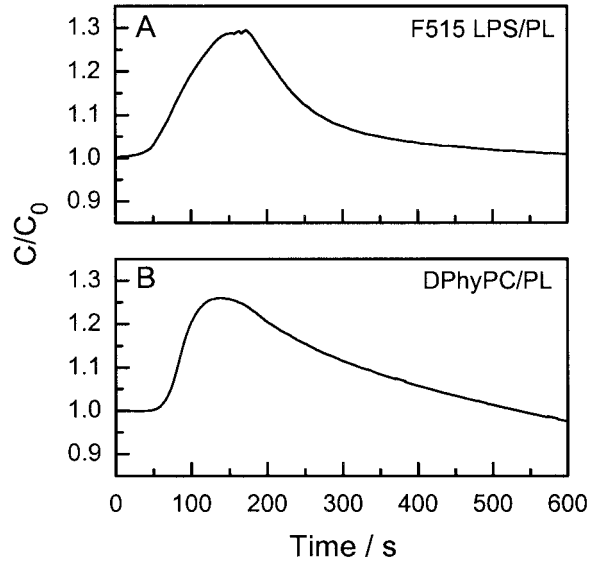

FIGURE 6 $hCAP18_{106-137}$-induced changes in membrane capacitance. Capacitance versus after addition of 2 μg/ml (500 nM) $hCAP18_{106-137}$ to asymmetric bilayers (the side of peptide addition is named first). (*A*) F515 LPS/PL bilayer; (*B*) DPhyPC/PL. Bathing solution: 100 mM KCl, 5 mM $MgCl_2$, 5 mM HEPES at pH 7 and 37°C.

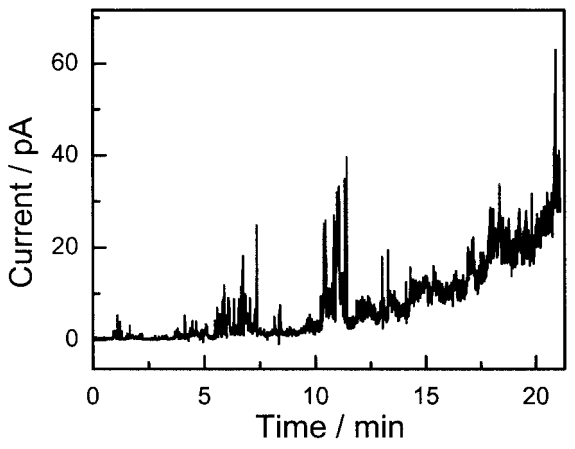

FIGURE 7 $hCAP18_{106-137}$-induced current fluctuations after addition of 500 nM $hCAP18_{106-137}$ to the LPS side of a F515 LPS/PL bilayer at a clamp voltage of 40 mV. Bathing solution: 100 mM KCl, 5 mM $MgCl_2$, 5 mM HEPES at pH 7, and 37°C.

DISCUSSION

The antimicrobial activity of polycationic peptides and their mode of interaction with lipid membranes mimicking the bacterial cell envelope has been investigated intensively over the past few years (Oren and Shai, 1998; Wu et al., 1999; Hancock and Scott, 2000). Cathelicidins belong to this group of antibacterial polycationic peptides. They are synthesized in neutrophilic granulocytes of mammals (Zanetti et al., 1995). The bioactivity of the human and rabbit CAP18 was found in their C-terminal domain. From earlier investigations into the interaction between $rCAP18_{106-137}$ and monolayers, liposomes, planar membranes, and different species of Gram-negative bacteria we proposed an interaction model (Gutsmann et al., 1999, 2000) that comprises the following steps: after the accumulation of the proteins at the membrane surface and intercalation into the membrane, the orientation of the proteins changes under the influence of an LPS-specific clamp voltage from a nonconductive to a conductive transmembrane state. CAP18 oligo- or multimers then form transient lesions with heterogeneous characteristics and limited lifetimes.

The aim of the present study was to elucidate the influence of structural properties of polycationic peptides derived from CAP18 on their interaction with the OM of Gram-negative bacteria. To allow the variation of defined parameters, we used simplified reconstitution systems of the bacterial membrane instead of the complex natural system. These reconstitution systems are composed of monolayers, liposomes, and planar bilayers composed of zwitterionic PC to mimic the cytoplasmic membrane and LPS to mimic the OM of Gram-negative bacteria. The interaction between the peptides and these systems was investigated with various biophysical techniques. The conclusions from the biophysical data and their correlation with physicochemical properties of the peptides permit us to propose a more complete picture of the role of structural properties of the peptides in the interaction with the bacterial OM.

The seven fragments derived from human and rabbit CAP18 represent only one possible selection of CAP18-derived peptides. This ensemble was selected because the peptides vary in their antibacterial activity and physicochemical properties; in particular, the number of charged residues was modified. To get a deeper insight into the importance of single amino acids, further experiments would be required.

Conclusions from biophysical techniques

Monolayers at the air/water interface of a Langmuir trough represent the most artificial membrane system used in this study. To investigate the binding of the seven CAP18-derived peptides to monolayers composed of LPS (step 1 in the scheme of Fig. 8), $^{45}Ca^{2+}$-displacement experiments were performed. The intercalation of the peptides into F515 LPS monolayers (step 2 in Fig. 8), which is defined here as the capability of a peptide to increase the distance between lipid molecules , was determined from the increase of the film area at a constant pressure of 30 mN m^{-1} (Figs. 5 and 2 *D*). Obviously, all fragments displace Ca^{2+} from the monolayers and, thus, bind to LPS (Fig. 2 *C*). However, the two peptides $hCAP18_{98-117}$ and $mhCAP18_{104-123}$ displace only 60% of the Ca^{2+} (Fig. 4). From this it can be deduced that they bind only to the outer region of the head group of F515 LPS, possibly to the two Kdo (3-desoxy-d-*manno*-oct-2-ulosonic acid). This binding does not lead to a significant increase in film area (Fig. 2 *D*). From Fig. 9 *A* we deduce that at $A < 0.15$ nm^2 a slight increase in the area occupied by a single peptide molecule leads to a significant decrease in $D_{50}(^{45}Ca^{2+})$, whereas at $A > 0.15$ nm^2 the further decrease in $D_{50}(^{45}Ca^{2+})$ is only small. These data show that Ca^{2+}-displacement and intercalation experiments with monolayers produce complementary results. Furthermore, from the Ca^{2+}-displacement experiments and from the determination of the inner membrane potential of planar bilayers (Gutsmann et al., 1999), binding to and accumulation of the peptides at the membrane can be determined (Fig. 8).

In a further step toward mimicry of the biological system we investigated intercalation of the various peptides into lipid bilayers. In these experiments we performed FRET experiments on symmetrically composed liposomes and capacitance measurements on asymmetric planar bilayers. After peptide addition, the FRET signal of double-labeled F515 LPS liposomes increased exponentially (Fig. 9 *B*), and the change of the capacitance of planar F515 LPS/PL membranes increased linearly (Fig. 9 *C*) with increasing area of the peptide molecules as determined in F515 LPS monolayer experiments. Moreover, in the PC membrane systems an increase of the monolayer area (Table 2), of the FRET signal of double-labeled liposomes (Fig. 3), and of the capacitance of planar bilayers (Fig. 6 *B*) was induced only

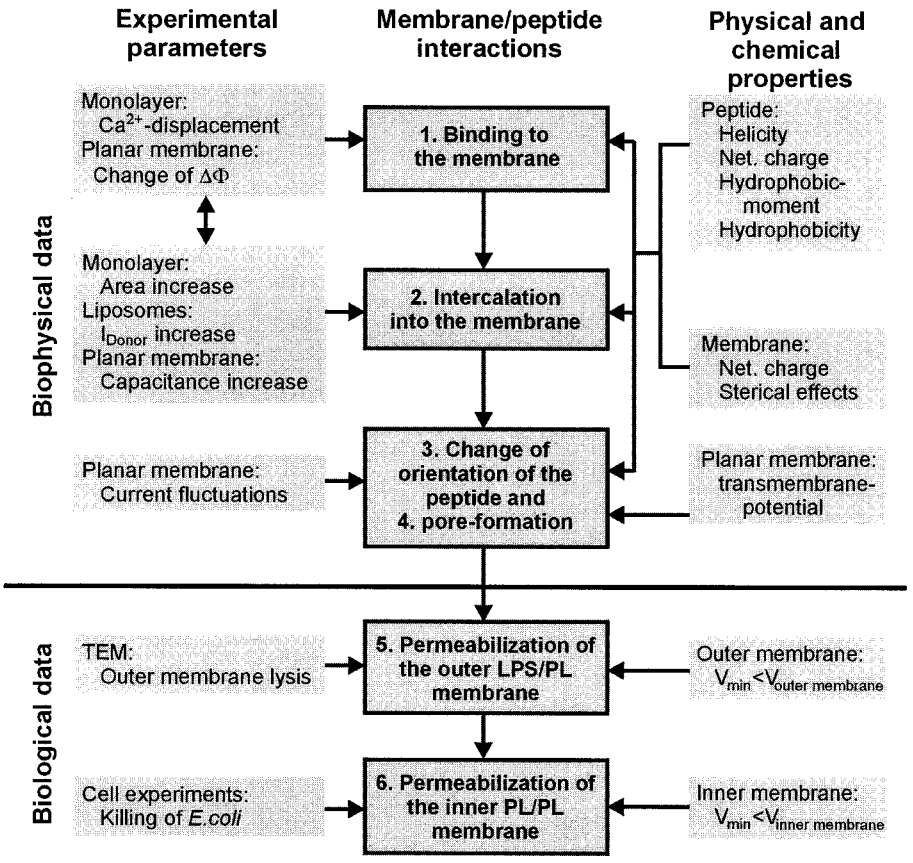

FIGURE 8 Schematic of the interactions induced by CAP18-derived peptides leading to killing of Gram-negative bacteria. In the middle column the membrane peptide interactions leading finally to the killing are summarized. These interactions were determined from the results of different biophysical and biological experiments and their respective parameters, which are depicted in the left column. The single steps in the action of the peptides on membranes are governed by specific parameters of the peptides and even of the composition of the membranes (*right column*).

by peptide hCAP18$_{104-135}$. Thus, these different techniques applied to different membranes systems lead to comparable results with respect to the intercalation of the peptides into F515 LPS and PC matrices, respectively (step 2 in Fig. 8).

All seven fragments induced the formation of voltage-dependent lesions (Fig. 7) with comparable characteristics concerning lifetime and size. Current fluctuations induced by the lesions are indicative of a change of peptide orientation and pore formation in the membrane (steps 3 and 4 in Fig. 8). The minimal clamp voltages (V_{min}) necessary for the induction of these lesions differ significantly for the peptides (Fig. 2 *F*). V_{min} decreases logarithmically in dependence on increasing change of the capacitance of F515 LPS/PL membranes (Fig. 9 *D*) and with that also with the area occupied by one molecule of the respective peptide. Thus, the transmembrane potential reflects an important property of bilayers in the context of the formation of voltage-dependent lesions.

Correlation of biophysical data and physicochemical properties of the peptides

The interaction between the peptides and uncharged or even zwitterionic lipid matrices results from hydrophobic interactions (i.e., minimization of Gibb's free energy) and does not depend on the net charge or the distribution of the charges within the peptides (Amano et al., 1985). Among the seven peptides only hCAP18$_{104-135}$ intercalated into PC matrices; however, binding of the other peptides to the outer region of PC membrane surfaces cannot be excluded. To define the contribution of different parameters of the peptides to this interaction we compared those of hCAP18$_{104-135}$ with those of rCAP18$_{106-137}$. Both peptides have a relatively high hydrophobic moment of ~0.3 (Fig. 1 *C*), a high overall helicity of ~80% (Fig. 1 *B*), a comparable hydrophobicity (Fig. 1 *D*), and an identical angle of hydrophilic residues of 140° (data not shown). The hydrophobic interaction with membranes may be amplified by evenly distributed hydrophobic residues within a peptide (resulting in a low hydrophobic gradient). hCAP18$_{104-135}$ exhibits a significantly reduced inner molecular hydrophobic gradient as compared with rCAP18$_{106-137}$, but this holds also for mhCAP18$_{104-123}$, which does not intercalate into PC membranes. Thus, these classical overall properties of peptides may be essential for the action on neutral membranes, as has been shown for KLAL-model peptides (Dathe et al., 1996), but they do not allow an explanation of the strong interaction between hCAP18$_{104-135}$ and PC considering of the lack of interaction of the other six peptides. Therefore, we propose that also small arrays of residues or even single amino acid residues influence the interaction of these peptides with zwitterionic membranes. In this context, the observation of

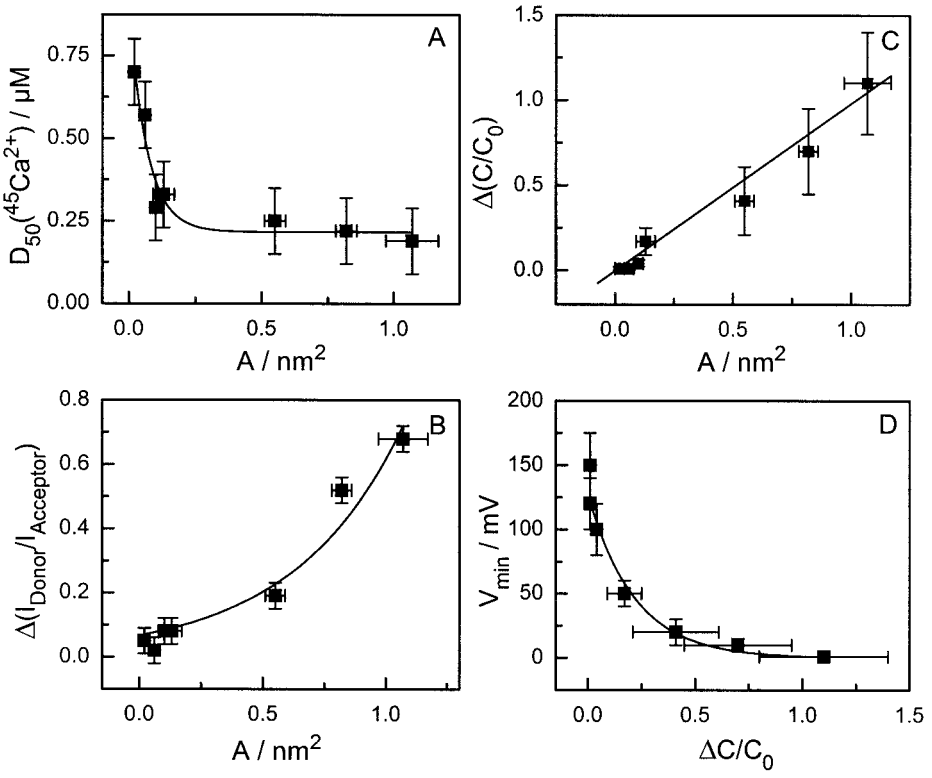

FIGURE 9 Correlation between data obtained from different biophysical techniques. (*A*) Peptide concentrations necessary to displace 50% Ca^{2+} from F515 LPS monolayers; (*B*) Changes of the FRET signal of F515 LPS liposomes; (*C*) Changes of the capacitance of F515 LPS/PL planar membranes induced by CAP18-derived peptides versus area occupied by one respective peptide molecule; (*D*) Changes of the capacitance of F515 LPS/PL planar membranes induced by CAP18-derived peptides versus minimal clamp voltage necessary to induce formation of lesions.

Oren et al. (1999) that LL-37 is self-associated when bound to PC vesicles may be important, because complexes of peptides might have different properties from those of single molecules with respect to the parameters under investigation.

The structural parameters of polycationic peptides responsible for the interaction with negatively charged membranes, in particular, the outer membrane of Gram-negative bacteria, are well known. The majority of antibacterial peptides are cationic, whereas hemolytic peptides can have any charge (Amano et al., 1985,) and thus, the intercalation of the former into negatively charged membranes is determined by a sensitive balance of electrostatic and hydrophobic interactions (Matsuzaki, 1999; Dathe et al., 1997). In the following, we will discuss the importance of the four peptide parameters (Fig. 1) on the interaction with negatively charged LPS membranes.

Travis et al. (2000) showed a positive correlation between antimicrobial activity of cathelicidin-derived peptides and their net positive charge. The importance of the net charge (Fig. 1 *A*) is also evident from our results: binding and intercalation of the peptides into F515 LPS matrices was mainly determined by electrostatic interactions. In the case of $hCAP18_{104-135}$, the hydrophobic interaction also plays an important role and results in a stronger interaction with F515 LPS matrices as observed for $rCAP18_{106-137}$, which contains twice the number of net positive charges. However, the role of the electrostatic interaction between $hCAP18_{104-135}$ and F515 LPS monolayers is indicated by the observed influence of divalent cations on the area occupied by one peptide molecule that increased in the presence of Mg^{2+} (Table 2).

The degree of helicity is not the sole parameter describing antibacterial activity (Travis et al., 2000); however, the pronounced α-helicity of CAP18 is important for its antimicrobial activity (Chen et al., 1995). The predicted α-helical content of the seven CAP18-derived peptides (Fig. 1 *B*) is significantly higher for the three peptides that intercalate strongest into the LPS monolayers, liposomes, and planar membranes (Fig. 2).

Matsuzaki et al. (1995) did not find a correlation between hydrophobic moment and lytic activity of different antimicrobial peptides. Pathak et al. (1995) and other groups, however, showed a log-correlation between hydrophobic moment and antibacterial activity. Studies with KLAL-model peptides revealed that the hydrophobic moment plays only a minor role in the permeabilization of highly charged lipids but influences substantially their effect on neutral lipid membranes (Dathe and Wieprecht, 1999). As pointed out above, the net positive charge of $hCAP18_{104-135}$ is not the only parameter responsible for its pronounced membrane interaction, and other parameters such as hydrophobic effects must play a modulating role. The hydrophobic moment does not reflect all hydrophobic effects. However, it has been shown to have more influence on the antimicrobial activity of peptides derived from the antisense sequence of magainin 2 than either mean hydrophobicity or α-helical content (Pathak et al., 1995).

The mode of interaction of antibacterial peptides, e.g., magainins and tachyplesins, with bilayers depends strongly on the physicochemical properties of the target membrane (Matsuzaki, 1999). In earlier investigations we observed that the chemical structure of LPSs of different bacterial strains is responsible for their sensitivity or resistance against $rCAP18_{106-137}$ (Gutsmann et al., 1999, 2000) and polymyxin B (Wiese et al., 1998).

Correlation of biophysical and biological data

Polycationic peptides kill Gram-negative bacteria in a two-step mechanism. In a first step, they permeabilize the OM (step 5 in Fig. 8) for their own self-promoted translocation, as described for the action of polymyxin B (Hancock, 1984). In a second step, they permeate the IM (step 6 in Fig. 8), thus allowing ions to equilibrate across the cytoplasmic membrane and to reduce or destroy the membrane potential (depolarization).

In the in vitro experiments on bacterial killing of *E. coli* J5 (Fig. 2 *A*) antibacterial activity of the peptides $rCAP18_{106-125}$, $rCAP18_{106-137}$, and $hCAP18_{104-135}$ was observed, whereas the other peptides were completely inactive. Nevertheless, some of the inactive peptides intercalate into LPS membranes (Fig. 2) and induce lesions (Fig. 7). Thus, it seems that one step in the peptide-membrane interaction, which causes permeabilization of the OM, discriminates between active and inactive peptides. In Gram-negative bacteria, the periplasmic space is highly anionic, mainly due to the presence of anionic membrane-derived oligosaccharides (MDOs). MDOs contribute to the Donnan potential V_{OM} across the OM, which was determined for *E. coli* in the presence of an external cation concentration of 100 mM to be 26 mV (inside negative) (Sen et al., 1988). According to Fig. 10, all active peptides induced lesions in F515 LPS/PL membranes at clamp voltages below V_{OM}, and all inactive have a $V_{min} > V_{OM}$. Moreover, the IC_{50} values for the active peptides correlate linearly with V_{min} of the respective peptides. Thus, it seems to be likely that V_{min} discriminates between active and inactive antibacterial peptides (Fig. 8). We have previously shown that V_{min} also discriminates between sensitive and resistant bacterial strains against $rCAP18_{106-137}$ (Gutsmann et al., 1999).

The observation that the active CAP18-derived peptides also induce lesions in DPhyPC/PL membranes at clamp voltages of <60 mV (outside positive), which is significantly lower than the potential gradient of ~ 150 mV across the cytoplasmic membrane (Bakker and Mangerich, 1981), clearly shows that the peptides can induce permeabilization of the IM, which then results in bacterial killing (Fig. 8).

Our data provide clear evidence that the interaction between antibacterial peptides and membranes is determined by the physicochemical properties of the peptides as well as by the chemical properties and the transmembrane potential of the target membranes. These findings are corroborated by observations of Lysenko et al. (2000), who showed that bacterial mimicry of host membranes based on the expression of phosphorylcholine contributes to resistance of *Haemophilus influenzae* to LL-37/hCAP18.

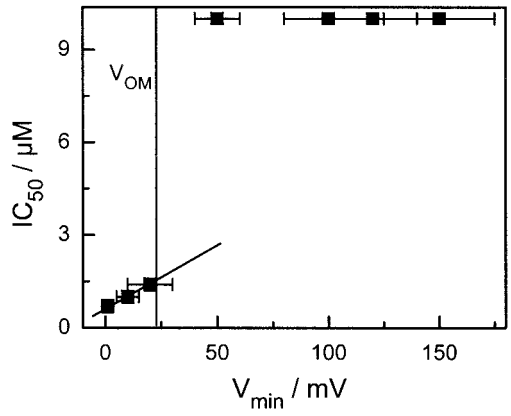

FIGURE 10 Correlation between the minimal clamp voltage required for the induction of lesions (V_{min}) by the peptides and the respective IC_{50} values of bacterial killing (*E. coli* J5). The transmembrane potential of the outer membrane of Gram-negative bacteria (V_{OM}) of 26 mV at a cation concentration of 100 mM is depicted. The IC_{50} values of the four peptides with $V_{min} > 26$ mV represent the highest concentration used in the experiments, and thus, the respective IC_{50} concentrations might be significantly higher.

The intercalation of CAP18-derived peptides into negatively charged membranes is mainly driven by electrostatic forces and modulated by hydrophobic interactions. We could, however, not define a responsibility of certain structural parameters for the strong hydrophobic interaction between $hCAP18_{104-135}$ and lipid membranes. The importance of the voltage dependence of lesion formation by CAP18-derived peptides in connection with the transmembrane potential of naturally occurring membranes was supported by our data.

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