Modification of the membrane lipid phosphatidyglycerol (PG) of \textit{Staphylococcus aureus} by enzymatic transfer of a L-lysine residue leading to lysyl-PG converts the net charge of PG from $-1$ to $+1$ and is thought to confer resistance to cationic antimicrobial peptides (AMPs). Lysyl-PG synthesis and translocation to the outer leaflet of the bacterial membrane are achieved by the membrane protein MprF. Consequently, mutants lacking a functional \textit{mprF} gene are in particular vulnerable to the action of AMPs. Hence, we aim at elucidating whether and to which extent lysyl-PG modulates membrane binding, insertion, and permeabilization by various AMPs. Lysyl-PG was incorporated into artificial lipid bilayers, mimicking the cytoplasmic membrane of \textit{S. aureus}. Moreover, we determined the activity of the peptides against a clinical isolate of \textit{S. aureus} strain SA113 and two mutants lacking a functional \textit{mprF} gene and visualized peptide-induced ultrastructural changes of bacteria by transmission electron microscopy. The studied peptides were: (i) NK-2, an $\alpha$-helical fragment of mammalian NK-lys, (ii) arenicin-1, a lugworm $\beta$-sheet peptide, and (iii) bee venom melittin. Biophysical data obtained by FRET spectroscopy, Fourier transform infrared spectroscopy, and electrical measurements with planar lipid bilayers were correlated with the biological activities of the peptides. They strongly support the hypothesis that peptide-membrane interactions are a prerequisite for eradication of \textit{S. aureus}. However, degree and mode of modulation of membrane properties such as fluidity, capacitance, and conductivity were unique for each of the peptides. Altogether, our data support and underline the significance of lysyl-PG for \textit{S. aureus} resistance to AMPs.

Antimicrobial peptides (AMPs)\(^2\) are part of the innate immunity and provide the first line of defense against pathogenic microorganisms (1). They have a positive net charge, are 20–40 amino acid residues in length, and exhibit an amphipathic secondary structure. These features enable the interaction and permeabilization of bacterial membranes. The disturbance of the bacterial membrane may be the lethal hit itself or may allow the entrance of the peptides to reach secondary intracellular targets (2). Due to their unique mode of action, AMPs have gained high interest as new drugs to overcome bacterial resistance to classical antibiotics (3). The primary target of AMPs is the cell membrane of bacteria. It is widely accepted that the negatively charged bacterial surface plays a major role in the attraction of the cationic peptides. Biophysical studies demonstrated explicitly that model membranes consisting of anionic lipids, such as phosphatidyglycerol (PG), are particularly vulnerable to AMPs, whereas membranes of the eukaryotic zwitterionic phospholipid lecithin (phosphatidylcholine) are refractory to the peptides (4, 5). Indeed, model membranes, if their compositions really reflect those of biological membranes, can be powerful tools to unravel molecular principles of peptide-membrane interactions (6–10) and to explain the molecular basis for bacterial sensitivity and resistance to AMPs (11–13).

The opportunistic pathogen \textit{Staphylococcus aureus} is among the bacteria that are difficult to treat, particularly by cationic antibiotics and AMPs (14, 15). This has been attributed partially to the modification of PG in the cytoplasmic membrane of \textit{S. aureus} by the addition of a L-lysine residue, leading to lysyl-phosphatidyglycerol (lysyl-PG) (14). This reaction modifies the net charge of PG from $-1$ to $+1$ and hence should impair the initial electrostatic interaction of the cationic peptides with the bacterial membrane. The lipid portion of the cytoplasmic membrane of \textit{S. aureus} is composed of PG and cardiolipin (16, 17), and it has been estimated that one-third of PG in the membrane is modified by lysine. PG lysination is achieved by the membrane protein multiple peptide resistance factor (MprF), which consists of a lysyl-PG synthase domain (18) transferring lysine from lysyl-tRNA to PG and a lysyl-PG translocase, which facilitates flipping of lysyl-PG to the outer membrane leaflet (19).
**S. aureus Strains**—We used a clinical isolate of *S. aureus* (35), as well as wild type SA113 (ATCC 35565), a SA113-derived strain lacking the complete *mprF* gene (ΔmprF), and one ΔmprF-derived strain expressing only the synthase domain of MprF but lacking the translocase domain (ΔmprFpRBsyn) (19). Bacteria were grown overnight in Luria-Bertani (LB) medium (Merck, Darmstadt, Germany) composed of 1% *Tryptone, 0.5%* [**S. aureus Resistance to Antimicrobial Peptides**](#)

**EXPERIMENTAL PROCEDURES**

**Peptides**—Peptides were synthesized either with a free carboxyl group or with an amidated C terminus by the Fmoc (N-(9-fluorenylmethoxycarbonyl) solid-phase peptide synthesis technique on an automatic peptide synthesizer (model 433 A; Applied Biosystems) as described previously (28, 36). Disulfide linkage of terminal cysteine residues was performed in dimethyl sulfoxide (DMSO) as described (28). Peptide stocks (1 mM) in 0.01% TFA were stored at −20 °C.

**Lipids and Reagents**—Synthetic phospholipids 1,2-dipalmityl-sn-glycero-3-phospho-(1′-rac-glycerol) (DPPG), 1,2-dipalmitoyl-sn-glycero-3-[phospho-rac-(3-lysyl(1-glycerol))] (lysyl-DPPG), 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DOPG), and 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(3-lysyl(1-glycerol))] (lysyl-DOPG), as well as natural *ω*-phosphatidylycerol (PG), both from *Escherichia coli* were purchased from Avanti Polar Lipids (Alabaster, AL). Fluorescently labeled phospholipids *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (rhodamine-PE) were from Molecular Probes (Eugene, OR). Lipid stock solutions were prepared in chloroform. All lysyl-PG solutions were used immediately.

**S. aureus Strains**—Peptides were diluted from stock solutions at the desired concentration in assay buffer (20 mM HEPES, pH 7.0, with or without an additional 150 mM NaCl), and 180 µl of these solutions were filled into the wells of the first row of a microtiter plate. For a 2-fold serial dilution, 90 µl of each peptide solution were transferred to the well in the next row, which was filled with 90 µl of buffer before. Subsequently, a suspension of exponential phase bacteria in LB was added (10 µl, containing 10⁶ CFU) to the peptide solution (90 µl). The constantly shaken plates were incubated in a wet chamber overnight at 37 °C. Bacterial growth was monitored by measuring the optical density at 620 nm in a microtiter plate reader (Rainbow, Tecan, Crailsheim, Germany). The minimal inhibitory concentration (MIC) was defined as the lowest peptide concentration at which no bacterial growth was measurable.
Portions of each well (10 μl) were diluted with buffer and plated out in duplicates on LB-agar plates. After incubation overnight at 37 °C, bacterial colonies were counted. The minimal bacterial concentration was defined as the lowest peptide concentration at which no colony growth was observed.

**Electron Microscopy**—Transmission electron microscopy was performed as described previously in detail (26). Bacteria (5 × 10⁶ cfu/ml) were incubated with indicated concentrations of peptides in buffer (20 mM HEPES, 150 mM NaCl, pH 7.0) supplemented with 10% LB medium for 30 min at 37 °C. Images shown are representative for the respective sample.

**Preparation of Liposomes for FTIR and FRET Measurements**—Lipids dissolved in chloroform were dried under a gentle stream of nitrogen. The resulting lipid film was dispersed in buffer (see under “FRET and FTIR Spectroscopy”), sonicated for 30 min, and subjected to 3–4 temperature cycles from 4 to 60 °C with an incubation period of 30 min at each step. Lipid dispersions were stored at 4 °C overnight before use.

**FRET Spectroscopy**—Intercalation of peptides into liposome membranes was determined in 20 mM HEPES, 150 mM NaCl, pH 7.4, at 37 °C by FRET spectroscopy applied as a probe dilution assay (37). Peptides were added to liposomes, which were labeled with 1% of the donor NBD-PE and 1% of the acceptor rhodamine-PE. NBD excitation wavelength was 470 nm. Intercalation was monitored as the increase of the ratio of the donor fluorescence intensity I_Donor at 531 nm to that of the acceptor intensity I_Acceptor at 593 nm in dependence on time. This ratio depends on the Förster efficiency; therefore, a rising value means that the mean distance separation of donor and acceptor dyes is rising.

**FTIR Spectroscopy**—The infrared spectroscopic measurements were performed on an IFS-55 spectrometer (Bruker). Pure lipids dispersed in 20 mM HEPES buffer, pH 7.0, as well as lipids mixed with peptides solubilized in the same buffer in a molar ratio of 1:0.3, were placed in a CaF₂ cuvette with a Teflon spacer. Consecutive heating and cooling scans were performed automatically between 10 and 70 °C with a heating rate of 0.6 °C/min. Every 3 °C, 50 interferograms were accumulated, apodized, Fourier transformed, and converted to absorbance spectra. The peak position of the asymmetric methylene stretching vibration was plotted versus temperature. Phase transition temperatures were derived by determination of the maximum (minimum) of the first derivative of the heating (cooling) scans.

**Preparation of Planar Bilayers and Electrical Measurements**—Planar bilayers were prepared according to the Montal-Mueller technique (38) as described earlier (39). Briefly, symmetric and asymmetric bilayers were formed by opposing two lipid monolayers, prepared in separated compartments on aqueous sub-phases from chloroformic solutions of the lipids, at a small aperture (~150 μm) in a thin Teflon septum (thickness 25 μm). The inner leaflet of the cytoplasmic membrane of S. aureus was reconstituted by a phospholipid mixture consisting of an equimolar ratio of PE and PG. For electrical measurements, planar membranes were voltage-clamped via a pair of Ag/AgCl electrodes (type IVM E-255, Science Products, Hofheim, Germany) connected to the headstage of an L/M-PCA patch clamp amplifier (List-Medical, Darmstadt, Germany). In all experiments, the compartment to which peptide was added is indicated first, and the compartment opposite to the addition was grounded. In comparison with the natural system, a positive clamp voltage represents a membrane with negative potential on the inside. All measurements were performed at a temperature of 37 °C in 5 mM HEPES, 100 mM KCl, 5 mM MgCl₂, pH 7.0. Two electrical parameters were determined: (i) peptide-induced changes of the membrane capacitance and (ii) current through peptide-induced lesions. Assuming a plate capacitor model, the membrane capacitance yields information about the area, thickness, and composition of the bilayer. Using small positive and negative jumps of the clamp voltage, the maximum voltage required to determine membrane capacitance is <1 mV. Therefore, peptide membrane interaction can be investigated without the influence of a significant external voltage. This method allows the determination of changes in membrane capacitance with a precision of <1 picofarad. The diameter (d, nm) of pores or lesions induced by the AMPs was calculated under the assumption of a membrane of 6-nm thickness and a cylindrical pore structure using the following formula: d = 2√(L²/πσ), with L = measured conductivity and σ = buffer conductivity.

**RESULTS**

**Interaction of Peptides with Intact Bacteria**

**Antibacterial Activity**—We determined the capability of synthetic cationic peptides (Table 1) to inhibit the growth (MIC) and to kill various strains of S. aureus (minimal bactericidal concentration). Strains with a functional mprF gene, i.e. wild type SA113 and a clinical isolate, were considerably less sensitive to the action of the peptides. Deletion of the mprF gene (ΔmprF) rendered SA113 into a highly susceptible strain. A strain with a rescued synthase activity but still lacking the translocase domain (ΔmprFpRBsyn) was undistinguishable from strain ΔmprF, providing strong evidence that exposure of lysyl-PG to the environment is essential for conferring resistance to antimicrobial peptides. The activity of melittin was only marginally affected by the lysine modification of PG. Among the other peptides tested, arenincin was the most effective. Moreover, the activity of arenincin-1 was most strongly modulated by the expression of lysyl-PG. NK-2 was of moderate activity against strains with a functional mprF gene. NK11 was almost completely inactive. To elucidate the structure/function relationship of peptide NK-2 for an anti-S. aureus activity, we tested derivatives with the sole cysteine residue substituted by serine (C7S/NK27) or alanine (C7A) residues as well as three peptides with deletions of four adjacent residues close to the N terminus (NK23a), in the center of the molecule (NK23b), and close to the C terminus of NK-2 (NK23c). Substitution of the cysteine residue improved the activity in particular against the clinical isolate. Deletion of the central residues considerably reduced the antibacterial activity, whereas deletion of C-terminal residues led to an enhancement of activity.

**Ultrastructure of Bacteria**—The direct influence of peptides on the structural integrity of S. aureus (clinical isolate) was visualized by transmission electron microscopy (Fig. 1). More-
over, aliquots of peptide-treated bacteria were plated out on LB-agar plates, and colonies grown overnight were counted. Upon a 30-min incubation of *S. aureus* with peptides at the indicated concentrations, 80–100% of bacteria were killed, with the exception of NK11, where all bacteria survived. Peptide concentrations were chosen based on their MIC data (Table 1) to obtain a comparable killing rate. It is apparent that all peptides (except NK11, Fig. 1D) elicited morphological changes of *S. aureus*; however, the characteristics of structural changes were unique for each family of peptides. NK-2- and C7S-treated bacteria exhibited higher electron density in the cytoplasm than control bacteria with areas of very low electron density attached to the membrane, suggesting peptide-induced release of cytoplasmic material (Fig. 1, B and C). Bacteria incubated with melittin showed a heterogeneous electron density in the cytoplasm, and also, extracellular material emerging from the cell membrane. *S. aureus* incubated with arenicin peptides had a frayed surface (Fig. 1, F and H), an effect that was not

**TABLE 1**
Sequence and antibacterial activity of synthetic peptides used in this study

Peptide C7S was formerly referred to as NK27 (26). Antibacterial activity is expressed as MIC (minimal inhibitory concentration) and in MBC (minimal bactericidal concentration, shown in parentheses) in μg/ml. The NaCl concentration in the assay buffer is indicated. Ar-1, arenicin-1; -OH and -NH₂ indicate a free (carboxylated) and an amidated C terminus, respectively. Amino acid residues that are modified with respect to the lead peptide are underlined. *, >99% killing; #, data were taken from Ref. 28.

**S. aureus strains**

| Peptide | Sequence | SA113 wt 0 mM | 150 μM | ΔmprF 0 mM | 150 μM | ΔmprFpRBsyn 0 mM | 150 μM | clin. isolate 0 mM | 150 μM |
|---------|----------|-------------|--------|-----------|--------|------------------|--------|-------------------|--------|
| NK-2    | KILRGVCCKIMRTFLRISRKDLIGK-NH₂ | 32 (256) | >256 (>256) | 8 (16) | 8 (16) | 8 (16)* | 8 (16)* | 64 (128) | 64 (128) |
| C7A     | KILRGVCCKIMRTFLRISRKDLIGK-NH₂ | 16 (32) | >256 (>256) | 8 (16) | 8 (16) | 8 (16) | 8 (16) | 16 (32) | 16 (64) |
| C7S/NK27| KILRGVCCKIMRTFLRISRKDLIGK-NH₂ | 32 (128) | >256 (>256) | 4 (16) | 4 (16) | 4 (8) | 4 (16) | 64 (128) | 64 (128) |
| NK23a   | KI_SKIMRTFLRISRKDLIGK-NH₂ | 64 (128) | >256 (>256) | 4 (16) | 4 (8) | 4 (8) | 4 (8)* | 32 (128) | 32 (64) |
| NK23b   | KILRGVCCKIM RTFLRISRKDLIGK-NH₂ | 256 (256) | >256 (>256) | 8 (32)* | 32 (64) | 8 (16)* | 32 (64)* | 64 (256)* | 128 (256)* |
| NK23c   | KILRGVCCKIMRTFLRRILTGK-NH₂ | 32 (128) | >256 (>256) | 4 (8)* | 4 (32) | 4 (8) | 4 (8)* | 16 (64) | 16 (64) |
| NK11    | KI_SK R ILTGK-NH₂ | >256 (>256) | >256 (>256) | 256 (256) | >256 (>256) | 256 (256) | >256 (>256) | >256 (>256) | >256 (>256) |
| Melittin | GIGAVKLVTTLGLPALSQSRKRRQQ-NH₂ | 4 (16) | 16 (64) | 4 (8) | 4 (16) | 4 (8) | 4 (8) | 4 (8)* | 8 (16) |
| Ar-1    | RWCVYAYVRBGVLVRYRCW-OH | 16 (64) | 128 (256) | 0.5 (2) | 1 (4) | 0.5 (2) | 2 (8)* | 32 (64)* | 16 (32)* |
| C/S-Ar-1| RWVYAYVRBGVLVRYRCW-OH | 64 (256) | >256 (>256) | 8 (16) | 8 (32) | 16 (32)* | 128 (256)* | 32 (64)* | 32 (128)* |
| R/K-Ar-1| RWVYAYVRBGVLVRYRCW-OH | 32 (256)* | >256 (>256) | 0.5 (4) | 1 (8) | 0.5 (2) | 4 (8)* | 64 (128)* | 32 (128)* |

**FIGURE 1.** Transmission electron microscopy images of peptide-treated *S. aureus* (clinical isolate). A, no peptide. B, NK-2 (20 μM). C, C7S (20 μM). D, NK11 (200 μM). E, melittin (12 μM). F, Ar-1 (20 μM). G, C/S-Ar-1 (20 μM). H, R/K-Ar-1 (20 μM). Each bar represents 0.5 μm.
observed for the linear derivative C/S-Ar-1 lacking the disulfide bond (Fig. 1G). It appeared just as if these peptides dissolve membrane components from the bacteria or promote the release of intracellular material.

**Interaction of Peptides with Artificial Membranes Composed of PG and Lysyl-PG**

NK-2, melittin, Ar-1, and the most interesting derivatives were selected for an in-depth biophysical investigation with artificial membrane systems. The lipid compositions of these model systems mirrored the cytoplasmic membrane lipid composition of the various *S. aureus* strains. The membrane mimetic of wild type SA113 strain was composed of a mixture of negatively charged PG and positively charged lysyl-PG. The artificial membrane mimicking the mutant strain lacking the *mprF* gene, and thus lacking lysyl-PG in the membrane, was composed of PG alone. For the formation of planar lipid bilayers, it was necessary to dilute PG and lysyl-PG into 50% of zwitterionic PE. The fatty acid compositions of our reconstituted membranes were dictated by the respective model systems.

**Membrane Intercalation of Peptides Monitored by FRET Spectroscopy**—FRET spectroscopy served as a sensitive tool to detect membrane intercalation of antimicrobial peptides. For this, peptides were added to liposomes consisting of DOPG alone (Fig. 2, A and C) and of an equimolar mixture of DOPG and lysyl-DOPG (Fig. 2, B and D). All lipid vesicles were doped with donor and acceptor dyes, peptides were added, and the emission intensities of both dyes were monitored over time. An increase of the fluorescence intensity of the donor (I$_{\text{Donor}}$) and a simultaneous decrease of the fluorescence intensity of the acceptor dye (I$_{\text{Acceptor}}$), i.e. a reduced FRET efficacy, indicated an increase in the overall mean distance between the labeled phospholipids and corresponded to an insertion of peptides into the lipid bilayer. For clarity, the I$_{\text{Donor}}$/I$_{\text{Acceptor}}$ ratio is shown.

Upon the addition of peptides to liposomes, we observed an immediate increase of the I$_{\text{Donor}}$/I$_{\text{Acceptor}}$ ratio, demonstrating a rapid interaction kinetic. Intercalation of NK-2 was pronounced into pure DOPG bilayers (Fig. 2A) and slightly impaired into lysyl-DOPG-containing bilayers (Fig. 2B). Moreover, the mixed lipid system was destabilized by the peptide, indicated by a slowly decreasing I$_{\text{Donor}}$/I$_{\text{Acceptor}}$ ratio for 0.2 and 0.4 μM NK-2. It is likely that NK-2 initially bound to negatively charged PG, formed peptide-enriched domains, and thus excluded the dyes from these domains. NK11 interaction was significant with DOPG vesicles but negligible with liposomes containing lysyl-DOPG. A direct peptide-fluorophore interaction can be ruled out for NK-2 and peptides derived thereof by the lack of any detectable interaction with labeled phosphatidylcholine vesicles (26). Contradictory to the susceptibility tests (Table 1), the intercalation of melittin and Ar-1 was enhanced into liposome membranes containing lysyl-DOPG. Derivatives of NK-2 and Ar-1, i.e. C7S and C/S-Ar-1, showed an almost identical behavior as the parent peptides (data not shown).

**Influence of Peptides on the Acyl Chain Fluidity of Phospholipids**—The frequency of the symmetric methylene (−CH$_2$−) stretching vibration is a marker for the fluidity of the acyl chains of membrane lipids and was assessed by FTIR spec-
troscopy. For this, synthetic phosphatidylglycerol (DPPG) with saturated palmitoyl chains with a defined \( L_{\beta} \) (gel) to \( L_{\alpha} \) (liquid crystalline) phase transition temperature of 40 – 41 °C (Fig. 3A) has been used. For lysyl-DPPG, the phase transition temperature was broadened and shifted to 48 – 49 °C. A similar behavior was observed for the equimolar mixture of DPPG and lysyl-DPPG with the phase transition temperature at 46 – 47 °C (Fig. 3A). However, a pronounced hysteresis for lysyl-DPPG-containing lipid preparations has been observed, exhibiting a phase transition that was 4 – 5 °C lower for the cooling scan than for the heating scan (not shown). Peptides that exhibited activity against \( S. aureus \) destabilized the gel phase of DPPG, suggesting a disturbance of the acyl chain packing. Among them, melittin had the strongest effect. NK11, on the other hand, rigidified the gel phase and shifted the phase transition of DPPG to higher temperature, which is indicative for a superficial binding to the negatively charged lipid headgroups without penetrating the acyl chain region (Fig. 3B). Virtually no influence on the phase behavior of a DPPG:lysyl-DPPG mixture was observed for C7S, NK11, Ar-1, and C/S-Ar-1. Melittin had qualitatively the same effect as on the DPPG membrane; however, it was less pronounced. Peptide NK-2 behaved oppositely on the mixed membrane by showing a stabilizing effect on the gel phase and a fluidizing effect on the liquid crystalline phase (Fig. 3C).

**Interaction of Peptides with Planar Lipid Bilayers** — The planar lipid bilayer membrane model enables the monitoring of peptide binding and peptide-induced membrane permeabilization by the measurement of membrane capacitance and resistance, respectively. For this setup, symmetric bilayers of an equimolar mixture of PG and PE (i.e., the reconstitution of the cytoplasmic membrane of the \( \Delta nmrF \) strain), as well as asymmetric bilayers (to mimic the wild type membrane) whose cis monolayer consisted of PG:lysyl-DOPG:PE (25:25:50, by mol) and whose trans monolayer consisted of PG:PE (50:50, by mol), were used. The course of a typical experiment was as follows. (i) The membrane was formed from two lipid monolayers on bathing solutions separated by a Teflon foil with a 120 – 140-\( \mu \)m aperture. (ii) If the membrane remained stable for at least 5 min, peptide was added to the cis side of the membrane, and membrane capacitance was monitored for a couple of minutes. (iii) A defined clamp voltage was applied, and current flow through the membrane was monitored until the membrane collapsed. An applied positive voltage complies with the natural situation (negative potential at the cytoplasmic side of the membrane) because in our setup, the trans chamber was grounded.

Injection of NK-2 into the bathing solution close to PG:PE membranes induced a drop-in capacitance of 17 – 21% and the formation of heterogeneous lesions after setting a clamp voltage of 20 mV (Fig. 4A). Moreover, the number of lesions accumulated over time and caused rapid membrane collapse. Contrarily, only a slight decrease of 3 – 5% in membrane capacitance was observed after the addition of NK-2 to the cis side of an asymmetric lysyl-PG-containing membrane. Membrane lesions were usually induced at higher voltages (50 – 100 mV), and the period until peptide-induced membrane collapse was significantly delayed (Fig. 4B). The characteristics of lesions, however, were indistinguishable for both types of membranes. No lesion formation was observed upon the addition of 0.5 \( \mu \)M NK11 to the bilayers. However, a slight decrease in capacitance of PG:PE bilayer indicated membrane binding (data not shown).

Melittin induced a pronounced decrease in capacitance and rapid permeabilization of symmetric PG:PE bilayers already at a concentration of 0.1 \( \mu \)M and 20 mV clamp voltage (Fig. 4C). In bilayers comprising lysyl-PG, an initial increase in capacitance of ~5% after the addition of melittin followed by a slow decrease back to the value before peptide addition have been observed. In contrast to other peptide-lipid systems, no significant decrease below that value was observed. Proximate to the change in capacitance, a rapid destabilization of the membrane occurred. In those experiments where the membrane remained stable, heterogeneous lesions accumulated at low clamp voltages (20 mV) until the membrane collapsed (Fig. 4D).

Interaction of Ar-1 with both types of membranes induced a similar pronounced decrease of membrane capacitance (6 – 28%), indicating that membrane binding was not significantly affected by substituting 50% of anionic PG by cationic lysyl-PG. Nevertheless, the formation of conducting lesions was impaired in the presence of lysyl-PG (Fig. 4F). Symmetric PG:PE membranes collapsed briefly after the addition of 0.5 \( \mu \)M Ar-1 and a clamp voltage of 20 mV, whereas current flow through asymmetric lysyl-PG-containing bilayers was detected at 50 mV or higher clamp voltages (Fig. 4F). The type of the formed lesions in these experiments was heterogeneous without defined conductivity levels. However, distinct conductivity levels, indicating defined pore structures, occurred in PG:lysyl-DOPG:PE bilayers.
PG:PE membranes if the bilayer formation was done after the addition of the peptide Ar-1 to the bathing solution. The observed conductivity levels could be assigned to a basic event \((n/H_{11005})\) with a conductivity of 0.73 nanosiemens, corresponding to a pore diameter of \(0.6 \text{ nm}\) and \(2^n\) of the first open state \((n = 0, 1, 2, 3, \ldots)\) (Fig. 5). No defined pore structures were observed for any of the other peptides under the same conditions (data not shown).

**DISCUSSION**

Deletion of the \textit{mprF} gene in \textit{S. aureus} strain SA113 transforms a rather refractory bacterial strain to be extremely vulnerable to peptides NK-2 and Ar-1 and to derivatives thereof. In contrast, the activity of bee venom melittin is only marginally affected. Concurrent findings with other antimicrobial peptides have been published earlier (14, 15). Beyond that, our data stress the involvement of the outer membrane leaflet exposing both cationic lysyl-PG and anionic PG to the bacterial surroundings because the origin of \textit{mprF}-based resistance to the tested peptides was accomplished by both enzymatic activities of MprF. Rescue of the synthase activity of MprF, leading to strain \textit{ΔmprFpRBsyn}, was insufficient to re-establish resistance. In this strain, lysyl-PG is generated but not efficiently translo-
cated to the outer membrane leaflet (19). Hence, ΔmprFpRBsyn
exposes a predominantly negatively charged surface. Based on
these findings, it appeared important to investigate the con-tribu-
tion of a lysyl-PG mixture in lipid bilayers for peptide-mem-
brane interactions. Apart from whole bacteria, reconstituted
membranes constitute well defined systems. They permit the
application of biophysical techniques to explore the underlying
molecular principles of the interplay between the (reconsti-
tuted) bacterial surface and the applied antibacterial com-
 pound, i.e. the peptides.

Here we successfully applied model membranes composed
of distinct mixtures of PG and lysyl-PG to mimic the cytoplas-
mic membranes of S. aureus strains differing in the expression
of functional MprF protein and, for the first time, analyzed the
modulation of their physico-chemical properties by several cat-
ionic peptides. Complete omission of lysyl-PG from the recon-
stituted membranes, which would correspond to the deletion of
the mprF gene in bacteria, dramatically influenced peptide-
membrane intercalation, peptide-mediated changes of the acyl
chain order (phase transition and fluidity), as well as peptide-
induced membrane permeabilization by the formation of
(mostly) heterogeneous lesions and changes in membrane
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S. aureus Resistance to Antimicrobial Peptides

activities of various antimicrobial peptides on the bacteria, thus
emphasizing the power of reconstituted model membranes
to decipher the mode of action of membrane-interacting
compounds.

Moreover, the pharmaceutical potential of our peptides is
highlighted by the successful design of a shortened NK-2 vari-
ant (NK23c) with an improved activity against SA113 strains
and a clinically isolated S. aureus strain. This promising result
courages the development of short peptides for a therapeutic
application.

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