Interaction of Cationic Antimicrobial Peptides with Model Membranes*

Received for publication, May 30, 2001, and in revised form, July 10, 2001
Published, JBC Papers in Press, July 25, 2001, DOI 10.1074/jbc.M104925200

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A series of natural and synthetic cationic antimicrobial peptides from various structural classes, including α-helical, β-sheet, extended, and cyclic, were examined for their ability to interact with model membranes, assessing penetration of phospholipid monolayers and induction of lipid flip-flop, membrane leakage, and peptide translocation across the bilayer of large unilamellar liposomes, at a range of peptide/lipid ratios. All peptides were able to penetrate into monolayers made with negatively charged phospholipids, but only two interacted weakly with neutral lipids. Peptide-mediated lipid flip-flop generally occurred at peptide concentrations that were 3- to 5-fold lower than those causing leakage of calcine across the membrane, regardless of peptide structure. With the exception of two α-helical peptides V681n and V25p, the extent of peptide-induced calcine release from large unilamellar liposomes was generally low at peptide/lipid molar ratios below 1:50. Peptide translocation across bilayers was found to be higher for the β-sheet peptide polyphemusin, intermediate for α-helical peptides, and low for extended peptides. Overall, whereas all studied cationic antimicrobial peptides interacted with membranes, they were quite heterogeneous in their impact on these membranes.

Cationic antimicrobial peptides are important components of innate immunity, and their distribution throughout the animal kingdom is widespread, including bacteria, fungi, plants, insects, birds, crustaceans, amphibians, and mammals (1, 2). The naturally occurring peptides are generally 12- to 50-amino acids-long and folded into a variety of different structures, including α-helices, β-sheets, extended helices, and loops (3). Despite this structural variation and extensive sequence variability, most antimicrobial cationic peptides share two unique features, in that they are polycationic, with a net positive charge of more than +2, and fold into amphipathic structures, with both a hydrophobic and a hydrophilic domain (3). These characteristics allow them to interact with the negatively charged surface molecule lipopolysaccharide of Gram-negative bacteria and to interact with and insert into the negatively charged cytoplasmic membranes of most bacteria. Antimicrobial peptides exhibit rapid killing, often within minutes in vitro, and a broad spectrum of killing activity against various targets, including Gram-negative and Gram-positive bacteria, fungi, enveloped viruses, parasites, and even tumor cells (4, 5). Therefore, improved variants of naturally occurring antimicrobial peptides may provide a feasible alternative to conventional antibiotics, especially because of the emergence of resistant bacterial strains worldwide (6, 7).

Although there are many studies addressing the issue, the molecular mechanism underlying antimicrobial peptide-mediated cell death is still a matter of debate. A considerable body of data indicates that most antimicrobial peptides interact with the cytoplasmic membrane rather than by interacting with a specific protein receptor (8). For example, certain membrane-active peptides, such as cecropins, magainins, and melittins, etc., have been demonstrated to permeabilize model membrane systems, cause leakage of fluorescent dyes from unilamellar liposomes, or induce ion transport across lipid bilayers (9, 10). This has led to the general conclusion that lysis or leakage of essential molecules due to formation of channels in the cytoplasmic membrane is the mechanism of killing. However, such model membrane experiments can sometimes be criticized, because they use very high peptide/lipid ratios. Consistent with this, intact cell experiments are generally inconsistent with the concept of peptide lysis of bacteria (11), and even the breakdown of the cytoplasmic membrane permeability barrier at the minimal inhibitory concentration (MIC) has been questioned (11, 12). It is noteworthy that if a high enough concentration of virtually any cationic peptide is used, the cytoplasmic membrane does become leaky, but no formal relationship between killing and permeabilization has been established for many peptides. Although some reports have shown that peptides can become oriented perpendicular to the bilayer at very high peptide/lipid ratios, most studies from NMR, Raman, and fluorescence measurements indicated that cationic peptides initially bind parallel to the lipid bilayer, probably around the interface of head groups and fatty acyl chains (13–16). Thus it has been proposed that such interfacial peptides might enhance membrane permeabilization by disrupting lipid organization and packing instead of forming a formal pore (17). Conversely it has been suggested that peptides form informal “aggregate channels” containing lipid and peptide and that using this as an intermediate, some peptides can translocate across the cytoplasmic membrane (16, 18). Therefore, inhibition of DNA,

* This work was supported in part by funding from the Canadian Bacterial Disease Network and by the SPArx program of the Canadian Cystic Fibrosis Foundation (to R. H., who is also the recipient of a Canada Research Chair). A. R. is the recipient of a Natural Sciences and Engineering Research Council of Canada postdoctoral award. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: MIC, minimal inhibitory concentration; PC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; PG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol; PE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; ePG, phosphatidylglycerol from egg yolk; ePC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine from egg yolk; CL, cardiolipin; C2-NBD-PC, 1-palmitoyl-2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-caproyl]-l-α-phosphatidylcholine; DNS-PE, dansyl phosphatidylethanolamine.

35714 This paper is available on line at http://www.jbc.org
RNA, and/or protein biosynthesis has also been proposed as an alternative mechanism resulting in cell death (11, 19, 20). Regardless of this debate, it is agreed by all researchers that the interaction of most peptides with the membrane, involving charge/charge and hydrophobic interactions, is a necessary precursor to cell death (21).

The interactions of antimicrobial cationic peptides with model phospholipid membranes have been extensively studied for more than two decades. However, the molecular mechanism of peptide-membrane interaction is still controversial. Studies on the frog skin peptide magainin 2 indicate that this peptide causes lipid flip-flop, coupled with pore formation and peptide translocation across the bilayer (22). This finding is interesting and has shed some light on the molecular mechanism of peptide-membrane interaction. Unfortunately, such studies have been largely limited to magainin and structurally related peptides, and most experiments were carried out using high peptide/lipids ratios of 1:10 to 1:50 (22, 23).

In the present study we have tried to work at lower peptide/lipid ratios and asked whether peptides with different structures and activities have the same mechanism of action. To provide structural diversity we have selected peptides from two previous structure-activity relationship studies. One group included α-helical peptides derived from a cecropin-melittin hybrid without proline or with one or two prolines, represented here by V881α, V25α and V8α, respectively (24). The other group included the extended, boat-shaped, bovine neutrophil L-form). Lines indicate disulphide bonds between cysteine residues.

### EXPERIMENTAL PROCEDURES

**Peptides and Reagents—**All peptides, except for gramicidin S and polymyxin B, were synthesized by Fmoc (7-9-fluorenylmethoxycarbonyl) solid-phase peptide synthesis using a model 432A peptide synthesizer (Applied Biosystems, Inc., Foster City, CA) at the University of British Columbia Nucleic Acid/Protein Service. The amino acid sequence and properties of cationic antimicrobial peptides included in this study

| Peptide          | Amino acid sequence | Length | Net charge | % Hydrophobic amino acids | Molecular weight |
|------------------|---------------------|--------|------------|--------------------------|-----------------|
| Gramicidin S     | Cyclic (LOVFP*LOVFP) | 10     | +2         | 80                       | 1214            |
| Polymyxin B      | Cycled isoctanoyl BTBB(BF*LBBT) | 10 | +6         | 40                        | 963             |
| Indolicidin      | ILPWKKWPPWPRNHRH₂   | 13     | +4         | 77                        | 1906            |
| CP11CN           | ILKKWIPWFWPRNHRH₂   | 12     | +6         | 77                        | 1916            |
| CP10α            | ILAWKWAWWWRNHRH₂   | 13     | +4         | 77                        | 1913            |
| V881α            | KWKSFLLKTFSAVKTQTVHIAISS | 26 | +6         | 58                        | 2920            |
| V25α             | KWKSFLLRTLSPKTVTQTVHIAISS | 26 | +6         | 58                        | 2928            |
| V8α              | KWKSFLLRTKSPVTQTVFQTTPISS | 26 | +6         | 58                        | 3062            |
| Polymyxin I      | RRWCWRFCVRGYCGRCYRCR-NH₂ | 18 | +8         | 61                        | 2458            |

*a One-letter amino acid code with the following additions: bold face indicates residues that are positively charged at neutral pH values; parentheses represent amino acids that are cyclic; O, ornithine; B, diminobutyrate. *b Represents the D-enantiomers (all other amino acids are of the L-form). Lines indicate disulphide bonds between cysteine residues.

*b Charge includes the amino terminal amino group and the carboxyl terminal carboxyl group (except when amidated).

### RESULTS

**Langumir Monolayers—**Lipid monolayers at an air/water interface provide a simple, sensitive model for mimicking biological membranes, and many studies have shown that the monolayer technique is a powerful tool to assess membrane insertion of proteins and peptides. The primary phospholipids of Escherichia coli cells comprise a mixture of the neutral lipid phosphatidylethanolamine and the anionic lipids phosphatidylglycerol and cardiolipin, at the ratio of 78:4:7:14:4, in addition to various minor lipid species (30). We prepared monolayers from PE/EG/CL (78:4:14:4) to mimic the E. coli cytoplasmic membrane and tested the ability of the peptides to interact with such monolayers. Molecules that interact only
with the head groups of monolayer lipids typically induce minimal changes in surface pressure. In contrast, insertion into the hydrophobic region of the lipid monolayer can cause a significant increase in monolayer surface pressure. Thus when a protein or peptide is injected into the aqueous subphase bathing a monolayer, the degree of surface pressure change can be used to resolve whether peptide-membrane interactions include insertion and disturbance of the fatty acyl core of the membrane. Fig. 1A shows the variation in surface pressure as a function of peptide concentration. A significant penetration of peptides into the hydrophobic portion of the monolayer was indicated by values of 2 mN/m (31). As observed previously for polyphemusin I (27), all peptides induced increases in surface pressure that were a sigmoidal function of peptide concentration, a result consistent with a cooperative interaction of the peptide molecules with the monolayer. CP10A appeared to be a very effective peptide at modulating the surface pressure increase as indicated by a plateau value of ~13 mN/m (Fig. 1A), only surpassed by gramicidin S, which gave a maximal Δπ of 16 mN/m (28). V681n, V25p, and indolicidin appeared to be slightly less effective at modulating surface pressure increase resulting in maximal Δπ values between 10 and 12 mN/m, whereas V8pp showed modest activity with a maximal Δπ of 9 mN/m (Fig. 1A). CP11CN showed a plateau Δπ around 7.5 mN/m (Fig. 1A), which is nearly identical to the surface pressure increase induced by polyphemusin I (27). These experiments were repeated at least three times and were very reproducible with differences that did not exceed 1 mN/m from experiment to experiment.

Lipid Avidity Assessment—The lipid avidity of each peptide was monitored by the extent of the surface pressure change upon addition of 1 µg/ml of peptide to the subphase bathing monolayers of pure PC, PE, ePG, or CL. As shown in Fig. 2B, all peptides selectively interacted with negatively charged phos-
pholipids ePG and CL and generally had a greater effect on PG than CL monolayers. Most peptides (including polymyxin B; see Ref. 28) were not able to penetrate monolayers composed entirely of neutral lipids such as PE or PC, except indolicidin (CP10CN) and its helical variant (CP10A), which, like gramicidin S (28), displayed modest surface pressure increases of nearly 4 mN/m upon binding to PE and PC monolayers (Fig. 1B).

**Lipid Flip-Flop**—The spontaneous flip-flop rate of the fluorescent zwitterionic lipid probe, C_{18}-NBD-PC, has been shown to be extremely small, and no measurable transfer from one monolayer to the other occurred even after 48 h of incubation in asymmetrically labeled liposomes (22). Peptide-induced lipid flip-flop was measured here using unilamellar PG/PC liposomes that were asymmetrically labeled with 0.5 mol % C_{6}-NBD-PC in the inner leaflet. Peptides were added to unilamellar liposomes, followed by the water-soluble quencher sodium dithionite (20 μl of 1 M dithionite solution in 2 ml of total volume). The extent of peptide-mediated lipid flip-flop was indicated by the percentage of the NBD groups being transposed from the inner leaflet to the outer leaflet where they would then be quenched by dithionite. The peptide-induced flip-flop of the fluorescent lipid was thus recorded as a decrease of fluorescence intensity of the NBD group within the 10-min observation time period. We assessed lipid flip-flop on the time scale of 0–10 min to avoid errors because of the possible slow permeation of the quencher (dithionite) through the membrane that might occur over longer reaction times. The percent flip-flop value was defined by the following equation: percent flip-flop = \( \frac{F_0 - F_T}{F_0} \times 100 \), where \( F_0 \), \( F_p \), and \( F_T \) represent the fluorescence intensity in asymmetrically labeled unilamellar liposomes without the peptide, with peptide, and with Triton X-100, respectively. Fig. 2 demonstrates the kinetics of increase in flip-flop for three peptides.

The two α-helical peptides, V25, and V681n, were most active at mediating lipid flip-flop. The flip-flop kinetics were similar for both peptides and depended on both concentration and time. At low peptide concentrations the flip-flop rate was slow, but as the peptide concentration increased the rate of increase in flip-flop also increased. V25 caused more than 95%
lipid flip-flop within 300 s at 3 g/ml (Fig. 2A), whereas V681n caused nearly 90% within 300 s at 4 g/ml (data not shown). Conversely, the kinetics of flip-flop caused by CP10A (Fig. 2C) and polyphemusin I (data not shown) were similar being relatively slower at any given concentration than observed for V25p and V681n. For example, CP10A resulted in only 70% flip-flop within 600 s at 5 g/ml (Fig. 2C), whereas polyphemusin I caused around 70% flip-flop at 5–5 g/ml. In contrast, indolicidin, CP11CN, gramicidin S, the double-bend α-helical peptide V8pp, and polymyxin B were totally inactive at concentrations of 1–5 μg/ml at mediating lipid flip-flop when 180 μm liposomes were used (e.g. see Fig. 2E).

The extent of flip-flop after 10 min was plotted as a function of peptide concentration for eight different peptides at a range of peptide/lipid ratios, averaged over three independent experiments (Fig. 3). Individual results were quite consistent, with standard errors of around 10%. In general the helical peptides V25p and V681n, the β-hairpin peptide, polyphemusin I, and the short helical indolicidin variant CP10A showed very good activity, at low peptide concentrations (peptide/lipid ratios of 1:100 to 1:400), in mediating lipid exchange between the two leaflets of a bilayer membrane (Fig. 3, A–D). This activity was clearly concentration-dependent in an apparent sigmoidal fashion. None of the other peptides, including the double-bend α-helical peptide V8pp, the extended structured peptides indolicidin and CP11CN, and the cyclic peptides gramicidin S and polymyxin B, induced any detectable lipid flip-flop at peptide/lipid ratios less than 1:100 (data not shown).

To see whether this represented an intrinsic difference between these groups of peptides or a weaker response for the latter group, the peptide/lipid ratio was increased above 1:50, and lipid flip-flop rates were reassessed. Gramicidin S initiated
lipid flip-flop at peptide/lipid ratios of 1:50 and reached a maximum (more than 90%) at peptide/lipid ratios above 1:15 (Fig. 3E). The double-proline peptide V8pp and indolicidin induced about 50 and 40% lipid flip-flop at peptide/lipid ratios of 1:15 and 1:25, respectively (Fig. 3F, 3H). Neither polymyxin B (data not shown) nor the indolicidin variant CP11CN (Fig. 3G) caused any detectable level of lipid flip-flop even at a peptide/lipid ratio below 1:10.

Calcein Release—To see whether lipid flip-flop was because of bilayer disruption, membrane leakage was assessed by de-quenching of calcein fluorescence (which is self-quenched at the high calcein concentrations inside liposomes) after release from unilamellar PG/PC liposomes. The maximum fluorescence intensity corresponding to 100% leakage was determined by the addition of 10% (w/v) Triton X-100. The percent leakage was calculated as follows: % leakage = 100 × (Fp - Fg)/(Fp - Fg), where Fg and Fp denote the fluorescence intensity before and after peptide addition, and Fp represents the fluorescence intensity after addition of Triton X-100.

The kinetics of calcein release is shown in Fig. 2 for three peptides using 180 μM liposomes. Both V25p (Fig. 2B) and V681n (data not shown) demonstrated similar kinetics of calcein release. At concentrations of 1–2 μg/ml, there was an immediate minor increase in peptide-mediated calcein release, but this did not increase over time. At high peptide concentrations of 4–5 μg/ml, a clearly time-dependent increase was observed (Fig. 2B) after an initial rapid release. Neither CP10A (Fig. 2D) nor polyphemusin I (data not shown) were effective at causing calcein release from unilamellar liposomes, and only at 5 μg/ml was any more than 20% calcein release observed (Fig. 2D). Indolicidin, CP11CN, V8pp, gramicidin S, and polymyxin B were unable to induce calcein release at the tested concentrations using 180 μM liposomes (e.g., see Fig. 2F).

These results were summarized for eight peptides at several peptide/lipid ratios for three independent experiments (Fig. 3). Calcein release induced by most peptides demonstrated a sigmoidal dependence on peptide concentration. There was little calcein release at low peptide concentrations, indicating that there was a threshold peptide concentration for stimulating calcein release, although the threshold concentrations differed from peptide to peptide.
among the peptides. Both V681\textsubscript{n} and V25\textsubscript{p} induced significant membrane damage at peptide/lipid ratios above 1:260 (Fig. 3, A and B). Nevertheless, for V681\textsubscript{m} and V25\textsubscript{p}, the concentration resulting in 50% calcein release was 2.5- and 5-fold higher than the concentration that caused 50% lipid flip-flop (Fig. 3, A and B). The threshold peptide concentration causing calcein release for CP10A and polyphemusin I was around 4 \(\mu\)g/ml, a concentration that lead to 50 and 70% lipid flip-flop, respectively (Fig. 3, C and D).

At higher peptide/lipid ratios, achieved at a lipid concentration of 40 \(\mu\)M, gramicidin S induced 50% calcein release at a concentration of 3.5 \(\mu\)g/ml whereas only 1.5 \(\mu\)g/ml caused more than 50% lipid flip-flop (Fig. 3E). Both V8\textsubscript{pp} (Fig. 3F) and indolicidin (Fig. 3H) showed a weak ability to induce calcein leakage that paralleled the induction of lipid flip-flop experiment. However, neither CP11CN (Fig. 3G) nor polymyxin B (data not shown) induced a detectable level of calcein release.

**Peptide Translocation**—It is still a matter of debate as to whether a peptide, after it inserts into a bilayer, remains only on the exterior surface, forms a stable transmembrane structure, or passes through and dissociates from the membrane to attack cytoplasmic targets. One reason is the paucity of reliable methods to study peptide translocation. One method utilizes lipid-linked probes that distribute asymmetrically across unilamellar liposomes bilayers and are capable of quenching the fluorescence of tryptophan through resonance energy transfer. However, this type of assay can only be performed if the peptide of interest does not cause lipid flip-flop (32). We chose to employ an enzyme digestion method that is based on measurements of the ability of peptides on the outside of unilamellar liposomes to gain access to the aqueous phase on the inner side, implying translocation across the membrane. The extent of peptide translocation across a bilayer was estimated by monitoring the uptake and enzymatic digestion of peptides in ePC/ePG (1:1) unilamellar liposomes, containing the fluorescent probe DNS-PE. The liposomes were prepared to incorporate \(\alpha\)-chymotrypsin with external enzyme being deactivated with a trypsin-chymotrypsin inhibitor added outside the liposomes. Resonance energy transfer from the tryptophan residue of the peptide to the dansyl group of DNS-PE resulted in an initial increase in fluorescence upon binding of the peptide to the membrane. If the peptide became translocated it would become digested by the \(\alpha\)-chymotrypsin encapsulated in the liposomes, leading to desorption of the peptide fragments from the bilayer. The extent of translocation, indicated by the resulting loss of energy transfer to DNS-PE upon excitation of tryptophan residues, and the consequent decrease in fluorescence intensity, was determined as follows: % translocation = \(100 \times (F_{\text{max}} - F_{\phi}) / (F_{\text{max}} - F_{\phi})\), where \(F_{\text{max}}\), \(F_{\phi}\), and \(F_{\phi}\) are the fluorescence intensities immediately after peptide addition, at any particular time, and before peptide addition, respectively. The translocation experiments were performed using peptide/lipid molar ratios ranging from 1:40 to 1:200. No dose dependence was observed within this range (data not shown). Fig. 4 demonstrates the time-dependent normalized fluorescence intensity of DNS-PE containing liposomes after addition of 2 \(\mu\)M peptide to 200 \(\mu\)M liposomes. In the time frame of the experiment (8 min) and at peptide/lipid ratios of 1:100, translocation and digestion were greatest for polyphemusin I (56–70%), followed by CP10a (20–35%), V681\textsubscript{m} (17–30%), V25\textsubscript{m} (12–25%), and V8\textsubscript{pp} (13–24%). Indolicidin and CP11CN showed very little translocation with 5 and 2% total fluorescence decrease, respectively. Unfortunately polymyxin B and gramicidin S could not be assessed in this system, because they do not have tryptophan residues and are protease-resistant.

**DISCUSSION**

Kobayashi et al. (23) recently identified two different types of activities of \(\alpha\)-helical cationic antimicrobial peptides interacting with model membranes. An analogue of magainin 2 was relatively effective at promoting flip-flop and causing leakage from membrane unilamellar liposomes but translocated poorly across lipid bilayers. In contrast, buforin 2 demonstrated a weak ability to promote leakage and flip-flop but translocated relatively well across bilayers. In our work we have applied these and other assays, using a broad range of cationic antimicrobial peptides, and demonstrate a far more complicated pattern of membrane interaction. The results for these assays and others published previously by us for these peptides are summarized in Table II and expressed in terms of efficiency such that higher numbers indicate greater efficiency (the individual columns are not, however, directly comparable, except for the lipid flip-flop and calcein release columns). These results indicate that these peptides have an overlapping set of membrane-interaction abilities but that for any given assay these are expressed in a concentration-dependent manner that varies from peptide to peptide.

One fact that seemed immediately clear was that there was no direct relationship between antibacterial activity and any particular measurement of membrane activity, even when permeabilization (depolarization) of the cytoplasmic membrane in intact bacteria was assessed. This is possibly not surprising, because the action of cationic antimicrobial peptides against bacteria involves an amalgam of factors, including uptake across the outer membrane (for Gram-negative bacteria) that involves either a stimulatory action (for those peptides with enhanced activity versus E. coli and other Gram-negatives) and/or an inhibitory action (for peptides like CP10A and gramicidin S with enhanced activity versus the Gram-positive bacteria like Staphylococcus aureus). Other possible factors would include energized efflux (33), nonspecific binding to polyanions, including capsules (3), and affinity for non-membrane targets (11, 19, 20). Overall, there is no doubt that cationic antimicrobial peptides interact with membranes, although they are by no means potent membrane-active compounds, because it required between 0.2 and 7 (or more) mol of peptide per 100 mol of lipid to observe measurable effects on lipid flip-flop and two to five times more to observe calcein release in model liposome systems. Indeed, a general criticism of our research and virtu-
aly all other papers describing the interaction of these peptides with membrane systems is the rather high levels of peptide to which membranes must be exposed to observe these effects. There is, in fact, no definitive evidence that such concentrations are present at the surface of the cytoplasmic membrane during bacterial killing. Nevertheless these results indicate that there is no simple relationship between in vivo membrane activity and bactericidal action.

In examining the various results for these peptides (Table II), there was clearly no formal relationship between structure or charge and activity. However, the peptides did fall into three broad groups with respect to membrane interaction. The first group, comprising V88p, indolicidin, CP11CN, and polymyxin B, was relatively weak in inducing all membrane activities, including monolayer insertion (except indolicidin), lipid flip-flop, in vitro membrane depolarization, calcein release, formation of channels in planar bilayers, and peptide translocation (except V88p, V88p is thus qualitatively similar to buforin; see Ref. 23). For these peptides, it is hard to ascribe an action on membranes as the primary killing mechanism. Indeed for indolicidin, a different mechanism of action on RNA synthesis has been suggested (11). With MICs ranging from 0.5 to 16 ng/ml versus E. coli and 8 to >64 ng/ml versus S. aureus, it is clear that these peptides can still be quite potent versus Gram-negative bacteria. A second group, consisting of a single peptide, gramicidin S, is relatively weak at inducing lipid flip-flop and calcein release but is one of the most potent peptides in terms of insertion into monolayers, cytoplasmic membrane depolarization, and channel formation in planar bilayers (Table II). Consistent with this, at a range of peptide/lipid ratios, published reports indicate that gramicidin S has almost no effect on the gel to liquid crystalline phase transition temperature, enthalpy, or cooperativity of PC multilamellar liposomes, and the organization of PC bilayers was not significantly perturbed by the presence of the peptide, as monitored by NMR spectroscopy (34). The most likely explanation for these results is that gramicidin S stacks in the membrane in a rather non-perturbing fashion to form small channels that permit transit of ions (12) or protons (to depolarize the cytoplasmic membrane) but not molecules as large as calcein. However, it must again be questioned as to whether this is the actual mechanism of bactericidal activity, as membrane depolarization by gramicidin S occurs at concentrations well below the effective bactericidal concentration (12, 28). Indeed uncouplers like carbonyl cyanide-m-chlorophenyl hydravzone, which depolarize bacterial cells, are bacteriostatic not bactericidal.

A third group of peptides includes three α-helical peptides (V881p, V25p, and CP10A) of 13–26 amino acids in length and the 18-amino acid β-hairpin peptide polymyxin 1. These peptides generally demonstrate superior ability to interact with membranes, although specific peptides are somewhat less potent in specific assays (e.g. CP10A is relatively less effective at inducing lipid flip-flop and calcein release, and V25p has a reduced ability to depolarize the bacterial cytoplasmic membrane). Does this, then, indicate that the primary action of these peptides is on the bacterial cytoplasmic membrane? From the data presented in Fig. 4, it is clear that these peptides all demonstrate an ability to translocate across a lipid bilayer. Indeed polymyxin 1, which has the best MICs of any of the peptides we have studied to date (see Ref. 27 and Table II), also demonstrated a superior ability to translocate across lipid bilayers (Fig. 4). Indeed the strong membrane depolarization observed within 10 min of addition of 0.2 ng/ml of polymyxin 1 to E. coli (27) was not accompanied by a substantial change in cell viability over 60 min. Conversely, V25p caused a significant but incomplete depolarization of the cytoplasmic membrane of E. coli (Table II), a result confirmed by the observation of only partial permeabilization of the cytoplasmic membrane to the β-galactosidase substrate, ortho nitrophenyl galactoside, even at 8-fold the MIC (24). Thus even with this group of peptides, we cannot definitively state that bacterial killing is because of an action on the cytoplasmic membrane.

It has been demonstrated recently by neutron diffraction that protegrin and magainin form two-dimensional monoclinic lattices (interpreted as stable pores) when added to bilayers (35). However these experiments were done at very high peptide/lipid ratios of 1:30. In contrast, we were able to observe
significant increases in lipid flip-flop at peptide/lipid ratios of 1:400 or less for certain peptides (Fig. 3, A–D), and planar bilayer experiments revealed no evidence of stable regular channels for most cationic antimicrobial peptides (12, 24). An interesting exception was provided by gramicidin S, which, consistent with the data presented here, tended to form rather consistent 375-pS channels with a 0.5–6-s lifetime.

If one assumes that peptides do not form defined channels, there are two current hypotheses as to how cationic antimicrobial peptides interact with membrane. The initial step for both is the association of the peptide with the Guoy-Chapman-Stern (electrostatic) layer of the membrane through interactions of the positively charged peptide with the negatively charged head groups of (phospho)lipids, followed by a rapid induction of folding and insertion of the peptide into the membrane interface (between the head groups and the hydrophobic core), such that peptides become oriented parallel to the plane of the membrane. In this paper the insertion step was studied by Langmuir balance monolayer assays. Thereafter the two hypotheses diverge. The carpet model (17) suggests that complete coating of the membrane by peptide leads to reorientation of peptide molecules perpendicular to the membrane and collapse of membrane integrity. We have argued previously against the hypothesis, because (a) channel-like events are clearly seen in planar bilayer experiments at concentrations of peptide where there is absolutely no evidence of membrane instability, and (b) assessment of changes in cellular membrane potential as a function of concentration of peptide indicates that there is no “threshold” concentration for collapse of membrane integrity (12). The observation here that many peptides induce lipid flip-flop at a concentration below that leading to calcein release is also consistent with this view.

A second hypothesis indicates that when peptides achieve a sufficient concentration, under the influence of a large transmembrane electrical potential gradient, the peptides reorient to form informal aggregates containing peptide and lipid and spanning the membrane (12, 16). Such aggregate channels (18) might explain the variable size and duration of membrane conductance events observed in planar bilayers. In addition, the collapse of these channels (occurring within 10 to 30 s), would permit peptide and lipid molecules to transit to the opposite monolayer as observed in lipid flip-flop and peptide translocation experiments (see Ref. 22 and Figs. 2 and 4). Killing of bacterial cells in this latter scenario might be accomplished by leakage of essential molecules through aggregate channels (although it is not known if this is a lethal event in bacteria, because electron microscopy clearly reveals substantial retention of cellular and cytoplasmic integrity). Conversely it might be because of the translocation of peptide (20) and inhibition of multiple targets (11). It is worth mentioning, however, that V88L (like buforin) translocates substantially at a peptide/lipid ratio of 1:100, at which concentration it does not cause lipid flip-flop or calcein release. Thus there may be more than one method that peptides can use to translocate across bilayers. Similarly, the studies described here were done with pure lipid membranes, whereas biological membranes are far more complex, and we do not know how this would influence activity. In conclusion, whereas all studied cationic antimicrobial peptides interact with membranes, they are quite heterogeneous in their impact on these membranes.

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