

MiniReview

Role of membranes in the activities of antimicrobial cationic peptides

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Abstract

Cationic amphiphilic peptides that are found throughout nature have very broad-spectrum activities against microbes. The initial sites of interaction are with microbial membranes. Although dogma suggests that their lethal action involves disruption of the cytoplasmic membranes, a number of cationic peptides can traverse intact membranes to interact with internal targets. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Small cationic amphiphilic peptides are being increasingly recognized as significant players in the process of innate immunity of hosts against microbes [1–3]. More than two dozen peptides can be observed in a single host [4]. They have at least two types of roles in innate immunity. First, they are known to kill microbes, an activity that is enhanced by the synergy exhibited between individual cationic peptides within a host, and by synergy between these peptides and other host factors, such as lysozyme [5]. Second, certain peptides have been shown to interact directly with host cells to stimulate the increased expression of specific host gene products, including specific chemokines, chemokine receptors, integrins, transcriptional factors etc., and thus probably have a non-antimicrobial, effector function in innate immunity against microbes [5]. Because of their ability to kill a broad spectrum of microbes, they are being developed as a novel class of antimicrobials, having progressed to phase III clinical trials as topical antibiotics [1].

For the purpose of this review, we restrict our comments to peptides of 12–40 amino acids with net positive

changes of between three and eight, due to an excess of basic residues such as lysine and arginine, and around 50% hydrophobic amino acids. Around 500 such natural peptide molecules are currently known [4], while thousands of synthetic variants have been synthesized. However, the actual number studied in depth is a small fraction of these. Structural studies have revealed a broad distribution of structures, including β -sheet structures stabilized by 2–3 disulfide bonds (sometimes containing a short α -helical segment), amphipathic α -helices, extended structures with a predominance of one or two amino acid residues (W, P, H), and cyclized peptides [1]. The α -helical and extended peptides adopt these structures only after interaction with membranes or membrane-like environments. Regardless of the secondary structure, the three-dimensional folding of the protein results in the hydrophilic/charged amino acids segregating in space from the hydrophobic residues, leading to either an amphipathic structure [6], or a structure with two charged regions spatially separated by a hydrophobic segment [7]. Such a structure is ideal for interacting with the membrane interface, the transition zone between the hydrophilic (and in the case of bacteria, net negatively charged) head groups and the hydrophobic fatty acyl chains. While this would seem to present a rather simple picture, it is not at all obvious how peptides can progress from this membrane-parallel state to a situation in which cells can be killed, and it is the intention of this review to attempt to discriminate what is known from what is speculated.

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2. Antimicrobial and other activities

Cationic amphiphilic peptides can have an astonishing range of activities, including antibiotic activity versus both Gram-positive and Gram-negative bacteria, broad-spectrum antifungal activity, anti-viral activity, an ability to kill neoplastic cells (anti-cancer activity) and a variety of non-killing activities, including chemotactic activity, ability to stimulate chemokine production, wound healing etc. [1,8]. We concentrate here on antibiotic activity.

Gram-negative bacteria differ from Gram-positive bacteria in having a smaller cell wall peptidoglycan layer, but possessing an outer membrane in addition to the common cytoplasmic membrane. Generally speaking, the possession of an outer membrane that functions as a size-selective, sieve-like permeability barrier, in conjunction with secondary protective mechanisms such as active antibiotic efflux and the periplasmic enzyme β -lactamase, makes Gram-negative bacteria, as a class, more difficult to target new antibacterial agents towards, and more intrinsically resistant to most antibiotics [8]. Conversely, antimicrobial cationic peptides, because they utilize a separate antibiotic uptake pathway across the outer membrane, termed self-promoted uptake [9], often work as well or better against Gram-negative than Gram-positive bacteria.

Another difference from conventional antibiotics is the rapid bactericidal action of cationic peptides at concentrations around the MIC. Thus at 4-fold the MIC, such peptides can kill up to 99.999% of bacteria within 5 min [1,10]. This has been suggested to reflect the physical mechanism of action of such peptides (cf. conventional antibiotics) that are generally enzyme inhibitors. However, one note of caution is provided by the observation that cationic peptides associate rapidly with cells, and although not covalently associated therewith become extremely difficult to remove. Thus killing curves may reflect the time at which sufficient peptide has associated with cells, to lead to eventual loss of colony forming ability. Our attempts to determine if this is true were inconclusive, although dilution of cells into broth containing the polyanionic inhibitor alginate did not affect subsequent loss of viability.

It is worth considering how bacterial cells can actually be killed. Progressive knockout of genes in e.g. *Pseudomonas aeruginosa* has led to the conclusion that as many as 17% of the total gene complement (>900 genes) may be required for survival under laboratory growth conditions (K. Stover, Chiron Corporation, Seattle, personal communication). However, even for these genes it requires their irreversible inactivation to kill bacteria. Certainly one can envisage that cells would be killed by known antibiotic modes of action such as blockage of cell wall peptidoglycan biosynthesis in growing cells, triggering of intrinsic autolysins and double stranded DNA breakage due to inhibition of DNA gyrase, as well as other mechanisms such as cytoplasmic membrane rupture, triggering of the *mazEF* bacterial apoptosis pathway [11], cross-linking of DNA,

blockage of cell division, prevention of DNA segregation into daughter cells, and dissociation of DNA from the membrane. It also seems possible that some antibiotic substances may have more than one inhibitory event leading to killing, as previously proposed for cationic aminoglycosides [12]. Nonetheless there are a complex array of possible modes of action that must be considered for any bactericidal antibiotic like the cationic antimicrobial peptides.

3. Concentration is important

As described in detail below, virtually all cationic amphiphilic peptides cause severe membrane perturbations if high enough concentrations are administered. However, if we are to learn the relevant mechanism of action at the minimal effective bactericidal concentration (MBC), experiments must be performed at this concentration. Fig. 1 illustrates how this may be important for a peptide which leads to complete and rapid disruption of membrane potential and all macromolecular synthesis at concentrations 10-fold higher than the MBC, but only macromolecular synthesis is inhibited at the MBC. Another consideration has to be whether the observed impairments really reflect a bactericidal event as cytoplasmic membrane permeabilization leading to loss of the proton gradient (by uncouplers such as carbonyl cyanide *m*-chlorophenyl hydrazone), and RNA (by rifampin) or protein (by tetracycline) synthesis inhibition is in fact bacteriostatic and reversible.

4. Interaction with model membranes

The most common mechanistic studies performed for

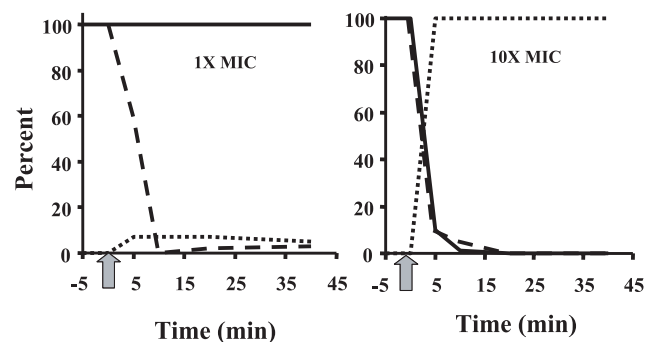


Fig. 1. Concentration-dependent influence of a pleurocidin–dermaseptin hybrid termed P-Der on *E. coli*. After addition of the peptide at time zero (denoted by the arrow), at concentrations equal to (left panel) and 10-fold (right panel) the MIC, residual cell viability (bold lines, assessed by colony counting), rate of RNA synthesis (dashed line, assessed by measuring the incorporation of radiolabelled uracil into RNA) and membrane potential (dotted lines, assessed using the membrane potential sensitive fluorophore diSC₃5) were measured. Results are taken from experiments performed by Aleks Patrzykat and Carol Friedrich and have been submitted for publication.

cationic antimicrobial peptides involve studies using isolated lipid membranes reconstructed from pure lipids or lipid mixtures (e.g. [13]). Such experiments are usually predicated on the knowledge that cationic amphiphilic peptides interact with membranes, and are often used as stand alone ‘proof’ that such cationic peptides kill cells by acting on the bacterial cytoplasmic membrane. However, this is an incorrect use of such studies, since model membranes usually fail to capture biological features such as lipid heterogeneity, the presence of membrane proteins, the relevant features (proteins, negatively charged membrane derived oligosaccharides, osmolarity, pH) in the periplasm adjacent to the membrane, the membrane potential and pH gradients across the cytoplasmic membrane, the presence of efflux pumps, some of which can accommodate cationic peptides, the binding sink provided by high concentrations of polyanionic polymers (DNA, RNA, proteins) in the cytoplasm, etc. Furthermore, it is very difficult to establish that the concentrations used in model membrane studies are biologically relevant, under circumstances where concentration is, as stated above, highly relevant. Nevertheless, such model membrane studies are useful in helping to define peptide–membrane interaction mechanisms.

4.1. Planar bilayer studies

The basic planar bilayer method involves the measurement of increases in the conductance of ions across a planar bilayer membrane when a voltage is established across the membrane and peptide is added to one side of the bilayer (Fig. 2A). With well-behaved channel forming

molecules like e.g. bacterial porins, beautiful stepwise increases in conductance signal the entrance into the bilayer of individual water-filled channels (Fig. 2B). In contrast, most antimicrobial peptides require quite high voltages to

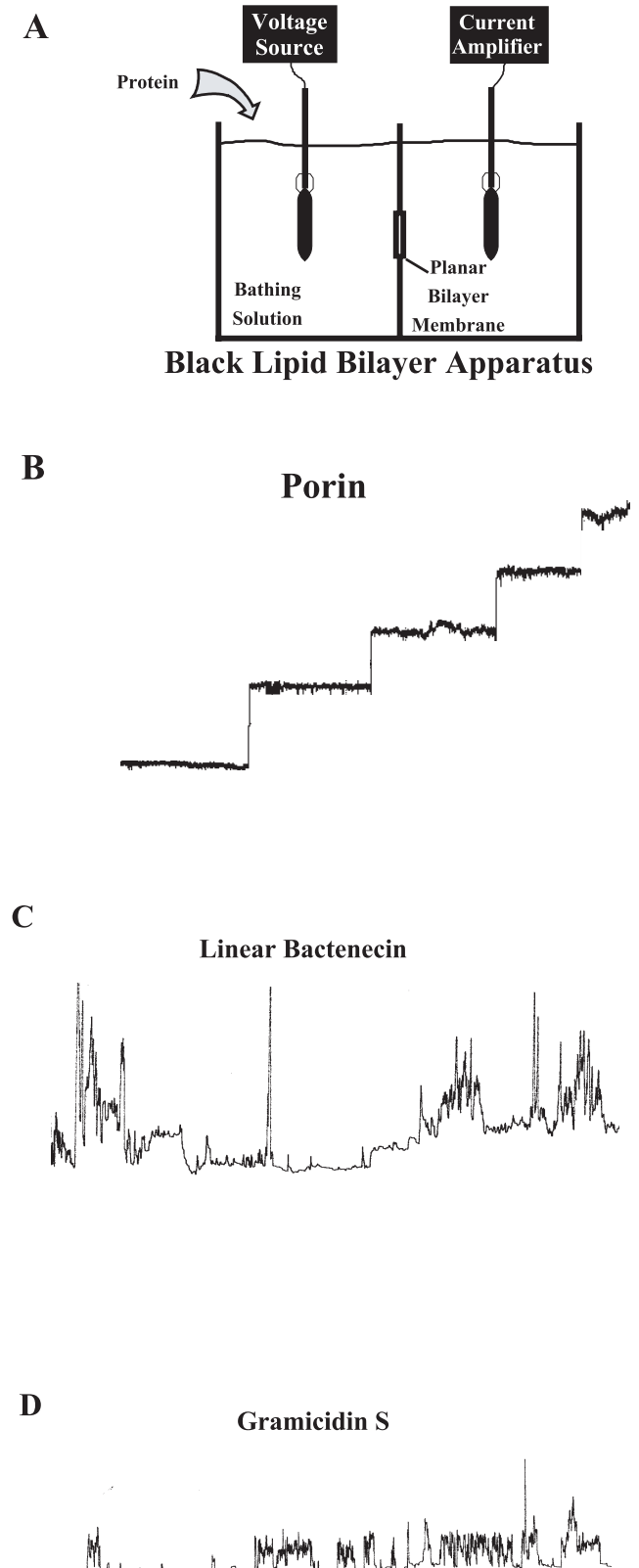


Fig. 2. Planar lipid bilayers as a tool for assessing channel forming ability by cationic antimicrobial peptides. A: Description of the apparatus. Lipid bilayer membranes are painted across a 2 mm² circular hole in a Teflon divider separating two compartments containing a bathing solution of 1 M KCl. Proteins or peptides are added to either compartment. Voltages are then applied across this membrane through Calomel electrodes connected by a salt bridge and the resultant current boosted 10⁹–10¹⁰-fold by a current amplifier, monitored on a Tektronix model 7633 oscilloscope and recorded on a Rikadenki R-01 strip chart recorder. The resultant current increases are due to incorporation of the proteins or peptides into the membranes, providing conduits for ions since naked membranes are rather impermeable to ions. B: Results observed when a bacterial outer membrane porin is added to the bathing solution (applied voltage 50 mV). Large channels of rather consistent size and substantial stability are observed. C: Results observed when a typical cationic antimicrobial peptide is added to the system (applied voltage –180 mV). Increases in current are observed that vary substantially in magnitude and duration. D: Results observed with a few cationic antimicrobial peptides, e.g. gramicidin S (applied voltage –180 mV). Channels still vary substantially in lifetime but appear to have rather consistent sizes. These may be true channels of regular composition. Since this peptide is rather small (a cyclic 10-mer), these results fit the concept of a toroidal or aggregate channel rather than a barrel-stave channel [14,19,20]. The baseline in C and D is flat since in the absence of channels the membrane lets through a small but consistent current, and since the channels observed are transient.

give rise to conductance events that are rather untidy, varying substantially in duration from microseconds to seconds and in magnitude (Fig. 2C) [14,15]. Few exceptions have been observed to this pattern [16]. For example, gramicidin S, a cyclic 10-mer, forms rather regular-sized channels (Fig. 2D), while other peptides such as bactenecin, that interact poorly with bacterial cytoplasmic membranes, demonstrate very poor ability to increase conductance even at an applied voltage of -280 mV [14].

Despite some correlations with results obtained from intact cell depolarization studies [14], there are substantial discrepancies with other types of models. For example, (a) most studies of membrane activity with liposomes do not employ a membrane potential (required to see ‘channels’) but observe membrane perturbation anyway, and (b) membrane disruption is rarely observed in planar bilayers. Nevertheless, similar high peptide concentrations are required in both systems. Possibly the discrepancies lie in the flat planar nature of the membranes and the rather restricted types of lipids that can be used in the planar bilayer system. Nevertheless, it could be argued that having a large transmembrane potential across a membrane is a significant feature in peptide activity.

4.2. Liposomal systems

Numerous papers have described the interactions of cationic antimicrobial peptides with liposomes, and this review will not be able to discuss or cite all of them. Instead we direct the reader to certain reviews [13,17–20] and pro-

vide an overview here. Studies with liposome systems have on balance suggested that the appropriate lipid compositions to use involve negatively charged lipids, such as phosphatidylglycerol, or a mixture of lipids such as phosphatidylglycerol and zwitterionic phosphatidylcholine (the latter reflecting the bacterial cytoplasmic membrane), reconstituted as large unilamellar liposomes. Under these circumstances, three types of activities have been measured (Fig. 3). Most authors incorporate an impermeable probe such as the fluorescent dye calcein into the intravesicular space of liposomes, and assess the ability of peptides to promote calcein release at varying peptide to lipid molar

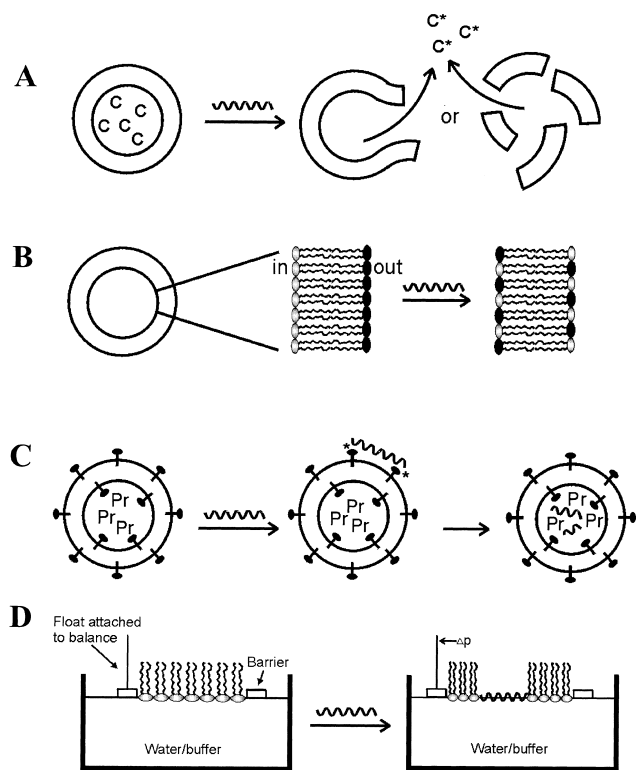


Fig. 3. Model membrane methods for assessing peptide interaction with membranes. A: Calcein release. Unilamellar liposomes are reconstituted such that they contain calcein at high enough concentrations such that calcein fluorescence is self-quenched. Membrane leakage is assessed by dequenching (increase) of calcein fluorescence after release from the unilamellar liposomes. B: Lipid flip-flop. The spontaneous flip-flop rate of the fluorescent zwitterionic lipid probe, C₆-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 labelled liposomes. Therefore peptide-induced lipid flip-flop can be measured using unilamellar liposomes that are asymmetrically labelled with 0.5 mole% C₆-NBD-PC in the inner leaflet (asymmetric labelling is accomplished by the quenching of external NBD fluorescence with the water-soluble quencher sodium dithionite). Peptides are added to unilamellar liposomes, and the extent of peptide-mediated lipid flip-flop is indicated by the percentage of the NBD groups being transposed from the inner leaflet to the outer leaflet where they are quenched by dithionite. C: Translocation. The extent of peptide translocation across a bilayer may be estimated by monitoring the uptake and enzymatic digestion of peptides in unilamellar liposomes, containing the fluorescent lipid DNS-PE. The liposomes are prepared to incorporate the protease α -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 results in an initial increase in fluorescence upon binding of the peptide to the liposomes. If the peptide becomes translocated it is digested by the α -chymotrypsin encapsulated in the liposomes, leading to desorption of the peptide fragments from the bilayer, leading to a loss of energy transfer to DNS-PE upon excitation of tryptophan residues in the peptide. The extent of translocation is thus measured by the consequent decrease in fluorescence intensity. D: Monolayer assays. Lipid monolayers are formed by applying the appropriate lipids dissolved in hexane or chloroform to water contained in a circular Teflon trough with a float on one side attached to a sensitive device for measuring pressure. Monolayers with the head groups in the water phase and the hydrophobic tails pointing towards the air are allowed to equilibrate until a stable surface pressure is obtained (<0.2 mN m⁻¹ drift in surface pressure $\Delta\pi$). A small port in the side of the trough enables injection of peptides into the aqueous subphase without disruption of the monolayer. The subphase is gently mixed with a magnetic stir bar. Molecules that interact only with the head groups of monolayer lipids typically induce minimal changes in surface pressure. In contrast, cationic amphiphilic peptides insert into the hydrophobic region of the lipid monolayer causing a significant increase in monolayer surface pressure. The degree of surface pressure change ($\Delta\pi$) can be used to assess the extent of insertion and disturbance of the fatty acyl core of the membrane.

ratios, usually ranging from 1:10 to 1:500 (Fig. 3A). In the perspective of Shai and collaborators [17], the entire surface of the liposome must become covered with a 'carpet' of peptides oriented parallel to the plane of the membrane in order for the membrane permeability barrier to be destroyed and calcein to leak out. Although this model appears reasonable for certain peptides, it does not really explain how peptides such as the indolicidin CP11-CN or polymyxin B cause no observable calcein release event at peptide to lipid ratios of 1:14, but have MICs in the same range as the α -helical peptide V25p (0.5–16 $\mu\text{g ml}^{-1}$ against most bacteria), which causes 50% calcein release at peptide to lipid ratios of 1:105 [20]. Alternative hypotheses leading to general membrane disruption are described below.

A second method, pioneered by Matsuzaki [21] for assessing cationic peptide interaction with liposomes, is measurement of lipid flip-flop, the movement of lipids from one monolayer in the bilayer to the other (Fig. 3B). Matsuzaki and colleagues demonstrated that magainin 2 efficiently promotes lipid flip-flop at concentrations similar to those causing release of markers from liposomes and they used this to propose that a peptide lipid complex was involved in forming transient channels. In contrast, buforin, a non-membrane-active peptide, caused little lipid flip-flop at concentrations at which it is translocated across the bilayer [21].

Our own observations [20] are that lipid flip-flop generally occurs at quite low peptide concentrations for very membrane-active peptides ($< 2 \mu\text{g ml}^{-1}$; a peptide to lipid ratio of around 1:250). Furthermore, for such peptides lipid flip-flop occurs at peptide concentrations that are at least 2–5-fold lower than those causing calcein release. In contrast, peptides that were weakly membrane active caused lipid flip-flop and calcein release at similar high peptide concentrations.

A third liposome methodology applied to relatively few peptides is the measurement of peptide translocation (Fig. 3C). Such assays are technically difficult in liposomal systems, but have been applied to a few peptides. There is substantial evidence that the frog peptide buforin (MIC = 4 $\mu\text{g ml}^{-1}$ vs. *Escherichia coli*) penetrates membranes without causing substantial disruption, and consistent with this it can efficiently translocate across lipid bilayers. In contrast, magainin 2 is a peptide that has relatively weak activity (MIC = 100 $\mu\text{g ml}^{-1}$ vs. *E. coli*), but is disruptive for membranes and did not translocate at the concentrations tested. We followed this up by examining translocation, at a peptide to lipid ratio of 1:100, of seven different peptides. Five of these peptides translocated with reasonable efficiency, but importantly, these included peptides with a range of efficiencies in promoting calcein release. Thus while buforin and magainin 2 represent two extremes of bilayer penetrating and non-penetrating peptides, other peptides lie in between these extremes [20,21].

4.3. Monolayer and related studies

The Langmuir method involves spreading lipids in a monolayer on an air–water interface (with the head groups facing down towards the water layer) and then assessing the ability of membrane-active substances injected into the water layer to insert into the monolayer leading to an increase in surface pressure (Fig. 3D). This method indicates that all tested peptides insert into monolayers with varying efficiency and generally with profound lipid specificity whereby anionic lipid layers made from phosphatidylglycerol or cardiolipin are much preferred over lipid layers made from the zwitterionic lipids phosphatidylcholine or phosphatidylethanolamine, generally consistent with the results of liposome specificity studies [20,22]. Titrating peptides into the water layer leads to peptide insertion into the monolayer and a progressive increase in surface pressure that is sigmoidal, indicating cooperativity. The maximum surface pressure change plateaus at different levels dependent on the particular peptide and cannot be easily correlated with other membrane interaction parameters. The most straightforward interpretation is that all peptides have the ability to insert into the outer monolayer of a lipid bilayer.

Consistent with this interpretation, fluorescence studies on tryptophan-containing cationic amphipathic peptides indicate that these tryptophan residues will insert into liposome membranes (as indicated by a blue shift in fluorescence and an enhanced quantum yield) [23]. Once in the membrane, the tryptophans become inaccessible to water-soluble fluorescence quenchers. This ability to insert into lipid bilayers can also be assessed by circular dichroism (CD) polarimetry [23]. Addition of peptides to liposomes results in the majority or all of the peptide molecules changing their CD spectra, a result that reflects a change of the peptide to a structured (or more structured) form. Oriented circular dichroism [24], modelling, and depth-dependent quenching of tryptophan fluorescence [7] are all consistent with the conclusion that these peptides, regardless of structure, adopt a position at the interface of the lipid head groups and fatty acyl chains, parallel to the surface of the membrane.

5. Interaction with intact bacteria

The ultimate method for studying how cationic amphiphilic peptides act, is to utilize intact cells. Unfortunately there are relatively few published studies. Perhaps the most open question in the field is what determines differences in activity against individual bacteria. This has not really been addressed, although electron micrographs of *Staphylococcus aureus* and *Staphylococcus epidermidis* treated with 10-fold the MIC of several peptides demonstrated substantially different appearances of the two different peptide-treated bacterial strains [25]. This could re-

flect different mechanisms of action/interaction of the same peptide with different bacteria.

5.1. Electron micrographs

Electron microscopy is an excellent method for visualizing the action of peptides against bacteria. However, to make effects more obvious, the tendency has been to utilize concentrations well above the MIC for periods of 30 min–1 h. Given the importance of concentration (Fig. 1) in interaction mechanisms, one must interpret such results with caution. Generally speaking, the published micrographs of peptide action demonstrate that most cells in a population remain intact [26,31]. For *E. coli* the most obvious alteration is usually an expansion of the outer membrane in the form of elongated blebs (finger-like extensions) [26]. Lysed (empty) cells are usually fairly rare (1–10% of total), and where visible the peptidoglycan layer and cytoplasmic membrane appear intact. In the Gram-positive bacterium *S. aureus*, the usual defects are cytoplasmic invaginations called mesosomes (the basis for these is poorly understood, although they are seen after inhibition with certain antibiotics) [25]. In *S. epidermidis* we have observed different effects with different peptides, including one or more of nuclear condensation, aberrant and false septum formation, cell wall fraying, partial cell wall lysis/thinning, and occasionally mesosomes.

5.2. Membrane permeabilization

Lehrer and colleagues were the first to demonstrate membrane permeabilization [27]. They utilized the ability of the normally impermeable substrate *o*-nitrophenyl galactoside (ONPG) to be hydrolysed by a cytoplasmic enzyme β -galactosidase as a test of increased permeability. Since this time, numerous peptides have been tested. In the studies of Lehrer and colleagues, permeabilization of *E. coli* to ONPG by a defensin mixture occurred with a similar kinetics to killing. Silvestro et al. subsequently demonstrated that the rate of permeabilization to ONPG was a function of peptide concentration over a very wide range of concentrations (i.e. 90.25–10 μ M cecropin A) [28]. A limitation of this assay is that it is currently only available for *E. coli*.

A second assay of cytoplasmic membrane permeabilization is the diSC₃₅ assay [14]. Basically this uses a lipophilic cation that fluoresces strongly in free solution, but when concentrated in the membrane, under the influence of the usual membrane potential of –140 mV maintained across the cytoplasmic membrane, it self-quenches its own fluorescence. Addition of a substance, such as a cationic antimicrobial peptide, that permeabilizes the cytoplasmic membrane results in a loss of membrane potential, that in turn causes the diSC₃₅ to leave the membrane and demonstrate enhanced fluorescence. This assay has been adapted to use in *E. coli* [14,28], *P. aeruginosa* [29],

S. aureus [25], and *Micrococcus flavus* (diSC₂₅) [30]. Using this assay, it has been demonstrated that individual peptides, even within a particular structural class, show substantial heterogeneity. Thus the α -helical peptide CP-29 permeabilizes (depolarizes) the cytoplasmic membrane of *E. coli* maximally at the MIC, while its close homologue CP-26 does not permeabilize at all at the MIC [14]. Similarly other peptides range from those that depolarize the membrane at concentrations well below the MIC [29] to those that only fully depolarize the membrane at 4–10-fold the MIC [14]. Polymyxin B, a cationic lipopeptide, represents an extreme case in that it only permeabilizes the *P. aeruginosa* cytoplasmic membrane at 50-fold the MIC [29]. Using uptake of ¹⁴C-labelled tetraphenylphosphonium, it was demonstrated in a menadione auxotroph of *S. aureus* that a high transmembrane potential was not sufficient by itself to restore susceptibility to thrombin-induced platelet microbicidal peptide [32].

6. Modes of action

There is some dispute about the role of membrane disruption/permeabilization in determining the mechanism of action of cationic amphiphilic peptides. As discussed above, some cationic peptides can clearly influence macromolecular synthesis at concentrations which do not cause a breakdown of the trans-cytoplasmic membrane potential gradient. Conversely some peptides clearly lodge within membranes and others permeabilize the cytoplasmic membrane maximally at the minimal bactericidal concentration. Clearly membrane interaction studies indicate considerable heterogeneity in the way individual peptides interact with various model membrane systems. Similarly disruption of the cytoplasmic membrane leading to depolarization is not necessarily a lethal event. Thus we favour the idea that there are multiple potential targets in bacteria (comprising especially anionic and/or hydrophobic molecules), e.g. membranes, cell division, DNA, RNA or protein synthesis, autolysin activation, etc. Individual peptides would then tend to ‘select’ one or more of these as their preferred target(s).

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