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Mechanisms of Antimicrobial Peptide Action and Resistance

MICHAEL R. YEAMAN AND NANNETTE Y. YOUNT

Division of Infectious Diseases, Harbor-University of California Los Angeles (UCLA) Medical Center; St. John's Cardiovascular Research Center, Harbor-UCLA Research and Education Institute, Torrance, California; and UCLA School of Medicine, Los Angeles, California

Address correspondence to: Dr. Michael R. Yeaman, UCLA School of Medicine, Division of Infectious Diseases, St. John's Cardiovascular Research Center, Harbor-UCLA Research and Education Institute, 1124 West Carson Street, RB-2, Torrance, CA 90502. E-mail: mryeaman@ucla.edu

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***Abstract*——Antimicrobial peptides have been isolated and characterized from tissues and organisms representing virtually every kingdom and phylum, ranging from prokaryotes to humans. Yet, recurrent structural and functional themes in mechanisms of action and resistance are observed among peptides of widely diverse source and composition. Biochemical distinctions among the peptides themselves, target versus host cells, and the microenvironments in which these counterparts convene, likely provide for varying degrees of selective toxicity among diverse antimicrobial peptide types. Moreover, many antimicrobial peptides employ sophisticated and dynamic mechanisms of action to effect rapid and potent activities consistent with their likely roles in antimicrobial host defense. In balance, successful microbial pathogens have evolved multifaceted and effective countermeasures to avoid exposure to and subvert mechanisms of antimicrobial peptides. A clearer recognition of these opposing themes will significantly advance our understanding of how antimicrobial peptides function in defense against infection. Furthermore, this understanding may provide new models and strategies for developing novel antimicrobial agents, that may also augment immunity, restore potency or amplify the mechanisms of conventional antibiotics, and minimize antimicrobial resistance mechanisms among pathogens. From these perspectives, the intention of this review is to illustrate the contemporary structural and functional themes among mechanisms of antimicrobial peptide action and resistance.**

I. Introduction

Human subtlety will never devise an invention more beautiful, more simple, or more direct than does Nature—because in her inventions, nothing is lacking—and nothing is superfluous. . .

Leonardo da Vinci

Antimicrobial peptides represent ancient host defense effector molecules present in organisms across the evolutionary spectrum. Fundamental differences exist between microbial and mammalian cells that may represent targets for antimicrobial peptides. Among these, significant distinctions include membrane composition and architecture, energetics such as transmembrane potential and polarization, and structural features including sterols, lipopolysaccharide and peptidoglycan. Disparities such as these appear to translate to varying degrees of selective toxicity among distinct antimicrobial peptides, relating to peptide and target cell properties, as well as the biological settings in which the two interact.

Although hundreds of antimicrobial peptides have now been characterized as having widely diverse sequences, these peptides have been classified into relatively few conformational paradigms. Therefore, it may be argued that a high degree of degeneracy exists within the conformation code governing structure-activity relationships among antimicrobial peptides. Many of these molecules, within and beyond conformational classes, exhibit mechanisms of action that are highly complex and non-identical. Moreover, new evidence points to targets that lie interior to the cytoplasmic membrane as being important in antimicrobial mechanisms of these peptides. Thus, the assumption that antimicrobial peptides are uniform and indiscriminant membrane detergents is obsolete. Recognition of the sophisticated and thematic structure-activity relationships underlying distinct mechanisms

of action among antimicrobial peptides will facilitate a more complete appreciation of their likely multiple roles in antimicrobial host defense.

Antimicrobial peptides have evolved as integral components of strategic and carefully regulated mechanisms of immunity to infection. However, microbial pathogens have not been passive to this evolutionary procession. Rather, prokaryotic and eukaryotic pathogens devote a considerable portion of their genomes to expressing complex and coordinately regulated countermeasures designed to subvert antimicrobial peptide targeting and mechanisms of action.

A clearer understanding of these parallel systems will advance two important, yet elusive goals. First, an awareness of the mechanisms employed by antimicrobial peptides will significantly improve our understanding of how these molecules act to defend against infection. Second, insights into these strategies will facilitate new opportunities and approaches to discover and develop pharmacologic agents that enhance or optimize immune mechanisms and suppress the ability of pathogens to subvert these mechanisms.

II. Mechanisms of Antimicrobial Peptide Target Specificity and Selective Toxicity

Polypeptides that exert antimicrobial activity have been isolated from essentially every tissue in which they have been sought. This intriguing observation has contributed to divergent interpretations regarding the potential functions of many of these peptides in antimicrobial host defense: peptides that may have little or no relevance in antimicrobial host defense can be demonstrated to inhibit or kill microorganisms in defined or austere conditions in vitro—versus—complementary peptides of varying structures, tissue sources, antimicrobial mechanisms, potencies, and/or spectra function in consort to provide optimal host defense against infection.

A pivotal consideration in this regard is the degree to which an antimicrobial peptide distinguishes between microbial and host cells in settings of potential toxicity. Evidence continues to mount in support of the concept that inherent structures or functions of microbial versus host cells contribute to selective antimicrobial discretion of some peptides. Alternatively, antimicrobial peptide access to potentially vulnerable host tissues may be limited by localization and/or or highly regulated expression. The following discussion highlights these themes as supported by recent studies.

A. Comparative Membrane Architecture and Energy

All biological membranes are in effect composed of a fluid mosaic of proteins and phospholipids. In some organisms, sterols and glycerides also contribute to the surface topology and biochemical architecture of biomembranes. Yet, fundamental differences exist between microbial and host membranes that represent potentially selective targets for antimicrobial peptides. Moreover, central to the potential pharmacologic application of antimicrobial peptides is the degree to which they differentiate, or may be engineered to differentiate, between microbial targets and normal host cells.

1. Membrane Composition, Hydrophobicity, and Charge. The elementary component of essentially all biomembranes is the phospholipid bilayer. By definition, such bilayers are amphipathic, having both hydrophobic and hydrophilic domains. However, based on composition and influenced by cell energetics, biomembranes of prokaryotic versus eukaryotic cells differ significantly. For example, phosphatidylcholine (PC[1]) and phosphatidylethanolamine (PE) normally have no net charge. Moreover, sphingomyelin (SM), a close analog of PC containing a palmitoyl residue, is also neutrally charged. In many membrane systems, the amounts of PC and SM are inversely related. Sterols such as cholesterol and ergesterol, found in eukaryotic but rarely in prokaryotic membranes, are also generally neutral. In contrast, hydroxylated phospholipids phosphatidylglycerol (PG), cardiolipin (CL; effectively a dimer of PG), and phosphatidylserine (PS), sustain a net negative charge. From these perspectives, it follows that the net charge of a biomembrane is based largely upon its phospholipid stoichiometry and architecture (Fig. 1). Cell membranes composed predominantly of PG, CL, or PS tend to be highly electronegative; such compositions are found in many bacterial pathogens. On the contrary, bilayers enriched in the zwitterionic phospholipids PE, PC, or SP—commonly found in mammalian cytoplasmic membranes—are generally neutral in net charge. These characteristic membrane charge properties may also be compounded by differences in electrochemical gradients of prokaryotic versus eukaryotic cells (see below). Sterols within membranes may further differentiate mammalian and fungal cells from prokaryotes (Tytler et al., 1995; see below) as potential targets for antimicrobial peptides. Moreover, it is intriguing to note that peptides with primarily antifungal activity, such as many of those isolated from plants, tend to be relatively rich in polar neutral amino acids, suggesting a unique structure-activity relationship (Hancock and Chapple, 1999).

Human cells such as erythrocytes have membranes that are enriched in PC, PE, and SM. By comparison, membranes from nonhuman mammalian cells often contain much less PE but relatively high SM content. In sharp contrast, bacterial cytoplasmic membranes are generally much more electronegative, with dramatically higher proportions of anionic PG and CL, which are

[1]Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; PG, phosphatidylglycerol; CL, cardiolipin; PS, phosphatidylserine; LPS, lipopolysaccharide; CD, circular dichroism; FTIR, Fourier-transform infrared; PMP, platelet microbicidal protein; tPMP, thrombin-induced PMP; SCV, small colony variant.

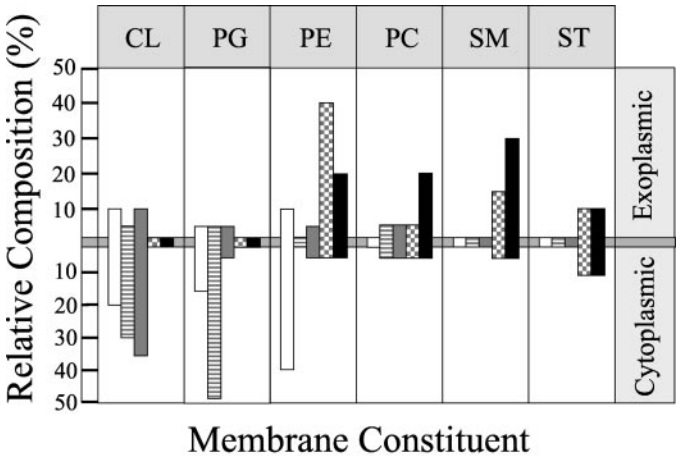

FIG 1. Comparative architecture of microbial and human cytoplasmic membranes. Cytoplasmic membranes of bacterial (*E. coli, S. aureus,* or *B. subtilis*) and fungal (*C. albicans*) pathogens are compared with that of the human erythrocyte in relative composition and distribution between inner and outer membrane leaflets. Membrane constituents ranging from anionic (left) to zwitterionic or neutral (right) are CL, PG, PE, PC, SM, and sterols (cholesterol or ergesterol, ST). Note the marked differences among microbial pathogens and human erythrocytes in phospholipid composition and asymmetry. These differences are believed to account for preferential antimicrobial peptide affinity for microbial versus host cells to the extent it exists for a given antimicrobial peptide. Key: open, *E. coli*; horizontal hatching, *S. aureus*; shaded, *B. subtilis*; checkered, *C. albicans*, solid, human erythrocyte.

typically present in extremely low levels, or altogether absent from mammalian membranes. Of note, Koppelman et al. (2001) have recently demonstrated that the cytoplasmic membrane of *Escherichia coli* is substantially more enriched in CL than previously known. Moreover, it appears that the membrane content of PG is reduced corresponding to increased CL content, preserving the highly anionic nature of this membrane. From these perspectives, composition likely provides an important determinant by which antimicrobial peptides target microbial versus host membranes.

Alternatively, microbial toxins reveal insights into selective targeting by peptides that preferentially target mammalian cell membranes but preserve microbial cells. Thiol-activated toxins made by a variety of bacterial pathogens are highly efficient in discerning target mammalian membranes that possess cholesterol, from those that do not. For example, streptolysin O, listeriolysin, perfringolysin, and pneumolysin appear to require membrane cholesterol, but no other membrane constituent, for targeting (Palmer, 2001). Thus, these toxins are relatively indiscriminant in lysing the cytoplasmic membranes of essentially any mammalian cell. It is unclear whether these toxins further distinguish between membranes containing cholesterol and ergesterol of mammalian or fungal cells, respectively.

2. Membrane Asymmetry. The compositional and architectural characteristics of prokaryotic and mammalian membranes are neither static nor symmetric. Distinctions between microbial and mammalian cells as targets for antimicrobial peptides include the configuration of phospholipid bilayer components. Recent evidence indicates that the distribution of phospholipids within cytoplasmic membranes is highly asymmetric. For example, only 2% of the total PE content in bovine erythrocytes is oriented toward the outer membrane leaflet (Florin-Christensen et al., 2001). Differences among asymmetric distribution, compositional stoichiometry, and saturation of phospholipid bilayers also significantly influence membrane phase transition and fluidity (Bayer et al., 2000; Verkleij and Post, 2000; McIntosh et al., 2001). These differences may extend to the inner and outer cytoplasmic membrane leaflets, or those of the outer membrane of Gram-negative bacteria or enveloped viral pathogens (which generally exhibit properties of their corresponding host cells). Accordingly, the charge and amphipathicity of the inner and outer membrane leaflets also vary considerably (Fig. 1). For example, in human erythrocytes, most glycosylated lipids (glycolipids), PC, PS, and SM are positioned on the exoplasmic membrane leaflet. Alternatively, when present, neutral or anionic phospholipids are typically localized on the cytoplasmic leaflet. Thus, differences in electronegativity resulting from leaflet asymmetry likely provide a further dimension influencing the relative affinity of antimicrobial peptides for biomembranes.

Interaction of cationic antimicrobial peptides with phospholipid membranes may also exaggerate dissymmetry and phospholipid remodeling in microbial membranes. For example, Lasch et al. (1998) demonstrated that a polylysine peptide induces bacterial 1,2-dimyristoyl-PE to segregate from lipopolysaccharide (LPS) into distinct, well defined domains. This observation suggests that interactions with antimicrobial peptides promote abnormal or exaggerated asymmetry within or between phospholipid leaflets comprising the bilayer in microbial membranes (also see *Section III.*). In this respect, a propensity for microbial versus host cell membranes to respond by dissociation, dispersion, or fusion may also contribute to selective toxicity of antimicrobial peptides.

Of note, relevant limitations regarding interpretations of antimicrobial peptide cytotoxicities against mammalian cells should be understood. Information derived from in vitro or ex vivo erythrocyte permeabilization or hemolysis assays should be considered limited to the degree it accurately represents antimicrobial peptide-selective toxicity against specific cell types and in complex biomatrices and physiologic settings in vivo. Therefore, the degree to which antimicrobial peptides permeabilize or lyse human erythrocytes may not realistically reflect their potential cytotoxicity in vivo.

3. Microbial Ligands for Antimicrobial Peptides. The fact that D-and L-amino acid versions of antimicrobial peptides generally show little selectivity in binding suggests that stereospecific receptors are not present on target microbial cells (see *Section III.*). However, certain structures may be crucial for selective affinity of peptides for microbial pathogens. For example, Teuber and

Bader (1976) demonstrated that radioactive mono-*N*-acetyl-^{14}C or native polymyxin B absorbed to isolated cytoplasmic and outer membranes of *Salmonella typhimurium* within 60 s of exposure. Moreover, polymyxin B exhibited sigmoidal binding kinetics, suggesting saturation of cytoplasmic and outer membranes, with approximately 30 and 60 nmol of peptide bound per milligram of membrane, respectively. Importantly, based on the stoichiometry of LPS, PG, CL, and PE in the membranes, these investigators calculated that the theoretical binding capacities of polymyxin B were almost identical to the binding properties if LPS, PG, and CL were modeled to function as specific receptors for this peptide. This robust concordance between theoretical and experimental approximations of polymyxin B binding capacities, along with parallel binding and killing kinetics, argues that membrane anionic constituents themselves function as pseudoreceptors for this cationic peptide. Thus, electronegative ligands (e.g., PG, CL, LPS) likely provide impetus for the initial interaction between cationic peptides and certain pathogens (also see *Sections III.* and *IV.*).

An interesting study by Edgerton et al. (1998) also implicates specific proteins on the candidal surface to be important in antimicrobial peptide binding. Salivary histatins exhibit in vitro antifungal activity against organisms such as *Candida albicans.* However, the interaction of these peptides with the fungal target cell does not appear to relate to general electrostatic or hydrophobic affinities. Rather, ^{125}I-labeled histatin binding assays suggested that *C. albicans* whole cells have saturable binding sites that are equally and competitively bound by histatins 3, 4, and 5. However, spheroplasts do not appear to exhibit such binding sites and are accordingly more than 10-fold less susceptible to histatins compared with whole cells. Preliminary experiments suggest the histatin binding ligand to be an estimated 67-kDa protein associated primarily with the intact surface of the organism.

4. Transmembrane Potential. Another fundamental difference between microbial and mammalian cells can be found in the charge separation between the extracellular and intracellular aspects of the cytoplasmic membrane. This electrochemical gradient, resulting from differing extents and rates of proton flux across the membrane, is termed the transmembrane potential ($\Delta\psi$). The difference in $\Delta\psi$ between certain microorganisms and host cells may provide a means of selective targeting of microorganisms by cationic antimicrobial peptides. For example, normal mammalian cells exhibit a $\Delta\psi$ ranging from -90 to -110 mV. However, bacterial pathogens in logarithmic phase growth commonly exhibit $\Delta\psi$ of -130 to -150 mV. Such significant differences in membrane electrochemistry have been hypothesized as additional parameters guiding selective toxicity of antimicrobial peptides, through a mechanism that has been termed self-promoted uptake (Hancock, 1997 [also see *Section III.*]).

B. Antimicrobial Peptide Structure-Based Selective Toxicity

Many antimicrobial peptides are believed to exist in relatively unstructured or extended conformations prior to interaction with target cells. Others are held in specific conformations by intramolecular bonds. Upon binding to pathogen membranes, peptides may undergo significant conformational dynamics to helical or other structures that effect antimicrobial activity (see below). There is mounting evidence supportive of the concept that inherent and/or dynamic conformations among antimicrobial peptides impact their selective toxicity. Furthermore, peptides may have distinct antimicrobial versus host cytotoxic conformers and/or undergo conformational phase transition, self-association, or oligomerization within target pathogen—but not host cell—membranes, as a means for selective toxicity (also see *Section III.*).

Tam et al. (2000) recently examined the influence of conformation on membranolytic selectivity of antimicrobial peptides. In these studies, antimicrobial activity and human cell cytotoxicity were assessed in conformationally restricted cyclic and noncyclic analogs of protegrin-1, an 18-amino acid cationic peptide exhibiting broad-spectrum antimicrobial activity. Antimicrobial assays in relatively low- and high-salt conditions revealed cyclic protegrins exert differential antimicrobial profiles against Gram-positive and Gram-negative bacteria, fungi, and human immunodeficiency virus-1. As compared to protegrin-1, the most constrained analog (a cyclic-tricystine protegrin termed ccPG-3) displayed a 10-fold decrease in hemolytic propensity to human cells and up to a 30-fold increased membranolytic selectivity against specific target pathogens. However, an analogous cyclic protegrin lacking a disulfide bond, or a cyclic mimic of protegrin-1 with one disulfide bond, exhibited antimicrobial and cytotoxic profiles equivalent to protegrin-1. Interestingly, circular dichroism showed that even cyclized protegrins stabilized by disulfide bonds display β-strand structure in water/trifluoroethanol or phosphate-buffered environments. These findings suggest that conformational dynamics subsequent to initial binding contribute to antimicrobial peptide activity in selected membrane environments.

Related studies by Unger et al. (2001) provide additional insights into the structural basis for selective toxicity of antimicrobial peptides. These investigators examined the interaction of linear versus cyclic counterparts of melittin and magainin analogs (peptides displaying non-identical selective toxicity toward mammalian cells) with membrane models in vitro. As compared with linear versions, the cyclized peptides were less efficient in initial binding to phospholipid membranes. However, at normalized bound concentrations, linear

and cyclic analogs retained equivalent potencies to induce membrane permeabilization. When bound to phospholipid membranes, these cyclized peptides reverted to ~75% of the helical structure of their linear analogs. Even more importantly, the cyclic melittin analog exhibited increased antibacterial activity, with reduced hemolytic propensity, whereas the cyclic magainin exhibited opposite biological functions. These observations were interpreted to suggest that conformation influences initial interactions of peptides with membranes, as well as ensuing disruptive actions on target membranes. Collectively, these findings emphasize the potential role for conformational dynamics subsequent to initial binding interactions in selective toxicity of antimicrobial peptides. In addition, the above studies lend insights into the potential for engineered conformational constraints to further dissociate antimicrobial activity from host cytotoxicity.

Recent studies by Oren and colleagues (1999) also shed light on the relationship between quaternary structure and selective toxicity among antimicrobial peptides. Human cathelicidin LL-37 is an antimicrobial peptide cytotoxic to both bacterial and mammalian cells. This peptide exists in equilibrium as monomers and oligomers in solution at low concentration but appears to undergo self-association within zwitterionic (mammalian-like) and electronegative (bacterial-like) artificial phospholipid membranes in vitro. Interestingly, in these models, LL-37 effected a detergent-like or carpet mechanism (see *Section III.*) in disrupting both membrane types, suggesting a structure-induced membrane perturbation in either setting. Supportive of this interpretation was the finding that the peptide conformed to a predominantly α-helical configuration oriented parallel with the surface of zwitterionic membranes. Thus, a propensity to assume an invariable helical conformation and multimerize within lipid membranes of differing compositions may reduce the ability of antimicrobial peptides to exert selective toxicity against microorganisms versus host cells.

Experiments focusing on cationic antimicrobial peptides of varying structures and origins extend this theme of peptide interaction with model membranes of distinct phospholipid compositions (Zhang et al., 2001). In these studies, test peptides were uniformly cationic but varied in conformation, including α-helical, β-sheet, extended, and cyclic motifs. Regardless of conformation, all test peptides interacted with and penetrated into lipid monolayers composed of anionic PG, as measured by the release of preloaded calcein dye. In comparison, only α-helical and extended peptides interacted with monolayers composed of more zwitterionic PC, albeit to a lesser extent than with the anionic lipids. Interestingly, a β-sheet peptide induced rapid phospholipid translocation (movement of lipid from the inner facet to the outer facet of the membrane) at concentrations less than required for membrane permeabilization. Similarly, Kol et al. (2001) demonstrated that the ability of peptides of comparable conformation to induce phospholipid translocation was greater for those containing proportionately more lysine or histidine residues, compared with tryptophan. From these examples, it appears that antimicrobial peptides not only interact with biomembranes of specific composition and asymmetry but may also promote remodeling of these membrane properties within target cells.

C. In Vivo Preferential Affinity for Microorganisms versus Mammalian Cells

Recently, Welling et al. (2001) tested the hypothesis that cationic antimicrobial peptides may discriminate between microbial cells and host tissues in vivo. Studies evaluated whether such peptides specifically accumulate in sites of infection, compared with sterile inflammatory lesions, due to preferential avidity for microorganisms. Peptide affinity and specificity for pathogens in vivo was assessed by intravenous injection of ^{99m}Tc-labeled synthetic derivatives of human ubiquicidin or lactoferrin into animals experimentally infected with *Staphylococcus aureus, Klebsiella pneumoniae,* or *C. albicans*. As controls, sterile inflammatory sites were induced by the introduction of heat-killed microorganisms or purified LPS into thigh muscle. Labeled human defensin, human polyclonal IgG, and ciprofloxacin were examined as comparative agents. The ^{99m}Tc-labeled peptides and defensin accumulated at a significantly higher rate and to a greater extent in bacteria- and *C. albicans*-infected lesions in mice and rabbits, compared with non-infected but inflamed tissues. These data were interpreted to indicate that the peptides distinguish between microorganisms and host tissues, and in doing so, accumulate at sites of infection in vivo.

In related studies, this same group examined the potential pharmacologic utility of antimicrobial peptides to localize to sites of infection (Welling et al., 2000). Biodistribution scintigraphy suggested that the ^{99m}Tc-labeled peptides were rapidly removed from the circulation by renal excretion. However, despite this rapid clearance, the radiolabeled peptides efficiently discriminated between infected and non-infected tissue, with up to 5-fold increased binding to target versus nontarget tissues within 1 h in rabbits. Collectively, these results indicate that antimicrobial peptides rapidly localize and accumulate at sites of infection, likely due to preferential affinity for peptides to associate with target microorganism surfaces rather than non-infected tissues.

D. Antimicrobial Peptide Localization to Restrict Exposure of Vulnerable Host Tissues

Selective toxicity among antimicrobial peptides—or the lack thereof—involves complex interactions between peptide and target cells as indicated above (also see *Section III.*). However, it is also likely that these peptides may be rendered less harmful to the host simply

through strategic localization or expression that minimizes their interaction with potentially vulnerable host tissues. For example, many antimicrobial peptides known in vertebrates are secreted onto relatively inert epithelial surfaces, such as the tracheal, lingual, or intestinal mucosa of mammals, or the skin of amphibians. In addition, this localization—along with rapidly inducible expression—places antimicrobial peptides in key positions to intervene at perhaps the earliest of opportunities to prevent microbial colonization or infection.

A similar, albeit more complex mechanism likely contributes to selective toxicity of antimicrobial peptides found in granules of phagocytic leukocytes. The fundamental antimicrobial functions of professional phagocytes include internalization of pathogens (phagocytosis), subjecting them to the harsh microenvironment of the phagolysosome. Neutrophils, monocytes, and macrophages of various mammalian species contain among the most potent antimicrobial peptides known—defensins (see below). However, defensins may also exhibit among the least selective toxicity of any host defense peptides, often exerting membrane permeabilizing and other harmful effects on microorganisms and mammalian cells alike. Phagocytes normally interiorize and expose pathogens to lethal concentrations of these peptides within the maturing phagolysosome, rather than degranulating these potentially injurious components into the extracellular milieu. Within the restricted confines of the phagolysosome, defensins and other antimicrobial peptides are present in very high relative concentrations, where they may act harshly and synergistically with one another, along with oxidative killing mechanisms. In this way, defensins may be constrained to granules of mammalian phagocytes to minimize their potential for host cytotoxicity. Moreover, Shafer et al. (1986) and Yeaman (1997) have suggested that antimicrobial activities of defensins and platelet microbicidal proteins are potentiated in mildly acidic conditions, such as those found in the maturing phagolysosome.

Beyond the scope of this review, some antimicrobial peptides may also perform other important functions contributing significantly to antimicrobial host defense, including interfering with host cell receptor access to pathogens, recruitment of leukocytes to sites of infection, as well as potentiate their antimicrobial activities (Yeaman, 1997; Yeaman and Bayer, 1999; Cole et al., 2001; Tang et al., 2002). For example, Zhang et al. (2002) have recently found that CD8+ cytotoxic T lymphocytes elaborate α-defensins 1, 2, and 3 in contributing to host defense against human immunodeficiency virus-1. Conceivably, these peptides act directly to alter or damage the human immunodeficiency virus virion, or indirectly by interfering with receptor targeting, eventual uncoating or replication, and/or enhanced intracellular destruction. Thus, the extracellular secretion of antimicrobial peptides at concentrations or in settings that do not result in host toxicity may play important roles in immunity. Through such strategies, the antimicrobial functions of peptides and phagocytes may be mutually amplified, while minimizing the potential for concomitant host cell toxicity.

E. Themes in Target Affinity and Selective Toxicity of Antimicrobial Peptides

Antimicrobial peptides display highly variable abilities to discriminate between microbial targets versus normal host cells. The governing rules for differences in selective toxicity among such peptides remain to be fully elucidated. However, several themes relating to the structural and functional properties of peptides as they relate to their potential targets include: 1) compositional divergence conveying differential electrostatic affinities for microbial versus host cells; 2) conformational dynamics that promote peptide activation or self-association in microbial membranes, but not others; 3) target cell energetics that accelerate or retard peptide interactions with target versus host membranes, respectively; and 4) limitations in the access of antimicrobial peptides with poor selective toxicity to potentially vulnerable host tissues.

III. Mechanisms of Antimicrobial Peptide Action

A striking feature among antimicrobial peptides as a group is their overall conservation of structure and charge themes across diverse phyla. Whether synthesized non-ribosomally with D- and L-amino acids, or from genetically encoded messenger RNA, antimicrobial peptides form amphipathic structures and are often cationic at physiological pH. As outlined above, amphipathicity and net charge are characteristics understandably conserved among many antimicrobial peptides. Furthermore, charge affinity is likely an important means conferring selectivity to antimicrobial peptides. In the context of these paradigms, the following discussion highlights current concepts relating to the molecular basis of antimicrobial peptide mechanisms of action.

A. Structural Determinants of Antimicrobial Peptide Activity

An essential requirement for any antimicrobial host defense or therapeutic agent is that it has a selective toxicity for the microbial target relative to the host. Ideally, such compounds have affinity for one or more microbial determinants that are easily accessible, common to a broad spectrum of microbes, and relatively immutable. Nature has apparently yielded a class of molecules that meets these constraints in the evolution of antimicrobial peptides. Antimicrobial peptides initially target microbial cells, and thus fulfill criteria outlined above for identifying molecular determinants of pathogens that are accessible and broadly conserved. As a group, antimicrobial peptides have amphipathic features that mirror phospholipids, thus allowing them to

interact with and exploit vulnerabilities inherent in essential microbial structures such as cell membranes. In the following section, several aspects of antimicrobial peptide structure relevant to antimicrobial activity and selective toxicity are considered thematically. Specifically, structural parameters such as conformation (χ), charge (Q), hydrophobicity (H), hydrophobic moment (M_H), amphipathicity (A), and polar angle (θ), are examined in some detail. It is important to note that these molecular determinants are interdependent, and therefore, modification of one parameter often leads to compensatory alterations in others. This holistic view of peptide structure-activity relationship relates to each of these key properties influencing mechanisms of action of antimicrobial peptides (Fig. 2). The following discussion is considered in this context.

1. Conformation (χ). Although antimicrobial peptides differ widely in sequence and source, several themes in their three-dimensional topology appear predominant, and peptides have been categorized accordingly. The two largest groups are the α-helical and β-sheet peptides, whereas the majority of remaining peptides can be classified as those that are enriched in one or more amino acid residues [e.g., proline-arginine or tryptophan-rich (Hancock, 1997)]. Other classification schemes are based upon peptide source (e.g., neutrophils or other leukocytes), precursor (e.g., cathelicidin, derivatives of cathelin), extent of intramolecular bonds (e.g., cysteine array or cyclization in peptides), or other parameters.

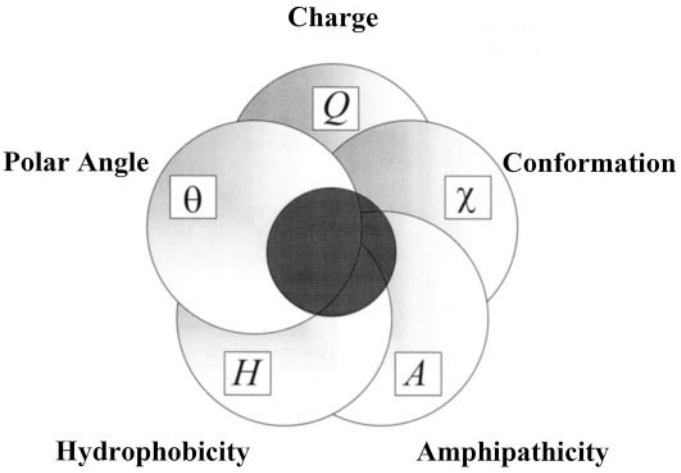

FIG 2. Interrelationship among structural determinants in antimicrobial peptides. Fundamental composition and amino acid sequence influences not only the biochemical properties of the peptide [e.g., charge (Q), amphipathicity (A), and hydrophobicity (H)], but also govern their three-dimensional configuration [e.g., conformation (χ), polar angle (θ), and overall stereo geometry]. Therefore, changes in composition, sequence, and intramolecular bonds may profoundly effect the structure-activity relationships of antimicrobial peptides in solution, upon binding to target membranes, or as they may undergo conformational phase transition to activated states. Moreover, these features may be specific for distinct peptides as they interact with specific pathogens or in specific physiologic microenvironments. Therefore, optimal antimicrobial peptide efficacy lies in the relevant coordination of these relationships (shaded area) as they relate to microbial target versus host cells in a particular context of infection.

The α-helical antimicrobial peptides are abundant in the extracellular fluids of insects and frogs and frequently exist as extended or unstructured conformers in solution. Many of these peptides only become helical upon interaction with amphipathic phospholipid membranes. The β-sheet peptides represent a highly diverse group of molecules at the level of primary structure. Despite such differences, these peptides share common features, including amphipathic composition, with distinct hydrophilic and hydrophobic surfaces. Less is known about the structures adopted by the proline-arginine-rich and tryptophan-rich peptides. However, examples of conformations distinct from prototypic α or β structures have also been identified. For example, certain proline-arginine-rich peptides, and tryptophan-rich indolicidin, conform to polyproline helical type II structures (Boman et al., 1993; Cabiaux et al., 1994), and tritrypticin may form a basket-shaped turn structure (Schibli et al., 1999).

2. Charge (Q). Many of the antimicrobial peptides characterized to date display a net positive charge, ranging from +2 to +9, and may contain highly defined cationic domain(s). Cationicity is undoubtedly important for the initial electrostatic attraction of antimicrobial peptides to negatively charged phospholipid membranes of bacteria and other microorganisms (see Fig. 2), and mutual electroaffinity likely confers selective antimicrobial targeting relative to host tissues. The fact that bacterial membranes are rich in the acidic phospholipids PG, PS, and CL confers their overall negative charge. Moreover, LPS and teichoic or teichuronic acids of Gram-negative and Gram-positive bacteria, impart additional negative charge to the surfaces of these respective organisms. Target cell $\Delta\psi$ is typically up to 50% greater in prokaryotes than in most mammalian cells. Thus, it has been proposed that such a chemiosmotic potential may act in an electrophoretic manner to concentrate positively charged peptides on microbial surfaces (also see *Section II.*).

Based on these considerations, it is not surprising that there is a strong correlation between peptide cationicity and antimicrobial activity, as has been demonstrated in a number of studies (Bessalle et al., 1992; Matsuzaki et al., 1996; Dathe et al., 1997). However, this relationship is not entirely linear, with examples of direct, indirect, or inverse relationships between these variables (Bessalle et al., 1992; Blondelle and Houghten, 1992). Within a certain range, increasing peptide cationicity is generally associated with increasing antimicrobial potency. Studies with magainin 2 analogs, in which other parameters such as peptide hydrophobicity and helicity were kept constant, have shown that increasing the charge from +3 to +5 results in increasing antibacterial activities against Gram-negative and Gram-positive pathogens (Dathe et al., 2001). However, there is a limit beyond which increasing positive charge no longer confers increased activity. For the magainins described

above, a net charge of +6 to +7 led to an increased hemolytic propensity and a loss of antimicrobial activity (Dathe et al., 2001). This decrease in antimicrobial activity may result in part from excessively strong peptide interactions with phospholipid head groups, thereby preventing translocation of the peptide into the cell interior.

3. Amphipathicity (A) and Hydrophobic Moment (M_H). Nearly all antimicrobial peptides form amphipathic structures upon interaction with target membranes. Amphipathicity can be achieved via a multitude of protein conformations; however, one of the simplest and perhaps most elegant is the amphipathic helix. The amphipathic α-helix has a periodicity of three to four residues and is optimal for interaction with amphipathic biomembranes. While the extent of amphipathic helicity influences peptide activity against negatively charged membranes, it may have an even more pronounced effect in rendering peptides hemolytic against zwitterionic or neutral membranes. Thus, a high degree of helicity and/or amphipathicity yielding a segregated hydrophobic domain, is correlated with increased toxicity toward cells composed of neutral phospholipids (Dathe and Wieprecht, 1999).

Amphipathicity reflects the relative abundance and polarization of hydrophobic and hydrophilic domains within a protein (Fig. 2). This attribute is somewhat difficult to describe in a formulaic manner. One quantitative measure of amphipathicity is the hydrophobic moment, M_H, calculated as the vectorial sum of individual amino acid hydrophobicities, normalized to an ideal helix (Eisenberg, 1984). Increasing hydrophobic moment results in a significant increase in the permeabilizing and hemolytic activities of model peptides against target membranes. For example, Pathak et al. (1995) suggested that amphipathicity was more important than hydrophobicity or α-helical content in governing antimicrobial peptide activity. In a similar study with magainin analogs, the relative role of hydrophobic moment on membrane binding and permeabilization was examined (Wieprecht et al., 1997). Increases in peptide hydrophobic moment had no effect on calcein release from large unilammelar vesicles composed entirely of PG. However, increased hydrophobic moment produced a significant increase in dye release from membranes composed of PC and PG at a 3:1 ratio. Moreover, circular dichroism (CD)-derived binding isotherms revealed a significant increase in membrane affinity for analogs with the highest hydrophobic moment. Relatively small increases in hydrophobic moment resulted in 8-fold reductions in the amount of peptide required for hemolysis of human erythrocytes. Thus, increasing hydrophobic moment appears to have only modest effects on peptide interactions with highly negative membranes. However, for more neutral membranes where electrostatic peptide-lipid interactions are minimized, hydrophobic moment interactions may play a more predominant role regarding host cell toxicity.

The β-sheet antimicrobial peptides are also amphipathic. This amphipathicity is characterized by a variable number of β-strands, with relatively few or no helical domains, organized to create both polar and nonpolar surfaces. These β-strands are frequently antiparallel, and are stabilized by a series of disulfide bonds, with as many as eight cysteines in some peptides [e.g., plant defensins (Sitaram and Nagaraj, 1999); and mussel mytilins (Dimarcq et al., 1998)], or by cyclization of the peptide backbone (e.g., protegrins, gramicidin, or θ-defensins). The conformational rigidity observed in many β-sheet antimicrobial peptides in aqueous solution may also promote multimerization, limiting exposure of hydrophobic facets to hydrophilic environments. This configuration contrasts with that of higher degrees of freedom among the α-helical peptides in similar solutions. A number of β-sheet peptides have been shown to exist as dimers in aqueous solution, including the human defensin HNP-3, as determined by X-ray crystallography. The proposed mechanisms by which HNP-3 and other defensins or antimicrobial peptides perturb target membranes involve amphipathicity and hydrophobic moment. For example, insertion of the hydrophobic peptide face into the lipid bilayer, and association of the charged arginine side chains with polar lipid head groups, relies upon three-dimensional separation of hydrophobic and charge. Once associated with the membrane, the amphipathic nature of β-sheet peptides likely enables their formation of transmembrane channels. Several models have been proposed to explain the exact mechanism by which these peptides may form and traverse the channel (see below); however, the precise conformation adopted by such peptides in the hydrophobic membrane environment remains to be determined. However, as in α-helical peptides, it is now apparent that highly segregated amphipathicity strongly influences β-sheet peptide disruption of neutral membranes. These findings have led to studies demonstrating that residue-specific modifications in hydrophobicity enhance selectivity among cationic peptides. For example, studies using synthetic derivatives of gramicidin S have revealed that reductions in hydrophobicity significantly increase selective toxicity against microorganisms, with approximately 10,000-fold increase in the estimated therapeutic index of such peptides (Kondejewski et al., 1999).

4. Hydrophobicity (H). Peptide hydrophobicity, defined as the percentage of hydrophobic residues within a peptide, is approximately 50% for most antimicrobial peptides. Hydrophobicity is an essential feature for antimicrobial peptide membrane interactions, as it governs the extent to which a peptide can partition into the lipid bilayer. Although hydrophobicity is required for effective membrane permeabilization, increasing levels of hydrophobicity are strongly correlated with mamma-

lian cell toxicity and loss of antimicrobial specificity. Therefore, many antimicrobial peptides are moderately hydrophobic, such that they optimize activity against microbial cell membranes.

The relationship between peptide hydrophobicity and membrane permeabilization was examined in an interesting study by Wieprect and coworkers (1997). In their investigations, charge, helicity, and hydrophobic moment were kept essentially constant in a series of magainin analogs. However, the hydrophobicity of these peptides was varied as defined by the Eisenberg consensus scale of hydrophobicity (Eisenberg, 1984). Notably, mean peptide hydrophobicity had no effect on membrane binding and permeabilization of vesicles composed entirely of PG. In marked contrast, peptide hydrophobicity had a significant effect on the binding and permeabilization of vesicles composed of PC and PG at a 3:1 ratio. The most hydrophobic peptide exhibited an ~60-fold greater permeabilizing activity for PC/PG (3:1) vesicles, compared with the least hydrophobic peptide. For vesicles composed entirely of PC, the effect was even more striking, as the most hydrophobic peptide was nearly 300-fold more active than the least hydrophobic peptide. These data were further corroborated by determinations of the apparent binding constants (K_{app}) for these peptides to vesicles composed of PC and PG at the 3:1 ratio. The most hydrophobic peptides bound vesicles with a K_{app} of 105,000 M^{-1}, compared with a K_{app} of 7,400 M^{-1} for the least hydrophobic peptide. This difference in binding affinity exemplifies the extent to which hydrophobicity influences membrane binding and permeabilization. Furthermore, differences in membrane perturbation were achieved with relatively minor changes in net peptide hydrophobicity, indicating the relative significance of hydrophobic features on these interactions.

5. Polar Angle (θ). Polar angle is a measurement of the relative proportion of polar versus nonpolar facets of a peptide conformed to an amphipathic helix. For example, in a hypothetical α-helical peptide, in which one facet is exclusively composed of hydrophobic residues and the other solely composed of charged residues, the polar angle would be 180°. A reduced segregation between these domains or an increased hydrophobic proportion of the helix would proportionately reduce the polar angle. In numerous studies of native and synthetic peptides, a smaller polar angle (and therefore a greater hydrophobic surface) is associated with increased capacity to permeabilize membranes (Dathe et al., 1997; Wieprecht et al., 1997; Uematsu and Matsuzaki, 2000). The polar angle has also been shown to correlate with the overall stability and half-life of peptide-induced membrane pores. In a recent study by Uematsu Matsuzaki (2000), the effects of polar angle on membrane permeabilization and pore formation were compared. Two model peptides with polar angles of 100° and 180° showed functional similarities with native α-helical antimicrobial peptides in forming amphipathic helices, selective targeting of negatively charged membranes, and creating toroid or lipid-containing pores (see below). Results from these studies indicated that peptides with smaller polar angles induced greater membrane permeabilization, translocation, and pore formation rates (Uematsu and Matsuzaki, 2000). However, although the rate of pore formation was greater for peptides with smaller polar angles, the rate of pore collapse was higher. These results suggest that peptides with smaller polar angles achieve less stable pore structures compared with peptides having larger polar angles. Greater stability of pores formed by the latter peptides could result from larger charged surfaces, and/or more peptide molecules per channel. These concepts are consistent with those observed in native peptides, showing that peptide PGLa ($\theta = 100°$) is more easily translocated than magainin 2 ($\theta = 180°$; Matsuzaki, 1998). These results indicate that hydrophobic and hydrophilic stereogeometries in antimicrobial peptides play significant roles influencing the process and consequences of membrane interaction and disruption.

B. Common Themes in Structural Determinants of Antimicrobial Peptides

The existence of a broad diversity in antimicrobial peptide sequences and structures underscores the reality that no single antimicrobial peptide sequence has emerged as singularly effective against all pathogens in all settings. Moreover, Nature may have sustained such diversity as a strategy to prevent or delay evolution of microbial resistance to antimicrobial peptides. Nonetheless, a circumspect analysis of structural parameters associated with differential antimicrobial activity versus host cell toxicity among peptides reveals several themes. Conservation in secondary structure may be key to three-dimensional configurations facilitating antimicrobial activity of distinct peptides. Generally, extremes of certain features, such as charge, amphipathicity, hydrophobic moment, or polar angle may disfavor peptide antimicrobial activity and selective toxicity. A minimum threshold of charge, perhaps as low as +2, appears necessary for antimicrobial peptide selectivity toward microorganisms. This property is likely important for a number of reasons: 1) initial electrostatic attraction to negatively charged microbial membranes; 2) potential to displace membrane-associated cations; and 3) a strong trans-negative $\Delta\psi$ of many microorganisms may facilitate cationic peptide transitions in orientation on the membrane, entry into the polar membrane core, and/or translocate peptides from exoplasmic to cytoplasmic membrane facets. A moderate level of amphipathicity, independent of or in context of polarization of charge, appears to be more favorable in these respects. Segregation of charge and hydrophobicity paralleling the inherent amphipathicity of the target lipid bilayer may also promote peptide integration into and disruption of the microbial membrane. A third theme is that selectivity

among membrane-lytic peptides may rely on moderate degrees of hydrophobicity, as excessive hydrophobicity may increase selectivity for zwitterionic membranes, increasing mammalian cytotoxicity. Thus, selective antimicrobial activity results from a delicate balance among three-dimensional hydrophobic and electrostatic interactions between an antimicrobial peptide and its target (Dathe et al., 1996, 1997).

C. Initial Peptide Interactions with Membrane Targets

As outlined above, antimicrobial peptides are inherently structured to target and interact with biomembranes. More importantly, the initial interaction with the target surface significantly influences subsequent peptide dynamics and membrane-disrupting effects. As discussed below, the basis of this initial interaction integrates biochemical as well as biophysical aspects of the peptide and the target membrane.

1. Electrostatic Interactions. There is widespread acceptance that the initial mechanism by which antimicrobial peptides target microbes occurs via an electrostatic interaction. For example, cationic antimicrobial peptides and negatively charged lipid membranes of bacteria provide for a mutual and vigorous attraction. This supposition has been borne out by numerous studies in which a strong correlation between peptide charge and membrane binding activity has been demonstrated (Bessalle et al., 1992; Vaz Gomes et al., 1993; Matsuzaki et al., 1997; Dathe et al., 2001). This view is also supported by the conservation of positive charge within many antimicrobial peptides isolated from organisms across the evolutionary spectrum. The facts that electrostatic forces are active over relatively long molecular distances and that lysine and arginine interactions with phosphate groups in lipid bilayers are particularly strong (Mavri and Vogel, 1996) likely contributes to the initial attraction and membrane-targeting step of many antimicrobial peptides.

The precise mechanism by which electrostatic attraction drives peptide-membrane interaction has been examined in a number of studies (Bessalle et al., 1992; Vaz Gomes et al., 1993; Matsuzaki et al., 1997; Dathe et al., 2001). In the case of Gram-negative organisms, Hancock (1997) has suggested a mechanism of peptide interaction with membranes termed self-promoted uptake. This mechanism, similar to that known for aminoglycoside antibiotics, contends that the initial action of the peptide involves a competitive displacement of LPS-associated divalent cations stabilizing the outer membrane. Such LPS displacement is likely to be energetically favorable given that the binding affinity of a typical antimicrobial peptide for LPS is ~3 orders of magnitude greater than that of divalent cations. This hypothesis is supported by studies with polymyxin-resistant *pmrA* strains of *S. typhimurium*. The LPS phosphate moiety in these strains is highly substituted with 4-amino-4-deoxy-L-arabinose, providing the bacteria a reduced overall negative charge and corresponding increased resistance to cationic antimicrobial peptides (Helander et al., 1994, 1995). Similarly, other modifications to LPS, such as acylation of lipid A, have been shown to inhibit the translocation of cationic antimicrobial peptides across the plasma membrane (see *Section IV*.). In comparison, Gram-positive organisms lack an outer membrane or LPS; however their cell envelopes are enriched in negatively charged teichoic and teichuronic acids. The significance of these anionic structures with respect to cationic antimicrobial peptide activity has been demonstrated using a mutant strain of *S. aureus* in which cell wall teichoic acid modification resulted in an increased negative surface charge and was associated with an increased sensitivity to killing by positively charged antimicrobial peptides (Peschel et al., 1999).

The strong electrochemical gradient ($\Delta\psi$) of most bacterial membranes likely compounds the biophysical forces driving the interaction between cationic peptides and target pathogens. Studies supporting this theory have shown that a membrane potential as low as −20 mV increases the binding constant of the cationic peptide tachyplesin 200-fold (Matsuzaki, 1997). Similarly, experiments with model membranes have demonstrated that a threshold $\Delta\psi$ is required for nisin activity (Breukink and Kruijff, 1999). Other antimicrobial peptides likewise require a substantial electrochemical potential for optimal activity (Yeaman et al., 1998). Thus, the strong bacterial $\Delta\psi$ relative to that of mammalian cells may be a significant factor contributing to charge-mediated peptide selectivity.

2. Receptor-Mediated Membrane Interactions. Early studies using all D-enantiomers of native and model peptides demonstrated equivalent antimicrobial activities of D- and L-isoforms. Thus, the prevailing dogma supported a non-receptor type interaction for antimicrobial peptides with most pathogen membranes (Bessalle et al., 1990; Wade et al., 1990). Since then, several studies suggest there may be important exceptions to this generalization. Perhaps the most well characterized example is that of nisin, a small, cyclic, non-ribosomally produced peptide that has been used in the food industry for several decades. Nisin exhibits antimicrobial activity in the nanomolar range and specifically binds to bacterial lipid II, a membrane bound component involved in peptidoglycan synthesis. When exposed to nisin, vesicles containing lipid II exhibit an ~1000-fold increase in fluorescein leakage compared with vesicles lacking lipid II (Breukink and Kruijff, 1999). It has been proposed that this specificity in nisin activity relates to a specific receptor-like interaction with lipid II and the proximity it confers to this peptide relative to the microorganism. Notably, lipid II is believed integral to peptidoglycan synthesis, and nisin is considerably more active against peptidoglycan-rich Gram-positive organisms than Gram-negative organisms (Breukink and Kruijff, 1999). Likewise, Brotz et al. (1998) have recently demonstrated

that the lantibiotic mersacidin interferes with transglycosylation and peptidoglycan synthesis in Gram-positive bacteria by direct targeting of lipid II. In addition, tachyplesin has been demonstrated to have a specific affinity for LPS (Hirakura et al., 2002). Moreover, a number of studies have now shown non-equivalent activities for native all-L peptides, versus their all-D enantiomers (Fehlbaum et al., 1996; Vunnam et al., 1997). For example, in intriguing studies using PR-39, a proline- and arginine-rich peptide of porcine origin, the all-D enantiomer showed 1000-fold differences in species-specific activity against bacterial organisms (Vunnam et al., 1997). These studies suggest receptor-type interactions may be important for some peptides in targeting specific epitopes on the microbial surface.

D. Events Subsequent to Initial Membrane Binding

Perhaps one of the more controversial issues within the field revolves around the fate of antimicrobial peptides following their initial interaction with biological membranes. The mechanism(s) by which peptides may permeabilize and traverse microbial membranes are not entirely clear and likely vary for different peptides. Uncertainty stems in part from technical difficulties associated with first-principle determinations or molecular modeling of peptide-lipid interactions. Attempts to crystallize antimicrobial peptides within a native lipid environment have been largely unsuccessful, and other methods of structure determination have various limitations. Conventional CD is an excellent tool for determining peptide secondary structures, such as α-helices. However it necessitates the use of optically clear solutions and provides little information as to the relative size of conformer regions or their location (Blondelle et al., 1999; Sitaram and Nagaraj, 1999). Similarly, infrared spectroscopy (e.g., FTIR) is an important tool particularly well suited to study β-sheet peptide conformations, but also has technical limitations. Fluorescence spectroscopy is convenient, and its high level of sensitivity allows for a small sample size. However, data are often highly dependent on the solvent or membrane mimetic system used. Nuclear magnetic resonance (NMR) studies also offer a powerful means to obtain structural information at the single residue or domain levels but can be limited by relatively slow rates of molecular reorientation. Recently developed methods include reverse phase-high pressure liquid chromatography-based and surface plasmon resonance; however, these techniques are limited to the extent they represent protein-whole microorganism interactions. Therefore, at the present time, the most comprehensive assessments of peptide-lipid structure often come from a combinatorial approach wherein a variety of methodologies are employed, and the results are considered collectively.

The following discussion considers events subsequent to peptide-target binding that may significantly influence peptide mechanisms of action and/or selective toxicity. Functional themes are reviewed using examples of prevailing models for these processes, which are proposed to occur via specific and nonspecific mechanisms. These data should be interpreted in the context of the specific biophysical methods employed; the particular conditions and assays utilized for assessing peptide antimicrobial activities are beyond the scope of this review. However, it should be understood that the potencies, spectra, and/or mechanisms of antimicrobial peptide action could be highly dependent upon conditions of testing. For example, media pH, osmotic and ionic strength, temperature, and viscosity (e.g., in peptide diffusion assays)—individually and in combination—may significantly influence peptide antimicrobial activities.

1. Threshold Concentration. At some point following initial membrane binding, peptides enter a second stage of membrane interaction, frequently referred to as the threshold concentration. In this phase, peptides begin to enter and traverse the lipid bilayer via a number of possible mechanisms, ultimately extending their antimicrobial action to targets interior to the cell membrane. Conceptually, the threshold concentration necessary to drive such events results from accumulation of peptides on the target surface. Parameters that likely influence this threshold include peptide concentration, propensity to self-assemble or multimerize, as well as phospholipid membrane composition, fluidity, and head group size (Yang et al., 2000). Additionally, it is important to note that individual peptide-membrane interactions can vary such that one type of peptide may act via multiple mechanisms dependent on conformation dynamics of the peptide or target membrane remodeling.

Another factor likely influencing threshold concentration and peptide parallel-to-transmembrane surface orientation is the considerable trans-negative $\Delta\psi$ of many bacterial membranes. It is postulated that membrane potential oriented in this way electrophoretically draws cationic peptides into the nonpolar membrane environment, effectively reducing the energy barrier for pore formation. For example, nisin, which requires a considerable $\Delta\psi$ for activity, has been shown to lose its voltage dependence when an N-terminal lysine is replaced with leucine (Breukink and Kruijff, 1999). This finding is consistent with the model of nisin cell penetration, in which the N-terminal region of the peptide is initially drawn into the membrane. Analogous mechanisms of action appear to be recapitulated by many cationic antimicrobial peptides.

2. Conformational Phase Transition. A key event occurring after membrane binding is the process of peptide structural or conformational phase transition, most well documented for α-helical antimicrobial peptides. Numerous studies using various biophysical methodologies show that many antimicrobial peptides are disordered in aqueous environments, exhibiting extended or random coil conformations in this setting (Bello et al., 1982; Dathe and Wieprecht, 1999). However, many such pep-

tides rapidly assume highly structured amphipathic α-helical conformation upon interaction with phospholipid bilayers or in membrane mimetic solvents. Interestingly, a number of peptides require a negatively charged bilayer to undergo this transition. For example, the frog skin peptide PGLa, disordered when exposed to membranes composed of the zwitterionic PC and SM membranes, adopts a helical structure in the presence of membranes composed of PG and PE (Latal et al., 1997). Similarly, magainins only undergo a helical transition when interacting with anionic membranes as demonstated by CD, (Matsuzaki et al., 1989, 1991), vibrational/Raman-FTIR (Williams et al., 1990; Hirsh et al., 1996) and NMR (Bechinger et al., 1993; Hirsh et al., 1996). Examination of cecropin analogs revealed that the extent of α-helical conformation is proportionately dependent on the amount of negatively charged phospholipid within the model membrane (Wang et al., 1998). One mechanism by which such a relationship may promote peptide order relies on the inherent phospholipid packing within the bilayer. For example, interactions of the peptide with the phospholipid head groups may promote an optimal periodicity within the charged residues of the peptide, promoting folding of the α-helix. As discussed above, this change in conformation would also likely alter peptide hydrophobic moment and polar angle. Another potentially important aspect of the conformational phase transition is that it may prevent indiscriminant membranolytic activity until the peptide identifies an appropriate target surface. Thus, a lack of bioactive structure at nontarget sites may be an important means by which antimicrobial peptides minimize host-cell toxicity.

In comparison, β-sheet antimicrobial peptides are typically much more ordered in aqueous solution and membrane environments, due to constraints imposed by disulfide bonds or cyclization of the peptide backbone. For example, the secondary structure of tachyplesin, a cyclic β-sheet peptide that contains a type-II β-turn, is largely unchanged as the peptide moves from an aqueous environment to that of a membrane-mimetic (Oishi et al., 1997). Thus, secondary structures of cystine-stabilized β-sheet peptides are likely relatively stable upon interaction with target cell membranes. However, it is possible that the quaternary peptide structures proposed for some β-sheet peptides in aqueous solution are dissociated upon interaction with the membrane surface. In contrast to α-helical peptides, the potential monomerization of such peptides could also facilitate antimicrobial mechanisms or selective toxicity.

3. Self-Association and Multimerization. Considerable evidence suggests that antimicrobial peptides may self-associate or multimerize following initial interactions with target membranes. These peptide-peptide and peptide-lipid interactions within membranes likely create complex structures associated with specific antimicrobial peptide mechanisms of action. However, the potential for a peptide to form quaternary structures is fundamentally related to the inherent composition and conformation of the peptide in its monomeric form. For example, peptides with well defined hydrophobic and hydrophilic domains may efficiently orient these facets toward respective membrane constituents, or corresponding domains in adjacent peptides. Such orientations may facilitate amphipathic peptides partitioning more deeply into the hydrophobic membrane core than would likely occur otherwise. Assembly of peptide complexes in this way may create the existence of transmembrane pores or channels, which meay be selective or non-selective. For example, peptide structures may assume configurations in which hydrophobic surfaces are aligned toward the membrane such that a hydrophilic channel is lined only by polar and charged facets of individual peptides.

A number of models for antimicrobial peptide membrane permeabilization have been proposed. Given the variability in microbial membrane ultrastructure, a given peptide may act via different mechanisms in distinct membrane environments. The models described below here have been largely derived from results examining activities of individual peptides or analogs against artificial membrane systems. It should be pointed out that there is no universal consensus among investigators in this regard. Therefore, the following models are compared with illustrate advances in proposed mechanisms of antimicrobial peptide action.

4. The Barrel-Stave Mechanism. The term barrel-stave describes the overall topology of a membrane channel formed in this mechanism of membrane permeabilization. In this model, a variable number of channel-forming peptides are positioned in a "barrel-like" ring around an aqueous pore. The "stave" term refers to individual transmembrane spokes within this barrel, which may be composed of individual peptides or peptide complexes. In this mechanism, the hydrophobic surfaces of α-helical or β-sheet peptides face outward, toward the acyl chains of the membrane, whereas the hydrophilic surfaces form the pore lining (Ehrenstein and Lecar, 1977; Breukink and Kruijff, 1999). The initial step in barrel-stave pore formation involves peptide binding at the membrane surface, most likely as monomers. Upon binding, the peptide may undergo a conformational phase transition, forcing polar-phospholipid head groups aside to induce localized membrane thinning. At this point, the hydrophobic portion of the peptide is inserted into the membrane to an extent corresponding to the hydrophobicity of the membrane outer leaflet. Positioning of the positively charged amino acids near the phospholipid head groups facilitates this process. When bound peptide reaches a threshold concentration, peptide monomers self-aggregate and insert deeper into the hydrophobic membrane core. Aggregation allows for a minimal exposure of the peptide hydrophilic residues to the hydrophobic membrane interior, as the peptides

adopt a transmembrane configuration. Continued accretion of peptide monomers results in further expansion of the membrane pore. Upon phospholipid translocation or relaxation of the pore, peptides are transported to the inner membrane leaflet aspect due to the concentration gradient of surface-bound peptide, as well as trans-negative $\Delta\psi$. An example of such a mechanism of action has been proposed for alamethicin (Sansom, 1991; Beven et al., 1999; Yang et al., 2001). Alamethicin-induced membrane conductance has been measured to proceed as a pattern of multistate conductance levels. This finding suggests the existence of pores with openings of various diameters, corresponding to channels composed of four or more transmembrane-spanning peptides. However, there remain relatively few peptides for which there is compelling evidence of a barrel-stave mechanism, despite this model having been proposed more than a decade ago. More recent studies often support the toroid pore model (see below; Yang et al., 2000, 2001). These newer data may reflect refinements in methodology and offer a clearer understanding of biophysical properties of transmembrane pores or channels that may incorporate lipid and peptide moieties.

5. The Toroid Pore or Wormhole Mechanism. One of the most well characterized peptide-membrane interactions is that of the toroid pore. A primary difference between the toroid pore and barrel-stave models is that in the former, lipids are intercalated with peptide in the transmembrane channel. Therefore, this structure has been referred to as a supramolecular complex and represents a membrane-spanning pore lined with polar peptide surfaces as well as phospholipid head groups. The toroid pore model has been deduced principally from experiments using α-helical peptides, including magainins and PGLa. In this model, peptides in the extracellular environment take on an α-helical structure as they interact with the charged and hydrophobic bacterial membrane. Helices are initially oriented parallel to the membrane surface as confirmed by NMR, fluorescence quenching, and CD (Hara et al., 2001). The hydrophobic residues of the bound peptides displace the polar head groups, creating a breach in the hydrophobic region and inducing positive curvature strain in the membrane (Hara et al., 2001). The introduction of strain and thinning further destabilizes the membrane surface integrity, making it more vulnerable to ensuing peptide interactions. At a threshold peptide-to-lipid ratio (e.g., estimated to be 1:30 for magainin), peptides orient perpendicular to the membrane. At this point, helices may begin to self-associate, such that their polar residues are no longer exposed to the membrane hydrocarbon chains. This transient and multimeric composite forms the dynamic peptide-lipid supramolecular or toroidal pore complex. The biophysical sequence of toroid pore formation by antimicrobial peptides has recently been examined in studies by Yang et al. (2000). Upon disintegration of the pore, some peptide becomes translocated to the cytoplasmic leaflet of the membrane (Uematsu and Matsuzaki, 2000), suggesting that toroid pore disassembly may be a key mechanism by which peptides enter the microbial cytoplasm to access potential intracellular targets.

Characteristic features of toroid pores include finite lifespan, discrete size, ion selectivity, and an inverse relationship between stability and peptide charge. Moreover, these properties affect the function of the pore itself. For example, the pore induced by magainin 2 has been estimated to be 2 to 3 nm and restrict transit of fluorescent particles depending on molecular weight (Ludtke et al., 1996; Matsuzaki et al., 1998). Peptide charge may also affect pore stability via intermolecular repulsion between positively charged side chains. Therefore, more positively charged peptides have been shown to induce pores with shorter half-lives (Matsuzaki, 1999).

6. The Carpet Mechanism. Models of nonspecific membrane permeabilization by antimicrobial peptides traditionally include diffuse effects that have been equated with detergents. In this sense, some peptides may act against microorganisms through a relatively diffuse manner, termed the carpet mechanism. However, peptides that employ this mechanism are not indiscriminate membrane detergents. In the carpet model, a high density of peptides accumulates on the target membrane surface. Phospholipid displacement changes in membrane fluidity and/or reductions in membrane barrier properties subsequently lead to membrane disruption. As in other models, peptides initially bind to the membrane mainly via electrostatic interactions, carpeting the phospholipid bilayer (Shai and Oren, 2001). However, no specific quaternary structure ensues in the carpet mechanism. Thus, when a threshold peptide density or concentration is reached, the membrane is subjected to unfavorable energetics, and membrane integrity is lost. From this perspective, membrane dissolution occurs in a dispersion-like manner that does not involve channel formation, and peptides do not necessarily insert into the hydrophobic membrane core. For example, cecropin P1, derived from moth hemolymph, appears to target microorganisms in this manner. Attenuated total reflectance FTIR spectroscopy indicates that this peptide initially orients parallel to the membrane and does not enter the hydrophobic environment. This orientation destabilizes phospholipid packing and causes membrane disruption due to a concentrated layer of peptide monomers on the surface (Sitaram and Nagaraj, 1999). Likewise, fluorescence spectroscopy indicates that the tryptophan-rich peptide indolicidin does not enter the bilayer to any significant degree, yet this peptide is an efficient antimicrobial agent (Rozek et al., 2000). It has been noted that most of the studies focusing on the carpet mechanism of membrane disruption utilize membrane models rich in PS (Matsuzaki, 1998, 1999). As with any mechanism of action, it is possible that alter-

nate results may be obtained using different membrane models or assay conditions.

E. Mechanisms of Cell Death

Another area of intensive focus regarding antimicrobial peptide biology relates to the precise mechanisms by which antimicrobial peptides cause cell death. A long-held paradigm for microbicidal action has been that peptides kill microorganisms by causing multiple and insurmountable defects in target microbial cell membranes. In this respect, peptides may create membrane pores in the organism as described above, leading to leakage of ions and metabolites, ensuing depolarization, loss of membrane-coupled respiration and biopolymer synthesis, and ultimately cell death. It is likely that these effects contribute to mechanisms by which antimicrobial peptides exert their effects. However, a mounting body of evidence supports additional or complementary mechanisms, wherein membrane permeabilization alone appears insufficient to cause cell death. Data supporting this latter concept come from studies documenting a clear dissociation between membrane perturbation and cell death. In these cases, cell killing may proceed with relatively little membrane disruption per se, due rather to disruption of intracellular processes.

1. Membrane Dysfunction. The cytoplasmic membrane is responsible for mediating many essential functions in microbial pathogens. Such functions include selective permeability and maintenance of gradients, cellular energetics driven by electron transport and oxidative phosphorylation in bacteria and mitochondria in eukaryotic pathogens, synthesis and cross-linking of peptidoglycan, chitin, or other biopolymers, motility, and processing or display of adhesins or other key virulence determinants. Conceivably, outer and/or cytoplasmic membrane dysfunctions caused by antimicrobial peptides may globally interfere with one or more of these functions, leading to cell death directly or indirectly.

Studies addressing the mechanisms of antimicrobial-peptide-mediated cell death indicate that, for some peptides, cell killing may begin as quickly as 2 to 3 min after initial exposure (Lehrer et al., 1989; Tossi et al., 1997). This swift cell death is attributed to rapid global consequences of membrane depolarization, loss of ion and metabolite gradients, and the cessation of other essential functions such as respiration (Blondelle et al., 1999; Hancock and Chapple, 1999). In Gram-negative bacteria, antimicrobial peptides likely interact independently with the outer and inner membranes. For example, Lehrer et al. (1989) demonstrated that human defensins sequentially permeabilize the outer then inner membrane. This sequence has been shown to occur in a variety of studies where penetration of the cytoplasmic membrane is correlated with the onset of cell death as measured using otherwise impermeant substrates. Thus, the lethal consequences of defensin exposure are correlated specifically with perturbation of the inner membrane. For Gram-positive cells, exposure to antimicrobial peptides results in immediate increases in water and ion flow, an efflux of K^+ ions, swelling and osmotic dysregulation (Juretic et al., 1989; Ohta et al., 1992; Matsuzaki et al., 1997). These concepts relating to the rapid and generalized membrane effects of antimicrobial peptides are the subject of other excellent reviews (Kagan et al., 1994; Lehrer and Ganz, 1996).

The influence of bacterial membrane energetics on susceptibility to antimicrobial peptides was recently examined by Yeaman and colleagues. In these studies, the effects of platelet microbicidal protein-2 (PMP-2), thrombin-induced PMP-1 (tPMP-1), and human neutrophil defensin hNP-1 were assessed against isogenic *S. aureus* strains exhibiting inherent $\Delta\psi$ of -150 mV versus -100 mV (Yeaman et al., 1998). Investigations focused on the relationship among $\Delta\psi$, membrane permeabilization, depolarization, and lethality. In this study, the profile of membrane depolarization appeared to be specific to each peptide, and was linked to a relationship between mechanism and $\Delta\psi$ of the different *S. aureus* strains. However, membrane permeabilization, depolarization, and cell killing by platelet microbicidal proteins were uniformly greater against the strain bearing the increased $\Delta\psi$ (-150 mV). In contrast, the staphylocidal activity of defensin hNP-1 was not significantly different against these two *S. aureus* strains.

Given that microbial cell membranes are responsible for multiple and essential functions, it is not surprising that cell death due to antimicrobial peptides has been primarily attributed to membrane dysfunction. However, it should be emphasized that membrane perturbation alone may not be sufficient to effect killing of microbial pathogens by antimicrobial peptides. This point has been underscored in two recent investigations. Koo et al. (2001) showed that permeabilization alone does not invariably result in staphylococcal death due antimicrobial peptides. In these studies, diverse peptides with varying staphylocidal potencies exhibited disparate extents of membrane permeabilization and cell killing. These differences suggest that diversity exists in mechanisms of action with respect to the relationship between membrane perturbation and staphylocidal activity of distinct peptides. Similar studies showed that gramicidin S rapidly depolarizes *Pseudomonas aeruginosa* cytoplasmic membranes as indicated by reduction in $diSC_35$ fluorescence (Zhang et al., 2000). However, these cells were relatively resistant to killing by this agent. In contrast, polymyxins B and E1 failed to cause significant $diSC_35$ deflorescence but rapidly killed the test organism. These observations support the concept that membrane perturbation and cell killing may be independent events that occur individually or complementary to other mechanisms of antimicrobial peptide action. This idea is consistent with the hypothesis that organisms capable of evoking rapid responses to peptide-induced stress may be particularly well adapted to

reverse or compensate for membrane dysfunction to circumvent irreversible cell death (see *Section IV.*).

2. Inhibition of Extracellular Biopolymer Synthesis. Inhibition of peptidoglycan, chitin, or other macromolecular synthesis may also be an important mechanism of antimicrobial peptide action. For example, peptidoglycan biosynthesis is integrally related to membrane integrity and function. Peptidoglycan precursors are activated and transported across the cytoplasmic membrane, and cross-linking occurs in the immediate proximity of this setting. As described above, cationic or other peptides likely perturb membrane and peptidoglycan synthesis integrity, such that direct or indirect inhibition of peptidoglycan precursor synthesis, translocation, and/or cross-linking may result. Given their greater peptidoglycan content, Gram-positive organisms may be particularly susceptible to this putative mechanism of action; however, testing of this hypothesis awaits further investigation. However, seminal plasmin, an antimicrobial protein from bovine seminal plasma, inhibits peptidoglycan synthesis in *E. coli* (Chitnis and Prasad, 1990). Interestingly, interference with peptidoglycan synthesis was observed to precede and occur independently of growth inhibition. Likewise, fungal biopolymers such as chitin may be similarly or uniquely vulnerable to inhibition by antifungal peptides.

3. Inhibition of Intracellular Functions. Although membrane perturbation almost certainly contributes to antimicrobial peptide mechanisms of action, recent studies suggest that disruption of key intracellular processes may contribute to or be required for cell death (Lehrer et al., 1989; Park et al., 1998; Sharma et al., 1999). These concepts imply a temporal and functional dissociation of membrane permeabilization, depolarization, and target cell viability. In some cases, microorganisms may survive for extended periods of time following membrane permeabilization, suggesting that non-membranolytic mechanisms are responsible for cell death. In studies by Xiong and coworkers using tPMPs, *S. aureus* cells remained viable long after rapid membrane permeabilization. tPMP-mediated inhibition of DNA and/or RNA synthesis corresponded temporally with cell death but was not observed until 30 or more minutes after membrane permeabilization (Xiong et al., 2002). Interestingly, staphylocidal effects did not appear to result from global cellular dysfunctions, since protein synthesis was inhibited to an equivalent extent in strains susceptible or resistant to tPMP-1. Moreover, pre-exposure to agents that selectively inhibit protein synthesis (30 S or 50 S subunit inhibitors) or DNA metabolism (DNA gyrase) mitigated subsequent tPMP-1 induced killing of an otherwise susceptible *S. aureus* strain in vitro. These findings implicate a direct inhibition of nucleic acid synthesis by tPMPs. The relatively strong negative charge of nucleic acids is consistent with the hypothesis that cationic peptides bind to and inhibit these molecules, not unlike histone proteins. Kragol et al. (2001) recently showed that the insect antibacterial peptides pyrrhocoricin, drosocin, and apidaecin inhibit the bacterial heat shock protein DnaK, and inhibition of this protein is associated with cell death. It is possible that pyrrhocoricin may also prevent chaperone-assisted folding of proteins in susceptible organisms. Similarly, buforin II has been reported to penetrate microbial cell membranes and interfere with intracellular functions (Park et al., 1998). The antimicrobial peptide, microcin B17, is also believed to inhibit an intracellular target within *E. coli*. This peptide has been suggested to specifically inhibit DNA replication by targeting DNA gyrase. The specificity of this intracellular mechanism of cell death was demonstrated in a recent study in which mutants resistant to killing by microcin B17 were found to have a single point mutation in DNA gyrase (del Castillo et al., 2001; also, see below).

Antimicrobial peptides may also penetrate fungal pathogens to effect lethal mechanisms. For example, the glycine-rich antifungal peptide tenacin-3 quickly enters the *C. albicans* cytoplasm via an energy-dependent mechanism that is influenced by target cell metabolic status and ionic environment (Kim et al., 2001). Yet, this peptide does not appear to induce membrane permeabilization or depolarization in doing so. Subsequent to internalization, tenecin-3 is uniformly dispersed within the cytoplasm, temporally corresponding to loss of cell viability. Antimicrobial peptides may also target and inhibit intracellular organelles found within fungal pathogens. Given their phylogenetic, structural, and functional similarities, it is reasonable to hypothesize that antimicrobial peptides interact with mitochondria in a manner very similar to bacteria. Moreover, specific evidence has been generated to support this concept. Helmerhorst et al. (1999) found that exposure to the cationic peptide histatin-5 caused a depletion in mitochondrial $\Delta\psi$ in *C. albicans*. Furthermore, fluorescein isothiocyanate-labeled peptide colocalized with a specific mitochondrial dye, and the uptake of histatin-5 was mitigated by inhibitors of respiration in vitro. These data were interpreted to suggest that this peptide internalizes within *C. albicans* and specifically targets to the energized mitochondrion.

The above observations suggest that peptide-mediated cell death may occur as a result of several independent or cooperative mechanisms of action; the latter phenomenon has been referred to as a "multi-hit process" (Zhang et al., 2000). Furthermore, peptides may kill the same species via more than one mechanism of action, depending on individual factors such as growth phase, tissue localization, and the presence or absence of other immune mechanisms or synergistic exogenous antimicrobial agents. From these perspectives, antimicrobial peptides may have multiple and complementary mechanisms of action necessary to inhibit or kill a wide variety of pathogens in diverse physiologic settings while suppressing the ability of the pathogen to avoid these mechanisms.

F. Synergy among Antimicrobial Peptides

To minimize experimental variability, microbiological and biophysical studies typically examine the biological activities of individual antimicrobial peptides in isolation. However, as it inevitably occurs in Nature, antimicrobial peptides may interact simultaneously with microbial pathogens in a variety of settings, including complex mixtures within phagolysosomes or into equally complex extracellular milieus. Therefore, antimicrobial peptides likely interact with one another, with microorganisms, and with host molecules prior to or at these sites. At present, a number of studies suggest that such heterologous peptide interactions may indeed be important to overall antimicrobial activity. For example, Tang et al. (2002) found that two antimicrobial peptides from human platelets, platelet factor-4 and connective tissue activating peptide-3, synergistically inhibit *E. coli*. These peptides appear to be generated simultaneously from activated platelets, thus their synergy is believed relevant in vivo. Likewise, studies of magainin 2 and PGLa from *Xenopus laevis* skin suggest such a similarly favorable interaction. The minimum inhibitory concentration for either peptide alone was $\sim$ 40 μg/ml; however, their minimum inhibitory concentration in combination was reduced by 20-fold (Westerhoff et al., 1995). Furthermore, in chemical cross-linking studies, these peptides form a parallel heterodimeric complex with a 1:1 stoichiometry (Hara et al., 2001). Pores formed by this heterodimer are more stable than those formed by either peptide alone. Synergism has also been demonstrated with magainin 2 plus tachyplesin (Kobayashi et al., 1991), the frog dermaseptins (Mor et al., 1994), and with helical antimicrobial peptides in combination with nalidixic acid (Zhang et al., 1999). Yan and Hancock (2001) have demonstrated that various antimicrobial peptides function synergistically with lysozyme in vitro. Taken together, the above findings substantiate multiple mechanisms by which antimicrobial peptides effect target cell killing. Undoubtedly, as experimental methodologies become more refined, future studies will more clearly assess the complex interactions among multiple antimicrobial peptides and their targets in situ.

G. Themes in Mechanisms of Action of Antimicrobial Peptides

As outlined above, antimicrobial peptides exert multiple and simultaneous effects that likely account for their generally rapid and potent antimicrobial activities. These actions are often microbicidal, resulting from irreversible and overwhelming disruption in essential cellular structure and/or function. In concept, antimicrobial peptide mechanisms of action adopt parallel themes despite wide diversities among source, composition, and conformation. These common themes seem to integrate the following events: 1) initial interaction with target cells due to electrostatic, hydrophobic, or other affinities based on biochemical and biophysical correspondence; 2) conformational phase transition in the framework of the target membrane (e.g., transition to α-helical conformational dynamics); 3) accumulation to a threshold stoichiometry facilitating active peptide monomer or multimer nonspecific membrane disruption (i.e., carpet mechanism), or self-association and ensuing pore or channel formation (i.e., barrel-stave or toroid pore mechanisms); 4) transient or prolonged membrane disruption yielding permeabilization, depolarization, and related perturbations that may cause direct and indirect dysfunction; and 5) peptide translocation across the membrane to access and inhibit intracellular targets (see Fig. 3).

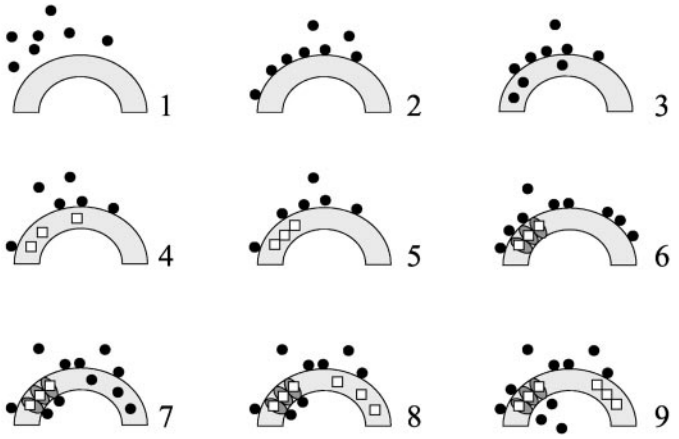

FIG 3. Integrative model of antimicrobial peptide mechanisms of action. Recurring themes in antimicrobial peptide mechanisms and the sequence of events associated with inhibition or killing of pathogens include: 1) initial electrostatic and hydrogen bond attraction; 2) subsequent hydrophobic interactions as the peptides contact the target surface; 3) accumulation and threshold concentration driving initial peptide conformational dynamics and early membrane deformation; 4) further peptide conformational phase transition and insertion within the membrane core; 5) self-association and multimerization; 6) formation of quaternary peptide complexes such as barrel-stave or toroid pore configurations; 7) translocation of peptide to the inner facet of the cytoplasmic membrane; 8) ongoing peptide accumulation and interactions as described above; and 9) access and targeting of essential intracellular structures and functions. Native antimicrobial peptides are indicated as dark spheres, whereas activated or conformation-transformed peptides are depicted as light squares.

IV. Mechanisms of Antimicrobial Peptide Resistance

Nature hates monoply. . .every excess causes a defect—every defect an excess. . .
Ralph Waldo Emerson

Microbial pathogens occupy and exploit a diverse variety of tissues and niches where they must confront antimicrobial peptide-mediated host defenses to survive. Thus, it is unrealistic to expect that no microbial pathogens are able to resist antimicrobial peptides. Rather, it is essential to understand whether a pathogen resists a given peptide, and if so, through constitutive or inducible mechanisms. As with any anti-infective agent, the answer to this question may have important implica-

tions regarding the potential use of antimicrobial peptides as agents or models in development of novel therapeutic agents to prevent or treat infection (Fig. 4).

A. Constitutive and Inducible Resistance

Pathogens capable of surviving exposure to antimicrobial peptides appear to employ two fundamentally distinct strategies: constitutive resistance versus inducible resistance. Constitutive (passive) mechanisms of resistance refer to inherent properties of an organism that confer resistance and are normally expressed even in the absence of peptide exposure. Alternatively, inducible (adaptive) resistance mechanisms include those triggered in response to the antimicrobial peptide or the target cell stresses it causes. In many respects, these strategies exist as a continuum of coordinate response systems that provide pathogens with the greatest likelihood of survival in diverse contexts containing antimicrobial peptides.

B. Constitutive (Passive) Resistance

An interesting observation yet to be fully explained is that of the inherent ability of certain microorganisms to resist killing by diverse types of antimicrobial peptides. For example, in a variety of studies, *Serratia, Proteus,* and *Providencia* species often prove to be refractory to inhibition or killing by cationic peptides (Viljanen and Vaara, 1984). *Burkholderia* (formerly *Pseudomonas*) species also exhibit exceptionally broad resistance to antimicrobial peptides in vitro (Manniello et al., 1978). These examples illustrate the likelihood that certain microbial pathogens are inherently more resistant to antimicrobial peptides due to stable structural or functional properties or pathogenesis strategies.

1. Inherent Mechanisms of Resistance to Antimicrobial Peptides. The molecular basis for comprehensive peptide resistance is not clear. However, several intriguing observations may provide insights into the possible reasons. At some point in their mechanism of action, antimicrobial peptides interact with the outermost sur-

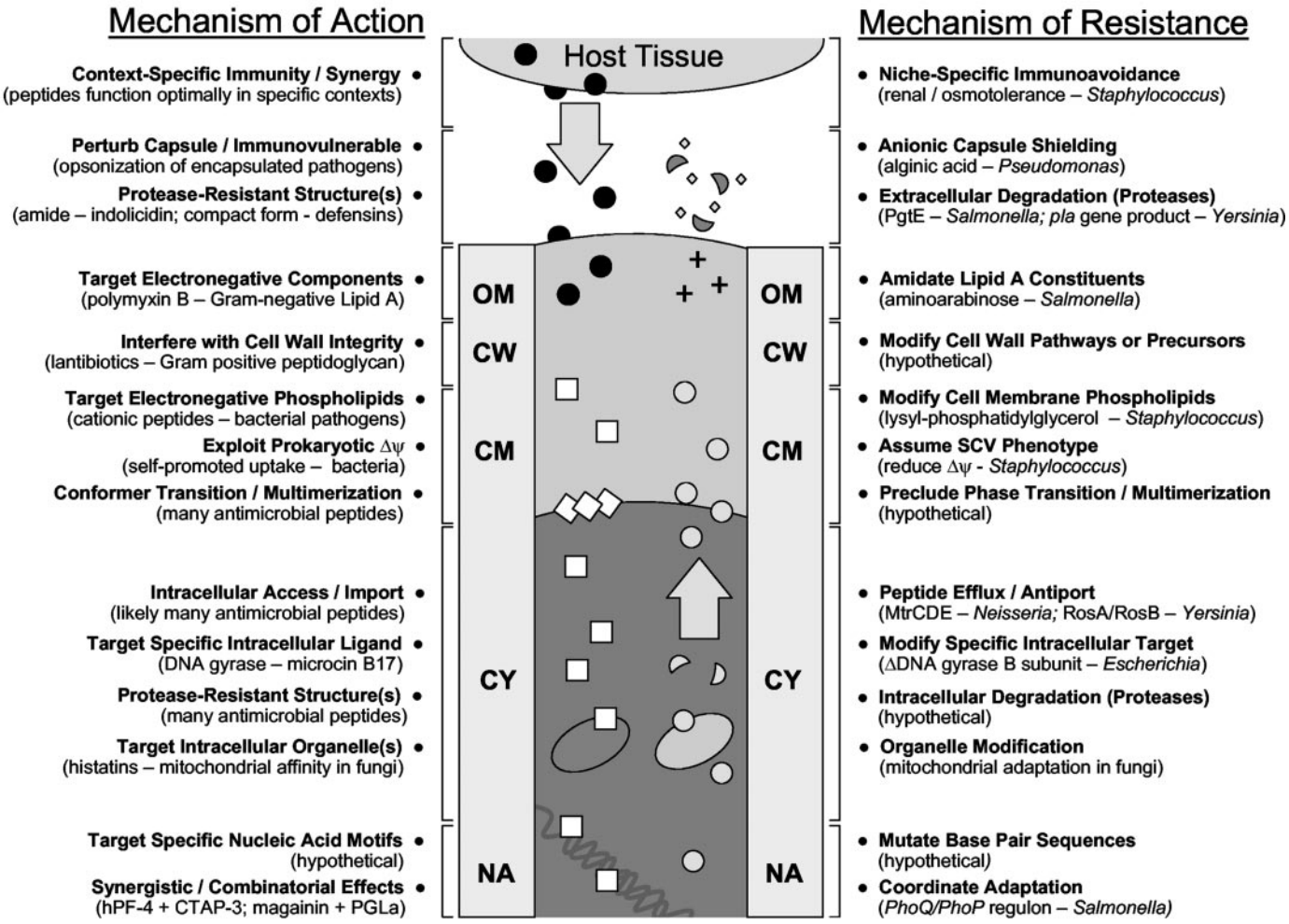

FIG 4. Parallels in antimicrobial peptide mechanisms of action and resistance. Key: OM, outer membrane; CW, cell wall; CM, cytoplasmic membrane; CY, cytoplasm; NA, nucleic acid. Specific mechanisms of antimicrobial peptide action are denoted with symbols consistent with Fig. 3. Other symbols used are as follows: ovals, organelles; double helix, nucleic acids; light spheres, inactive peptides; crescents, degraded peptides. Specific examples of mechanisms of action or resistance, listed in parentheses, are representative only and should not be considered exhaustive.

face of the target pathogen. Thus, it is conceivable that such surfaces inherently lack electrostatic affinity for, or may even repel, cationic antimicrobial peptides. As will be described below, certain staphylococcus species constitutively express membranes with reduced negative charge. In *Enterococcus*, resistance to antimicrobial peptides has been associated with unusual susceptibility and resistance patterns to conventional antibiotics that target their cell membrane or wall. For example, Cashman et al. (1998) demonstrated that *Enterococcus* species exhibit broad resistance to a panel of cationic antimicrobial peptides and heavy metal ions. Furthermore, these investigators identified an inverse correlation between glycopeptide susceptibility and resistance to cationic antimicrobial peptides or oxidized metal ions among enterococcal clinical isolates. This finding suggested that mechanisms conferring inherent resistance to antimicrobial peptides may predispose some pathogens to the inhibitory effects of unrelated agents.

Phylogenetic observations also support the concept that distinct microorganisms possess constitutive structural features that confer antimicrobial peptide resistance. In an interesting study, Nahaie et al. (1984) examined the constitutive phospholipid composition of a group of *Staphylococcus* species. Most species examined displayed polar lipid profiles consisting predominantly of PG and di-PG (CL). However, among the organisms tested, *S. aureus* was unique in having a lipid composition enriched in unsaturated menaquinones with eight isoprene units, and lysyl-PG, a derivative of PG that is considerably less electronegative.

Insights consistent with the above interpretations have been derived from studies examining stable resistance in *S. aureus* to tPMPs. For example, Wu et al. (1994) demonstrated that *S. aureus* strains resistant to tPMP-1 in vitro exhibit an enhanced propensity to cause human endocarditis. Ensuing studies (Dhawan et al., 1997; Bayer et al.,1998) likewise demonstrated that *S. aureus* strains resistant to tPMP-1 produce more extensive experimental endocarditis and metastatic sequelae than isogenic susceptible counterparts. Bayer et al. (2000) then compared parameters of membrane structure and function in genetically related *S. aureus* strains differing in tPMP-1 susceptibility or resistance. Results demonstrated that a tPMP-1-resistant strain exhibits a significant increase in unsaturated membrane lipids, compared with its tPMP-1-susceptible counterpart. The resistant strain had correspondingly higher degrees of membrane fluidity as assessed by fluorescence polarization. These data suggest that constitutive alterations in cytoplasmic membrane structure or function may be key to inherent antimicrobial peptide resistance in *S. aureus*. Importantly, analogous modifications in the outer membrane of some Gram-negative bacteria are also hypothesized to preserve membrane integrity in the presence of antimicrobial peptides (Guo et al., 1998). Thus, intrinsic characteristics of microbial phospholipid membranes are likely inseparably related to constitutive antimicrobial peptide resistance.

2. Altered Membrane Energetics. The activities of several types of antimicrobial peptides have been shown to be influenced by target cell growth phase or transmembrane potential. For example, the type-I (highly cationic) α-defensins appear to exert equivalent antimicrobial potency against metabolically energetic or quiescent bacteria (Lehrer and Ganz, 1996). However, type-II defensins exert maximal antimicrobial activity against highly energized cells. These distinctions illustrate the concept that antimicrobial peptides may have significantly reduced potencies against organisms with inherently low $\Delta\psi$ or that have the capability to adapt to such a status.

As described above, *S. aureus* strains with constitutive reductions in $\Delta\psi$ display reduced susceptibilities to some but not all antimicrobial peptides (Yeaman et al., 1998). These results suggest that the ability of *S. aureus* to subvert peptide-induced membrane dysfunction and cell death likely involves altered membrane energetics as they relate to peptides with specific mechanisms of action. More broadly, these observations reflect selective antimicrobial versus mammalian cell toxicities of peptides corresponding to target cell $\Delta\psi$. Similar relationships in antimicrobial peptide resistance have also been observed in the fungal pathogen, *C. albicans*. Gyurko and colleagues studied the histatin-5 susceptibility of petite mutants (analogous to small colony variants in bacteria; see below) of *C. albicans,* deficient in respiration resulting from mutations in mitochondrial DNA (Gyurko et al., 2000). These mutants were significantly more resistant to histatin-5 when compared with their parental counterpart. In addition, histatin-5 killing activity was significantly reduced against the parental strain when exposed to inhibitors of respiration and at low temperature. These findings suggest that the anticandidal activities of histatin-5 require a threshold level of cellular energetics involving mitochondrial ATP synthesis. It follows that fungal pathogens may suppress or resist antimicrobial peptide mechanisms by assuming a dormant metabolic status (see below).

The above themes derive from the likelihood that transmembrane potential is important for some antimicrobial peptides as they interact with and execute mechanisms of action against microbial pathogens. These observations may reveal insights into both selective toxicity and potential antimicrobial spectra of such peptides. Nonetheless, it is also highly likely that some microbial pathogens employ regulation of energy status as a means of subverting the mechanisms of antimicrobial peptides.

3. Electrostatic Shielding. Many virulent bacterial or fungal pathogens rely upon elaboration of a capsule as a means of adherence to tissue or avoidance of opsonization and phagocytosis. Thus, capsule production is an important virulence factor Prticularly among microor-

ganisms that colonize or infect the mammalian bloodstream, respiratory tract, and gastrointestinal mucosa. Yet, there is relative little information available from which to assess the role of pathogen capsule or glycocalyx expression related to resistance to antimicrobial peptides. Capsular compositions vary widely among different organisms. However, the glycocalyx of many microbial pathogens is often composed of an anionic complex of carbohydrate and phosphate. Thus, it is reasonable to hypothesize that matrices such as these sequester cationic antimicrobial peptides, preventing them from accessing their intended targets.

Pseudomonas aeruginosa exhibits an unusual propensity to infect tissues in which dysfunctional salt transport results in abnormal tissue physiology, abnormal phagocyte function, and increased local ionicity. Examples of such settings are found in burn wounds, and airways of cystic fibrosis patients. In such microenvironments, chronic pseudomonal infection and persistent inflammation often leads to fibrotic transformation of tissue and may trigger fulminant sepsis. Alginic acid is a highly anionic capsular exopolysaccharide produced by virulent strains of *P. aeruginosa*. Friedrich et al. (1999) have shown that purified alginate interferes with the antimicrobial activities of cationic antimicrobial peptides in vitro. Thus, the electronegative alginate glycocalyx is believed to sequester cationic antimicrobial peptides present in mucosal secretions, before they can reach the pseudomonal membrane, thereby conferring resistance to peptide-mediated killing. The observation that many cationic antimicrobial peptides are inhibited in the presence of high concentrations of mono- and divalent cations may also augment the protective effect of alginate. Along with capsular shielding, *P. aeruginosa* employs mechanisms of resistance to antimicrobial peptides through inherent modifications of outer membrane structures (see below). These facts suggest shielding against antimicrobial peptides is one of several mechanisms by which *P. aeruginosa* avoids antimicrobial peptides and causes persistent infections in patients with cystic fibrosis. Similar mechanisms of antimicrobial peptide resistance may be employed by other respiratory tract or mucosal pathogens that elaborate capsules or biofilms, including *K. pneumoniae, Haemophilus influenzae, Legionella pneumophila, Streptococcus pneumoniae, S. aureus,* and *Bacillus anthracis.*

4. Niche-Specific Resistance. The concept of niche-specific resistance to antimicrobial peptides integrates many aspects of constitutive resistance described above. This model is based on the hypothesis that some pathogens may resist antimicrobial peptides to which they would otherwise be susceptible, simply by virtue of their affinity for or exploitation of certain anatomical or physiological niches. Several examples can be used to illustrate this concept.

As outlined above, reductions in cellular energetics appear to negatively influence the activity of antimicrobial peptides that rely on $\Delta\psi$ for target affinity and/or mechanism of action. Proctor and coworkers have shown that *S. aureus* may utilize such a strategy to survive within the interior of vascular endothelial cells (Proctor et al., 1994; Proctor and Peters, 1998). Small colony variants (SCVs) of *S. aureus* typically exhibit defects in electron transport, and their uptake of cationic antimicrobial agents is generally reduced. Interestingly, development of SCVs is apparently advantageous in the microenvironment within vascular endothelial cells (Vesga et al., 1996). It is important to emphasize that intracellular *S. aureus* appears to adopt the SCV phenotype at a rate approximately 10^4-fold more frequently than identical cells not subjected to this milieu. Moreover, as detailed above, an *S. aureus* mutant with inherently lower $\Delta\psi$ was significantly more resistant to antimicrobial peptide effects compared with its parental counterpart. Taken together, this evidence suggests that *S. aureus* cells may enter host cells, reversibly assume an SCV phenotype to circumvent killing by antimicrobial peptides (as well as other antimicrobial agents), and thereby persist within this setting to await future pathogenesis strategies or opportunities.

Other examples of niche-specific resistance to antimicrobial peptides relate to the potential for organisms to exploit specific anatomic settings as a means of circumventing the effects of antimicrobial peptides. For example, as indicated above, *P. aeruginosa* is an opportunistic pathogen that preferentially colonizes tissues having abnormal osmotic or ionic strength. Results consistent with this theme have also been obtained from studies examining *S. aureus* or *C. albicans* proliferation within distinct tissues in an experimental model of infective endocarditis (Yeaman et al., 1996; Dhawan et al., 1997, 2000). In these studies, organisms susceptible to tPMP-1 exhibited reduced propensity to proliferate within cardiac vegetations or splenic abscesses compared with isogenic tPMP-1-resistant strains. However, in the kidney, this discrepancy in proliferation between tPMP-1-susceptible and -resistant strains was absent. These results suggest that the protective role of tPMP-1 may be diminished in relatively high ionic strength microenvironments, such as the kidney, compared with cardiac or splenic tissues. Differences in other immune mechanisms in these settings may also contribute to this outcome. Observations such as these underscore the likelihood that some pathogens likely exploit specific tissues or physiologic microenvironments to subvert the host defense contributions of antimicrobial peptides.

C. Inducible (Adaptive) Resistance

Most pathogens encounter numerous potentially lethal host defense mechanisms, including antimicrobial peptides, which must be negotiated for the pathogen to survive and proliferate. Thus, many pathogenic microorganisms have evolved an array of inducible countermeasures intended to suppress or subvert the effects of

these host defense mechanisms. Adaptive responses may range from rapidly inducible activation of virulence factors and responses acutely required for survival to chronic strategies that eventuate toward permanent modifications. Moreover, most mechanisms of peptide resistance represent microbial responses that are diametrically opposed to mechanisms of peptide action. The following discussion considers themes in antimicrobial peptide resistance from these perspectives.

1. Coordinate Microbial Responses to Antimicrobial Peptide Stress. Exposure to antimicrobial peptides represents a potentially lethal stress condition, against which rapid and conserved response pathways have evolved. This cascade of responses is often referred to as the starvation/stress response. For example, in bacteria, inducible resistance to antimicrobial peptides is largely controlled through sophisticated sensor-transducer response systems. Among the first to observe this phenomenon were Fields et al. (1989), who determined that a specific genetic locus of the pathogen *S. typhimurium* is integral to intracellular survival in macrophages and virulence in mice by conferring resistance to a defensin peptide. Several two-component regulatory mechanisms are now recognized and believed to activate a diverse spectrum of adaptive survival responses in a variety of pathogens. Recently, Oh et al. (2000) demonstrated that sublethal levels of cecropins induce hyperosmotic stress response systems in *E. coli*. However, Hong and coworkers (2003) found that sublethal concentrations of cecropin A prompted a pattern of genomic response in this organism that is distinguishable from that of lethal concentrations, and distinct from global stress response systems such as heat-shock, hyperosmotic, or oxidative response paradigms. Thus, transcriptional profiling studies such as this may advance our understanding of coordinate responses to antimicrobial peptide-induced stress.

It is now known that activation of the PhoP/PhoQ regulon yields global protein, phospholipid, and lipopolysaccharide modifications in Gram-negative pathogens that mitigate actions of antimicrobial peptides. For example, numerous laboratories have shown that the PhoP/PhoQ system is integral to activating transcription of genes in *Salmonella* that encode inducible surface and secretory proteins, enzymes that modify lipopolysaccharide, lipid and protein constituents of the outer membrane, and proteases that likely degrade certain antimicrobial peptides (see below).

Gunn and Miller (1996) have shown the PhoP/PhoQ system to regulate phoP-activated genes (*pag*), and phoP-repressed genes (*prg*). The respective coordinate effects of these gene clusters are to coordinately upregulate expression of pathways that actively defend against antimicrobial peptides (e.g., proteases, synthetic and enzymatic modification of surface structures) and simultaneously suppress the expression of structural or functional features vulnerable to antimicrobial peptide actions. In parallel, a distinct two-component regulatory system, PmrA/PmrB, coordinates the expression of *pmrE* and *pmrH, F, I, J, K,* and *L* genes. These latter genes appear to be necessary for synthesis and addition of aminoarabinose onto lipid A in the Gram-negative outer membrane (Gunn et al., 1998), yielding greater cationic charge to the membrane, and increased resistance to cationic antimicrobial peptides.

The distinct PhoP/PhoQ and PmrAB regulatory systems also likely function in complement to degrade antimicrobial peptides and confer peptide resistance through surface and envelope modifications, respectively. For example, *pagB* interacts with PmrA/PmrB, activating this latter two-component regulon. Thus, the PhoP/PhoQ and PmrA/PmrB systems respond synergistically to protect *Salmonella* species against antimicrobial peptides. The PagB-activated *pmrA-pmrB* operon confers *Salmonella* resistance to numerous cationic antimicrobial peptides, including polymyxin B, azurocidin, bactericidal/permeability increasing factor (BPI or CAP57), protamine, and polylysine. Furthermore, PhoP/PhoQ regulates *pagP*, encoding an acyltransferase that catalyzes lipid A palmitoylation in *Salmonella*. This outer membrane modification is believed to reduce antimicrobial peptide accessibility to the cytoplasmic membrane. In addition, the *pgtE* gene encodes a protease that independently increases resistance to antimicrobial peptides (see below). Importantly, *pgtE* and *pagP* in *Salmonella* yields a reduction in susceptibility to cationic antimicrobial peptides greater than is observed in strains possessing either gene alone (Gunn and Miller, 1996). Thus, reduced peptide accumulation via lipid A modification, along with elaboration of proteases such as PgtE, likely act synergistically to subvert the actions of antimicrobial peptides.

These complex systems may offer *Salmonella* protection against antimicrobial peptides both within and beyond the phagolysosome. Other Gram-negative pathogens possess similar two-component mechanisms that sense and respond to circumvent antimicrobial peptide functions. For example, analogous responses to antimicrobial peptide exposure have been observed in other *Salmonella* species (Zhou et al., 2001) and *Proteus mirabilis* (McCoy et al., 2001). Moreover, aminoarabinose modification of lipid A has been observed in *P. aeruginosa* (Ernst et al., 1999). Likewise, mutational inactivation of a *pagP* homolog in *L. pneumophila* results in sensitivity to a synthetic cationic antimicrobial peptide (Robey et al., 2001). Consistent with diversity among mechanisms of antimicrobial peptide structure and function, acylation of lipid A appears to differentially influence resistance to distinct types of antimicrobial peptides. For example, acylated lipid A reduces Gram-negative bacterial susceptibility to human LL-37 (Guina et al., 2000) and protegrin-1 but not to defensin NP-1 (Belden and Miller, 1994; see below). Likewise, analogous regulatory pathways are believed operative in

Gram-positive bacterial resistance to antimicrobial peptides (Peschel and Collins, 2001). Determining the extent to which these mechanisms independently or collectively, influence virulence will require continued further evaluation of genetically altered pathogen strains in experimental systems and animal models.

2. Adaptive Mechanisms of Resistance to Antimicrobial Peptides. Constitutive and inducible mechanisms of resistance to antimicrobial peptides are becoming clearer with the advent of genetically modified pathogens and the availability of reagent quantities of native or synthetic antimicrobial peptides. As with constitutive responses, it is not surprising that many of the mechanisms responsible for inducible resistance involve modifications of the pathogen envelope and/or extracellular facet of the cytoplasmic membrane directly offsetting mechanisms of peptide action. The following examples are intended to highlight recent insights and advances in these areas.

3. Proteases and Peptidases. One of the clearer examples of protease-mediated resistance to antimicrobial peptides can be illustrated from studies examining well characterized Gram-negative bacterial pathogens. As described above, PhoP/PhoQ-like systems regulate many of the countermeasures employed by bacteria to resist antimicrobial peptides. Among these, the PgtE protein was recently demonstrated by Guina et al. (2000) to be an outer membrane endopeptidase in *Salmonella*. This molecule was identified as having structural features consistent with the outer membrane protease families OmpT or protease VII of *E. coli* (Sugimura and Nishihara, 1988), and Pla of *Yersinia* (Sodeinde et al., 1992). These proteases specifically cleave peptides between paired basic residues and at the carboxy-terminal aspect of basic amino acid residues that precede a nonpolar residue. Thus, a variety of amphipathic and cationic antimicrobial peptides are potential substrates of PgtE protease. These properties distinguish OmpT/PgtE-type proteases from trypsin-like enzymes, with the former being optimally active at pH 6.0, sensitive to the inhibitor diisopropyl fluorophosphate, and to the divalent cations Cu^{2+}, Fe^{+2}, and Zn^{2+} (Sugimura and Nishihara, 1988).

PgtE and its E. coli homolog OmpT have recently been demonstrated to promote resistance to antimicrobial peptides in *Salmonella*, as well as *Escherichia*. For example, Stumpe et al. (1998) showed that expression of OmpT increases survival of *E. coli* grown in the presence of protamine, a DNA-binding and cationic antimicrobial peptide from salmon sperm. Mutant strains defective in protease genes *degP, ptr,* or *ompT* were generated in *E. coli*, and tested for their ability to degrade protamine in vitro. Interestingly, only the strain lacking *ompT* became hypersusceptible to protamine. This susceptibility was abolished by complementation with a plasmid carrying *ompT*. Additionally, OmpT appeared to be capable of degrading protamine in low or high Mg^{2+} concentrations, although degradation of the peptide was slower in conditions of higher ionic strength. These findings suggest that OmpT functions to degrade antimicrobial peptides at the extracellular facet of the outer membrane of *E. coli.* This function could provide *E. coli* with an important survival advantage in settings defended by innate immunity. For example, *ompT* in *E. coli* has been implicated as a key virulence factor in human urinary tract infections. Interestingly, the ability of *E. coli* to elaborate PgtE to subvert protamine activity has been linked with rapid uptake of K^+ ions, mediated by expression of the *trkA* gene (Stumpe and Bakker, 1997). Thus, reaccumulation of K^+ by protamine-treated cells induces protease PgtE expression, in turn degrading protamine.

It is intriguing to note that, in *Salmonella*, a *pgtE* deletion strain exhibited increased in vitro sensitivity to a panel of α-helical peptides that contain predicted OmpT cleavage sites. However, PgtE protease does not confer to *Salmonella* increased resistance to antimicrobial peptides exhibiting amphipathic β-sheet conformation induced by intramolecular disulfide bonds (e.g., defensins or protegrins). Therefore the three-dimensional structures of the latter peptides likely create steric hindrance protective against the activity of PgtE protease. As above, PgtE-mediated resistance to antimicrobial peptides presumbably acts in consort with surface modification resistance mechanisms in *Salmonella* or other pathogens (see below).

Other protease have also been implicated in antimicrobial peptide resistance of *S. aureus* and *E. coli* (Ulvatne et al., 2002). For example, the heat-shock serine protease DegP appears to mediate reduced susceptibility of *E. coli* to lactoferricin B in vitro. In addition, many other microbial pathogens elicit proteases that are believed to be important virulence determinants. For example, *Yersinia* and *Streptococcus* species liberate secretory proteases that may be involved in protecting them from antimicrobial host defenses. In *Yersinia*, inactivation of the plasmid *pla* gene encoding a surface protease logarithmically increases the median lethal dose for mice (Sodeinde et al., 1992). It is notable that lesions produced by the pla^- mutant accumulated significantly more inflammatory cells compared with the pla^+ counterpart strain. Likewise, *S. pneumoniae* secretes extracellular proteases believed integral to immuno-avoidance. For example, logarithmic phase pneumococci degrade complement protein C3 rapidly, through cell-associated activity that is independent of the presence of the polysaccharide capsule (Angel et al., 1994). Staphylococci also produce a variety of extracellular proteases (e.g., V8 protease) that are believed important in pathogenesis. While specific antimicrobial proteases have yet to be identified from these pathogens, it is likely that cationic or other antimicrobial peptides are substrates of one or more such resistance mechanisms.

It should be noted that antimicrobial peptides with specific structures conferring resistance to proteolytic degradation have recently been identified. For example, indolicidin is a relatively unique antimicrobial peptide originating from the cytoplasmic granules of bovine neutrophils. This small cationic peptide amide is composed of 13 amino acids, five of which (nearly 40% of the peptide) are tryptophan residues. The unusual composition of indolicidin differentiates it from many of the helical and sheet conformations commonly found in other antimicrobial peptides. Recently, Osapay et al. (2000) identified a synthetic modified form of indolicidin, termed X-indolicidin, produced by deprotonation of two indole side chains, yielding an intrachain ditryptophan configuration in which the Trp-6 and Trp-9 residues are covalently linked. Compared with indolicidin, X-indolicidin was more resistant to trypsin and chymotrypsin digestion, suggesting that ditryptophan stabilizes an indolicidin conformer resistant to certain proteases. However, although this synthetic form of indolicidin displayed unusual fluorescence and UV absorbance characteristics, it exhibited an identical sequence and antimicrobial activity compared with native indolicidin. Although these concepts have yet to be demonstrated in a physiological setting, observations such as these may provide key insights into the development of novel antimicrobial peptides that avoid proteases generated by microorganisms as a means of circumventing antimicrobial host defenses, or optimize peptide half-life necessary for therapeutic applications. Naturally occurring antimicrobial peptides may also possess inherent properties that resist proteolytic degradation. For example, Oren et al. (1999) have shown that LL-37, a cathelicidin peptide isolated from humans, is resistant to proteolytic degradation in solution, and when bound to artificial membranes simulating mammalian (zwitterionic) or bacterial (anionic) targets. Furthermore, their findings suggest a role for the N terminus in proteolytic resistance of this peptide. Collectively, the above studies indicate that antimicrobial peptides resistant to microbial degradation may exist naturally, or may be engineered in the development of novel therapeutic candidates.

4. Extracellular Structural Modifications. Antimicrobial peptides initially target and interact with microbial structures exterior to the cytoplasmic membrane. Thus, microbial pathogens have evolved mechanisms by which these targets may be modified to resist peptide targeting and circumvent the ensuing antimicrobial mechanisms.

Coordinate systems commonly regulate expression of countermeasures needed for pathogen survival in response to antimicrobial peptide exposure. Conceptually, this feedback mechanism must be rapidly inducible and triggered in part by the presence or action of the peptide itself. For example, significant fluctuations in Mg^{2+} or Ca^{2+} concentrations (intracellular or transcellular) induced by antimicrobial peptides have been suggested to activate the *Salmonella* PhoP/PhoQ locus (Groisman, 2001). Cationic antimicrobial peptides likely displace or mobilize these ions or perturb their transmembrane gradient as part of their mechanism of action against organisms unable to respond defensively. Therefore, it is likely that one mechanism of action of these peptides involves the inactivation and/or dysregulation of these or other resistance countermeasures (Groisman et al., 1997). The recent finding that the gene *mig-14* in *Salmonella enterica* is strongly induced by polymyxin B and protegrin-1 and functions in response to *phoP* suggests that bacterial resistance to antimicrobial peptides involves complex cascades of mechanisms (Brodsky et al., 2002).

Modifications of lipid A and LPS in Gram-negative Enterobacteriaceae have also been identified as a common mechanism of antimicrobial peptide resistance. These inducible responses include lipid A acylation (Guo et al., 1998), 4-amino-4-deoxy-L-arabinose and palmitate derivation of lipid A in *E. coli* similar to that seen in *Salmonella* (Zhou et al., 1999), aminoarabinose versions of LPS in *Pseudomonas* strains associated with cystic fibrosis (Ernst et al., 1999), and myristylation of LPS (Ernst et al., 1999). These mechanisms of resistance and regulatory mechanisms governing their expression are the topics of other excellent reviews (Groisman, 2001; Ohl and Miller, 2001). Taken together, these observations emphasize the numerous ways in which microbial pathogens may vary their surfaces to subvert cationic antimicrobial peptide binding or mechanisms of action.

Analogous to virulence regulons in other bacterial pathogens, the *Bvg* locus is believed to be integral to coordinating virulence factor expression and immunoavoidance strategies in *Bordetella* species. Banemann et al. (1998) have shown that mutation of the *bvgS* gene in *Bordetella bronchiseptica* renders mutants significantly more susceptible to antimicrobial peptides. The specific loci inactivated by transposon insertion were found to be highly homologous to the *Bordetella pertussis* genes integral to LPS biosynthesis, *wlbA* and *wlbL*. Moreover, these peptide-sensitive *B. bronchiseptica* mutants exhibited loss of LPS O-specific side chains. These findings suggest that O-specific glycosylation in LPS of *Bordetella* species may be important in resistance to antimicrobial peptides as this pathogen interacts with mucosal immunity. Analogous mechanisms likely confer reduced susceptibility to cationic antimicrobial peptides in Gram-positive bacterial pathogens (Peschel et al., 2000; Ganz, 2001).

More generally, evidence is accumulating to support the hypothesis that microbial pathogens colonizing specific biological niches modify their envelope to subvert antimicrobial peptide susceptibility. For example, Lysenko and coworkers found that *H. influenzae* expresses unusually high levels of phosphorylcholine to circumvent killing by antimicrobial peptides (Lysenko et al.,

2000). Phosphorylcholine is a component of LPS in some bacteria, and may mimic PC present in mammalian cell membranes. *H. influenzae* was shown to exhibit resistance to cationic antimicrobial peptide exposure correlating with the relative amount of phosphorylcholine present on its surface. Interestingly, these bacteria resisted killing by antimicrobial peptides only when provided choline, which is necessary for phosphorylcholine modification of LPS. Such envelope modifications may represent strategic themes in microbial resistance to respiratory tract antimicrobial peptide-based immune mechanisms that selectively target differences between host versus microbial membranes. In addition these findings emphasize the concept that microbial resistance to antimicrobial peptides may be difficult to reproduce in vitro, since resistance in vivo is likely dependent on strategies and conditions relevant to immunoavoidance in specific physiological niches.

5. Resistance Modifications of the Cytoplasmic Membrane. Among the earliest observations that microbial pathogens adaptively modify their cytoplasmic membrane to resist cationic antimicrobial peptides were made by Dorrer and Teuber (1977). In their studies, a shift of *Pseudomonas fluorescens* from phosphate-rich into phosphate-limited medium yielded a dramatic decline in cytoplasmic membrane PE, PG, and CL composition. Equally important was the observation that, concomitant with these changes in anionic phospholipids, a cationic ornithine-amide constituent emerged in membranes. Increasing resistance to polymyxin B paralleled the steady increase in the amount of this lipid. Furthermore, intact cells, in addition to isolated cytoplasmic or outer membranes of resistant organisms, displayed significantly reduced binding capacities for polymyxin B. These findings were taken as evidence that cationic polymyxin B exerts its antibiotic activity in part via high affinity binding to comparatively electronegative bacterial membrane constituents.

Recent studies have also demonstrated highly regulated antimicrobial resistance mechanisms involving cytoplasmic membrane modification in Gram-positive bacterial pathogens (Peschel et al., 2001). Enhanced *S. aureus* resistance to defensins and protegrins has been linked to lysine modification of PG present in the cytoplasmic membrane. The production of lysyl-PG was dependent on the presence and function of *mprF* in *S. aureus.* Moreover, an *mprF* mutant strain was more susceptible to killing by human neutrophils and exhibited reduced virulence in mice compared with the parental strain. Lysine-derivatized PG reduces net electronegativity of the cytoplasmic membrane and presumably diminishes affinity for or increases repulsion of cationic peptides. Interestingly, *mprF* in *S. aureus* is a gene believed to have close analogs in other human pathogens, including *Mycobacterium tuberculosis, P. aeruginosa,* as well as *Enterococcus faecalis.* These observations are consistent with increases in *S. aureus* cytoplasmic membrane fluidity shown to correlate with reduced susceptibility to tPMPs in vitro (Bayer et al., 2000; see above).

6. Efflux-Dependent Resistance Mechanisms. Efflux has also emerged as a mechanism by which microbial pathogens may resist antimicrobial peptides. In *Neisseria gonorrhoeae*, Shafer et al. (1998) have shown that resistance to antibacterial peptides of diverse structure is mediated in part by an energy-dependent efflux system termed mtr. Evidence also indicates the MtrCDE complex ejects antibiotics, dyes, and detergents, suggesting this mechanism protects the pathogen against mucosal or other endogenous or exogenous antimicrobials within and beyond the genitourinary tract. A similar mechanism has been shown to confer antimicrobial peptide resistance to *Yersinia* (Bengoechea and Skurnik, 2000). In this latter pathogen, efflux of antimicrobial peptides appears to involve a potassium antiporter system formed by the RosA and RosB proteins (Stumpe and Bakker, 1997; also see above). Importantly, the *RosA/RosB* gene regulon appears to be inducible upon exposure to antimicrobial peptides and may enhance the survival of the organism in the acidic and antimicrobial peptide-rich environment of the phagolysosome.

Efflux systems have also been associated with resistance to antimicrobial peptides in Gram-positive bacteria and fungal pathogens. For example, the plasmid-encoded gene *qacA* mediates staphylococcal resistance to multiple organic cations via a proton motive force-dependent efflux pump. Kupferwasser et al. (1999) demonstrated that an *S. aureus* plasmid containing *qacA* confers resistance to tPMP-1 in an otherwise susceptible parental strain. Specific deletions that inactivated the *qacA* gene construct reversed tPMP-1 resistance. Notably, the expression of *qacA* did not appear to impart cross-resistance to other structurally distinct cationic peptides, including a defensin, protamine, or the lantibiotics pep5 or nisin. Moreover, the presence of the *qacA* gene product may be sufficient to confer such resistance, without energy-dependent efflux per se. This possibility suggests that the QacA protein may modify the composition of the cytoplasmic membrane such that it is less amenable to disturbance by tPMP-1. Additionally, ABC-type transporters have been implicated in fungal resistance to antimicrobial peptides and other antifungal agents (Andrade et al., 2000). Taken together, the above findings indicate that microbial pathogens have evolved structure-specific and energy-dependent mechanisms to subvert actions of antimicrobial peptides.

7. Modification of Intracellular Targets. A temporal and functional separation between initial membrane interaction and subsequent cell death supports the concept that antimicrobial peptides access and inhibit essential microbial targets interior to the cytoplasmic membrane. Accordingly, new data indicate the existence of complex mechanisms that specifically modify these intracellular targets to confer resistance. For example,

del Castillo and colleagues have identified a mutation in the *gyrB* gene that is associated with a significant reduction of *E. coli* susceptibility to microcin B17, an antimicrobial peptide believed to inhibit DNA replication (del Castillo et al., 2001). This mutation yields replacement of tryptophan 751 by arginine in the GyrB polypeptide, ostensibly reducing microcin B17 targeted inhibition of DNA gyrase. These studies represent areas of research focusing on the growing awareness that antimicrobial peptides exert mechanisms of action that transcend their initial interaction with phospholipid bilayers.

V. Prospectus: Therapeutic Targets of Antimicrobial Peptides

Recent studies have significantly advanced our understanding of the mechanisms of antimicrobial peptide action and resistance. Such advances have revealed new insights into potentially vulnerable microbial structures and functions that may facilitate the discovery and development of novel anti-infective agents or strategies. For example, new efforts are increasingly focused on targeting sensitive microbial structures or functions, disabling pathogen adaptive response mechanisms, and exploiting specific contexts or virulence factors characteristic of infection. These approaches may take advantage of unique situations associated with pathogenesis or host response to govern and optimize antimicrobial peptide targeting and selective toxicity. The following comments highlight concepts emerging in these areas.

A. Reconstitution or Potentiation of Conventional Antibiotic Efficacy

The most obvious potential therapeutic applications for antimicrobial peptides or derived mimetics relate to their use to reconstitute or amplify the antimicrobial efficacies of conventional antibiotics. For example, given their propensity to permeabilize target microbial membranes, antimicrobial peptides may facilitate conventional agents in overcoming access-based resistance mechanisms such as reduced uptake or enhanced efflux. Alternatively, peptides that interact with intracellular processes or targets could be engineered or selected to noncompetitively augment the targets and mechanisms of classical antibiotics. Moreover, the potential for synergistic activities among antimicrobial peptides in combination is only recently becoming more fully appreciated (e.g., Tang et al., 2002). While many convincing examples of these favorable interactions have been observed in vitro, the challenge remains to understand and apply the mechanistic foundations thereof, which will guide the identification and formulation of optimal peptide-antibiotic and/or peptide-peptide combinations or their equivalents in vivo.

B. Unique and Specific Microbial Targets

Structural and functional attributes unique to antimicrobial peptide interactions with pathogens offer new insights for development of novel anti-infective agents derived from these ancient host defense molecules. Molecular determinants that are emerging as potential targets for antimicrobial peptide strategies include microbial receptors, metabolic processes, energetics, or essential pathways, virulence factors such as surface adhesins and envelope proteins, as well as intracellular targets such as ribosomes, mitochondria, or nucleic acids. In addition, antimicrobial peptides may be useful in potentiating microbial targets vulnerable to related immune mechanisms. For example, opsonophagocytic enhancement of organisms exposed to antimicrobial peptides has been hypothesized to augment the ability of phagocytes to kill microorganisms intracellularly (Yeaman, 1997). Furthermore, increasing awareness of the close structural and functional relationship between antimicrobial peptides and certain cytokines suggests a convergence of their roles in antimicrobial host defense. For example, the chemokines RANTES, platelet factor-4, and IP-10 are among the cytokines that have now been shown to exhibit antimicrobial activity in vitro (Yeaman, 1997; Cole et al., 2001; Tang et al., 2002). Exploiting these developments will require further dissection of the molecular basis underlying peptide differentiation of appropriate microbial targets from those of hosts, emphasizing selective activity without concomitant host cytotoxicity. In these respects, experimental approaches integrated with molecular modeling of critical structure-activity relationships in the mechanisms of antimicrobial peptide activities will continue to play important roles.

C. Targeting Strategic Microbial Response Pathways

It is likely that antimicrobial peptides target constitutive and inducible properties of pathogens as targets of their mechanisms of action. For example, modification of characteristic membrane energetics, surface ligands, or expression of virulence factors may be avenues exploited by antimicrobial peptides in host defense. In addition, antimicrobial peptide-induced responses such as these almost certainly evoke global changes in pathogen status and virulence capability. For example, responses necessary for survival upon exposure to antimicrobial peptides may prompt organisms to dramatically compromise virulence factor or surface feature expression, which may be required for adhesion, colonization, or immunoavoidance. Likewise, the unregulated activation of signal transduction pathways or response regulons upon exposure to antimicrobial peptides or their analogs may lead to pathogen incapacitation and eventual cell death due to global dysregulation. Even if nonlethal, these effects may render pathogens at greatly

increased vulnerability to clearance by other host defense mechanisms.

D. Engineering New Anti-Infectives Based on Peptide Structure and Function

Antimicrobial peptides have been in use to prevent or treat infections for many decades. For example, polymyxins, gramicidins, and bacitracin can be found in many topical applications. Lantibiotics, antimicrobial peptides derived from bacteria, have been used to preserve livestock feed for many years. Clinical trials assessing the efficacies of topical and systemic peptide anti-infectives are underway but have yet to receive approval for use. Thus, the concept that antimicrobial peptides may be utilized to prevent or treat disease is not novel. However, advances in understanding the structural and mechanistic aspects of antimicrobial peptides may accelerate the development of improved anti-infective agents. For example, a clearer recognition of how antimicrobial peptides differentiate between pathogen and host cells holds the promise of designing agents with greater selective toxicity. In this respect, efforts to identify and constrain peptides to antimicrobial conformations may allow the engineering of novel agents with potent efficacy against even the most antibiotic-resistant pathogens, without concomitant host cytotoxicity. Examples include use of native peptides, their engineered derivatives or mimetics (Shankaramma et al., 2002; Yeaman et al., 2002), and/or non-peptide small molecules that recapitulate strategic and/or favorable structure-activity relationships. Beyond these direct applications, the identification of specific peptide mechanisms of action may also reveal vulnerable targets suitable for exploitation by novel small molecule agents with favorable pharmacologic properties.

As with all new agents, pharmacologic and production issues will require optimization if antimicrobial peptides or their mimetic derivatives are to become standard therapeutic agents (Zasloff, 2002). For example, historically, the development of peptide agents has been limited by concerns relating to manufacturing methods, costs, and quality control. Recent advances in eukaryotic expression systems, synthesis platforms, and evaluation methods have greatly reduced, but not altogether resolved, these challenges. In addition, uncertainties related to the potential systemic use of peptides require more complete study. However, recent findings demonstrate that synthetic antimicrobial peptides can be designed to exert potential antimicrobial effects in complex biomatrices, including blood and blood fractions (Yeaman et al., 2002). Mimetic peptides, designed in part based on antimicrobial peptides from platelets (PMPs and tPMPs) exerted dramatic efficacy against serum-resistant E. coli in human blood, plasma, and serum. It is important to emphasize that antimicrobial efficacy was retained even when peptides were incubated in these biomatrices for up to 2 h before introduction of the organism. In some conditions, peptide microbicidal activities exceeded that of gentamicin tested in parallel in these complex conditions. Moreover, the peptides appeared to favorably interact with endogenous antimicrobial components present in blood and blood fractions. These promising results illustrate the potential advantages of developing antimicrobial peptides or analogs thereof mindful of contexts corresponding to their source, so as to optimize their natural structure-activity relationships and antimicrobial spectra. In this sense, peptides may necessitate new and unique approaches regarding dosage and administration to optimize distribution and clearance, degradation and immunogenicity, as well as the molecular basis for potential acute or chronic untoward effects. A more subtle but nonetheless important element of this perspective will rely on a greater acceptance by the medical and commercial sectors for new approaches to managing infections caused by pathogens resistant to conventional modalities. Yet, progress is being made, and the reality of burgeoning resistance to conventional antimicrobial agents will drive further advances. Thus, antimicrobial peptide structure and function as conserved by Nature over an evolutionary timespan offers hope for discovery and development of improved agents to prevent or treat infectious diseases caused by pathogens that resist conventional antimicrobial agents.

VI. Summary

Research focusing on the structures and functions of antimicrobial peptides from diverse sources has burgeoned in recent years. Investigations in this area have identified compelling themes among mechanisms of antimicrobial peptide action and resistance. Composition and conformation yield signature three-dimensional distributions of charge and hydrophobicity among antimicrobial peptides. Differences in biochemical and biophysical properties of microbial versus host cells, and the settings in which these cells are exposed to peptides, provide an additional basis for selective toxicity of antimicrobial peptides. Many antimicrobial peptides employ dynamic mechanisms of action that go beyond the phospholipid bilayer to effect rapid and potent activities. These structure-activity themes of antimicrobial peptides are consistent with their likely multiple roles in antimicrobial host defense. However, microbial pathogens have evolved constitutive or inducible countermeasures to subvert antimicrobial peptide mechanisms of action. Many such resistance pathways are highly coordinated and triggered by exposure to antimicrobial peptides themselves. Thus, a more thorough understanding of the balance between the opposing mechanisms of action and resistance among antimicrobial peptides will further reveal how these molecules function to defend against infection. These insights may provide novel strategies or templates from which novel agents may be

developed to improve the prevention or treatment of infections, particularly those caused by pathogens resistant to conventional antibiotics. Thus, pharmacologic agents may be discovered and developed that target strategic microbial structures or functions, suppress pathogen resistance to host defenses, and restore or potentiate the activities of conventional antibiotics against drug-resistant pathogens. From these perspectives, the mechanisms of antimicrobial peptide action and resistance may hold many secrets yet to be uncovered or fully appreciated.

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