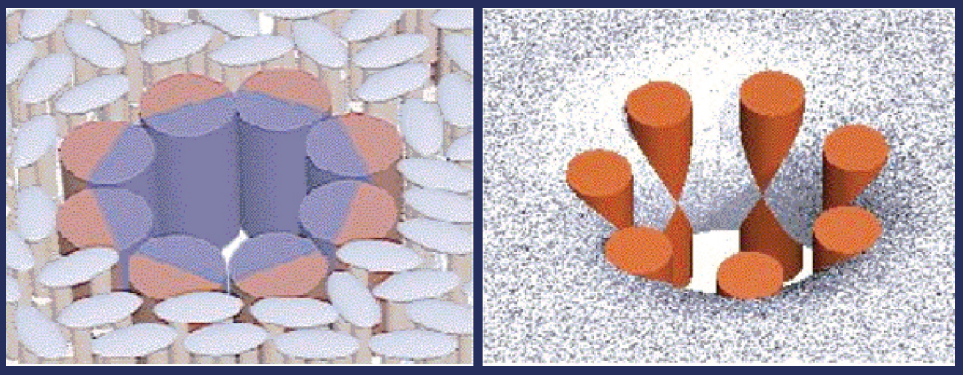
Mode of Action of Antimicrobial Peptides



Hongxia Zhao

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MODE OF ACTION OF ANTIMICROBIAL PEPTIDES

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I. Zhao, H., Mattila, J. P., Holopainen, J. M., and Kinnunen, P. K. J. (2001) Comparison of the membrane association of two antimicrobial peptides, magainin 2 and indolicidin. *Biophys. J.* 81, 2979-2991.

II. Zhao, H., Rinaldi, C. R., Di Giulio, A., Simmaco, M., and Kinnunen, P. K. J. (2002) Interactions of the antimicrobial peptides temporins with model membranes. Comparison of temporins B and L. *Biochemistry* 41, 4425-4436.

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IV. Zhao, H. and Kinnunen, P. K. J. (2003) Modulation of phospholipase A_2 activity by antimicrobial peptides. *Antimicrob. Agents Chemother.* 47, 965-971.

ABBREVIATIONS

(6,7)-Br_2-PC	1-Palmitoyl-2-(6,7-dibromostearoyl)phosphatidylcholine
(9,10)-Br_2-PC	1-Palmitoyl-2-(9,10-dibromostearoyl)phosphatidylcholine
(11,12)-Br_2-PC	1-Palmitoyl-2-(11,12-dibromostearoyl)phosphatidylcholine
CD	Circular dichroism
d	Distance from the center of the bilayer
DPH	Diphenylhexatriene
F	Fluorescence emission intensity
HMC	Hoffman modulation contrast
I_e	Excimer fluorescence intensity of pyrene at 480 nm
I_m	Monomer fluorescence intensity of pyrene at 398 nm
K_{sv}	Stern-Volmer quenching constant
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LUVs	Large unilamellar vesicles
PC	Phosphatidylcholine
PG	Phosphatidylglycerol
PLA_2	Phospholipase A_2
$sPLA_2$	Secretory phospholipase A_2
POPG	1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol
PPDPC	1-Palmitoyl-2-[10-(pyren-1-yl)decanoyl]-*sn*-glycero-3-phosphocholine
PPDPG	1-Palmitoyl-2-[10-(pyren-1-yl)decanoyl]-*sn*-glycero-3-phospho-*rac*-glycerol
PPHPC	1-Palmitoyl-2-[6-(pyre-1-yl)]hexanoyl-*sn*-glycero-3-phosphatidylcholine
PPHPM	1-Palmitoyl-2-[6-(pyre-1-yl)]hexanoyl-*sn*-glycero-3-phosphatidylmonomethylester
r	Anisotropy of diphenylhexatriene
RFI_m	Relative fluorescence intensity of pyrene monomers
RFI_e	Relative fluorescence intensity of pyrene excimers
$R(I_e/I_m)$	Relative excimer to monomer ratio of pyrene fluorescence
SOPC	1-Stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine

SR-1	(2S,3R) -2,3-dimethoxy-1,4-bis(N-hexadecyl-N,N-dimethylammonnium)butane dibromide
SUVs	Small unilamellar vesicles
X	Mole fraction of the indicated lipid
π	Surface pressure
π_c	Critical surface pressure abolishing the intercalation of the peptides into the lipid monolayers
π_0	Initial surface pressure
$\Delta\pi$	Increment in surface pressure
$\Delta I_m/\Delta[M]$	Slopes for the increase in I_m due to peptides before reaching saturating responses in RFI_m

ABSTRACT

The antimicrobial peptides are cytolytic peptides that serve in the vertebrates and invertebrates for both offensive and defensive purposes. They can rapidly kill a broad range of microbes and have additional activities that impact on the quality and effectiveness of innate responses and inflammation. Furthermore, the challenge of bacterial resistance to conventional antibiotics and the unique mode of action of antimicrobial peptides have made such peptides promising candidates for the development of a new class of antibiotics. Accordingly, the molecular basis of their action is of considerable interest and requires to be elucidated. The present study thus focused on the interactions of the four antimicrobial peptides, magainin 2, indolicidin, temporin B and L with model biomembranes, as well as their potential to impact the activity of secretory phospholipase A_2. In brief, the results show that these peptides are highly membrane active and they readily intercalate into lipid monolayers. The penetration was significantly enhanced by the presence of acidic phospholipids. Similarly, the effects of the antimicrobial peptides on lipid dynamics were significantly stronger in bilayers containing acidic phospholipids and they increased the acyl chain order and caused lipid segregation. However, the effects of magainin 2, indolicidin, temporin B and L on lipid dynamics in bilayers were attenuated by the presence of cholesterol. Interestingly, all the four antimicrobial peptides induced an endocytosis-like process in the acidic phospholipid containing giant liposomes although the formed aggregated structures were different. The phosphatidylcholine (PC) membranes were unaffected by magainin 2, indolicidin, and temporin B. Likewise, magainin 2 and temporin B had little impact on giant liposomes composed of PC/cholesterol (X_{chol}=0.1). In contrast, temporin L induced pronounced vesiculation of both PC and PC/cholesterol giant vesicles and indolicidin had a significant effect on PC/cholesterol membranes. The results indicate that temporin L and indolicidin interact also with neutral membranes, in keeping with their hemolytic activities. Indeed, temporin L inserted into both zwitterionic and acidic membranes with similar depth of ≈ 8.0 ± 0.5 Å from the center of the bilayer, with defined α-helical structure. However, in the presence of cholesterol the partitioning of temporin L into lipid bilayers was reduced and the insertion of this peptide into the membrane was shallow, with a distance of 9.5 ± 0.5 Å from the center of the bilayer.

Increasing evidence indicates that antimicrobial peptides act through a variety of mechanisms other than disruption of membrane integrity, and that their role in host defense goes well beyond direct killing of microorganisms. Indeed, the four antimicrobial peptides, magainin 2, indolicidin, temporin B and L caused pronounced effects on the activities of secretory phospholipase A_2 ($sPLA_2$) from bee venom and human lacrimal fluid. The activity of $sPLA_2$ towards anionic liposomes was significantly enhanced by the antimicrobial peptides while their impact on the hydrolysis of the zwitterionic membranes by $sPLA_2$ was dependent on the concentration of Ca^{2+} and the origin of $sPLA_2$. The effects of the four antimicrobial peptides on $sPLA_2$ activity were suggested to depend on lipid compositions in a manner showing a bacterial membrane to be susceptible to enhanced hydrolysis while the host cell membrane would remain intact. Interestingly, inclusion of a cationic gemini surfactant in the vesicles showed essentially similar pattern on $sPLA_2$ activity, indicating that modulation of the enzyme activity by the antimicrobial peptides may involve charge properties of the substrate surface. The results highlight the potential of concerted action between $sPLA_2$ and antimicrobial peptides as part of the innate response to infections, particularly when more antibiotic resistant bacterial strains are emerging.

1 INTRODUCTION

All organisms need protection against microorganisms and their ability to prevent the onset of infection is believed to depend on their innate immune response. The inducible effectors of the innate immune response are rapidly induced (within minutes or hours), relatively non-specific, and have conserved molecular patterns of recognition of stimulatory molecules including bacterial lipopolysaccharide (LPS) and lipoteichoic acid (LTA). In contrast, the adaptive humoral and cellular immune responses have minimal impact on whether these pathogens will cause infections in unimmune animals because a primary response involving clonal expansion of slow-growing B and T cells takes up to several days (three days for secondary response) before it is even noticeable. The effectors of the innate immune response have traditionally been thought to include phagocytic cells, such as neutrophils and macrophages, other leukocytic cells (e.g. mast cells), and serum proteins such as complement. During the past two decades, living organisms of all types have been found to produce a large repertoire of antimicrobial peptides that play an important role in innate immunity to microbial invasion. In higher organisms they are mainly produced on epithelial surfaces and in phagocytic cells that play a crucial role in the innate as well as the adaptive defense systems (Ganz and Lehrer, 1995; Simmaco *et al*., 1998; Hancock and Scott, 2000; Yang et al., 2001). Antimicrobial peptides exhibit rapid killing, often within minutes in vitro, and a broad spectrum of activity against various targets, including Gram-positive and -negative bacteria, fungi, parasites, enveloped viruses, and tumor cells (Baker et al., 1993; Hancock and Scott, 2000; Chen et al., 2001; Fernandez-Lopez et al., 2001; Zasloff, 2002). In addition, their antimicrobial activity can be enhanced by the synergy between individual cationic peptides within a host, and between the peptides and other host factors, such as lysozyme (Hancock and Scott, 2000). Hundreds of antimicrobial peptides have been isolated so far and irrespective of their origin, spectrum of activity, and structure, most of these peptides share several common properties. They are generally composed of < 60 amino acid residues (mostly common L-amino acids), their net charge is positive, they are amphipathic, and in most cases they are membrane active. The interaction of antimicrobial peptides with membranes alters the organization of the bilayer and makes it permeable, causing membrane depolarization (Matsuzaki et al., 1997; Kourie and Shorthouse, 2000). This has led to the general conclusion that

the interaction of most antimicrobial peptides with membranes, involving electrostatic and hydrophobic interactions, is a necessary precursor to cell death. However, it should be underlined that alternative and/or coexistent antimicrobial mechanisms cannot be ruled out at this stage, and evidence is accumulating that some peptides might actually act by binding to intracellular targets, or by stimulating host defense-mechanisms (Li et al., 1995; Hancock and Rozek, 2002; Gennaro et al., 2002). Indeed, certain peptides have been found to interact directly with host cells to stimulate host gene products, including specific chemokines, chemokine receptors, integrins, transcriptional factors etc. (Hancock and Scott, 2000). Furthermore, it has been suggested that the cationic antimicrobial peptides have many potential roles in inflammatory responses, which represent an orchestration of the mechanisms of innate immunity (Hancock and Diamond, 2000).

The development of new families of antibiotics that can overcome the resistance problem has become a very important task because of the emergence of resistant bacterial strains worldwide (Bonomo, 2000). The killing mechanism of antimicrobial peptide is such different from that of the conventional antibiotics that antimicrobial peptides are considered as attractive substitute and/or additional drugs. The elucidation of the mechanisms of the action of antimicrobial peptides and their specific membrane damaging properties, viz. their selectivity to a specific target, is supposed to be a key for the rationale design of novel types of peptide-antibiotics (Lohner, 2001).

The purpose of this study was to elucidate the membrane activity and specificity of four antimicrobial peptides, magainin 2, indolicidin, temporin B and L. The effects of these antimicrobial peptides on the activity of secretory phospholipase A_2 was also studied. The peptides employed in our investigations were chosen as they represent different types of antimicrobial peptide families. These peptides differ in structural parameters such as charge, conformation, hydrophobicity, hydrophobic moment, and size. In this study, different membrane models, viz. monolayers, large and small unilamellar vesicles, as well as giant liposomes, respectively, were used to investigate the penetration of these four antimicrobial peptides into films, their effects on the dynamics of lipids in bilayers, and macroscopic, morphological changes in membranes. In addition, the structure, orientation, and depth of penetration of

temporin L into lipid bilayers were studied in more detail. Characterization of peptide-membrane interactions, membrane lysis by antimicrobial peptides, as well as the topology of the peptides in membranes provided insight into the mode of their action and the effect of different lipids on their function and selectivity. Furthermore, the potential of concerted action between antimicrobial peptides and $sPLA_2$, which could improve the efficiency of the innate response to infections, would highlight the application of antimicrobial peptides as therapeutic agents.

2 REVIEW OF THE LITERATURE

Antimicrobial peptides are widely distributed in nature and represent an ancient mechanism of host defense (Lehrer and Ganz, 1999). They were initially found in invertebrates (Boman, 1991), and later also in vertebrates, including humans (Gudmundsson et a., 1996). Antimicrobial peptides are promptly synthesized at low metabolic cost, easily stored in large amounts and readily available shortly after an infection, to rapidly neutralize a broad range of microbes. Most antimicrobial peptides tested so far, regardless of sequence, size, charge, diastereomerism, L- or D-amino acid composition and structure, kill bacteria in the micromolar range supporting a non-receptor mediated mechanism for their mode of action (McElhaney and Prenner, 1999). Numerous studies indicate that many antimicrobial peptides act directly on the membrane of the target cell (for reviews, see McElhaney and Prenner, 1999). The net positive charge of antimicrobial peptides causes their preferential binding to more than one negatively charged targets on bacteria, which may account for the selectivity of antimicrobial peptides (Hancock and Scott, 2000). It has been reported that not only the nature of the peptide but also the characteristics of cell membrane, as well as the metabolic state of the target cells determine the mechanisms of action of antimicrobial peptides (Liang and Kim, 1999). The action of cationic antimicrobial peptides is not limited to direct killing of microorganisms. Instead, they have an impressive variety of additional activities that impact particularly on the quality and effectiveness of innate responses and inflammation (Hancock and Diamond, 2000). The advantage for antimicrobial peptides as defense weapons, is that, given the non-specificity of their mechanism, it is not easy for microbial pathogens to develop resistant mutants to overcome peptide intervention. Understanding how, when and where they function has become of considerable interest which could lead to the development of novel therapeutic agents, especially in the context of the growing microbial resistance to conventional antibiotics. Some antimicrobial peptides are being developed for use either as antibiotics for topical use in healthcare or as preservatives in the food industry (Chen et al., 2000; Nes and Holo, 2000; Zasloff, 2000; van't Hof et al., 2001; Cleveland et al., 2001). We are encouraged to believe that antimicrobial peptides have great potential to be the next breakthrough and first truly novel class of antibiotics.

The purpose of this review is to summarize the biological activity and diversity of antimicrobial peptides, the mechanisms of their action and selectivity, and to discuss their potential as therapeutics.

2.1 Biological Activity

The antimicrobial peptides display a broad spectrum of activity. Many antimicrobial peptides not only kill bacteria, they are cytotoxic also to fungi (Fehlbaum et al., 1996; Kieffer et al., 2003), protozoa (Arrighi et al., 2002), malignant cells (Cruciani et al., 1991; Baker et al., 1993; Lindholm et al., 2002), and even enveloped viruses like HIV, herpes simplex virus, and vesicular stomatitis virus (Tamamura et al., 1998; Robinson et al., 1998). The defining characteristic of the above targets of antimicrobial peptides is their possession of a distinct cell membrane. Antimicrobial peptides exhibit selectivity against different microorganisms, of which the molecular basis is not completely understood. On the one hand, many antimicrobial peptides display broad-spectrum activity against Gram-negative bacteria, Gram-positive bacteria, and fungi (Miyasaki and Lehrer, 1998). On the other hand, some peptides, e.g. andropin (Samakovlis et al., 1991) and insect defensins (Meister et al., 1997) preferentially kill Gram-positive bacteria, while others preferentially kill Gram-negative bacteria, e.g. apidaecin (Casteels and Tempst, 1994), drosocin (Bulet et al., 1996), and cecropin P1 (Boman et al., 1991). Peptides that preferentially kill fungi have also been described, e.g. drosomycin (Meister et al., 1997) and plant antimicrobial peptides (Tailor et al., 1997). Normal human cells are relatively resistant, but it should be mentioned that certain cationic antimicrobial peptides, such as melittin from bees (Perez-Paya et al., 1994), mastoparan from wasps (Delatorre et al., 2001), charybdotoxin from scorpions (Tenenholz et al., 2000), and temporin L from frogs (Rinalti et al., 2002) are potent toxins.

The antimicrobial peptides, magainins and their analogs have been found to be able to lyse hematopoietic tumor and solid tumor cells with little toxic effect on normal blood lymphocytes (Cruciani et al., 1991; Baker et al., 1993). The mechanism of antitumor activity of magainins was suggested to target cell membrane by a non-receptor pathway (Baker et al., 1993). Tachyplesin is an antimicrobial peptide present in leukocytes of the horse crab (*Tachypleus tridentatus,* Hirakura et al., 2002). A synthetic tachyplesin conjugated to the integrin binding sequence RGD showed that it

inhibited the proliferation of both cultured tumor and endothelia cells by disrupting their membranes and inducing apoptosis (Chen et al., 2001). Similarly, a designed novel peptide DPI was found to be able to trigger rapid apoptosis in tumor cells (Mai et al., 2001). In contrast, temporin L, a 13 amino acid long peptide isolated from the skin of the european red frog, *Rana temporaria,* induced necrosis of tumor cells (Rinaldi et al., 2001; 2002). Cyclotides, members of macrocyclic cystine-knotted peptides exhibit antimicrobial, anticancer, and antiviral activities (Tam et al., 1999; Lindholm et al., 2002). The activity profile of cyclotides differed significantly from those of antitumor drugs in clinical use, which may indicate a new mode of anticancer action (Lindholm et al., 2002). Other antimicrobial peptides, such as defensins (Kagan et al., 1990), cecropin (Moore et al., 1994), lactoferricin (Vogel et al., 2002), and lactoferrin (Yoo et al., 1998) also have similar antitumor activity.

Several cationic amphipathic peptides also display antiviral activity in vitro. Defensins are able to neutralize herpes simplex virus (HSV), vesicular stomatitis virus, and influenza virus (Daher et al., 1986; Ganz and Lehrer, 1995). Tachyplesins and polyphemusins are active against vesicular stomatitis virus, influenza A virus, and HIV (Tamamura et al., 1996). Melittins (Wachinger et al., 1998), cecropins (Wachinger et al., 1998), and indolicidin (Robinson et al., 1998) also display anti-HIV activity. The generally accepted mechanism of antiviral action is a direct interaction of these peptides with the envelope of the virus, leading to permeation of the envelope and, eventually, lysis of the virus particle, analogous to the pore-formation model suggested for antibacterial activity. Robinson *et a*l. (1998) found that the antiviral activity of indolicidin is temperature-sensitive, which was regarded as indication of a membrane-mediated mechanism. Other mechanisms for antiviral action have also been proposed. Peptide T22, derived from polyphemusin II, inactivates HIV-1 *in vitro* by binding to both the gp120 protein of the virus and the CD4 receptor of the T-helper cells, which may block virus-cell fusion in an early stage of the infection (Tamamura *et al*., 1996). Melittin and cecropins have been found to inhibit the replication of HIV-1 by suppression of the long terminal repeat gene (Wachinger *et a*l., 1998).

2.2 Diversity of Antimicrobial Peptides

More than 500 antimicrobial peptides have been discovered so far from animals as well as plants (a good collection of sequences, besides a wealth of other useful

information, can be found at http://www.bbcm.univ.trieste.it/~tossi/pag1.htm). The diversity of sequences is such that the same peptide sequence is rarely recovered from two different species of animals, even those closely related (except for the peptides cleaved from highly conserved proteins, such as buforin II). However, both within the antimicrobial peptides from a single species, and even between certain classes of different peptides from diverse species, significant conservation of amino-acid sequences can be recognized in the preproregion of the precursor molecule. The design suggests that constrains exist on the sequences involved in the translation, secretion or intracellular trafficking of this class of membrane-disruptive peptides. This feature is well illustrated by cathelicidins (Zanetti, et al., 2000). The diversity of antimicrobial peptides probably reflects the species' adaption to the unique microbial environments that characterize the niche occupied, including the microbes associated with acceptable food sources (Simmaco et al., 1998; Boman, 2000). It appears reasonable to speculate that an individual could find itself in the midst of microbes against which the peptides of its species were ineffective, although the individual might suffer, the species itself could survive through emergence of individuals expressing beneficial mutations.

The diversity of antimicrobial peptides discovered is so great that it is difficult to categorize them except broadly on the basis of their secondary structure (Epand and Vogel, 1999; van't Hof et al., 2001). The fundamental structural principle is the ability of a peptide to adopt a shape in which clusters of hydrophobic and cationic amino acids are spatially organized in discrete parts of the molecule. It is common to classify antimicrobial peptides into four groups according to their secondary structure. Table 1 describes the main structural features of some well-studied antimicrobial peptides originating from a wide range of sources, selected as representative examples of their structural class. This classification follows that proposed by van't Hof et al. (2001):

Table 1. Main characteristics of some representative antimicrobial peptides

Group	Peptide	Sequence	Origin
I α-helix	temporin L temporin B magainin 2 SMAP29 LL-37 cecropin A	FVQWFSKFLGRIL LLPIVGNLLKSLL-Am GIGKFLHSAKKFGKAFVGEIMNS RGLRRLGRKIAHGVKKYGPTVLRIIRIAG LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES KWKLFKKIEKVGQNIRDGIKAGPAVAVVGQATQIAK-Am	*Rana temporaria* (European red frog skin) *Rana temporaria* (European red frog skin) *Xenopus laevis* (clawed toad skin) sheep myeloid humans, leukocytes, epithelia *Hyalophora cecropia* (moth)
II β-sheet	protegrin-1 tachyplesin-1 polyphemusin-1 androctonin human β-defensin-1	RGGRLC$_1$YC$_2$RRRFC$_1$VC$_2$VGR-Am KWC$_1$FRVC$_2$YRGIC$_2$YRRC$_1$R-Am RRWC$_1$FRVC$_2$YRGFC$_2$YRKC$_1$R-Am RSVC$_1$RQIKIC$_2$RRRGGC$_2$YYKC$_1$TNRPY DHYNC$_1$VSSGGQC$_2$LYSAC$_3$PIFTKIQGTC$_2$YRGKAKC$_1$C$_3$K	porcine leukocytes *Tachypleus gigas* (Asian horseshoe crab) *Limulus polyphemus* (Atlantic horseshoe crab) *Androctonus australis* (scorpion hemolymph) several human tissues
III unusual composition	indolicidin histatin-5 bactenecin-5 PR-39	ILPWKWPWWPWRR-Am DSHAKRHHGYKRKFHEKHHSHRGY RERPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPPFRPPLRFP RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP-Am	bovine neutrophils human saliva bovine neutrophils pig intestine
IV looped peptide	bactenecin-1 ranalexin thanatin brevinin 1E lactoferricin	RLC$_1$RIVVIRVC$_1$R FLGLIKIVPAMIC$_1$AVTKKC$_1$ GSKKPVPIIYC$_1$NRRTGKC$_1$QRM FLPLLAGLAANFLPKIFC$_1$KITRKC$_1$ FKC$_1$RRWQWRMKKLGAPSITC$_1$VRRAF	bovine neutrophils *Rana catesbeiana* (bullfrog skin) insect hemocytes *Rana esculenta* (European frog skin) cow and human milk

Note: -Am represents an amidated C-terminus and subscript numbers represent amino acids that are joined by disulfide bridges.

Group I: linear peptides with an α-helical structure (Fig. 1, B)

One of the larger and better studied classes of antimicrobial peptides is those forming cationic amphipathic helices, e.g. magainin, cecropin A, temporins, as well as a number of *de novo* designed antimicrobial peptides (Boman, 1995; Mangoni et al., 2000). These peptides adopt disordered structures in aqueous solution while fold into an α-helical conformation upon interaction with hydrophobic solvents or lipid surfaces. α-Helical peptides are often found to be amphipathic and can either absorb onto the membrane surface or insert into the membrane as a cluster of helical bundles. The majority of the cytotoxic amphipathic helical peptides are cationic and they do exhibit selective toxicity for microbes. One of the most studied of the cationic, antimicrobial, amphipathic helical peptides is magainin. This 23 amino acid peptide is secreted on the skin of the African clawed frog, *Xenopus laevis* (Zasloff et al., 1987). The properties of this peptide have recently been reviewed (Matsuzaki, 1998, 1999). The structural parameters of this group of peptides concerning modulation of their biological activities will be discussed in more detail below. There are also hydrophobic or slightly anionic α-helical peptides. Peptides that are not cationic exhibit less selectivity towards microbes compared with mammalian cells. An example of a well-studied hydrophobic and negatively charged cytotoxic peptide is alamethicin (Duclohier and Wroblewski, 2001; Kikukawa and Araiso, 2002). This helical peptide forms clusters of helices that traverse the bilayer and surround an aqueous pore transporting ions (Sansom, 1993).

Group II: conformationally more restrained peptides, predominantly consisting of β-strands connected by intramolecular disulfide bridges (Fig. 1, A)

In contrast to the linear α-helical peptides, β-sheet peptides are cyclic peptides constrained either by disulfide bonds, as in the case of human β-defensin-2 (Hancock, 2001), tachyplesins (Matsuzaki, 1999), protegrins (Harwig et al., 1995), and lactoferricin (Jones et al., 1994), or by cyclization of the peptide backbone, as in the case of gramicidin S (Prenner et al., 1999), polymyxin B (Zaltash et al., 2000), and tyrocidines (Bu et al., 2002). They largely exist in the β-sheet conformation in aqueous solution that may be further stabilized upon interactions with lipid surfaces.

Defensins are among the most characterized β-sheet-forming antimicrobial peptides. Different mechanisms involving either the perturbation of lipid bilayers or the formation of discrete channels have been suggested for these peptides based on high resolution crystallography (Hill et al., 1991) and 2D-NMR studies (Zhang et al., 1992). Studies aimed at understanding the importance of the disulfide bridges for their activity have been carried out. Detailed studies have indicated that replacement of the cysteine residues by certain amino acids like Ala, Asp, and Leu lead to inactivation, whereas analogs with aromatic residues Phe, Tyr, and hydrophobic amino acid like Leu, Met, and Val retained broad spectrum antimicrobial activity (Tamamura et al., 1998). Studies with tachyplesin analogs, where the SH groups were chemically protected to prevent cyclization or cysteines were replaced by Ala residues, suggested that the cyclic structure was essential for antimicrobial activity while it might not be crucial for membrane permeabilization (Matsuzaki et al., 1997; Tamamura et al., 1998; Rao, 1999).

Less is known about the mechanism by which this class of antimicrobial peptides causes membrane damage. A study of the orientation of protegrin in membranes using oriented circular dichroism has demonstrated two different states of the peptide to insert into a membrane, depending on the peptide concentration, the nature of the lipid, and the extent of hydration (Heller et al., 1998). The peptide tachyplesin, which has a cyclic anti-parallel-β-sheet structure held together by two disulfide bonds, permeabilizes both bacterial and artificial lipid membranes. It also translocates across lipid bilayers coupled with transient pore formation (Matsuzaki et al., 1997), suggesting a similar mode of action as the α-helical magainin. The critical parameter associated with the antimicrobial action of this peptide appears to be the maintenance of a certain hydrophilic-hydrophobic balance (Rao, 1999). The effect of substitution of a D-amino acid residue (Lys^4) in a cyclic β-structure peptide (VKLKV*d*-YPLKVKL*d*-YP), based on the sequence of gramicidin S, was determined and resulted in high antimicrobial activity coupled with low hemolytic activity (Kondejewski et al., 1999). The findings show that a highly amphipathic nature is not desirable in the design of constrained cyclic antimicrobial peptides and that an optimum amphipathicity can be defined by systematic enantiomeric substitutions.

Group III: linear peptides with an extended structure, characterized by overrepresentation of one or more amino acids (Fig. 1, D)

Certain antimicrobial peptides have an unusual amino acid composition, having a sequence that is rich in one or more specific amino acids. For example, the peptide histatin, which is produced in saliva, is highly rich in His residues (table 1, Brewer et al., 1998; Tsai and Bobek, 1998; Helmerhorst et al., 1999a). This peptide translocates across the yeast membrane and targets to the mitochondria, suggesting an unusual antifungal mechanism (Helmerhorst et al., 1999a). The peptides produced by porcine neutrophils are very rich in proline and arginine or proline and phenylalanine. They belong to the cathelicidin family of antimicrobial peptides and are called PR-39 and prophenin, respectively (Zhao et al., 1995; Linde et al., 2001). Tryptophan is generally not an abundant amino acid residue in peptides or proteins. This amino acid is of particular interest with regard to the partitioning of peptides into membranes because of its propensity to position itself near the membrane/water interface (Yau et al., 1998; Persson et al., 1998). Examples of antimicrobial peptides that are rich in Trp include tritrpticin (VRRFPWWWPFLRR) (Lawyer et al., 1996) and indolicidin (ILPWKWPWWPWRR-amide) (Selsted et al., 1992). Indolicidin was reported to adopt a turn conformation which greatly enhances its membrane activity (Ladokhin et al., 1999). Indolicidin has been shown to permeabilize the outer membrane of *E. coli* (Falla et al., 1996; Subbalakshmi et al., 1998) to form channels, as revealed by conductance measurements with planar bilayers (Falla et al., 1996; Wu et al., 1999). This peptide incorporates in a highly cooperative manner within the acyl chain region of the membrane (Ha et al., 2000) and its hemolytic activity is associated with the concentration required for its self-association (Ahmad et al., 1995). The unusual amino acid composition has led to studies directed towards delineation of the role of multiple tryptophan residues in its biological activity as well as interactions with model membranes. Several analogs have been synthesized in order to understand the charge requirements and also the importance of Try and Pro residues for activity (Falla et al., 1996; Subbalakshmi et al., 1996).

Group IV: peptides containing a looped structure (Fig. 1, C)

In contrast to other antimicrobial peptides, proline-arginine-rich peptides cannot form amphipathic structures due to the incompatibility of high concentration of proline residues in such structures and have been proposed to adopt a polyproline helical

type-II structure (Boman et al., 1993; Cabiaux et al., 1994). Lantibiotics contain small ring structures enclosed by a thioether bond and their structure and properties have recently been reviewed (Montville and Chen, 1998). One of the lantibiotics, nisin, is currently used as an antimicrobial agent for food preservation and this peptide has relatively high activity against Gram-positive bacteria due to its specific high affinity with Lipid II, a precursor in the bacterial cell wall synthesis (Breukink and de Kruijff, 1999; Breukink et al., 1999). Recently synthesized six- and eight residue cyclic D, L-α-peptides have been found to exhibit high efficacy to kill bacteria with low hemolytic activity (Fernadez-Lopez, et al., 2001). Upon binding to lipid membranes the cyclic peptides can stack to form hollow, β-sheet-like tubular structures increasing membrane permeability. With short size, easy to synthesize and being proteolytically stable, this class of peptides holds considerable potential in fighting existing and emerging infectious diseases.

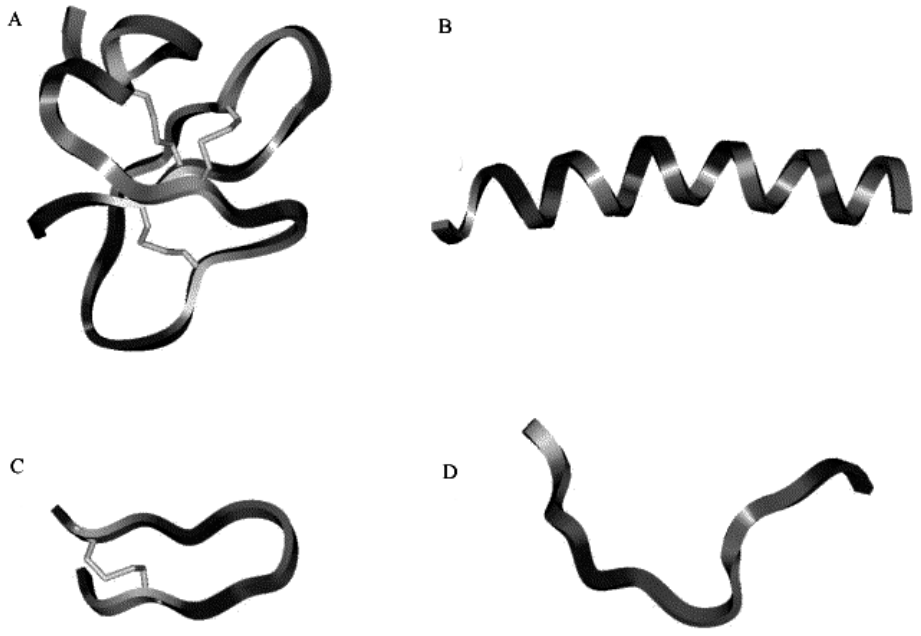


Fig. 1. Molecular models of the different structural classes of antimicrobial peptides (taken from Hancock, 2001). These models are based on two-dimensional nuclear magnetic resonance spectroscopy of the peptides in aqueous solution for human β-defensin-2 or a membrane mimetic condition for other peptides. (A) human β-defensin-2, which forms a triple-stranded β-sheet structure (containing a small α-helical segment at the N-terminus) stabilized by three cysteine disulphide bridges. (B) The amphipathic α-helical structure of magainin 2. (C) The β-turn loop structure of bovine bactenecin. (D) The extended boat-shaped structure of bovine indolicidin.

2.3 Induction and Regulation of Expression

Antimicrobial peptides in multicellular organisms are found on external surfaces such as the skin or the lungs or they are sequestered in granules of neutrophils, from where they can be released to kill microbes. Some antimicrobial peptides are synthesized constitutively, for instance the histatins (Tsai and Bobek, 1998) and human β-defensin-1 (Yang et al., 2002). Others are often induced in response of an infection (Hoffmann et al., 1999) and thus can be considered as acute-phase proteins, e.g. LL-37 (Frohm *et a*l., 1997) and human β-defensin-2 (Harder et al., 2000; Schutte and McCray, 2002; Yang et al., 2002). Interestingly, it has been found that plasmatic levels of both vasostatin-1 and secretolytin increase during surgery in patients undergoing cardiopulmonary bypass (Tasiemski et al., 2002). Vasostatin-1 and secretolytin, initially present in plasma at low levels, are released just after skin incision. Consequently, they can be added to enkelytin, an antibacterial peptide derived from proenkephalin A, for the panoply of components acting as a first protective barrier against hypothetical invasion of pathogens, which may occur during surgery.

The large majority of antimicrobial peptides synthesized by multicellular organisms are encoded by the genome. Insects and mammals typically express multiple antimicrobial peptides. For example, at least ten sheep genes encode antimicrobial peptides, including eight cathelicidins and two β-defensins (Huttner et al., 1998). The bovine genome contains genes for at least eleven cathelicidins (Scocchi et al., 1997) and over twenty β-defensins (Ryan et al., 1998). The antimicrobial peptides are produced mainly through regular processes of gene transcription and ribosomal translation, often followed by further proteolytic processing. Non-ribosomal biosynthetic antimicrobial peptides will be not addressed in detail and recent advances in this field are covered by a number of good review articles (Jack and Jung, 2000; Moffitt and Neilan, 2000; Sablon *et al*., 2000). Magainins are synthesized as long preproproteins containing six copies of the peptide. Proteolytic processing leads to the release of the individual magainin peptides (Ketchem et al., 1993). The 35–37 residue insect cecropins are synthesized as preproproteins of 62 amino acids and processing involves several protease activities (Gudmundsson et al., 1991). Interestingly, in many cationic antimicrobial peptides such as the temporins, the negative charge of the

carboxyl terminus is removed by an amidation process (Simmaco et al., 1996). Recently, it was found that several antimicrobial peptides are released by cleavage of intact proteins that may have no or limited antibacterial activity themselves. This has been demonstrated first for the milk protein lactoferrin (Bellamy et al., 1992). Proteolytic cleavage of the intact bovine protein by pepsin under acidic conditions releases a 25 residue peptide, lactoferrin B, which shows a significantly increased bacteriostatic potency compared to the intact protein (Bellamy et al., 1992).

Antimicrobial peptides can neutralize host responses to conserved bacterial signaling molecules such as endotoxic LPS from Gram-negative bacteria, or LTA from Gram-positive bacteria (O'Leary and Wilkinson, 1988). Such molecules interact with Toll-like receptors on the surface of host cells to trigger signaling cascades and cause upregulation of cytokines, such as tumor necrosis factor (TNF) and interleukin 6, chemokines, and dozens of other gene products (for a review, see Medzhitov, 2001). Differential responses are mediated by different Toll-like receptors. Accordingly, human Toll-like receptor-2 (TLR2) is activated by bacterial lipoprotein, Gram-positive cell walls, peptidoglycan and mycobacterial factors, whereas TLR4 is essential for the cellular responses to LPS from Gram-negative bacteria (Shimazu *et a*l., 1999, Takeuchi and Akira, 2001). Although many antimicrobial peptides are induced by bacterial molecules or upregulated by inflammatory stimuli involving a Toll-related IL-1 receptor (Williams et al., 1997; Tauszig et al., 2000), only a few genes encoding such inducible peptides were found. Homologous responsive elements (Meister *et al*., 1997) have been identified in *Drosophil*a. These were associated with the Rel proteins *dorsal, Dif,* and *relish* and the inhibitory protein *cactus,* similar as in the NF-kB/I-kB signaling pathway (for a review, see Imler and Hoffmann, 2000). Drosomycin gene expression is exclusively regulated *via* this pathway, diptericin and drosocin gene expressions are regulated by the independent, not yet well defined, *imd* pathway, whereas gene induction for cecropin, insect defensin and the antimicrobial protein attacin (Khush et al., 2001) requires contributions from both signaling pathways. These pathways explain the differential response of *Drosophila* to fungi, Gram-negative, and -positive bacteria. In plants, the production of inducible antimicrobial proteins is upregulated *via* similar signaling pathways. For instance, microbial products induce an intracellular proteolytic cascade in potato cells leading to binding of nuclear factors PBF-1 and PBR-2 to an elicitor-response element (ERE)

on the DNA (Subramaniam *et a*l., 1997). Again, differential responses against different types of microbes are observed (Cardinale *et a*l., 2000) and several fungal (Nürnberger *et a*l., 1994; Keller *et a*l., 1999) and bacterial (Gomez-Gomez *et a*l., 1999) response elicitors have been identified. Like animal cells, plant cells are able to communicate microbial infection to neighbor cells using plant hormones as functional analogues of interleukins. In plants two different hormone-dependent signaling pathways have been identified that lead to differential responses to distinct microorganisms. The salicylic acid signaling pathway induces antibacterial responses while the *cis*-jasmonic acid/ethylene signaling pathway induces antifungal responses (Thomma *et a*l., 1998; Pieterse and Van Loon, 1999).

2.4 Molecular Basis for Antimicrobial Peptide Cell Specificity

The most interesting property of antimicrobial peptides is their cell specificity by which they kill microbes but are nontoxic to mammalian cells. The relative insensitivity of eukaryotic cells to antimicrobial peptides is generally ascribed to differences in lipid composition between eukaryotic and prokaryotic cell membranes (Dathe and Wieprecht, 1999; Matsuzaki, 1999). It has been proposed that the net positive charge of the antimicrobial peptides accounts for their preferential binding to the negatively charged outer surface of bacteria, which is different from the predominantly zwitterionic surface of normal mammalian cells (Devaux, 1991; Dolis et al., 1997). Also a less negative membrane potential in eukaryotes than in prokaryotes plays an important role in their selectivity (Matsuzaki, 1999). Tumor cells have lost part of their lipid asymmetry and therefore exhibit a more anionic character on the external leaflet of their plasma membrane, which thus preferentially binds cationic antimicrobial peptides (Utsugi et al., 1991). Furthermore, the selectivity of some antimicrobial peptides towards tumor cells has been suggested to be due to a higher negative membrane potential over the cytoplasmic membranes of many types of cancer cells (Utsugi et al., 1991).

2.4.1 The potential role of lipopolysaccharide and the bacterial outer membrane

In order to develop cytolytic activity, the peptide has to ‘survive’ exposure to serum or other media, and pass different barriers such as the extracellular matrix, or the bacterial lipopolysaccharides, outer membrane and/or peptidoglycan layers (Andreu and Rivas, 1999). Once it reaches the cell membrane its structures and topologies are

in dynamic interchange (Lambotte et al., 1998; Sansom, 1998). The outer surface of Gram-negative bacteria contains lipopolysaccharides and that of Gram-positive bacteria, acidic polysaccharides (teichoic acids), giving the surface of bacteria a negative charge. Additionally, the phospholipids composing the cytoplasmic membrane of Gram-negative bacteria, and the single membrane of Gram-positive bacteria are negatively charged. In contrast, the outer leaflet of a normal mammalian cell is composed predominantly of zwitterionic phosphatidylcholine and sphingomyelin phospholipids (Devaux, 1991; Dolis et al., 1997). The role of the outer membrane and LPS on antimicrobial activity was investigated with magainin (Rana et al., 1991). Magainin 2 alters the thermotropic properties of the outer membrane-peptidoglycan complexes from wild-type *Salmonella typhimurium* and a series of LPS mutants, which display differential susceptibility to the bactericidal activity of cationic antibiotics. LPS mutants show a progressive loss of resistance to killing by magainin 2 as the length of the LPS polysaccharide moiety decreases. While disruption of outer membrane structure is not the primary factor leading to cell death (Hancock, 1997; Guo et al., 1998), the susceptibility of Gram-negative cells to magainin 2 has been proposed to be associated with factors that facilitate the transport of the peptides across the outer membrane, such as the magnitude and location of LPS charge, the concentration of LPS in the outer membrane, the outer membrane molecular architecture, and the presence or absence of the O-antigen side chain. Nevertheless, there is not always a direct correlation between binding of antimicrobial peptides to LPS and their antimicrobial activity. It has been suggested that the reduced antimicrobial activity of gramicidin S analogs results from increased affinity to outer membrane components of Gram-negative microorganisms, competing for binding to the inner membrane needed to cause bacteria death (Kondejewski et al., 1999). In addition, an antimicrobial peptide that binds as oligmers onto the surface of the bacteria will find it difficult to diffuse through the LPS layer into the target membrane. LPS and in particular lipid A play an important role in the pathophysiology of Gram-negative bacterial sepsis which elicits ‘endotoxic shock’ syndrome in the circulation in humans, often culminating in the death of the patient. In principle, agents that can bind LPS released from the bacterial cell walls in the bloodstream can neutralize its toxic action. Indeed, vertebrate and invertebrate have proteins in their circulatory system that can bind LPS and neutralize its endotoxic

activity. Since various cationic antimicrobial peptides are known to bind LPS, they may have a role in treating sepsis.

2.4.2 Role of membrane lipids

It is commonly known that a large diversity of phospholipids exists in biological membranes, which is related to specific membrane functions due to the different phospholipid composition and properties (Kinnunen, 1991; De Kruijff, 1997; Dowhan, 1997). Especially the physicochemical properties of phospholipids in microorganism membranes are of interest since it has been suggested that the lipid composition of bacterial membranes plays an important role in the interaction with antimicrobial peptides (Lohner and Prenner, 1999; Lohner, 2001). Cationic antimicrobial peptides preferentially bind to membranes containing acidic phospholipids such as PG or PS due to strong electrostatic interactions (Matsuzaki et al., 1991, 1995a, 1998). The enhanced binding due to the presence of negatively charged lipids could be explained by the Gouy-Chapman theory (Wenk and Seelig, 1998). This has been demonstrated with cecropins (Gazit and Shai, 1995), magainins (Matsuzaki et al., 1999), and others (Hetru et al., 2000; Oren et al., 1999a, 1999b). In several cases a direct correlation was found between the order of efficacy to permeate membranes and the order of potency to kill bacteria (Oren et al., 1997). For example, peptides with high permeating activity on PE/PG membranes matching the composition of the inner membrane of *E. coli*, were highly active on this bacterium. Likewise, the fluidity and curvature of lipid bilayers influence peptide-lipids interactions. Fluid bilayers are generally more susceptible to the peptides. For example, magainins more effectively permeate lipid bilayers in the liquid-crystalline phase than those in the gel phase (Matsuzaki et al., 1991). Cholesterol affects the fluidity and the dipole potential of phospholipid membranes, and therefore modulates the activity of membrane-inserted peptides (Matsuzaki et al., 1995a). In addition, the formation of hydrogen bonds between glutamates and cholesterol has been suggested to reduce antibiotic activity (Tytler et al., 1995). Interestingly, cholesterol delays the cell lytic effects of cecropins without abolishing them, suggesting that the peptide inserts more slowly into cholesterol-containing membranes (Silvestro et al., 1997). A slowing of the insertion process or a modulated association behavior might allow proteases to remove the toxins from the cellular surface (Resnick et al., 1991; Silvestro et al., 1997; Andreu and Rivas, 1999).

The importance of curvature strain in the functions of membrane associated peptides and proteins has attracted a great deal of attention (Epand, 1998). If the head group of a lipid possesses a size comparable to the cross-section of the acyl chains the lipid has a cylindrical shape and the lipids form a flat monolayer with zero curvature (e.g. phosphatidylcholine, Huttner and Schmidt, 2002). In contrast, a relatively larger polar headgroup results in a convex monolayer with positive curvature (e.g. lyso-phospholipids), whereas a smaller hydrophilic group causes concave bending of the monolayer with negative curvature (e.g. phosphatidylethanolamine). Incorporation of phosphatidylethanolamine was found to inhibit the magainin-induced pore formation in the inhibitory order of dioleoylphosphatidylethanolamine > dielaidoylphospha-tidylethanolamine (Matsuzaki et al., 1998). However, addition of a small amount of palmitoyllysophosphatidylcholine enhanced the peptide-induced permeabilization of phosphatidylglycerol bilayers. These results suggest that the peptide imposes positive curvature strain, facilitating the formation of a torus-type pore, and that the presence of negative curvature-inducing lipids inhibits pore formation. Similarly, the capacity of gramicidin S to induce the formation of cubic or other three dimensionally ordered inverted nonlamellar phases was suggested to increase with the intrinsic nonlamellar phase-preferring tendencies of the lipids (Prenner et al., 1997).

2.5 Mechanisms of Action of Antimicrobial Peptides

Many antimicrobial peptides bind in a similar manner to negatively charged membranes and permeate them, resulting in the formation of a pathway for ions and solutes (McElhaney and Prenner, 1999). Before reaching the phospholipid membrane peptides must transverse the negatively charged outer wall of Gram-negative bacteria containing LPS or through the outer cell wall of Gram-positive bacteria containing acidic polysaccharides. Hancock and coworkers described this process as a ‘self-promoted uptake’ with respect to Gram-negative microorganisms (Hancock, 1997). In this mechanism, the peptides initially interact with the surface LPS, competitively displacing the divalent polyanionic cations and partly neutralize LPS. This causes disruption of the outer membrane and peptides pass through the disrupted outer membrane and reach the negatively charged phospholipid cytoplasmic membrane. The membrane-active properties of such peptides have been extensively studied using model membranes (McElhaney and Prenner, 1999). The amphipathic peptides can

partition into cytoplasmic membrane through hydrophobic and electrostatic interactions, causing stress in the lipid bilayer. When the unfavorable energy reaches a threshold, the membrane barrier property is lost, which is the basis of the antimicrobial action of these peptides.

2.5.1 Model membranes

A molecular understanding of the interaction of antimicrobial peptides with membranes requires experimental knowledge of the structure of the membrane, the transmembrane location of bound peptides, the structures the peptides adopt, and the changes that occur in the membrane environment as a result of partitioning. The interactions between an antimicrobial peptide and membranes, can be studied either by an *in vivo* approach using intact cells or, alternatively, by extensive use of the *in vitro* experiments with isolated or model membranes. Natural membranes are composed of a great variety of lipids and proteins, thus studies carried out in cells allow characterization only of the global membrane damage phenomena. Most of our knowledge of the specific lipid-peptide interactions derived from studies on model membranes. Among them liposomes with different size and lipid composition represent the most promising tool. Hydration of most lipid films of various compositions produces multilamellar vesicles (MLVs, $\varnothing \approx 1$ μm) which can be sized by extrusion or sonication into large unilamellar vesicles (LUVs, $\varnothing \approx$ 50-200 nm) or small unilamellar vesicles (SUVs, $\varnothing <$ 50 nm), respectively (New, 1990). SUVs can also be prepared by ethanol injection and has been shown to be indistinguishable from those obtained by sonication (Batzri and Korn, 1973). MLVs are often applied for solid-state NMR or DSC experiments. Optically clear SUVs are suitable for spectroscopic measurements, especially for CD spectroscopy. Due to high degrees of curvature which impose strain on lipid packing of SUVs, LUVs are commonly used for most studies. However, these liposomes are submicroscopic and do not allow resolution of morphological features relevant to more macroscopic changes in biomembranes. The so-called giant vesicles (10-500 μm) which are spherical, closed molecular bilayers and entrap an aqueous compartment are instead good models for studies of undulation, budding, wounding, healing, and other manifestations of cell-like behavior induced by membrane-acting reagents (Menger, 1998; Angelova and Dimitrov, 1986; Angelova et al., 1999; Holopainen et al., 2000; Nurminen et al.,

2002). Due to their large size, giant vesicles can be observed by light microscopy, permitting direct visualization of processes involving supramolecular chemistry and biophysics (Luisi and Walde, 2000).

Besides the above model bilayer systems, phospholipid monolayers (for a review, see Brockman, 1999) is of interest due to their homogeneity, their stability and their planar geometry, where the lipid molecules have specific orientation (at an air/water interface, the heads of phospholipids point toward the water subphase and the acyl chains toward the air). Also the two dimensional molecular density and the ionic conditions of the subphase can be varied, as well as the temperature and composition. Since the amphipathic character of antimicrobial peptides makes them surface-active and their biological activity begins at lipid membrane interface, the monolayer technique is particularly suitable to study their physicochemical and biological properties. The interaction of peptides with phospholipids at the air/water interface provides a unique model in understanding the insertion mechanisms of antimicrobial peptide into cell membranes (for reviews, see Maget-Dana, 1999; Zhao et al., 2003).

2.5.2 Models presented for the mode of action of antimicrobial peptides

The action of antimicrobial peptides induces membrane defects such as phase separation or membrane thinning, pore formation, promotion of nonlamellar lipid structure or bilayer disruption, depending on the molecular properties of both peptide and lipid (Lohner and Prenner, 1999). These pathways are variously termed transmembrane pores, wormholes or toroidal pores, and channel aggregates (Bechinger, 1999; Matsuzaki, 1999; Shai, 1999; Hancock and Scott, 2000). An interesting ‘in plane diffusion’ model has also been proposed, where lipid-mediated channel formation is based on the curvature stain imposed on lipid membranes in the presence of intercalated amphipathic peptides. This model is independent of peptide aggregation, which has been reported to be entropically and electrostatically disfavored even in the presence of negatively charged phospholipids (Bechinger, 1999).

Two general mechanisms were originally proposed to describe the process of phospholipid membrane permeation by membrane-active peptides, the ‘barrel-stave’ (Ehrenstein and Lecar, 1977) and the ‘carpet’ (Pouny et al., 1992) mechanisms. Fig.

2, a cartoon illustrates both models suggested for membrane permeation. The third mechanism, the aggregate channel formation, was also proposed for peptides without causing significant membrane depolarization. The details of these models are as follows:

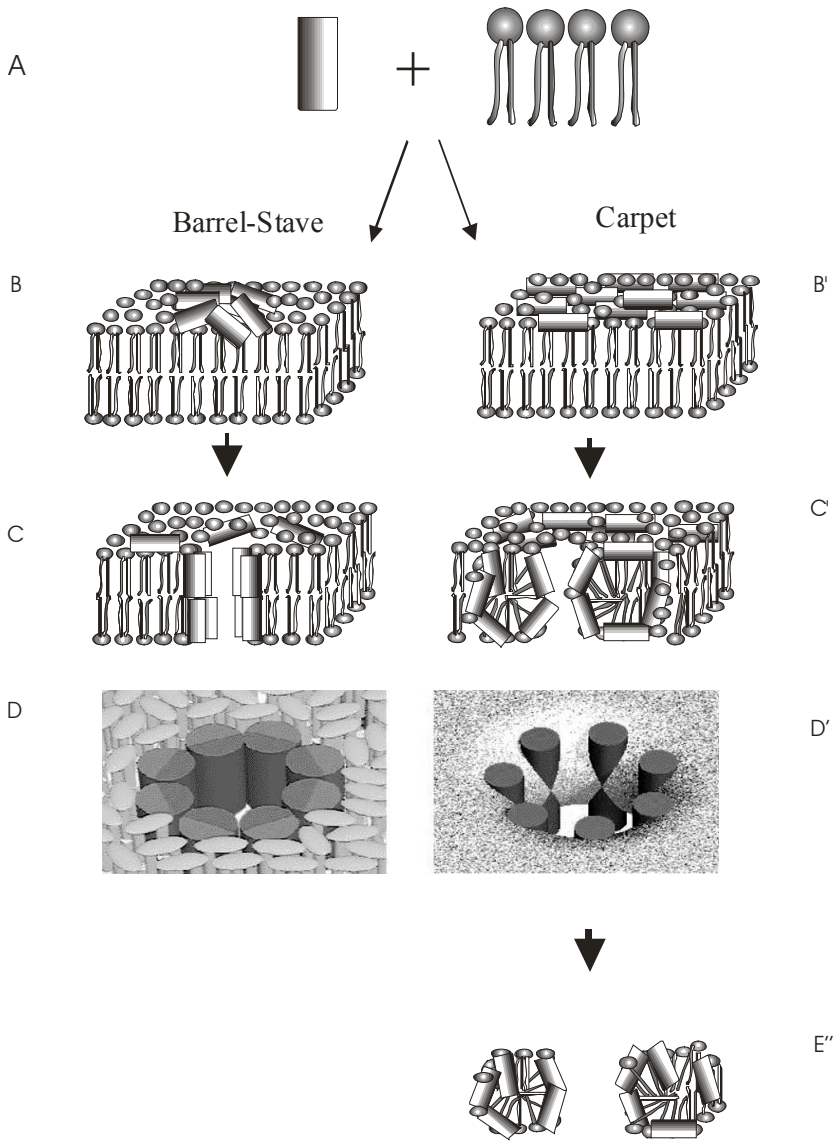


Fig. 2. A cartoon illustrating the barrel-stave (to the left) and the carpet (to the right) models presented for membrane permeation. In the barrel-stave model peptides first assemble on the surface of the membrane (*panel B*), then insert into the lipid core of the membrane following recruitment of additional monomers (*panel C*), forming pores (*panel D,* taken from Huang, 2000). In the carpet model the peptides are bound to the surface of the membrane with their hydrophobic surfaces facing the membrane and their hydrophilic surfaces facing the solvent (*panel B′*). When a threshold concentration of peptide monomers is reached, the membrane is permeated and transient pores can be formed (*panels C′ and D′, panel D′* was taken from Huang, 2000), a process that can lead also to membrane disintegration (*panel E′*). The spheres represent the phospholipid headgroups and two legs connected with these the two acyl chains. The molecules of antimicrobial peptides are illustrated as cylinders.

2.5.2.1 The barrel-stave model

The 'barrel stave' mechanism describes the formation of transmembrane channels/pores by bundles of peptides (Fig. 2, B-D). Progressive recruitment of additional peptide monomers leads to a steadily increasing pore size. Leakage of intracellular components through these pores subsequently causes cell death. To allow pore formation the inserted molecules should have distinct structures, such as, an amphipathic or hydrophobic α-helix, β-sheet, or both α-helix and β-sheet structures. A crucial step in the barrel stave mechanism requires peptides to recognize one another in the membrane bound state. Peptide assembly can occur on the surface or within the hydrophobic core of the membrane, since hydrophobic peptides can span membranes as monomers (Ben Efraim and Shai, 1997). In contrast, it is energetically unfavorable for a single amphipathic α-helix to transverse the membrane as a monomer. The low dielectric constant and inability to establish hydrogen bonds prohibits direct contact of the hydrophobic core of the membrane with the polar face of a single amphipathic α-helix. Therefore such monomers must associate on the surface of the membrane before insertion and the inserted peptides associated in membranes such that their non-polar side chains face the hydrophobic lipid core of the membrane and the hydrophilic surfaces of peptides point inward into the water-filled pore. An amphipathic α-helical peptide with a highly homogeneously charged hydrophilic face cannot form a transmembrane pore unless its charges become neutralized. It is therefore unlikely that antimicrobial peptides with a large number of lysines or arginines spread along the peptide chain will permeate the membrane by barrel-stave mechanism.

Because these peptides insert into the hydrophobic core of the membrane, they should have two important properties (Shai and Oren, 2001): (i) their interaction with the target membrane is driven predominantly by hydrophobic interactions. (ii) if they adopt amphipathic α-helical structure, their net charge along the peptide backbone should be close to neutral. Alternatively they can be composed of predominantly hydrophobic amino acids (Ben Efraim et al., 1993). As a consequence of these properties the peptides bind to phospholipid membranes irrespective of the membrane charge, and therefore, should be toxic to both bacteria and normal mammalian cells. Indeed, functional studies revealed that pardaxin (Rapaport and Shai, 1991),

alamethicin (Sansom, 1993), and the helix α5 of δ-endotoxin (Gazit et al., 1994) kill both bacteria and erythrocytes by the barrel-stave mechanism.

2.5.2.2 The carpet model

Early studies suggested that antimicrobial peptides kill microorganisms by forming transmembrane pores presumably via the barrel-stave mechanism (Matsuzaki et al., 1991). However, the interaction of certain antimicrobial and lytic peptides with membranes clearly differentiated from the barrel-stave pore formation and a new mechanism was suggested. The carpet model was proposed for the first time to describe the mode of action of dermaseptin S (Pouny et al., 1992), and later on was used to describe the mode of action of other antimicrobial peptides, such as dermaseptin natural analogues (Ghosh et al., 1997), cecropins (Gazit et al., 1995), the human antimicrobial peptide LL-37 (Oren et al., 1999a), caerin 1.1 (Wong et al., 1997), trichogin GA IV (Monaco et al., 1999), and diastereomers of lytic peptides (Oren et al., 1999b; Sharon et al., 1999; Oren and Shai, 2000). According to the carpet model, the peptides first bind onto the surface of the target microbial cell membrane and subsequently the membrane is covered by a 'carpet-like' cluster of peptides (Fig. 2, B'). Initial interaction with the negatively charged target membrane is electrically driven. In the second step, after a threshold concentration has been reached, the peptides cause membrane permeation (Fig. 2, C' and D'). This model further suggests that the membrane breaks into pieces and leads to lysis of the microbial cell when a threshold concentration of peptide is reached (Oren and Shai, 1998, Fig. 2, E'). High local concentration on the surface of the membrane depends upon the type of the target membrane and can occur either after all the surface of the membrane is covered with peptide monomers, or alternatively, antimicrobial peptides that associate on the surface of the membrane can form a local carpet. At an intermediate stage wormhole formation has been suggested to occur (Fig. 2, C' and D'). Pores like these may enable the passage of low molecular weight molecules prior to complete membrane lysis. The formation of so-called wormholes or toroidal pores was proposed to describe the mode of action of dermaseptin (Mor and Nicolas, 1994), magainin (Ludtke et al., 1996; Matsuzaki et al., 1995b, 1996; Yang et al., 2000), protegrin (Heller et al., 1998), and melittin (Yang et al., 2001). Neutral in-plane scattering data showed that pores of magainin molecules are almost twice as large as the alamethicin pores and

suggested that the lipid layer bends back on itself like the inside of a torus (Ludtke et al., 1996). This requires a lateral expansion in the polar headgroup region of the bilayer, which is filled up by individual peptide molecule. The positively charged amino acids are spread along the peptide chain of magainin and continuously in contact with the phospholipid head group during the process of peptide permeation, even when the peptide oriented perpendicular to the membrane plane. A structure of organized holes composed of lipids and peptides were recently reported (Yang et al., 2000). The presence of negatively charged lipids is important for a peptide carpet to form, as they help to reduce the repulsive electrostatic forces between positively charged peptides. In addition, high local peptide concentrations are achieved when cationic peptides phase-separate into domains rich in acidic phospholipids (Latal et al., 1997). The carpet model describes a situation in which these peptides are in contact with the phospholipid head group throughout the entire process of membrane permeation. Furthermore, a peptide that permeates the membrane via this mechanism can adopt different secondary structures, lengths, and can be either linear or cyclic upon its binding to the membrane. The net charge of the peptides needs to be highly positive and spread along the peptide chain that they only bind very weakly, or not bind zwitterionic membranes. However, highly positively charged antimicrobial peptides can lyse erythrocytes membranes if they form oligomers in solution (Shai and Oren, 2001).

In brief, peptides that act via the carpet mechanism and remain in contact with the acidic phospholipid head groups, should have the following properties (Shai and Oren, 2001): (i) their net charge needs to be highly positive and spread along the peptide chain. (ii) they should bind very weakly, or not bind zwitterionic membranes and as a consequence not be hemolytic. It should be noted however, that highly positively charged antimicrobial peptides can lyse erythrocytes if they form oligomers in solutions (iii) no preferable structure is required, as long as a certain level of hydrophobicity and number of positive charges are preserved.

2.5.2.3 The aggregate channel model

Both the carpet and the barrel-stave models predict that killing activity of antimicrobial peptides occurs with concomitant dissipation of the membrane potential, due to the collapse in membrane integrity. However, although permeabilization of the

cell membrane seems necessary, several studies indicate that permeabilization alone may not be enough to explain antimicrobial activity (Ganz and Lehrer, 1995). Planar lipid bilayer model studies showed that transient conductance events indicative of pore formation were only seen at high concentrations of peptides and high transmembrane voltages (Wu *et a*l., 1999). The magnitude of these conductance effects exhibited little correlation with the antimicrobial potency. Some peptides, e.g. bactenecin, did cause membrane depolarization at their minimal inhibitory concentrations. However, other peptides, *e.g.* gramicidin S, caused maximal membrane depolarization at a concentration well below their minimal inhibitory concentrations. This implicates that membrane depolarization by itself is not necessarily the crucial event in the killing of microorganisms and thus the aggregate channel model is proposed (Hancock and Chapple, 1999). After binding to the phospholipid head groups, the peptides insert into the membrane and then cluster into unstructured aggregates that span the membrane. These aggregates are proposed to have water molecules associated with them providing channels for leakage of ions and possibly larger molecules through the membrane. This model essentially differs from the other two: only short-lived transmembrane clusters of an undefined nature are formed, which allow the peptides to cross the membrane without causing significant membrane depolarization. Once inside, the peptides home to their intracellular targets to exert their killing activities. Recently, NMR study with indolicidin revealed an interesting fold, comprised of a hydrophobic core flanked by two dispersed, positively charged regions. Due to its central hydrophobic core, it was suggested that indolicidin inserts into the hydrophobic core of the membrane and forms channel aggregates (Rozek et al., 2000).

2.5.3 Alternative mechanisms of action: intracellular targets of antimicrobial peptides

Most antimicrobial peptides act directly on lipid bilayers of the target membrane, rather than on a receptor, as discussed in detail above. However, studies with several antimicrobial peptides have shown that all-L and all-D enantiomers are not of equal activity and the results appear to be strongly species dependent (Vunnam et al., 1997). For an example, the all-D isomers of apidaecin (Casteels and Tempst, 1994) and drosocin (Bulet *et al*., 1996) are no longer active, pointing to a stereo-specific interaction. A stereospecific complementarity between the peptide and a bacterial

receptor might exist, at least in certain bacterial species. Receptor-mediated signaling activities of some peptides have also been reported (Hoffman *et al*., 1999). Likewise, the antimicrobial peptides might target intracellular molecules, such as DNA or enzymes, since they are capable of spontaneously traversing bacterial outer and inner membranes. Recently, indolicidin was proposed to inhibit DNA synthesis leading to filamentation in *E. coli* (Subbalakshmi and Sitaram, 1998). Accordingly, increasing evidence indicates that antimicrobial peptides could also act through a variety of mechanisms other than disruption of membrane integrity, and that their role in host defense goes well beyond direct killing of microorganisms. For example, stimulation of host defense-mechanisms by antimicrobial peptides is becoming apparent in several cases. Among these antimicrobial peptides, dermaseptin, which was found to stimulate the release of myeloperoxidase and the production of reactive oxygen species by polymorphonuclear leukocytes (Ammar *et al*., 1998). Also defensins can enhance the recruitment of neutrophils to the infected tissue (Welling *et al*., 1998), thus increasing the ability of the host to resist local infections. Some antimicrobial peptides were found to interfere with the metabolic processes of microbes. Accordingly, the glycine-rich attacins block the transcription of the *omp* gene in *E. coli* (Carlsson *et al*., 1991), whereas magainins and cecropins induce selective transcription of its stress-related genes *micF* and *osmY* at sublethal concentrations (Oh *et a*l., 2000). Bac5 and Bac7 inhibit protein and RNA synthesis of *E. coli* and *Klebsiella pneumoniae* by inhibition of the respiration in addition to their potential to disturb the membranes of these bacteria (Skleravaja *et al*., 1990). PR-39 binds to the cell membrane of *E. coli* without causing permeabilization (Cabiaux *et a*l., 1994), but kills bacteria solely by stopping both DNA and protein synthesis (Boman *et a*l., 1993). Similarly, buforin II inhibits the cellular functions of *E. coli* by binding to DNA and RNA after penetrating the cell membranes (Park *et a*l., 1998). Another emerging view is that many peptides act synergistically with other host molecules with antimicrobial activity to kill microbes. Positive cooperativity has been reported between peptides and lysozyme, and between different antimicrobial peptides (McCafferty *et al*., 1999; Hancock and Scott, 2000; Kobayashi et al., 2001). More in general, it is becoming clear that there exists a certain degree of coupling between the innate and adaptive immune systems with antimicrobial peptides having a large impact on the quality and effectiveness of immune and inflammatory responses (Raj and Dentino, 2002). Although the present scenario is far to be complete, excellent

reviews are available that highlight recent examples of these intriguing themes (Hancock and Diamond, 2000; Hancock and Scott, 2000; Hancock, 2001).

2.6 The Potential of Structural Features of α-helical Antimicrobial Peptides to Modulate Activity on Model Membranes and Cells

Antimicrobial peptides that assume an amphipathic α-helical structure are widespread in nature and most studied. Their activity depends on several parameters, including the sequence, size, degree of structure formation, cationicity, hydrophobicity and amphipathicity, which can be used as starting points in the rational design of antimicrobial peptides with reduced hemolytic activity (Dathe and Wieprecht, 1999). Although the individual importance of each of these features is difficult to determine, as they all influence each other, some general trends have emerged from studies with model peptides. Early studies directed towards enhancing membrane activity and improving the antimicrobial effect were based on appropriate amino acid substitutions to increase peptide helicity. Helicity is essential for the activity against neutral (eukaryotic) membranes, but seems less important for the activity against negatively charged (prokaryotic) ones. Breaking the helix by insertion of proline residues resulted in slight decrease in antimicrobial activity, but a large decrease in hemolytic activity and an enhanced synergy with conventional antibiotics (Zhang *et a*l., 1999). However, some peptides, such as magainins, completely lost their antimicrobial activity after breaking the helix by substitutions with two consecutive D-amino acids, whereas the activity of melittin and pardaxin remained unaffected (Shai and Oren, 1996). Recently, titration calorimetry studies confirmed that the α-helix formation is a strong driving force for the binding to membranes (Wieprecht *et al*, 1999). The role played by this parameter modulating the peptide-membrane interaction has been further explored by several investigations. The study with the amphipathic model peptide KLAL (KLALKLALKALKAAKLA-NH_2) readily showed that the degree of helicity plays a negligible role in the binding between the peptide and a very negatively charged bilayer, whereas a strong effect is observed on membranes with reduced negative charge (Dathe *et al*., 1996). All these results seem in agreement with the effects observed on erythrocytes, because a decrease in helicity of KLAL analogs correlates with a remarkable reduction in hemolytic activity, whereas both Gram-negative and -positive bacteria are not much sensitive to the change of this parameter

(Dathe *et al*., 1996). Nevertheless, more recent data concerning some synthetic small peptides (10 amino acid long) asserted the necessity to widen these considerations, since helicity seems to condition the antimicrobial activity of these peptides in a significant way (Oh *et al*., 1999).

Amphipathicity: amphipathicity of a peptide is a measure for the spatial separation between hydrophilic and hydrophobic side chains. It is generally determined by calculating the hydrophobic moment, the vector sum of the hydrophobicities of each amino acid residue perpendicular to the axis of the helix. To mutually compare the amphipathicity of peptides with different lengths, in many studies the mean hydrophobic moment per residue is used (Eisenberg *et a*l., 1984). Although this method is generally in use, the hydrophobic potential, which takes into account the charge distribution along the axis of the helix including the gaps between individual amphipathic stretches, would be a more accurate measure. The concept that amphipathicity is a key feature of antimicrobial peptides has initiated the *de novo* design of a number of simple antimicrobial peptides that consist of repeating sequences of alternating hydrophobic and positively charged stretches (Cornut *et a*l., 1994; Javadpour *et a*l., 1996). Some investigations reported by Wieprecht and colleagues suggest that the hydrophobic moment influences much more the activity on neutral model membranes than on negatively charged bilayers (Wieprecht *et al*., 1997a; Dathe *et al*., 1997). An increase of this parameter in magainin 2 analogs causes an enhanced permeabilizing efficiency on phosphatidylcholine-rich bilayers, whereas the negatively charged model membranes are less affected. It suggests that hydrophobic interactions with acyl chains of lipids dominate the activity on neutral membranes and this parameter influences the antimicrobial specificity in a negative manner. Nevertheless, this last point of view is not in agreement with more recent data obtained by a C-terminal fragment of melittin (Subbalakshmi *et al*., 1999). The extent of its hydrophobic face causes a more remarkable increase in antibacterial activity compared to hemolytic activity, thus providing important directions to the design of small peptides with selective antimicrobial activity.

Hydrophobicity: hydrophobicity of the peptide is usually defined as the average of the numeric hydrophobicity values of all residues (Eisenberg *et a*l., 1984) and is a measure of the ability of a peptide to move from an aqueous to a hydrophobic phase.

This parameter measures the degree of peptide affinity for the lipid acyl chains at the core of model and biological membranes. Most data are in agreement to associate an increase in hydrophobicity with an enhanced permeabilizing activity on zwitterionic bilayers. High mean hydrophobicities thus have been correlated with high cytotoxic activities against the neutral membranes of mammalian cells (Javadpour *et a*l., 1996; Skerlavaj *et a*l., 1996). Studies on histatin- and magainin-derivatives confirmed that hemolytic activity was strongly correlated with mean hydrophobicity, but independent of amphipathicity or net peptide charge (Helmerhorst *et a*l., 1999b). Moreover, these investigations also suggest that hydrophobicity plays a secondary role in the interaction between peptides and negatively charged bilayers. The antimicrobial activity is thus not much influenced by this parameter, and besides it seems to present a negative correlation with the antibacterial specificity. All these observations have been confirmed by studies on brevinin 1E amide (Kwon *et al*., 1998) and some synthetic amphiphilic α-helical peptides (Kiyota *et al*., 1996).

Hydrophobic and hydrophilic angles: the angles subtended by the hydrophilic and hydrophobic faces of the peptide modulate the manner in which amphipathic peptides bind to membranes (Dathe and Wieprecht, 1999). Peptides with small hydrophilic angles and high mean hydrophobicities tend to form transmembrane pores, whereas peptides with equivalent hydrophobic and hydrophilic faces rather adopt a parallel orientation upon binding to membranes. Changes in the hydrophilic angle had little effect on the neutral lipid membranes of erythrocytes, whereas the permeabilization of highly charged model membranes was decreased upon increase of the polar angle. This has been found to be a very important factor in the overall peptide-bilayer interaction because it emphasizes the differences between binding and permeabilization. In the last years this aspect was elucidated by several investigations on model peptides designed from magainin 2 amide which underlined that the value of the hydrophilic angle (subtended by the positively charged helix face) correlates in an opposite manner with the peptide-membrane affinity and the membrane permeabilization (Wieprecht *et al*., 1997b). These synthetic peptides, having a different polar angle but no other structural modification, show that the binding is favoured by a large angle and by a negatively charged bilayer, whereas the permeabilization is enhanced by a small angle and by a low negative membrane

charge. Moreover both antimicrobial and hemolytic activity present a positive correlation with an increase of the angle. Nevertheless, these and other data show that the same increase causes a decrease in specificity against bacteria compared to erythrocytes (Wieprecht *et al*., 1997b; Dathe *et al*., 1997). These observations are partly confirmed by more recent investigations on two model peptides, θp100 and θp180, which differ only in their polar angles (Uematsu and Matsuzaki, 2000). Indeed, the binding affinity of θp180 (with a larger positive angle) for a negatively charged bilayer, is only slightly larger than that of θp100, whereas the latter is more effective in permeabilizing the same bilayer, thus confirming the positive correlation between a small polar angle and the ability of a given peptide to penetrate in a lipid bilayer to form a pore. Instead, the data on hemolytic and antimicrobial activity in this case do not seem completely in agreement with the previous observations (Uematsu and Matsuzaki, 2000), and that emphasizes the complexity of peptide interaction with a biological membrane.

Charge: most cytotoxic peptides are positively charged due to the presence of lysine and arginine residues (and, to a lesser extent, histidine) in their sequences. The net charge of these molecules usually ranges from +2 to +9 and can vary with pH as a result of the ionization state of various residues. Obviously, a positive charge facilitates the binding of antimcirobial peptides to negatively charged membranes. However, when the positive charge becomes too high, the membrane activity of the peptides may decrease because the strong electrostatic interactions anchor the peptide to the lipid head group region (Dathe and Wieprecht, 1999) or because the repulsion between the positively charged side chains, intra- or intermolecular, obstructs the formation of pores (Matsuzaki, 1999). Accordingly, there is no simple correlation between peptide charge and membrane activity. It has been shown that the presence of negatively charged lipids increases the local concentration of cationic peptides close to the membrane surface and thus also their apparent association constants (Matsuzaki et al., 1991; Silvestro et al., 1997). The charges of the peptide screen the negative surface charge density of the lipid, therefore, the electrostatic contribution to the binding isotherm is strongly dependent on the amount of bound peptide and saturates at apparent charge equality (Kuchinka and Seelig, 1989). The ability of amphipathic peptides to penetrate or to disrupt the membrane are dependent on the charge and the

size of the lipid head group (Wieprecht et al., 1997b; Vogt and Bechinger, 1999). Furthermore, the electrostatic interactions could arise from the presence of large inside-negative transmembrane potentials present in respiring prokaryotic cells but not in erythrocytes (Matsuzaki et al., 1995a). It has been suggested that transmembrane electric potentials affect the partitioning of polypeptides between the aqueous and the lipid phase (Stankowski et al., 1989), the orientations of peptides within membranes (Sansom, 1993), the peptide bilayer penetration depth, and peptide conformation (Mattila et al., 1999).

In summary, peptides with a moderately high positive charge, a large hydrophobic moment and a small hydrophilic angle tend to have high activity against microbial membranes, low hemolytic activity, and a preference for a carpet-like mechanism of action. In contrast, peptides with a low positive charge, a small amphipathic moment, and high intrinsic hydrophobicity show high activity to microbial as well as host membranes and a preference to form barrel-stave-like pores (Shai, 1999). However, because all the structural features discussed are strongly interrelated, it is difficult to predict the antimicrobial activity, selectivity, and mode of action of a given peptide. A striking example is presented by melittin, which consists of a hydrophobic N-terminal domain, responsible for its hemolytic activity, joined by a proline hinge to a highly selective positively charged amphipathic C-terminal domain. In this peptide, both extremes of the spectrum discussed above are combined in one molecule**.**

2.7 Potential as Therapeutics

2.7.1 Introduction of peptide antibiotics on the market

The widespread increase of bacterial resistance towards many conventional antibiotics has resulted in an intensive search for alternative antimicrobial agents (Bonomo, 2000). In this respect, antimicrobial peptides are on the brink of a breakthrough. Due to the increasing interests of antimicrobial peptides, many companies are making efforts to introduce the antimicrobial peptide products on the market.

Natural antimicrobial peptides have potential application in food preservation as they specifically kill microbial cells by destroying their unique membranes. Interest in LAB bacteriocins has been sparked by growing consumer demands for natural and

minimally-processed foods. LAB bacteriocins have well-documented lethal activity against foodborne pathogens and spoilage microorganisms (Cleveland et al., 2001), and can play a vital role in the design and application of food preservation technology (Leistner and Gorris 1995; Montville and Winkowski 1997). Currently, nisin is approved as a food preservative in more than 40 countries worldwide (Delves-Broughton 1990) and the use of pediocin PA-1 is covered by several European and US patents (Vandenbergh et al. 1989; Boudreaux and Matrozza 1992). Both nisin and pediocin PA-1 have applications in dairy and canned products. Studies of model food systems demonstrate that pediocin-like bacteriocins are better at killing pathogens in meat products, where nisin is ineffective (Leisner et al. 1996; Montville and Winkowski 1997).

Antimicrobial peptides tend to be involved in a local response to infections and the first clinical trials thus have been directed towards topical infections. Magainin Pharmaceuticals have taken the α-helical magainin variant peptide MSI-78 into phase-III clinical trials in studies of efficacy against polymicrobic foot-ulcer infections in diabetes. It was announced (http://www.pslgroup.com/dg/2168e.htm) that these trails demonstrated equivalency to orally administered ofloxacin, but with less side effect. Applied Microbiology has initiated a trial testing the efficacy of the bacterial lantibiotic peptide nisin against *Helicobacter pylori* stomach ulcers. The antibacterial activity of nisin may not be impressive, but its endotoxin neutralizing activity upon intravenous administration has led to a dramatically increased survival rate. Iseganan (IB-367, Intrabiotics, Mountain View, CA, USA), a protegrin-derivative (Mosca et al., 2000), has passed phase II clinical trials for application against oral mucositis successfully and the company has announced plans to launch Phase II/III clinical study to investigate iseganan HCl (Giles et al., 2002) in the prevention of ventilator-associated pneumonia (VAP). Another formulation of this company, iseganan HCl solution for inhalation, has completed phase I clinical trials in cystic fibrosis patients. Other companies, for examples, Periodontix Inc. (Watertown, MA, USA) has entered phase I clinical trials for the application of a histatin-derived peptide against oral candidiasis and Trimeris (Durham, NC, USA) has successfully completed a phase II clinical trial, in which peptide T-20 (Su et al., 1999; Cohen et al., 2002; Wei et al., 2002) reduced the viral load of HIV-infected patients with up to

97%. Also Demegen (Pittsburgh, PA, USA) has successfully completed animal studies with peptide D2A21 (Robertson et al., 1998) as therapeutic for several types of cancer and has been developing this peptide gel formulation as a wound healing product to treat infected burns and wounds. Demegen's P113L (histatin 5 fragment) Oral Rinse exhibits significant binding to oral mucosal membranes and has an excellent human safety profile in over 400 treated patients. Another product of Demegen, P113D derived from histatins (Sajjan et al., 2001), had been granted orphan drug status for the treatment of cystic fibrosis infections.

Interestingly, a number of evidence has shown efficacy of some antimicrobial peptides against systemic infections, including α-helical-peptide (SMAP29, table 1) efficacy against *P. aeruginosa* peritoneal infections, β-sheet-protegrin (table 1) efficacy against methicillin-resistant *S. aureus* (MASA), vancomycin-resistant *Enterococcus faecalis* (VRE) *and P. aeruginosa* infections, and indolicidin (table 1) in liposomal formulation against *Aspergillus* fungal infections (Ahmad et al., 1995; Steinberg et al., 1997; Saiman et al., 2001). Entomed (Illkirch, France) 's product, heliomicin (Lamberty et al., 2001) for systemic antifungal treatment is under preclinical stage. Human lactoferricin (table 1, AM Pharma, Bunnik, Netherland) and bactericidal/permeability-increasing protein (Xoma, Berkeley, CA, USA, Gray et al., 1989) have also been proved to have potential for systemic applications. This has indicated that antimicrobial peptides could be used as injectable antibiotics against serious bacterial and fungal infections that are resistant to conventional antibiotics. Indeed, Neuprex™, a systemic formulation of the recombinant BPI-derived peptide rBPI 21 (Xoma Corp., Berkeley, CA, USA, Horwitz et al., 1996), has proven to be very effective in treatment of meningococcal sepsis in phase II/III clinical trials and more than 1000 patients have received NEUPREX in clinical studies without any safety concerns.

2.7.2 Novel methods for production and application of antimicrobial peptides

For peptide therapeutics, in order to become real alternatives for conventional antibiotics and achieve similar broad applications, it is essential that their prices will be reduced significantly as long as cheap conventional antibiotics are on the market. Because most antimicrobial peptides are simple gene translation products, it is

relatively simple to produce them by recombinant expression methods at the place of action, thus avoiding problems associated with proteolysis and rapid clearing. At first, it may seem contradictory to express antimicrobial peptides in bacteria or yeast cells. However, this can be achieved if the producing microorganisms are resistant to the produced peptide antibiotic. A number of such applications are already known. In dairy products, the addition of bacteria producing lantibiotics as natural conserving agents has been common since the early fifties. A promising new alternative with a lower cost is large-scale production of biologically active proteins in transgenic plants or animals. In recent years, remarkable results have been obtained by producing transgenic plants that express elevated levels of antimicrobial peptides in leaves and seeds, thus potentially reducing the need for using environmentally hazardous antibacterial or antifungal crop protecting agents (Jach *et al*., 1995, De Bolle *et a*l., 1996). This expression may also provide these plants with built-in preservation agents that prolong their storage lives after harvesting. Recently, a modified intein expression system was used to produce pharmaceutical peptides in transgenic plants (Morassutti et al., 2002). Another approach to reduce the need of conventional crop protecting agents is to use plant hormones to induce the production of innate antimicrobial factors. Spraying of plants with *cis*-jasmonate-derivatives was successful in wind-tunnel experiments (Immanishi *et a*l., 1997) and field experiments are currently taking place. An alternative for transgenic techniques that may be applicable in animals or humans is gene therapy. In animal models, incorporation of LL-37 (Bals *et a*l., 1999) or histatin (O'Connell *et a*l., 1996) genes in mucosa or salivary glands, respectively, has been demonstrated to lead to an increased antimicrobial defense. Again, the latter applications are limited to external use, including the gastrointestinal tract. As most peptides will not survive passage through the gastrointestinal tract, treatment protocols for systemic infections with peptide therapeutics will be limited to injections for the time being. In specific applications, the rapid clearing of peptides can be reduced by mixing them with a muco-adhesive polymer (Ruissen *et a*l., 1999) or with acrylic bone cement (Yaniv *et al*., 1999). Novel formulations may also be necessary to modulate the action of antimicrobial peptides with little intrinsic selectivity. In an animal model for fungal infection, the high cytotoxicity of indolicidin for host cells was overcome by administration of liposomally encapsulated material (Ahmad *et al.,* 1995). In this respect, the development of vehicles delivering an efficiently high concentration of peptide

therapeutics at the right place, right time, and reasonable costs, has been becoming a challenge for pharmacologists.

2.7.3 Limitations as therapeutics

The future of antimicrobial peptides as antibiotics appears to be great and as mentioned above, a number of antimicrobial peptides with therapeutic potential have been developed in the past two decades. Antimicrobial peptides are generally considered to be highly selective antimicrobial agents. However, peptides do not discriminate absolutely between eukaryotes and prokaryotes, although the former are less sensitive. Several studies show that simple eukaryotes, such as yeasts, fungi, parasites (Jaynes et al., 1988; Ahmad et al., 1995), even as large ones as planaria (Zasloff, 2002), are effectively killed by cationic peptides. In addition, their unique pharmacological properties have limited their application to topical use. Recent publications describe also changes in bacterial cell wall components induced by environmental conditions, which may be involved in bacterial resistance towards antimicrobial peptides (Groisman *et a*l., 1997; Wösten and Groisman, 1999). Technical difficulties and high production costs have made the pharmaceutical industry reluctant to invest much effort in the development of antibiotic peptide therapeutics so far. The antimicrobial peptides are too expensive or with a too limited spectrum to be used on a large, commercially interesting scale. The biggest challenge of the near future will be to overcome the pharmacological limitations of these interesting molecules and to develop them into therapeutics.

2.7.3.1 Antimicrobial peptide resistance

As a major component of host innate immunity, antimicrobial peptides can directly contact and disrupt the bacterial membrane by permeating lipid bilayers, and ultimately lead to cell death (Tossi et al., 2000). However, it is virtually impossible to elicit resistance against some antimicrobial peptides (Hancock, 1997; Hancock and Lehrer, 1998). Bacterial pathogens have developed the means to curtail the effect of antimicrobial peptides. Direct degradation of antimicrobial peptides and modification of cell surface properties are two major strategies used by Gram-negative bacteria to resist the bactericidal activity of antimicrobial peptides. The former strategy is dependent on the production of outer membrane-associated proteases, which cleave antimicrobial peptides outside the cells and enable bacteria to evade killing. For

example, it has been found that *E. coli* outer membrane protease OmpT hydrolyzes the antimicrobial peptide protamine before it enters this bacterium (Stumpe et al., 1998). A PhoP-regulated outer membrane protease PgtE of *Salmonella typhimurium* also contributes to resistance to antimicrobial peptides (Guina et al., 2000). Because antimicrobial peptides act on bacterial membranes, Gram-negative bacteria can also protect themselves from attack by antimicrobial peptides via modification of their cell surface properties to prevent the binding of antimicrobial peptides to the outer membrane or decrease the permeability of the outer membrane (Ernst et al., 1999; Guo et al., 1997; Guo et al., 1998). For example, two-component regulatory systems in *S. typhimurium* including PhoP/PhoQ and PmrA/PmrB, promote antimicrobial peptide resistance by activating transcription of genes that are involved in the modification of lipid A, the bioactive component of LPS (Ernst et al., 1999; Guo et al., 1997). The *S. typhimurium* PhoP/PhoQ-activated gene pagP, which is essential for addition of palmitate to *Salmonella* lipid A, encodes an OMP with enzymatic activity involved in lipid A biosynthesis (Guo et al., 1998; Bishop et al., 2000). Such lipid A modification changes the fluidity of the outer membrane and decreases the permeability of the outer membrane enhancing the resistance of *S. typhimurium* to α-helical cationic antimicrobial peptides (Peschel, 2000). Whether other Gram-negative bacteria use PagP-like enzymes to mediate antimicrobial peptide resistance is largely unknown. However, PagP-mediated lipid A palmitoylation is likely a general mechanism for Gram-negative bacterial resistance to α-helical cationic antimicrobial peptides (Guo et al., 1998; Bishop et al., 2000). Furthermore, synergistic action of multiple resistance strategies can greatly decrease the bactericidal activity of antimicrobial peptides. One such example is that inactivation of both the protease gene (pgtE) and the lipid A modification gene (pagP) in *S. typhimurium* resulted in greater antimicrobial peptide sensitivity than that in mutants containing a single mutation in either gene (Guina et al., 2000).

2.7.3.2 Immunogenicity

Another feature of peptides that may interfere with their use as therapeutics is immunogenicity. Apparently, the mucosal antibody response to short peptides is not very strong. However, for systemic use immunogenic safety is not that obvious. In blood no high levels of antimicrobial peptides have been reported, and the release of

systemically working antimicrobial peptides generally is strictly controlled. Preliminary results *in vivo* suggest that antimicrobial peptides may work as single-shot therapeutics, so that the risk of prolonged systemic concentrations high enough to induce an IgG antibody response may be insignificant. However, certain cationic peptides are known to have a direct effect on mast cells, leading to histamine release without an allergenic response. Guinea pig antibacterial polypeptide CAP11, neutrophil defensins, and histatin 5 induce a strong histamine release (Yomogida et al., 1997; Yoshida et al., 2001). Furthermore, antimicrobial peptides may cross-react with other receptors. A number of neuropeptides and peptide hormones with their specific receptors are known. As they are small, often amphipathic, molecules, their receptor-binding sites are presumed to contain only a limited number of essential amino acids. The chance that a similar motif will be found in a peptide antibiotic may become significant upon use of large numbers of different antimicrobial peptides in considerably higher concentrations than endogenous peptide hormone levels. To this end, as these paragraphs illustrate, the pharmacology and pharmacokinetics of antimicrobial peptides are still unknown and many studies on these topics are required before the feasibility of peptide therapeutics will be generally accepted by the pharmaceutical industry.

3 AIMS OF THE STUDY

The purpose of the present work was to study the molecular mechanism of membrane activity of antimicrobial peptides and their target membrane specificity, as well as their alternative role in innate immunity. The long-term aim of the effort is to find a key for the rationale design of novel antimicrobial peptidomimetics.

The major aims are:

I. To assess the relative energies of peptide-lipid interactions and characterize lipid specificity of the antimicrobial peptides, magainin 2, indolicidin, temporin B and L.

II. To investigate the effects of the antimicrobial peptides on lipid dynamics, as well as the structure, orientation, and depth of penetration of temporin L in phospholipid bilayers.

III. To visualize and characterize the impact of the antimicrobial peptides on the 3-D topology of a novel biomimetic membrane model for cells.

IV. To elucidate the alternative mechanism of the antimicrobial peptides in host defense, viz. their potential to regulate the activity of secretory phospholipase A_2.

4 MATERIALS AND METHODS

4.1 Materials

Hepes, EDTA, magainin 2, and bee venom phospholipase A_2 were from Sigma and indolicidin from Bachem (Bubendorf, Switzerland). Synthetic temporin B were purchased from Tana Laboratories (Houston, TX) and temporin L was purchased from Synpep (Dublin, CA). The purities of the peptides (> 99%, > 95%, > 90%, and > 94% for magainin 2, indolicidin, temporin B and L, respectively) were analyzed by HPLC and their sequences were verified by both automated Edman degradation and mass spectrometry. Peptide concentrations were determined gravimetrically and by quantitative ion-exchange column chromatography and ninhydrin derivatization. Human lacrimal fluid was collected from healthy donors briefly exposed to the vapors of freshly minced onions. The collected fluid was stored at −20°C until used. 1-Stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), 1-palmitoyl-2-(6,7-dibromostearoyl) phosphatidylcholine (6,7-Br_2-PC), 1-palmitoyl-2-(9,10-dibromostearoyl) phosphatidylcholine (9,10-Br_2-PC), 1-palmitoyl-2-(11,12-dibromostearoyl) phosphatidylcholine (11,12-Br_2-PC), and β-cholesterol were from Avanti Polar Lipids (Alabaster, AL). The fluorescent phospholipid analogs 1-palmitoyl-2-[10-(pyren-1-yl)decanoyl]-*sn*-glycero-3-phospho-*rac*-glycerol (PPDPG), 1-palmitoyl-2-[10-(pyren-1-yl)decanoyl]-*sn*-glycero-3-phosphocholine (PPDPC), 1-palmitoyl-2-[6-(pyre-1-yl)]hexanoyl-*sn*-glycero-3-phosphatidylcholine (PPHPC) and 1-palmitoyl-2-[6-(pyre-1-yl)]hexanoyl-*sn*-glycero-3-phosphatidylmonomethylester (PPHPM) were from K&V Bioware (Espoo, Finland). Diphenylhexatriene (DPH) was from EGA Chemie (Steinheim, Germany). The gemini surfactant (2S,3R)-2,3-dimethoxy-1,4-bis(N-hexadecyl-N,N-dimethylammonnium)butanedibromide (SR-1) was synthesized as described previously (Cerichelli et al., 1996) and its purity was verified by NMR. This compound was kindly provided by Drs. S. Borocci and G. Mancini (University of Rome, Rome, Italy). The concentrations of the non-labeled lipids were determined gravimetrically with a high-precision electrobalance (Cahn, Cerritos, CA) and those of the pyrene containing phospholipids and DPH spectrophotometrically, by absorbance at 341 nm, using molar extinction coefficients of 38000 cm^{-1} for PPDPC

and PPDPG, 42000 cm^{-1} for PPHPC and PPHPM, and 88000cm^{-1} for DPH. The purity of the lipids was checked by thin layer chromatography on silicic acid coated plates (Merck, Darmstadt, Germany) developed with chloroform/methanol/water (65:25:4, v/v/v). Examination of the plates after iodine staining and, when appropriate, upon UV-illumination revealed no impurities.

4.2 Methods

4.2.1 Liposome preparation

4.2.1.1 Large unilamellar vesicles (LUVs)

The indicated lipids were mixed in chloroform after which the solvents were removed under a stream of nitrogen and the lipid residue subsequently maintained under reduced pressure for at least 2 h. The dry lipids were then hydrated at 50 °C and the resulting dispersions were extruded through a stack of two polycarbonate filters (100 nm pore-size, Millipore, Bedford, MA) using a Liposofast-low pressure homogenizer (Avestin, Ottawa, Canada) to obtain large unilamellar vesicles. The average diameters of these LUVs are between 111 to 117 nm (Wiedmer et al., 2001).

4.2.1.2 Small unilamellar vesicles (SUVs)

Appropriate amounts of the lipid stock solutions were mixed in chloroform to obtain the desired compositions and then dried under a stream of nitrogen followed by high vacuum for a minimum of 2h. The lipid residues were subsequently hydrated at 50 ^{0}C and the suspension was sonicated in a bath sonicator (Bedford, MA) until achieving clear solutions. The vesicles were used within the same day. For the purpose of measuring phospholipase A_2 activity SUVs were formed by rapidly injecting the indicated lipid ethanolic solution into the buffer (Batzri and Korn, 1973).

4.2.1.3 Giant vesicles

Giant liposomes were prepared as described elsewhere (Angelova and Dimitrov, 1986; Holopainen et al., 2000). Briefly, approx. 1-3 µl of the desired lipids dissolved in diethylether:methanol (9:1, v/v, final total lipid concentration 1 mM) were spread on the surface of two Pt electrodes and subsequently dried under a stream of nitrogen.

Possible residues of organic solvent were removed by evacuation in vacuum for one hour. A glass chamber with the attached electrodes and a quartz window bottom was placed on the stage of a Zeiss IM-35 inverted fluorescence microscope. An AC field (sinusoidal wave function with a frequency of 4 Hz and an amplitude of 0.2 V) was applied before adding 1.3 ml of 0.5 mM Hepes buffer, pH 7.4. During the first minute of hydration the voltage was increased to 1.0-1.2 V. The AC field was turned off after 2-4 hours and giant liposomes were observed with Hoffman modulation contrast (HMC) optics with a 10×/0.25 objective (Modulation Optics Inc., New York, USA). The size of giant liposomes was measured using calibration of the images by motions of the micropipette as proper multiples of the step length (50 nm) of the micromanipulator (MX831 with MC2000 controller, SD Instruments, Oregon, USA). Images were recorded with a Peltier-cooled 12-bit digital CCD camera (C4742-95, Hamamatsu, Japan) interfaced to a computer, and operated by the software (HiPic 5.0.1) provided by the manufacturer.

4.2.2 Microinjection techniques

Micropipettes with inner tip diameters of >0.5 μm were made from borosilicate capillaries (1.2 mm outer diameter) by a microprocessor-controlled horizontal puller (P-87, Sutter Instrument Co., Novato, CA, USA). Indicated amounts of the peptide solutions (0.5 mM in 10 mM Hepes, 0.1 mM EDTA, pH 7.0) were applied onto the outer surface of individual giant vesicles as series of single injection of approx. 20 fl each and delivered with a pneumatic microinjector (PLI-100, Medical Systems Corp., Greenvale, NY, USA). For easier handling only vesicles attached to the electrode surface were used. All experiments were performed at ambient temperature (approx. +24^{o}C) and were repeated at least 10 times.

4.2.3 Penetration of antimicrobial peptides into lipid monolayers

Penetration of peptides into monomolecular lipid films was measured using magnetically stirred circular Teflon wells (Multiwell plate, subphase volume 1.2 ml, Kibron Inc., Helsinki, Finland). Surface pressure (π) was monitored with a Wilhelmy wire attached to a microbalance (Delta Pi, Kibron Inc., Helsinki, Finland) connected to a Pentium PC. Lipids were mixed in the indicated molar ratios in chloroform and then spread onto the air-buffer (5 mM Hepes, 0.1 mM EDTA, pH 7.0) interface.

Subsequently, the lipid monolayers were allowed to equilibrate for approx. 15 min at different initial surface pressures (π_0) before the injection of the peptides into the subphase. The increments in π after the injection of peptides were complete in approx. 30 min and the difference between the initial surface pressure (π_0) and the value observed after the penetration of peptides into the films was taken as $\Delta\pi$. All measurements were performed at ambient temperature (approx. +24^{o}C).

4.2.4 Steady state fluorescence spectroscopy

4.2.4.1 Measurement of I_e/I_m

All fluorescence measurements were performed in quartz cuvettes with one cm path length. Fluorescence emission spectra for LUVs labeled with PPDPG or PPDPC (X=0.01) were measured with a Perkin-Elmer LS50B spectrofluorometer at 25 °C with a magnetically stirred, thermostated cuvette compartment using excitation wavelength of 344 nm. Band widths of 5 nm were used for both excitation and emission. The lipid concentration used was 25 μM. After addition of appropriate amounts of peptides, samples were equilibrated for 5 min before recording the spectra. Three scans were averaged and the emission intensities at ≈ 398 and 480 nm were taken for I_m and I_e, respectively. All measurements were repeated three times.

4.2.4.2. Fluorescence anisotropy of DPH

DPH were included into liposomes at X=0.002. The lipid concentration used was 25 μM with temperature maintained at 25 °C. Polarized emission was measured in L-format using Polaroid-type filters with Perkin-Elmer LS50B. Fluorescence anisotropy r for DPH was measured with excitation at 360 nm and emission at 450 nm, using 5 nm band widths and its values were calculated using routines of the software provided by Perkin-Elmer. All measurements were repeated three times.

4.2.4.3 Trp fluorescent emission spectra

LUVs were added to a solution of temporin L (5 μM, final concentration) in 5 mM Hepes, 0.1 mM EDTA, pH 7.0, with temperature maintained at 25 ^{0}C. After 3 h of equilibration fluorescence spectra was measured with a Perkin-Elmer LS 50B spectrometer with both emission and excitation bandpasses set at 5 nm. The

tryptophan residue of temporin L was excited at 280 nm and emission spectra were recorded from 290 to 450 nm, averaging five scans. Spectra were recorded as a function of the lipid/peptide molar ratio and corrected for the contribution of light scattering in the presence of vesicles. Blue shifts were calculated as the differences in wavelength of the maxima in emission spectra of lipid-peptide and peptide samples.

4.2.4.4 Quenching of Trp emission by acrylamide

To reduce absorbance by acrylamide excitation of Trp at 295 nm instead of 280 nm was used (De Kroon et al., 1990). Aliquots of the 3.0 M solution of this water soluble quencher were added to the peptide in the absence or presence of liposomes at peptide/lipid molar ratio of 1:100. The values obtained were corrected for dilution and the scatter contribution derived from acrylamide titration of a vesicle blank. The data were analyzed according to the Stern-Volmer equation (Eftink and Ghiron, 1976) :

$$F_0/F=1+K_{sv}[Q]$$

where F_0 and F are the fluorescence intensities in the absence and the presence of the quencher (Q), respectively and K_{sv} is the Stern-Volmer quenching constant, which is a measure for the accessibility of Trp to acrylamide.

4.2.4.5 Quenching of Trp emission by brominated phosphatidylcholines

The indicated LUVs were added to a temporin L solution (final concentration of 5 μM in 5 mM Hepes, 0.1 mM EDTA, pH 7.0) and the emission spectra were recorded after 3h, averaging five spectra. The differences in the quenching of Trp fluorescence by (6,7), (9,10), and (11,12)-Br_2-PC were used to calculate the probability for location of the fluorophore in the membrane using two methods, viz. the parallax method (Chattopadhyay and London, 1987) and distribution analysis (Ladokhin et al., 1993). In the first one, the depth of the Trp residue is calculated as

$$Z_{cf} = L_{c1} + [(-\ln (F_1/F_2)/\pi C)-L_{21}^2]/2L_{21}$$

where Z_{cf} is the distance of the fluorophore from the center of the bilayer, L_{c1} is the distance of the shallow quencher from the center of the bilayer, L_{21} is the distance between the shallow and deep quencher, F_1 is the fluorescence intensity in the presence of the shallow quencher, F_2 is the fluorescence intensity in the presence of the deep quencher, and C is the concentration of quencher in molecules/Å^2. In

distribution analysis, the depth of the Trp residue is calculated by fitting the data to the following equation:

$$\ln (F_0/F_h)\cdot c(h) = [S/\sigma(2\pi)^{1/2}]\cdot \exp [-(h-h_m)^2/2\sigma^2]$$

where F_0 is the intensity in the absence of the brominated phospholipids, F_h is a set of intensities measured as a function of vertical distance from the bilayer center to the quencher (h), $c(h)$ is the concentration of different quenchers, S is the area under the curve (a measure of the effectiveness of quenching), σ is the dispersion (a measure from the distribution of the depth in the bilayer), h_m is the most probable position of the fluorophore in the membrane bilayer, and h is the average bromine distances from the bilayer center, based on X-ray diffraction and taken to be 10.8, 8.3, 6.3 for (6,7)-, (9,10)-, and (11,12)-Br_2-PC, respectively (McIntosh and Holloway, 1987). When equal concentrations of the Br-lipids are used the value for c(h) is unity (Ladokhin, 1999).

4.2.5 Circular dichroism measurements

UV CD spectra from 250 nm to 190 nm were recorded with a CD spectrophotometer (Olis RSF 1000F, On-line Instrument Systems Inc., Bogart, GA, USA) with temperature maintained at 25 °C. A one mm path length quartz cell was used at final concentrations of 50 μM and 3 mM of the peptide and liposomes, respectively. Interference by circular differential scattering by liposomes was eliminated by substracting CD spectra for liposomes from those recorded in the presence of the peptide. Data are shown as mean residue molar ellipticity (deg cm^2dmol^{-1}) and represent the averages of five scans. The percentage of helical content was estimated from the molar ellipticity at 222 nm (θ_{222}), as described previously (Bernstein et al., 2000).

4.2.6 Assay for phospholipase A_2

Phospholipase A_2 activity was determined by a kinetic assay described previously (Mustonen and Kinnunen, 1991, 1992; Thuren et al., 1986, 1988). The indicated lipids were mixed in chloroform and then dried under a stream of nitrogen followed by high vacuum for a minimum of 2 h. The lipid residues were subsequently dissolved in ethanol to yield a lipid concentration of 50 μM and small unilamellar liposomes (SUVs) were formed by rapidly injecting this lipid ethanolic solution into the buffer

(Batzri and Korn, 1973). The lipid concentration was 1.25 μM for both PPHPC and PPHPM/POPG (5:5, molar ratio) liposomes in a total volume of 2 ml. After 15 min of equilibration the reactions were initiated by adding 50 ng bee venom $sPLA_2$ or 0.5 μl of human lacrimal fluid. The progress of phospholipid hydrolysis was followed by measuring the pyrene monomer intensity at 400 nm as a function of time at 37 ^{0}C. Fluorescence intensities were measured with a Perkin-Elmer LS 50B spectrometer with excitation wavelength of 344 nm and both emission and excitation bandpasses set at 4 nm. The assay was calibrated by adding known picomolar aliquots of (pyren-1-yl)hexanoate into the reaction mixture in the absence of enzyme while detecting pyrene monomer emission intensity. The activity of the enzyme was calculated from the initial velocity of the reaction kinetic curves and converted to the amount of product released per unit time. The assays were carried out both with and without added 5 mM Ca^{2+}. Under the former conditions approx. 10 μM residual Ca^{2+} was present, as determined by titration with EDTA.

5 RESULTS

5.1 Penetration of Antimicrobial Peptides into Lipid Monolayers

The four antimicrobial peptides, magainin 2, indolicidin, temporin B and L readily inserted into SOPC monolayers and the penetration of these peptides into the lipid monolayer was progressively augmented when the content of the acidic phospholipid POPG in the film was increased (publications I and II, Figs. 1). However, the impact of PG on their penetration was significantly different, with a distinct 'cross-over' point at ≈ 38, 32, and 44 mN/m for indolicidin, temporin B and L, respectively. In contrast, the slopes for $\Delta\pi$ vs π_0 for the penetration of magainin 2 remained unaltered by the presence of PG. The values for π_c abrogating the intercalation of magainin 2 into the monolayer were thus increased from approx. 35 mN/m (X_{POPG}=0) to approx. 39 (X_{POPG}=0.1), 43 (X_{POPG}=0.2), and 46 mN/m (X_{POPG}=0.4). Furthermore, the inclusion of cholesterol (X=0.10) into the SOPC film had no significant effect on the penetration of magainin 2 while suppressed the insertion of indolicidin, temporin B and L into the lipid monolayer. Interestingly, the kinetics of the increase in surface pressure for temporin B were distinctly different from magainin 2, indolicidin and temporin L (publication II, Fig.1, *panels A and B*). Accordingly, after the addition of temporin B into the subphase there was a fast transient peak in π, followed first by a relaxation and subsequently by a slow increase in π. These kinetics were independent of lipid composition and initial surface pressure varied in the range of 12 to 36 mN/m. In contrast, for magainin 2, indolicidin, and temporin L the value for surface pressure increased in a continuous manner, reaching a plateau within approx. 40 min. The kinetics were not investigated in more detail at this stage.

5.2 Effects of Antimicrobial Peptides on Lipid Dynamics in Bilayers

Consequences of the association of the peptides with liposomes were subsequently studied by recording the emission spectra for the pyrene-labeled fluorescent phospholipid and the anisotropy of DPH incorporated into LUVs. For SOPC LUVs the addition of magainin 2 caused significant quenching of pyrene fluorescence (Fig. 3, *panel A*). Upon increasing X_{POPG} progressively enhanced I_m (RFI_m) was observed. Magainin 2 caused only insignificant changes in I_e/I_m at X_{POPG}=0 and 0.1, whereas at X_{POPG}=0.2 and 0.4, decrements were evident (Fig. 3, *panel B*). In the presence of

cholesterol (X_{chol}=0.1), both I_m and I_e decreased dramatically by magainin 2, with insignificant changes in I_e/I_m (Fig. 3, *panels A* and *B*). Similarly to magainin 2, indolicidin caused a parallel decrease in both I_m and I_e for SOPC LUVs (Fig. 4, *panel A*). However, quenching of pyrene fluorescence was evident also when the acidic phospholipid POPG was present. The largest decrease in I_m was observed at X_{chol}=0.1 (Fig. 4, *panel A*). In spite of the decrease in I_m, I_e/I_m revealed no significant changes due to indolicidin (Fig. 4, *panel B*). Similarly to magainin 2 and indolicidin, temporin B and L influence the quantum yields of pyrene-labeled phospholipids. Quenching of pyrene monomer and excimer fluorescence by temporin B and L was evident in zwitterionic membranes although the decrement in emission was less in the presence of cholesterol (X_{chol}=0.1, Figs. 5 and 6, *panels A and C*). When the acidic phospholipid was present at X_{POPG}=0.1 temporin B caused only insignificant changes in I_m, and upon increasing X_{POPG} from 0.2 to 0.4 I_m increased in a progressive manner. Also I_e was increased by temporin B in the presence of POPG (Fig. 5, *panel C*). Interestingly, at X_{POPG}=0.4 the values for I_e increased progressively up to temporin B/lipid molar ratio of ≈ 1/15, whereas upon exceeding this stoichiometry I_e decreased. Temporin B caused I_e/I_m to increase for SOPC LUVs while only minor changes in I_e/I_m were observed in the presence of cholesterol (X_{chol}=0.1, Fig. 5, *panel B*). The increment in I_e/I_m was progressively enhanced by increasing X_{POPG} from 0.1 to 0.2. At X_{POPG}=0.4 I_e/I_m increased at a temporin B/lipid molar ratio of ≈ 1/30. However, above this peptide/lipid ratio, temporin B caused I_e/I_m to decrease and insignificant changes in I_e/I_m were observed above a peptide/lipid ratio of 1/6. In contrast to temporin B, temporin L decreased I_m also for LUVs containing POPG, with the largest effect at X_{POPG}=0.1 (Fig. 6, *panel A*). The decrement in I_m was progressively attenuated with increasing contents of POPG in the liposomes. Similarly to I_m the decrement in I_e due to temporin L was progressively attenuated with increasing X_{POPG} (Fig. 6, *panel C*). At X_{POPG}=0.1 a clear discontinuity was evident, corresponding to POPG/temporin L molar ratio of 1/1 and thus suggesting stoichiometric complex formation. I_e/I_m for SOPC LUVs slightly increased upon the addition of temporin L, whereas a significant increase was observed in the presence of POPG (Fig. 6, *panel B*). While minor difference of the increment in I_e/I_m due to temporin L was evident at X_{POPG}=0.1, 0.2, and 0.4 below a temporin L/lipid molar ratio of ≈ 1/10, the increments in I_e/I_m at X_{POPG}=0.2 and 0.4 were significantly higher than those at X_{POPG}=0.1 above a

temporin L/lipid stoichiometry of ≈ 1/8. Likewise, I_e/I_m increased further at X_{POPG}=0.4 above temporin L/lipid stoichiometry of 1/6 (Fig. 6, *panel B*).

The antimicrobial peptides, magainin 2, indolicidin, temporin B and L caused virtually no changes in r in the absence of the acidic phospholipid (Figs. 3 and 4, *panels C,* Figs. 5 and 6, *panels D,* respectively). However, progressively augmented lipid packing and acyl chain order upon the addition of the four antimicrobial peptides were evident with increasing X_{POPG}. Enhanced membrane order by the incorporation of cholesterol in the membrane is revealed by the increase in r before the addition of the peptides, as reported previously (van Ginkel et al., 1989). Magainin 2, temporin B and L had no further effect on r in the presence of cholesterol (X_{chol}=0.1). In contrast, the increment in the membrane acyl chain order due to indolicidin was observed in the presence of cholesterol (Fig. 4, *panel C*). Accordingly, lack of changes in acyl chain order or an increase in membrane order thus cannot cause the observed increase in I_e/I_m, revealing that peptide-induced segregation of lipids, viz. clustering of fluorescent probe in the membrane occur.

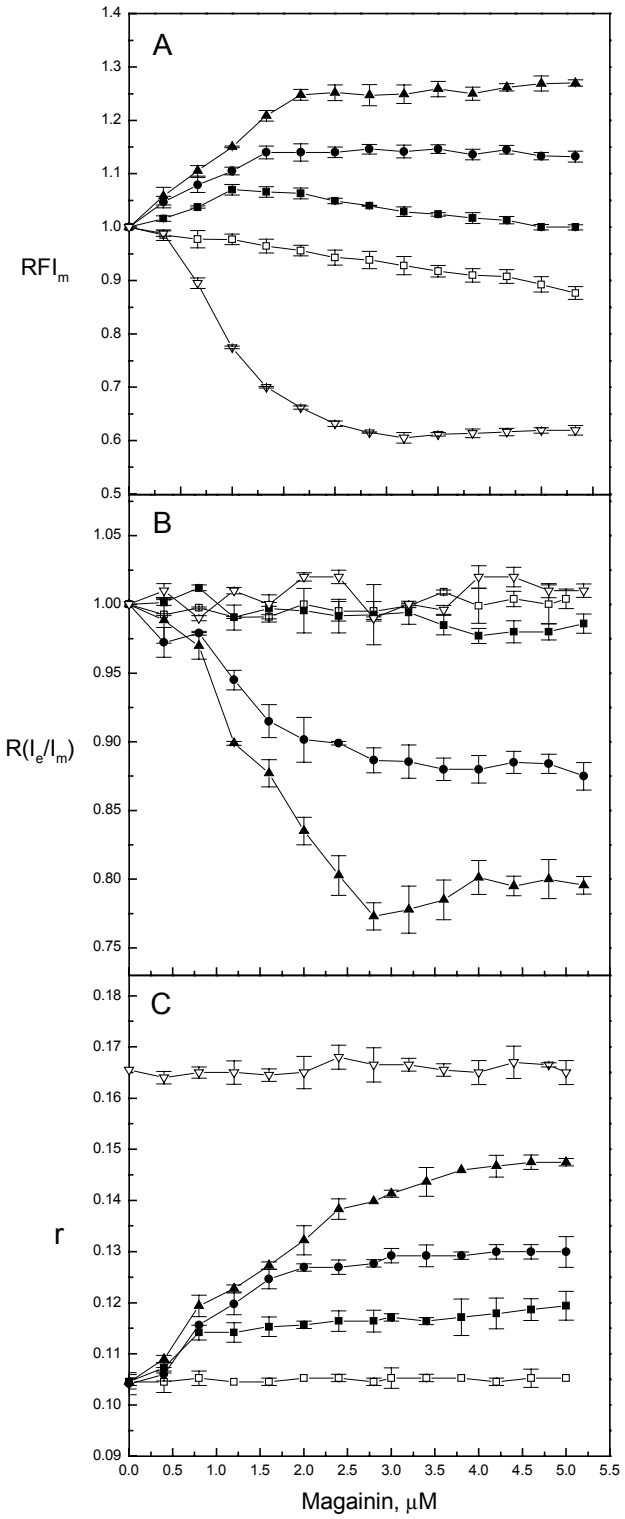


Fig. 3. Effects of magainin 2 on lipid dynamics in LUVs assessed by fluorescence of pyrene-labeled phospholipid analogs (X=0.01) and on the steady-state emission anisotropy r of DPH (X=0.002) (*panel C*), shown as changes in pyrene monomer emission intensity I_m (*panel A*) and excimer to monomer ratio $R(I_e/I_m)$ (*panel B*). PPDPC was used in SOPC and SOPC/cholesterol LUVs, and PPDPG in LUVs containing POPG. Liposomes were composed of SOPC with X_{POPG}=0 (□), 0.1 (■), 0.2 (●), and 0.4 (▲), and with X_{chol}=0.1 (∇). The concentration of lipids was 25 μM in a total volume of 2 ml of 5 mM Hepes, 0.1 mM EDTA, pH 7.0. The temperature was maintained at 25 °C with a circulating waterbath. Each data point represents the mean of triplicate measurements, with the error bars indicating ±S.D.

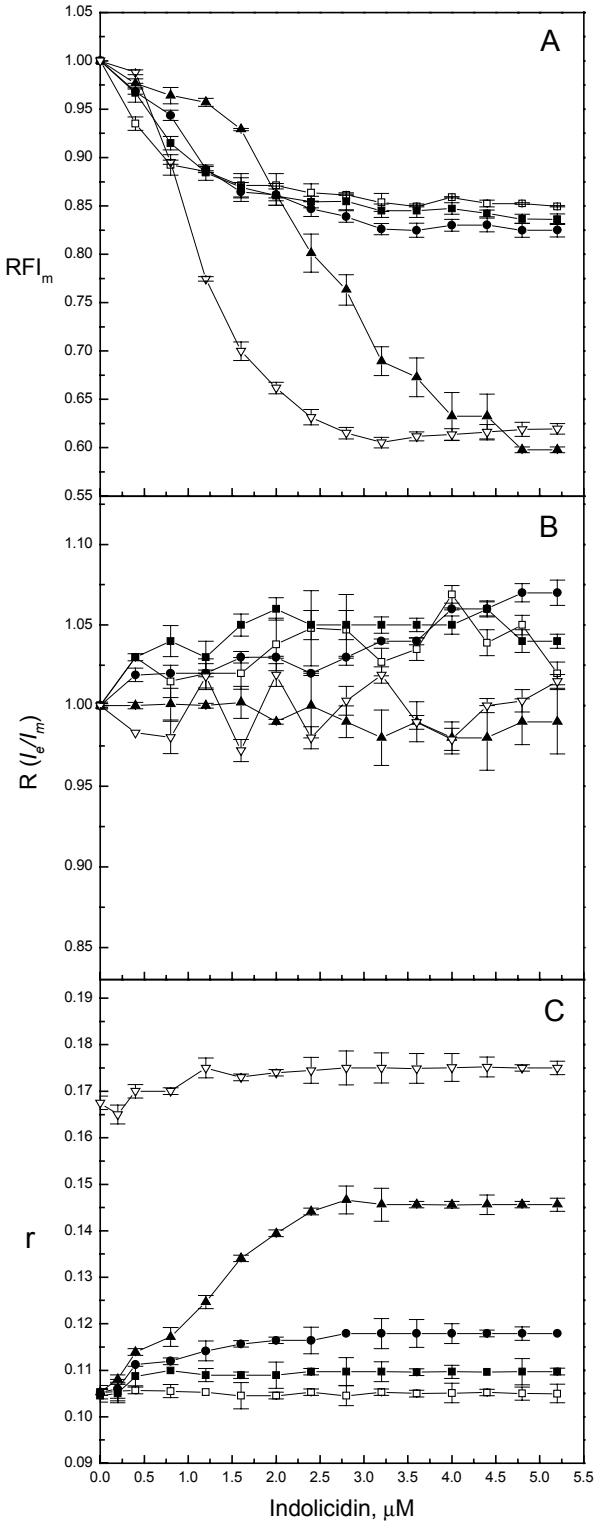


Fig. 4. Effects of indolicidin on lipid dynamics in LUVs assessed by fluorescence of pyrene-labeled phospholipid analogs (X=0.01) and on the steady-state emission anisotropy r of DPH (X=0.002) (*panel C*), shown as changes in pyrene monomer emission intensity I_m (*panel A*) and excimer to monomer ratio R(I_e/I_m) (*panel B*). Liposomes were composed of SOPC with X_{POPG}=0 (□), 0.1 (■), 0.2 (●), and 0.4 (▲), and with X_{chol}=0.1 (∇). Otherwise conditions were as described in the legend for Fig. 3.

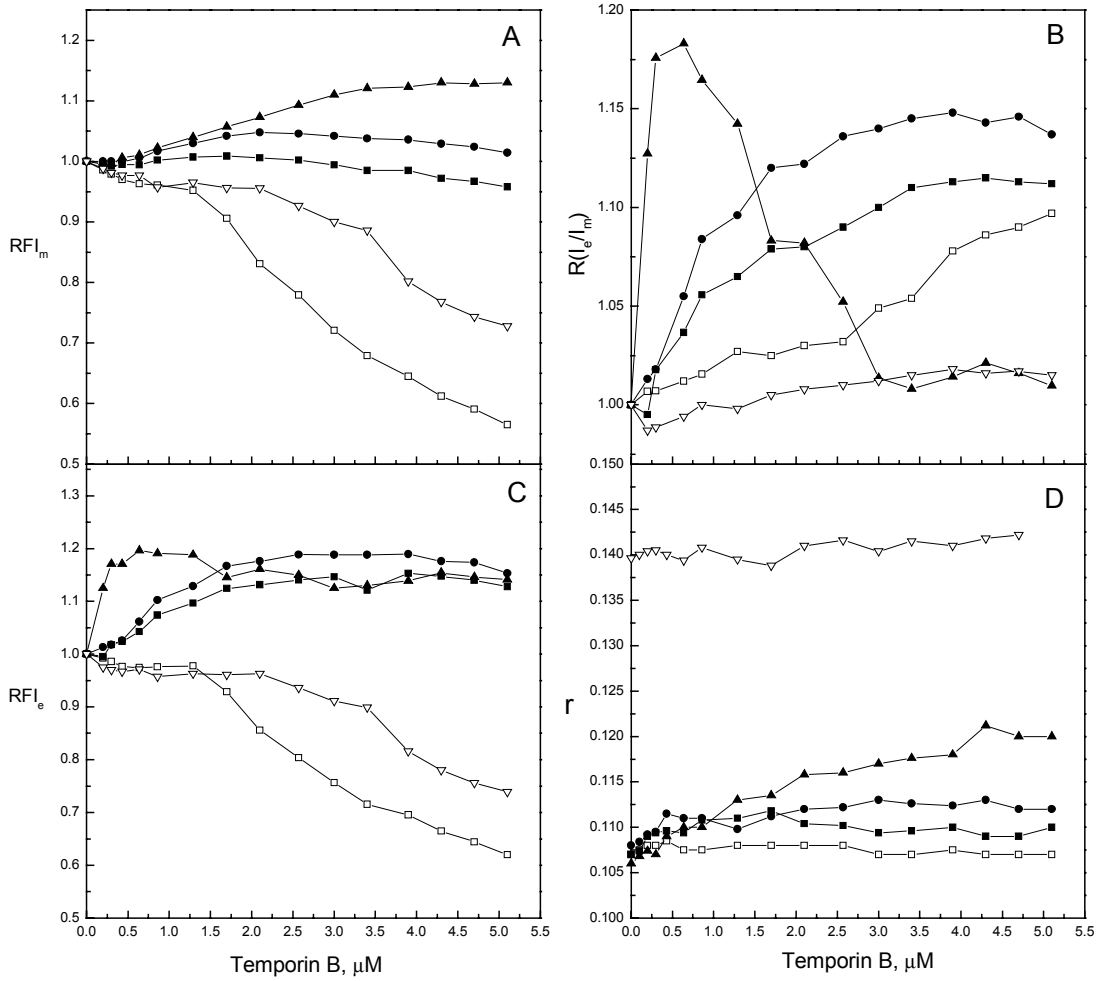


Fig. 5. Effects of temporin B on lipid dynamics in LUVs assessed by fluorescence of pyrene-labeled phospholipid analog PPDPC (X=0.01) and on the steady-state emission anisotropy r of DPH (X=0.002) (*panel D*), shown as changes in pyrene monomer and excimer emission intensity I_m (*panel A*), I_e (*panel C*), respectively, and excimer to monomer ratio $R(I_e/I_m)$ (*panel B*). Liposomes were composed of SOPC with X_{POPG}=0 (□), 0.1 (■), 0.2 (●), and 0.4 (▲), and with X_{chol}=0.1 (∇). The concentration of lipids was 20 μM. Otherwise conditions were as described in the legend for Fig. 3.

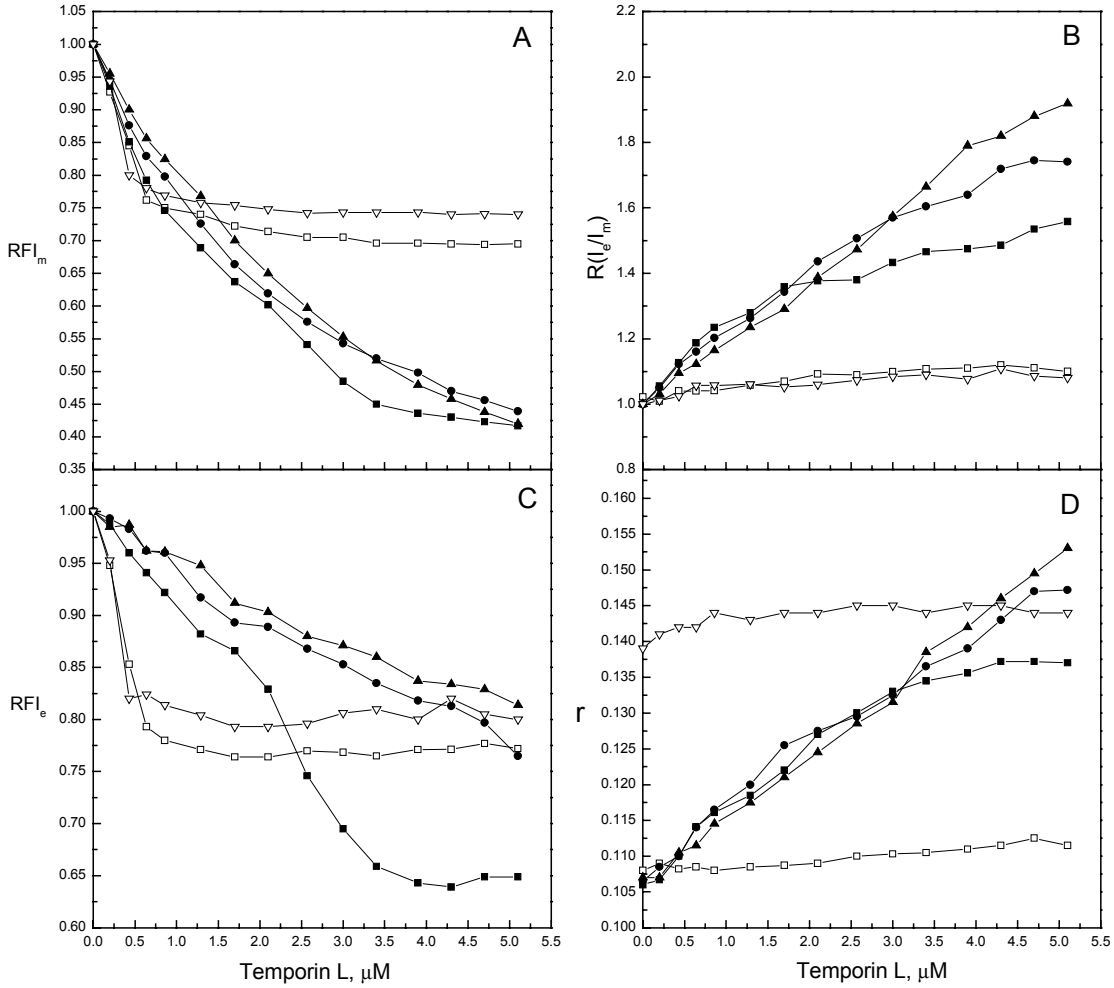


Fig. 6. Effects of temporin L on lipid dynamics in LUVs assessed by fluorescence of pyrene-labeled phospholipid analog PPDPC (X=0.01) and on the steady-state emission anisotropy r of DPH (X=0.002) (*panel D*), shown as changes in pyrene monomer and excimer emission intensity I_m (*panel A*), I_e (*panel C*), respectively, and excimer to monomer ratio $R(I_e/I_m)$ (*panel B*). Liposomes were composed of SOPC with X_{POPG}=0 (□), 0.1 (■), 0.2 (●), and 0.4 (▲), and with X_{chol}=0.1 (∇). The concentration of lipids was 20 μM. Otherwise conditions were as described in the legend for Fig. 3.

5.3 Effects of Antimicrobial Peptides on Membrane Topology

The impact of the antimicrobial peptide magainin 2, indolicidin, temporin B and L on the 3-D topology of macroscopic membranes were studied using giant liposomes with different lipid compositions. SOPC membranes were unaffected by magainin 2, indolicidin, and temporin B even when exposed to high amounts of the peptides (data not shown). Likewise, giant liposomes composed of SOPC/cholesterol (X_{chol}=0.1) were unaffected by magainin 2 and temporin B (data not shown). In contrast, indolicidin had a significant effect on SOPC/cholesterol giant vesicles (publication I,

Fig. 8) and temporin L induced pronounced vesiculation of zwitterionic membranes although the vesiculation of giant liposomes containing cholesterol was attenuated (publication II, Figs. 7 and 9). Similarly to the monolayer penetration and the effects of the four antimicrobial peptides on the bilayer dynamics, the impact of the peptides on the topology of giant liposomes was significantly enhanced by the presence of POPG (X_{POPG}=0.1, publication I, Figs. 6 and 7, and publication II, Figs.6 and 8). All the four antimicrobial peptides induced an endocytosis-like process in the acidic phospholipid containing giant liposomes although the aggregated structures were different. More specifically, the 'endocytotic' vesicles caused by magainin 2 and temporin B remained separated from each other inside the giant liposomes and aggregation of particles on the surface of the giant vesicles was observed (publication I and II, Figs. 6). In contrast, indolicidin and temporin L induced the formation of a tightly packed 3-D structure on acidic phospholipid membranes with progressive accumulation of a number of 'endocytotic' particles inside the giant liposomes upon the subsequent addition of the peptides (publication I, Fig. 7, and publication II, Fig. 8). Compared to magainin 2, indolicidin, and temporin B, temporin L caused strongest effects on the topology of giant liposomes. More specifically, in the presence of POPG the area of the giant liposome surface exposed to temporin L became dark, revealing a pronounced change in the refractive index and indicating the formation of a tightly packed 3-D structure (publication II, Fig. 8). Subsequently, a group of strongly aggregated vesicles emerged inside the cavity of the giant liposome. Further addition of temporin L caused the area of the dark domain as well as the size of the aggregated structure to increase. Interestingly, after the addition of approx. 60 fmol of temporin L the aggregate formed inside the giant liposome became very large (publication II, Fig. 8, *panels E and F*). Concomitant vesiculation of the neighboring giant liposomes was also observed.

5.4 Interaction of Temporin L with Model Membranes

5.4.1 Secondary structure of temporin L

The structure of temporin L in zwitterionic (SOPC) and negatively charged phospholipid (POPG) containing bilayers was investigated using circular dichroism (publication III, Fig. 1). In an aqueous solution, temporin L adopts a random coil conformation, revealed by the single minimum at 200 nm. However, in the presence

of SUVs the peptide is α-helical, with the characteristic double minima at 210 and 222 nm. Compared to the zwitterionic SOPC SUVs, the helical conformation is more pronounced in the presence of POPG, with the calculated helical content increasing from 69% to 89% at X_{POPG}= 0 and 0.4, respectively.

5.4.2 Binding of temporin L to liposomes

Temporin L in buffer has fluorescence emission maximum at 352 nm (publication III, Fig. 2, *panel A*), typical for Trp in a polar environment (Breukink et al., 1998). Upon the addition of SOPC liposomes, a blue shift of the fluorescence emission maximum of Trp^4 was observed, while the emission intensity (*F*) was attenuated (publication III, Fig. 2, *panel A*). In addition, the association of temporin L with SOPC vesicles caused decrement in *F* up to the highest lipid/peptide molar ratio used (publication III, Fig. 2, *panel D*). However, in the presence of POPG a pronounced increase in *F* and a blue shift were evident, the increase being progressively enhanced with increasing X_{POPG} in the membranes (publication III, Fig. 2, *panel B*). A sigmoidal dependence of the blue shift was evident at all lipid compositions studied and the blue shift was more pronounced in the presence of POPG (publication III, Fig. 2, *panel C*). Compared to SOPC LUVs smaller lipid concentrations were required to obtain the maximum effect on peptide fluorescence when POPG was present in the bilayer, thus indicating higher affinity of temporin L for liposomes containing the acidic phospholipid. Interestingly, in the presence of cholesterol *F* decreased at all lipid/peptide molar ratios measured whereas the decrement in *F* was augmented compared to SOPC LUVs (publication III, Fig. 2, *panel D*). Smallest blue shift was evident in the presence of cholesterol (X_{chol}= 0.10), suggesting cholesterol to attenuate the lipid binding of temporin L. In addition, under the latter conditions the location of residue Trp^4 in the bilayers appears to become more superfacial (see below).

5.4.3 Quenching of Trp by acrylamide

Fluorescence of Trp^4 decreased in a concentration-dependent manner by the addition of acrylamide to the peptide solution both in the absence and presence of liposomes, without other effects on the spectra (data not shown). However, in the presence of liposomes, less decrement in fluorescence intensity was evident, thus revealing that Trp^4 is less accessible to the quencher in the presence of LUVs (publication III, Fig.

3). Compared to the measurements in the absence of liposomes the values for the Stern-Volmer quenching constant (K_{sv}) were decreased in the presence of SOPC and SOPC/POPG LUVs (publication III, table 1), suggesting that Trp^4 was buried in the bilayers, becoming inaccessible for quenching by acrylamide. Comparison of K_{sv} values for POPG containing and pure SOPC liposomes indicates that the binding of temporin L to liposomes is enhanced by POPG. Accordingly, the values for K_{sv} were decreased in the presence of POPG with essentially no difference at X_{POPG}= 0.2 and 0.4. Interestingly, K_{sv} for cholesterol containing liposomes is only slightly less than in the absence of liposomes, yet significantly higher than the values measured for the other lipid compositions. These data thus suggest the affinity of temporin L for liposomes to increase in the order SOPC/cholesterol (X_{chol}= 0.1) < SOPC < SOPC/POPG (X_{POPG}= 0.1) < SOPC/POPG (X_{POPG}= 0.2) ≈ SOPC/POPG (X_{POPG}= 0.4).

5.4.4 Quenching of Trp by phosphatidylcholines brominated at different positions along the acyl chains (Br_2-PCs)

The intercalation depth of Trp^4 into the hydrophobic core of the bilayer can be assessed from the quenching efficiency of Br-labeled phospholipids. While this Trp was quenched significantly in a SOPC membrane by all three brominated lipids, (9,10)-Br_2-PC was most efficient (publication III, Fig. 4). Similar quenching profiles were evident for POPG containing membranes while the overall quenching efficiency was enhanced by the presence of the acidic phospholipid. Quenching of Trp emission as a function of lipid/peptide molar ratio revealed a sigmoidal dependence (publication III, Fig. 5). Interestingly, at X_{POPG}= 0.1 quenching of Trp^4 by (6, 7)-Br_2-PC is more efficient at low lipid/peptide molar ratios and decreased with increasing the concentration of liposomes. At X_{POPG}= 0.4 the extent of quenching was lower than at X_{POPG}=0.2 for all brominated lipids. According to the quenching of Trp by brominated phosphatidylcholines located in the bilayer with different distance from the center of the bilayer, the penetration depth of Trp^4 in bilayers was estimated by the parallax method (Chattopadhyay and London, 1987) and distribution analysis (Ladokhin et al., 1993; Ladokhin, 1997, 1999). The results revealed the Trp residue to be buried in both SOPC and SOPC/POPG membranes at a distance of ≈ 8.0 ± 0.5 Å from the center of the bilayer (publication III, Fig. 6). In addition, the insertion took place at all lipid/peptide molar ratios tested and there were only insignificant

differences in the penetration depth except at X_{POPG}= 0.1. At X_{POPG}= 0.1 the depth of bilayer penetration of Trp^4 was shallow at a lipid/peptide molar ratio of 10 while the peptide was immersed deeper upon increasing the concentration of liposomes. Interestingly, least effective quenching by brominated lipids was evident in the presence of cholesterol suggesting that temporin L was associated to the membrane surface, in keeping with the observed efficient quenching by acrylamide (publication III, Figs. 3-5). These data readily revealed that the partitioning of the peptide into LUVs was reduced by cholesterol and the insertion of this peptide into the membrane was shallow, with a distance of 9.5 ± 0.5 Å from the center of the bilayer for Trp^4 (publication III, Fig. 6).

5.5 Modulation of the Activity of Secretory Phospholipase A_2 by Antimicrobial Peptides

The antimicrobial peptides, magainin 2, indolicidin, and temporin B and L were found to modulate the hydrolytic activity of secreted phospholipase A_2 ($sPLA_2$) from bee venom and in human lacrimal fluid. More specifically, hydrolysis of phosphatidylcholine (PC) liposomes by bee venom $sPLA_2$ at 10 μM Ca^{2+} was attenuated by these peptides while augmented product formation was observed in the presence of 5 mM Ca^{2+} (publication IV, Fig. 1). The activity of $sPLA_2$ towards anionic liposomes was significantly enhanced by the antimicrobial peptides at low [Ca^{2+}] and was further enhanced in the presence of 5 mM Ca^{2+} (publication IV, Fig. 2). Similarly, with 5 mM Ca^{2+} the hydrolysis of anionic liposomes was enhanced significantly by human lacrimal fluid $sPLA_2$ while that of PC liposomes was attenuated (publication IV, Fig. 4). Interestingly, inclusion of a cationic gemini surfactant in the vesicles showed essentially similar pattern on $sPLA_2$ activity (publication IV, Fig. 5), suggesting that the modulation of the enzyme activity by the antimicrobial peptides may involve charge properties of the substrate surface.

6 DISCUSSION

6.1 Surface Activity of Antimicrobial Peptides

Monolayer experiments revealed magainin 2, indolicidin, temporin B and L to be highly membrane active. Although these antimicrobial peptides intercalated into zwitterionic phosphatidylcholine monolayers, they interact preferentially with acidic phospholipids, in keeping with their net cationic charge (Matsuzaki et al., 1991, 1995a, 1998; Ladokhin et al., 1997). Yet, the impact of PG on their penetration was significantly different, with a 'cross-over' point for indolicidin, temporin B and L. In contrast, the slopes for $\Delta\pi$ vs π_0 for the penetration of magainin 2 remained unaltered by the presence of PG. The data suggest that unlike in bilayers magainin 2 would not adopt the vertical orientation (i.e. the long axis of its α-helix perpendicular to the layer plane) in lipid monolayers. The possible significance of the 'cross-over' point to the mechanism(s) of action of these peptides warrants further studies. Qualitatively, it is of interest that the more surface active peptides, temporin L and indolicidin have higher values of 'cross-over' pressures. Intriguingly, the 'cross-over' point remains virtually unaltered irrespective of lipid composition (i.e. presence of PG), thus suggesting that it could be solely determined by surface pressure, irrespective of electrostatic interactions. A possible mechanism is that at 'cross-over' surface pressure, the peptides change their orientation in the monolayer in a pressure dependant manner, perhaps from a parallel to perpendicular orientation of its long axis with respect to the layer plane (publication I, Fig. 9, *panels B and C*), thus reducing the area/peptide and decreasing $\Delta\pi$. Furthermore, it is also possible that some PG molecules which are electrostatically bound to the peptides become oriented differently from the phospholipids in the monolayer (publication I, Fig. 9, *panel C*). In other words, the peptides with their associated PG would form a supramolecular complex, the orientation of which would be pressure dependent. The kinetics of the increase in π caused by temporin B was different from that for magainin 2, indolicidin and temporin L (publication II, Fig. 1, *panels A and B*), also suggesting a conformational and/or orientational change following the initial intercalation of the former peptide into the lipid film.

6.2 Impact of the Antimicrobial Peptides on Bilayer Lipid Dynamics

The effects of antimicrobial peptides magainin 2, indolicidin, temporin B and L on lipid dynamics in bilayers are pronounced. Although there are distinct differences in the effects of these peptides on bilayer lipid dynamics, also several similarities were evident. Accordingly, increment in DPH anisotropy r revealed that all these peptides increased acyl chain order in the presence of the acidic phospholipid POPG, the magnitude of this effect increasing with X_{POPG}. Likewise, lipid segregation was induced in the presence of the acidic phospholipid. All these effects imply the importance of the acidic phospholipid as well as the cationic charge of the peptides for the interaction of the latter with biomembranes. Interestingly, all the four antimicrobial peptides influence the quantum yields of pyrene-labelled lipids except for magainin 2 and temporin B where quenching of pyrene fluorescence was abolished in the presence of POPG while for temporin L and indolicidin quenching was observed regardless of lipid composition. Both π-π interactions between pyrene and Trp as well as contacts of the basic residues of the peptides with the fluorophore resulting in π-cation interaction (Dougherty, 1996) may be involved in the quenching. In addition, the microenvironment of the fluorophore could change due to the interaction of antimicrobial peptide with membranes.

Due to the strong interaction of peptides with the membranes, e.g. temporin B at X_{POPG}=0.40 (Fig. 5, *panel B*), different effects may contribute to the reduction of the excimer formation of pyrene lipid analogue, as follows. First, the membrane bound peptides could represent a mechanical barrier for the lateral diffusion of the fluorescent probe. In other words the membrane associated peptides could statically reduce excimer formation by occupying neighbouring positions of the probe and thus increase the path length for the diffusion of the pyrene-labeled lipid between collisions. Second, the rotational freedom of the lipid acyl chains adjacent to the peptides could be reduced due to a peptide-lipid interaction. Finally, lipids adjacent to the peptides could be partly immobilized and their exchange frequency (and that of the pyrene molecules in this area) thus be significantly lower than in the bulk lipid matrix.

6.3 Effects of the Antimicrobial Peptides on the Topology of Giant Liposomes

Interestingly, all peptides studied so far in our laboratory induce an endocytosis-like process in giant vesicles yet the differences observed in the aggregated structures suggest different modes of association of the four peptides with membranes. Vesiculation of giant liposomes required repeated applications of peptides and thus imply a concentration dependent mechanism of action. 'Pore' or 'channel' structure could form by inserted peptides with its associated phospholipids. Upon disintegration of nearby 'pores' or 'channels', a supramolecular complex of peptides and lipids may form, thus causing transient disruption of the bilayer. Simultaneously, some of the peptide molecules translocate into the inner leaflet of the membrane (Matsuzaki et al., 1995a, 1996), allowing the membrane to reorganize and 'heal'. Alternatively, the antimicrobial peptides could lack specific knob-into-hole packing, which is a general characteristic of helical bundle membrane proteins (Langosch and Heringa, 1998) and causes promiscuous aggregation. The latter may induce local phase transitions such as the separation of lipid-peptide micelles from the bilayers, or local changes of the membrane curvature due to the intercalating peptides, which would cause transient collapse of the bilayer structure. These processes could relate to the vesiculation of giant vesicles induced by the four antimicrobial peptides observed in our study.

The microscopy images revealed the formation of the macroscopic aggregates to be asymmetric, i.e. to occur inside the giant vesicles. Similarly to our studies on the formation of ceramide by sphingomyelinase (Holopainen et al., 2000), this vectorial nature of the action of the antimicrobial peptides suggests that their asymmetric application onto the outer surface results in an augmented bending rigidity as well as negative spontaneous curvature in the membrane. However, elucidation of the mechanism(s) requires further studies and the nature of the aggregates visible by microscopy is of interest. To this end, the possibility that the lamellar liquid crystalline phase would not represent thermodynamic equilibrium has been proposed. Accordingly, dimyristoylphosphoglycerol has been demonstrated to transform into the so-called 'sponge' phase (Schneider et al., 1999). The factors controlling the formation of this phase remain poorly understood, however, lipid headgroup interactions appear to be important. An intriguing possibility is that in addition to their other effects on bilayers these antimicrobial peptides could trigger a transition of the

bacterial lipid, phosphatidylglycerol from a lamellar liquid crystalline state into the 'sponge' phase, the latter representing the thermodynamic equilibrium. On the level of thermodynamics this would also provide the driving force for the macroscopic aggregation of the peptide-lipid complexes observed in giant liposomes. However, also in this case the mechanistic basis would warrant further studies.

6.4 Mode of Action of Temporin L on Model Membranes

The characteristics of quenching of the Trp residue of temporin L by acrylamide and brominated phospholipid in the presence of SOPC liposomes demonstrate that this peptide inserts in part into the hydrophobic lipid phase of the bilayer. However, Trp^4 fluorescence intensity of temporin L decreased upon its transfer from the buffer into a SOPC membrane and the fluorescence intensity was further decreased in the presence of cholesterol (publication III, Fig. 2, *panels A and D*). Temporin L could aggregate upon binding to zwitterionic membranes. Accordingly, in these aggregates the Trp residues may be in close proximity so as to result in Trp self-quenching and quenching by the positive charges of the peptide, as suggested for nisin (Breukink et al., 1998). Similar interpretation may apply to the decrement of fluorescence in POPG containing membranes at lipid/peptide molar ratio < 10 (publication III, *panel D*). Yet, quenching could also be due to the charged headgroup of POPG interacting directly with the π-orbitals of Trp (De Kroon et al., 1990). The third and perhaps the most likely possibility is π-orbital-cation interaction (Dougherty et al., 1996), which is suggested by the close proximity of Trp^4 and Lys^7 in α-helical temporin L (publication III, Fig. 7).

Quenching by acrylamide reveals that Trp^4 of temporin L is shielded in the presence of liposomes. However, although at lipid/peptide molar ratio of 100 the data indicate the peptide to be quantitatively membrane bound this residue was not completely inaccessible to acrylamide. Experiments with brominated phospholipids further demonstrate most efficient quenching by (9,10)-Br_2-PC in both SOPC and SOPC/POPG containing membranes (publication III, Fig. 4). Yet, significant quenching was also due to (6,7)-Br_2-PC in which the bromines reside at a distance of 10.8 Å from the bilayer center. Combining these results with those from acrylamide quenching experiments, we may conclude that not all of the peptides are bound within

the lipid bilayer in a transbilayer state, and an equilibrium of orientational states (inserted and surface associated) may exist (Huang, 2000). Calculation of the penetration depth indicates that Trp^4 resides at a distance of 8.0 Å ± 0.5 Å from the bilayer center in SOPC and SOPC/POPG membranes (publication III, Fig. 6). The length of temporin L in α-helical conformation is approx. 19.5 Å, thus corresponding to about half of the thickness of a SOPC bilayer. In this α-helical peptide the distance of Trp^4 from the N-terminus is approx. 6 Å. Accordingly, the N-terminus of temporin L is likely to reside deepest in the lipid bilayer (publication III, Fig. 7). In the presence of cholesterol most efficient quenching was observed for (6,7)-Br_2-PC. Together with the acrylamide quenching data, this suggests that under these conditions most of the temporin L bound to this membrane is surface-associated. Notably, the effects of temporin L on membranes were significantly decreased in the presence of cholesterol (publication II). Cholesterol increases membrane acyl chain order and stabilizes the bilayer structure (Rietveld and Simons, 1998) which could prevent the penetration of temporin L into the lipid bilayer and decrease the extent of membrane perturbation by this peptide.

The interaction of antimicrobial peptides with bilayers alters the organization of membranes and makes them permeable. Several models have been proposed to describe the molecular events taking place during the peptide-induced leakage of the target cell (for a review, see Shai and Oren, 2001). Our studies reveal that temporin L inserts into the hydrophobic core of the membranes in a cooperative manner. Either 'toroidal' or 'barrel stave' model would thus be feasible (Shai and Oren, 2001). One α-helical monomer of temporin L could span one half of a bilayer. Accordingly, pore formation by this peptide is likely to require dimerization (Mangoni et al., 2000). The 'toroidal' model suggested for magainin 2 involves five peptide helices together with several surrounding phospholipids, forming a membrane-spanning pore (Ludtke et al., 1996). For this mechanism the concentration of bromines near the peptide would be rather different than in the bulk of the bilayer, particularly in POPG containing membranes. This can be rationalized by the Br_2-PCs being partially excluded from the predominantly negatively charged lipid domains where the peptide is preferentially bound. Such variation in the local distribution of the lipid probes could affect the analysis of the quenching data by the parallax method while distribution analysis

would remain unaffected (Ladokhin, 1999). However, calculation of the distance *d* of Trp^4 from the bilayer center by the parallax method and distribution analysis gave similar values for SOPC and POPG containing membranes, thus suggesting that partial segregation of lipids caused by temporin L has only insignificant effects on the quenching profiles by the brominated lipids. In combination with the data revealing that temporin L readily inserts into and perturbs zwitterionic SOPC bilayers, the 'barrel stave' model (Shai and Oren, 2001) could be the most likely mechanism for its action, as suggested for other temporins (Mangoni et al., 2000).

6.5 Modulation of The Activity of Secretory Phospholipase A_2 by Antimicrobial Peptides

A variety of membrane perturbants have been shown to affect the adsorption and action of PLA_2 (e.g. Jain et al., 1991; Mustonen and Kinnunen, 1991; Cajal and Jain, 1997). In keeping with this our present results demonstrate a profound impact of the four antimicrobial peptides on the activities of $sPLA_2$s. Interestingly, it has been shown that secreted PLA_2 ($sPLA_2$) has antibacterial activity and represents an important host defense molecule (Qu and Lehrer, 1998; Buckland and Wilton, 2000; Nevalainen et al., 2000). The global cationic nature of group II phospholipase A_2 (pI > 10.5) appears to facilitate penetration of the anionic bacterial cell wall, which is also characteristic for the antimicrobial peptides (Buckland and Wilton, 2000). In addition, the considerable preference of phospholipase A_2 and antimicrobial peptides for anionic phospholipid interface provide specificity toward anionic bacterial membranes as opposed to zwitterionic eukaryotic cell membranes. A bacterial membrane is thus susceptible to enhanced hydrolysis while the host cell membrane would remain intact. The results highlight the potential of concerted action between $sPLA_2$ and antimicrobial peptides as part of the innate response to infections, particularly when more antibiotic resistant bacterial strains are emerging.

In distinction from enzymes acting on monomeric soluble substrates, PLA_2s have access to their substrate only within the membrane phase and are sensitive to the curvature and physicochemical characteristics of the phospholipid membrane (Lehtonen and Kinnunen, 1995; Burack et al., 1997; Gadd and Biltonen, 2000). However, in this study a possible variation in the bilayer curvature would be unlikely

to influence the comparison of the measured effects due to progressively increasing concentrations of the added antimicrobial peptides on the enzyme activity. Importantly, the effects of the four antimicrobial peptides on the structure of anionic membranes in particular are pronounced (publications I and II). Reorientation of peptides and formation of 'channel'- or 'pore'-like structures could occur and the bilayer structure become locally destabilized. These peptides are likely to induce complex dynamic structures in the membranes with a variety of substrate configurations. 'Structural defects' in membranes caused by antimicrobial peptides could enhance the penetration of $sPLA_2$ and the hydrolysis of vesicles would proceed at a much higher rate with augmented catalytic turnover of the bound enzyme. Alternatively, the antimicrobial peptides could modulate $sPLA_2$s activity by substrate replenishment through peptide-mediated vesicle-vesicle contacts, similarly to melittin (Cajal and Jain, 1997). Different effects of these peptides on membrane properties may thus influence $sPLA_2$ activity to different degrees.

Due to the neutral charge and high curvature of PPHPC SUVs, the four antimicrobial peptides studied can be expected to bind weakly to the zwitterionic membrane surface without inserting deeply into the hydrophobic core of the bilayers. At low [Ca^{2+}] the coverage of the interface by the peptides would decrease the area available for the binding of $sPLA_2$, resulting in inhibition due to a smaller fraction of the enzyme being bound to the interface. Repulsion between Ca^{2+} and membrane bound positively charged antimicrobial peptides could also affect the binding of $sPLA_2$ to the membrane. However, in the presence of 5 mM Ca^{2+} the activity of bee venom $sPLA_2$ towards PPHPC was enhanced by all four antimicrobial peptides while under the same conditions the activity of lacrimal fluid $sPLA_2$ was attenuated. Bee venom and lacrimal fluid $sPLA_2$s belong to group III and group II $sPLA_2$s, respectively. Compared to the group III $sPLA_2$s the interface binding surface of group II $sPLA_2$s contains a significant larger number of basic amino acids and has a highly cationic character (Ghomashchi et al., 1998; Nevalainen et al., 2000). Furthermore, Ca^{2+} is essential for the activity of PLA_2s (Berg et al., 2001). Accordingly, the effects of cationic antimicrobial peptides on the binding of these two enzymes to the substrate could differ, resulting in different effects on the catalytic activities of bee venom and lacrimal fluid $sPLA_2$.

Interestingly, while magainin 2, indolicidin, and temporin B and L differ in structural parameters they had qualitatively identical effects on $sPLA_2$ activity. Intriguingly, comparison of zwitterionic and anionic vesicles as substrates for $sPLA_2$ has revealed an essentially similar pattern for the effects of adriamycin (Mustonen and Kinnunen, 1991), phorbol esters (Mustonen and Kinnunen, 1992), and polyamines (Thuren, et al., 1986) on the activity of PLA_2. Modulation of $sPLA_2$ activity via similar changes induced in the substrate by these peptides is thus suggested, implying a lack of a direct and specific effect on the enzyme. Importantly, also the cationic gemini surfactant SR-1 added to the vesicles showed a similar pattern on $sPLA_2$ activity (publication IV, Fig. 5), indicating that modulation of $sPLA_2$ reaction by the above cationic perturbants could involve alterations in the electric properties of the substrate surface. Subsequently, changes in the extent of the association of the enzyme to substrate could be involved. Due to the inherent complexity of the physicochemical characteristics of the dynamic phospholipid/water interface harbouring the site of the catalytic action of PLA_2 it is difficult to distinguish at this stage between the various possibilities outlined above. Furthermore, there is no reason to assume the above possibilities to be mutually exclusive. More thorough studies are thus warranted to establish the molecular level mechanism(s) involved. Yet, taken the importance of the development of novel means to fight emerging antibiotic resistant strains such efforts are clearly needed.

7 GENERAL DISCUSSION AND CONCLUSION

The antimicrobial peptides exert their effects on cells by interacting with membrane lipids and the influence of the lipid composition on their specificity is significant. The cationic nature of the antimicrobial peptides facilitate their binding and disrupting negatively charged membranes. The considerable preference of the peptides for anionic phospholipids provides specificity toward anionic bacterial membranes as opposed to zwitterionic eukaryotic cell membranes. In addition, cholesterol, which is not found in bacterial membranes, attenuates the interaction of antimicrobial peptides with membranes. The bacterial membrane is thus more susceptible to the antimicrobial peptides while the host cell membrane would remain intact. However, for the hemolytic peptides temporin L and indolicidin, they significantly bind and permeate also zwitterionic membranes though they have higher affinity to anionic phospholipid, further suggesting the important role of membrane lipids in the specificity of antimicrobial peptides.

Magainin 2, indolicidin, temporin B and L increase acyl chain order and cause segregation of acidic phospholipids, complexing them into supramolecular clusters. Likewise, in the presence of the negatively charged phospholipid the pronounced vesiculation and formation of endocytotic aggregates of giant liposomes caused by these antimicrobial peptides are dose-dependent. Furthermore, their antimicrobial action requires the initially asymmetric exposure of the target membranes to these peptides, and relates to the relaxation of the system back to thermodynamic equilibrium. All these effects are in keeping with cooperative membrane association being necessary for their antimicrobial action, involving transmembrane orientation of the peptides. However, the action of these antimicrobial peptides is not limited to permeabilization of the membranes and disruption of the membrane properties, they were also found to modulate the hydrolytic activity of secreted phospholipase A_2 by affecting the physical properties of the membrane. The concerted action between the $sPLA_2$ and antimicrobial peptides could improve the innate response to infections and thus further highlight the great potential of these peptides as therapeutics.

The presented mechanisms, some of which relate to each other, have been suggested to explain the molecular basis of antimicrobial peptides acting on membranes. In fact,

the same peptide might interact with phospholipid membranes in different ways depending on the peptide-to-lipid ratio, the lipid composition of the membrane, and other experimental conditions. With the help of biophysical and biochemical methods, the conformation, topology and dynamics of membrane-associated peptides and lipids can be investigated in considerable detail.

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9 REFERENCES

Ahmad, I., Perkins, W. R., Lupan, D. M., Selsted, M. E., and Janoff, A. S. (1995) Liposomal entrapment of the neutrophil-derived peptide indolicidin endows it with in vivo antifungal activity. *Biochim. Biophys. Acta* **1237**, 109-114.

Ammar, B., Perianin, A., Mor, A., Sarfati, G., Tissot, M., and Nicolas, P. (1998) Dermaseptin, a peptide antibiotic, stimulates microbicidal activities of polymorphonuclear leukocytes. *Biochem. Biophys. Res. Commu.* **247**, 870-875.

Andreu, D. and Rivas, L. (1999) Animal antimicrobial peptides: an overview. *Biopolymers* **47**, 415-433.

Angelova, M. I. and Dimitrov, D. S. (1986) Liposome electroformation. *Faraday Discuss. Chem. Soc.* **81**, 303-311.

Angelova, M. I., Hristova, N., and Tsoneva, I. (1999) DNA-induced endocytosis upon local microinjection to giant unilamellar cationic vesicles. *Eur. Biophys. J.* **28**, 142-150.

Arrighi, R. B. G., Nakamura, C., Miyake, J., Hurd, H., and Burgess, J. G. (2002) Design and activity of antimicrobial peptides against sporogonic-stage parasites causing murine malarias. *Antimicrob. Agents Chemother.* **46**, 2104-2110.

Baker, M. A., Maloy, W. L., Zasloff, M., and Jacob, L. S. (1993) Anticancer efficacy of magainin 2 and analogue peptides. *Cancer Res.* **53**, 3052-3057.

Bals, R., Weiner, D. J., Moscioni, A. D., Meegalla, R. L., and Wilson, J. M. (1999) Augmentation of innate host defense by expression of a cathelicidin antimicrobial peptide. *Infect. Immun.* **67**, 6084-6089.

Batzri, S. and Korn, E. D. (1973) Single bilayer liposomes prepared without sonication. *Biochim. Biophys. Acta* **298,** 1015-1019.

Bechinger, B. (1999) The structure, dynamics, and orientation of antimicrobial peptides in membranes by multidimensional solid-state NMR spectroscopy. *Biochim. Biophys. Acta* **1462**, 157-183.

Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K., and Tomita, M. (1992) Identification of the bactericidal domain of lactoferrin. *Biochim. Biophys. Acta* **1121**, 130-136.

Ben Efraim, I., Bach, D., and Shai, Y. (1993) Spectroscopic and functional characterization of the putative transmembrane segment of the minK potassium channel. *Biochemistry* **32**, 2371-2377.

Ben Efraim, I. and Shai, Y. (1997) The structure and organization of synthetic putative membranous segments of ROMK1 channel in phospholipid membranes. *Biophys. J.* **72**, 85-96.

Berg, O. G., Gelb, M. H., Tsai, M. D., and Jain, M. K. (2001) Interfacial enzymology: the secreted phospholipase A_2-paradigm. *Chem. Rev.* **101,** 2613-2653.

Bernstein, L. S., Grillo, A. A., Loranger, S. S., and Linder, M. E. (2000) RGS4 binds to membranes through an amphipathic alpha-helix. *J. Biol. Chem.* **275**, 18520-18526.

Bishop, R. E., Gibbons, H. S., Guina, T., Trent, M. S., Miller, S. I., and Raetz, C. R. (2000) Transfer of palmitate from phospholipids to lipid A in outer membranes of gram-negative bacteria. *EMBO J.* **19**, 5071-5080.

Boman, H. G. (1991) Antibacterial peptides: key components needed in immunity. *Cell* **65**, 205-207.

Boman, H. G., Faye, I., Gudmundsson, G. H., Lee, J. Y., and Lid-holm, D. A. (1991) Cell free immunity in *Cecropi*a. A model system for antibacterial proteins. *Eur. J. Biochem.* **210**, 23-31.

Boman, H. G., Agerberth, B., and Boman, A. (1993) Mechanisms of action on *Escherichia coli* of cecropin P1 and PR-39, two antibacterial peptides from pig intestine. *Infect. Immun.* **61**, 2978-2984.

Boman; H. G. (1995) Peptide antibiotics and their role in innate immunity. *Annu. Rev. Immunol.* **13**, 61-92.

Boman, H. G. (2000) Innate immunity and the normal microflora. *Immunol. Rev.* **173**, 5-16.

Bonomo, R. A. (2000) Multiple antibiotic-resistant bacteria in long-term-care facilities: an emerging problem in the practice of infectious diseases. *Clin. Infect. Dis.* **31**, 1414–1422.

Breukink, E., van Kraaij, C., van Dalen, A., Demel, R. A., Siezen, R. J., de Kruijff, B., and Kuipers, O. P. (1998) The orientation of nisin in membranes. *Biochemistry* **37**, 8153-8162.

Breukink, E. and de Kruijff, B. (1999) The lantibiotic nisin, a special case or not? *Biochim. Biophys. Acta* **1462**, 223-234.

Breukink, E., Wiedemann, I., Van Kraaij, C., Kuipers, O. P., Sahl, H.-G., and de Kruijff, B. (1999) Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. *Science* **286**, 2361-2364.

Brewer, D., Hunter, H., and Lajoie, G. (1998) NMR studies of the antimicrobial salivary peptides histatin 3 and histatin 5 in aqueous and nonaqueous solutions. *Biochem. Cell Biol.* **76**, 247-256.

Brockman, H. (1999) Lipid monolayers: why use half of a membrane to characterize protein-membrane interactions? *Curr. Opin. Struct. Biol.* **9**, 438-443.

Bu, X., Wu, X., Xie, G., and Guo, Z. (2002) Synthesis of Tyrocidine A and Its Analogues by Spontaneous Cyclization in Aqueous Solution. *Org. Lett.* **4**, 2893-2895.

Buckland, A. G. and Wilton, D. C. (2000) The antibacterial properties of secreted phospholipases A_2. *Biochim. Biophys. Acta* **1488**, 71-82.

Bulet, P., Urge, L., Ohresser, S., Hetru, C., and Otvös, L. (1996) Enlarged scale chemical synthesis and range of activity of drosocin, an O-glycosylated antibacterial peptide of *Drosophil*a. *Eur. J. Biochem.* **238**, 64 – 69.

Burack, W. R., Dibble, A. R., Allietta, M. M., and Biltonen, R. L. (1997) Changes in vesicle morphology induced by lateral phase separation modulate phospholipase A2 activity. *Biochemistry* **36**,10551-10557.

Cabiaux, V., Agerberth, B., Johansson, J., Homblé, F., Goormaghtigh, E., and Ruysschaert, J.-M. (1994) Secondary structure and membrane interaction of PR-39, a Pro+Arg-rich antibacterial peptide. *Eur. J. Biochem.* **224**, 1019-1027.

Cajal, Y. and Jain, M. K. (1997) Synergism between melittin and phospholipase A_2 from bee venom: apparent activation by intervesicle exchange of phospholipids. *Biochemistry* **36**, 3882-3893.

Cardinale, F., Jonak, C., Ligterink, W., Niehaus, K., Boller, T., and Hirt, H. (2000) Differential activation of four specific MAPK pathways by distinct elicitors. *J. Biol. Chem.* **275**, 36734-36740.

Carlsson, A., Engström, P., Palva, E. T., and Bennich, H. (1991) Attacin, an antibacterial protein from *Hyalophora cecropia*, inhibits synthesis of outer membrane proteins in *Escherichia coli* by interfering with *omp* gene transcription. *Infect. Immun.* **59**, 3040-3045.

Casteels, P. and Tempst, P. (1994) Apidaecin-type peptide antibiotics function through a non-poreforming mechanism involving stereospecificity. *Biochem. Biophys. Res. Commun.* **199**, 339-345.

Cerichelli, G., Luchetti, L., and Mancini, G. (1996) Surfactant control of the ortho/para ratio in the bromination of anilines. 3. *Tetrahedron* **52,** 2465-2470.

Chattopadhyay, A. and London, E. (1987) Parallax method for direct measurement of membrane penetration depth utilizing fluorescence quenching by spin-labeled phospholipids. *Biochemistry* **26**, 39-45.

Chen, J., Falla, T. J., Liu, H., Hurst, M. A., Fujii, C. A., Mosca, D. A., Embree, J. R., Loury, D. J., Radel, P. A., Cheng Chang, C., Gu, L., and Fiddes, J. C. (2000) Development of protegrins for the treatment and prevention of oral mucositis: structure-activity relationships of synthetic protegrin analogues. *Biopolymers* **55**, 88-98.

Chen, Y., Xu, X., Hong, S., Chen, J., Liu, N., Underhill, C. B., Creswell, K., and Zhang, L. (2001) RGD-tachyplesin inhibits tumor growth. *Cancer Res.* **61**, 2434-2438.

Cleveland, J., Montville, T. J., Nes, I. F., and Chikindas, M. L. (2001) Bacteriocins: safe, natural antimicrobials for food preservation. *J. Food Microbiol.* **71**, 1-20.

Cohen, C. J., Dusek, A., Green, J., Johns, E. L., Nelson, E., and Recny, M. A. (2002) Long-term treatment with subcutaneous T-20, a fusion inhibitor, in HIV-infected patients: patient satisfaction and impact on activities of daily living. *AIDS Patient Care Stds.* **16**, 327-335.

Cornut I., Büttner, K., Dasseux, J.-L., and Dufourq, J. (1994) Application to the *de novo* design of ideally amphipathic Leu, Lys peptides with hemolytic activity higher than that of melittin. *FEBS Lett.* **349**, 29-33.

Cruciani, R. A., Barker, J. L., Zasloff, M., Chen, H. C., and Colamonici, O. (1991) Antibiotic magainins exert cytolytic activity against transformed cell lines through channel formation. *Proc. Natl. Acad. Sci. USA* **88**, 3792-3796.

Daher, K. A., Selsted, M. E., and Lehrer, R. I. (1986) Direct inactivation of viruses by human granulocyte defensins. *J. Virol.* **60**, 1068-1074.

Dathe, M., Schumann, M., Wieprecht, T., Winkler, A., Beyermann, M., Krause, E. Matsuzaki, K., Murase, O., and Bienert, M. (1996) Peptide helicity and membrane surface charge modulate the balance of electrostatic and hydrophobic interactions with lipid bilayer and biological membranes. *Biochemistry* **35,** 12612-12622.

Dathe, M., Wieprecht, T., Nikolenko, H., Handel, L., Maloy, W.L., MacDonald, D.L., Beyermann, M., and Bienert, M. (1997) Hydrophobicity, hydrophobic moment and angle subtended by charged residues modulate antibacterial and hemolytic activity of amphipathic helical peptides. *FEBS Lett.* **403**, 208-212.

Dathe, M. and Wieprecht, T. (1999) Structural features of helical antimicrobial peptides: their potential to modulate activity on model membranes and biological cells. *Biochim. Biophys. Acta* **1462**, 71-87.

De Bolle, M. F. C., Osborn, R. W., Goderis, I. J., Noe, L., Acland, D., Hart, C. A., Torrekens, S., Van Leuven, F., and Broekaert, W. F. (1996) Antimicrobial peptides from Mirabilis jalapa and Amaranthus caudatus: expression, processing, localization and biological activity in transgenic tobacco. *Plant Mol. Biol.* **31**, 993-1008.

De Kroon, A. I. P. M., Soekarjo, M. W., De Gier, J., and De Kruijff, B. (1990) The role of charge and hydrophobicity in peptide-lipid interaction: a comparative study based on tryptophan fluorescence measurements combined with the use of aqueous and hydrophobic quenchers. *Biochemistry* **29**, 8229-8240.

De Kruijff, B. (1997) Biomembranes. Lipids beyond the bilayer. *Nature* **386**, 129-130.

Delatorre, P., Olivieri, J. R., Ruggiero Neto, J., Lorenzi, C. C. B., Canduri, F., Fadel, V., Konno, K., Palma, M. S., Yamane, T., and de Azevedo, W. F. (2001) Preliminary cryocrystallography analysis of an eumenine mastoparan toxin isolated from the venom of the wasp Anterhynchium flavomarginatum micado. *Biochim. Biophys. Acta* **1545**, 372-376.

Delves-Broughton, J. (1990) Nisin and its uses as a food preservative. *Food Technol.* **44**, 100-117.

Devaux, P. F. (1991) Static and dynamic asymmetry in cell membranes. *Biochemistry* **30,** 1163-1173.

Dolis, D., Moreau, C., Zachowski, C., and Devaux, P. F. (1997) Aminophospholipid translocase and proteins involved in transmembrane phospholipid traffic. *Biophys. Chem.* **68,** 221-231

Dougherty, D. A. (1996) Cation-pi interactions in chemistry and biology: a new view of benzene, Phe, Tyr, and Trp. *Science* **271**, 163-168.

Dowhan, W. (1997) Molecular basis for membrane phospholipid diversity: why are there so many lipids? *Annu. Rev. Biochem.* **66**, 199-232.

Duclohier, H. and Wroblewski, H. (2001) Voltage-dependent pore formation and antimicrobial activity by alamethicin and analogues. *J. Membr. Biol.* **184**, 1-12.

Eftink, M. R. and Ghiron, C. A. (1976) Fluorescence quenching of indole and model micelle systems. *J. Phys. Chem.* **80**, 486-493.

Eisenberg, D., Schwarz, E., Komaromy, M., and Wall, R. (1984) Analysis of membrane and surface protein sequences with the hydrophobic moment plot. *J. Mol. Biol.* **179**, 125-142.

Epand, R. M. (1998) Lipid polymorphism and lipid-protein interactions. *Biochim. Biochem. Acta* **1376**, 353-368.

Epand, R. M. and Vogel, H. J. (1999) Diversity of antimicrobial peptides and their mechanisms of action. *Biochim. Biophys. Acta* **1462**, 11-28.

Ernst, R. K., Guina, T., and Miller, S. I. (1999) How intracellular bacteria survive: surface modifications that promote resistance to host innate immune responses. *J. Infect. Dis.* **179** (Suppl. 2), S326-S330.

Falla, T. J., Karunaratne, D. N., and Hancock, R. E. W. (1996) Mode of action of the antimicrobial peptide indolicidin. *J. Biol. Chem.* **271**, 19298-19303.

Fehlbaum, P., Bulet, P., Chernysh, S., Briand, J.-P., Roussel, J.-P., Leitellier, L., Hetru, C., and Hoffmann, J. A. (1996) Structure-activity analysis of thanatin, a 21-residue inducible insect defense peptide with sequence homology to frog skin antimicrobial peptides. *Proc. Natl. Acad. Sci. USA* **93**, 1221-1225.

Fernandez-Lopez, S., Kim, H. S., Choi, E. C., Delgado, M., Granja, J. R., Khasanov, A., Kraehenbuehl, K., Long, G., Weinberger, D. A., Wilcoxen, K. M., and Ghadiri, M. R. (2001) Antibacterial agents based on the cyclic D,L-alpha-peptide architecture. *Nature* **412**, 452-455.

Frohm, M., Agerberth, B., Ahangari, G., Stahle-Backdahl, M., Liden, S., Wigzell, H., and Gudmundsson, G. H. (1997) The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders. *J. Biol. Chem.* **272**, 15258-15263.

Gadd, M. E. and Biltonen, R. L. (2000) Characterization of the interaction of phospholipase A_2 with phosphatidylcholine-phosphatidylglycerol mixed lipids. *Biochemistry* **39,** 9623-9631.

Ganz, T. and Lehrer, R. I. (1995) Defensins. *Pharmacol. Ther.* **66**, 191-205.

Gazit, E., Bach, D., Kerr, I. D., Sansom, M. S., Chejanovsky, N., and Shai, Y. (1994) The α-5 segment of Bacillus thuringiensis δ-endotoxin: in vitro activity, ion channel formation and molecular modeling. *Biochem. J.* **304**, 895-902.

Gazit, E., Boman, A., Boman, H. G., and Shai, Y (1995) Interaction of the mammalian antibacterial peptide cecropin P1 with phospholipid vesicles. *Biochemistry* **34**, 11479-11488.

Ghomashchi, F., Lin, Y., Hixon, M. S., Yu, B.-Z., Annand, R., Jain, M. K., and Gelb, M. H. (1998) Interfacial recognition by bee venom phospholipase A2: insights into nonelectrostatic molecular determinants by charge reversal mutagenesis. *Biochemistry* **37**, 6697-6710.

Ghosh, J. K., Shaool, D., Guillaud, P., Ciceron, L., Mazier, D., Kustanovich, I., Shai, Y., and Mor, A. (1997) Selective cytotoxicity of dermaseptin S3 toward intraerythrocytic Plasmodium falciparum and the underlying molecular basis. *J. Biol. Chem.* **272**, 31609-31616.

Giles, F. J., Redman, R., Yazji, S., and Bellm, L. (2002) Iseganan HCl: a novel antimicrobial agent. *Expert Opin. Investig. Drugs* **11**, 1161-1170.

Gomez-Gomez, L., Felix, G., and Boller, T. (1999) A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. *Plant J.* **18**, 277-284.

Gray, P. W., Flaggs, G., Leong, S. R., and Gumina, R. (1989) Cloning of the cDNA of a human neutrophil bactericidal protein. *J. Biol. Chem.* **264**, 9505-9509.

Groisman, E. A., Kayser, J., and Soncini, F. C. (1997) Regulation of polymyxin resistance and adaptation to low-Mg^{2+} environments. *J. Bacteriol.* **179**, 7040-7045.

Gudmundsson, G. H., Lidholm, D. A., Asling, B., Gan, R., and Boman, H. G. (1991) The cecropin locus. Cloning and expression of a gene cluster encoding three antibacterial peptides in Hyalophora cecropia. *J. Biol. Chem.* **266,** 11510-11517.

Gudmundsson, G. H., Agerberth, B., Odeberg, J., Bergman, T., Olsson, B., and Salcedo, R. (1996) The human gene FALL39 and processing of the cathelin precursor to the antibacterial peptide LL-37 in granulocytes. *Eur. J. Biochem.* **238**, 325-332.

Guina, T., Yi, E. C., Wang, H., Hackett, M., and Miller, S. I. (2000) A PhoP-regulated outer membrane protease of Salmonella enterica serovar typhimurium promotes resistance to alpha-helical antimicrobial peptides. *J. Bacteriol.* **182**, 4077-4086.

Guo, L., Lim, K. B., Gunn, J. S., Bainbridge, B., Darveau, R. P., Hackett, M., and Miller, S. I. (1997) Regulation of lipid A modifications by Salmonella typhimurium virulence genes phoP-phoQ. *Science* **276**, 250-253.

Guo, L., Lim, K. B., Poduje, C. M., Daniel, M., Gunn, J. S., Hackett, M., and Miller, S. I. (1998) Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. *Cell* **95**, 189-198.

Ha, T. H., Kim, C. H., Park, J. S., and Kim, K. (2000) Interaction of indolicidin with model lipid bilayer: quartz crystal microbalance and atomic force microscopy study. *Langmuir* **16**, 871-875.

Harwig, S. S., Swiderek, K. M., Lee, T. D., and Lehrer, R. I. (1995) Determination of disulphide bridges in PG-2, an antimicrobial peptide from porcine leukocytes. *J. Pept. Sci.* **1**, 207-215.

Hancock, R. E. W. (1997) Peptide antibiotics. *Lancet* **349**, 418-422.

Hancock, R. E. W. and Lehrer, R. (1998) Cationic peptides: a new source of antibiotics. *Trends Biotechnol.* **16**, 82-88.

Hancock, R. E. W. and Chapple, D. S. (1999) Peptide antibiotics. *Antimicrob. Agents Chemother.* **43**, 1317-1323.

Hancock, R. E. W. and Diamond, G. (2000) The role of cationic antimicrobial peptide in innate host defences. *Trends Microbiol.* **8**, 402-410.

Hancock, R. E. W. and Scott, M. G. (2000) The role of antimicrobial peptides in animal defences. *Proc. Natl. Acad. Sci. USA* **97**, 8856-8861.

Hancock, R. E. W. (2001) Cationic peptide: effectors in innate immunity and novel antimicrobials. *Lancet Infect. Dis.* **1**, 156-164.

Hancock, R. E. W. and Rozek, A. (2002) Role of membranes in the activities of antimicrobial cationic peptides. *FEMS Microbiol. Lett.* **206**, 143-149.

Harder, J., Meyer-Hoffert, U., Teran, L. M., Schwichtenberg, L., Bartels, J., Maune, S., and Schröder, J-M. (2000) Mucoid *Pseudomonas aeruginosa*, TNF-α, and IL-1β, but not IL-6, induce human β-defensin-2 in respiratory epithelia. *Am. J. Resir. Cell Mol. Biol.* **22**, 714-721.

Heller, W. T., Waring, A. J., Lehrer, R. I., and Huang, H. W. (1998) Multiple states of beta-sheet peptide protegrin in lipid bilayers. *Biochemistry* **37**, 17331-17338.

Helmerhorst, E. J., Breeuwer, P., van't Hof, W., Walgreen-Weterings, E., Oomen, L. C., Veerman, E. C., Amerongen, A. V., and Abee, T. (1999a) The cellular target of histatin 5 on Candida albicans is the energized mitochondrion. *J. Biol. Chem.* **274**, 7286-7291.

Helmerhorst, E. J., Reijnders, I. M., Van 't Hof, W., Veerman, E. C. I., and Nieuw Amerongen, A. V. (1999b). A critical evaluation of the lytic activity of cationic antimicrobial peptides against *Candida albicans* cells and human erythrocytes. *FEBS Lett.* **449**, 105 – 110.

Hetru, C., Letellier, L., Oren, Z., Hoffmann, J. A., and Shai, Y. (2000) Androctonin, a hydrophilic disulphide-bridged non-hemolytic anti-microbial peptide: a plausible mode of action. *Biochem. J.* **345**, 653-664.

Hill, C. P., Yee, J., Selsted, M. E., and Eisenberg, D. (1991) Crystal structure of defensin HNP-3, an amphiphilic dimer: mechanisms of membrane permeabilization. *Science* **251**, 1481-1485.

Hirakura, Y., Kobayashi, S., and Matsuzaki, K. (2002) Specific interactions of the antimicrobial peptide cyclic beta-sheet tachyplesin I with lipopolysaccharides, *Biochim. Biophys. Acta* **1562**, 32-36.

Hoffmann, J. A., Kafatos, F. C., Janeway, C. A., and Ezekowitz, R. A. (1999) Phylogentic perspectives in innate immunity. *Science* **284**, 1313-1318.

Holopainen, J. M., Angelova, M. I., and Kinnunen, P. K. J. (2000) Vectorial budding of vesicles by asymmetrical enzymatic formation of ceramide in giant liposomes. *Biophys. J.* **78**, 830-838.

Horwitz, A. H., Leugh, S. D., Abrahamson, S., Gazzano-Santoro, H., Liu, P. S., William, R. E., Carroll, S. F., and Theofan, G. (1996) Expression and characterization of cysteine-modified variants of an amino-terminal fragment of bactericidal/permeability-increasing protein. *Protein Expr. Putif.* **8**, 28-40.

Huang, H. W. (2000) Action of antimicrobial peptides: two-state model. *Biochemistry* **39**, 8347–8352.

Huttner, K. M., Lambeth, M. R., Burkin, H. R., Burkin, D. J., and Broad, T. E. (1998) Localization and genomic organization of sheep antimicrobial peptide genes. *Gene* **206**, 85-91.

Huttner, W. B., and Schmidt, A. A. (2002) Membrane curvature: a case of endofeelin. *Trends Cell Biol.* **12**, 155-158.

Imler, J.-L. and Hoffmann, J. A. (2000) Signaling mechanisms in the antimicrobial host defense of *Drosophila. Curr. Opin. Microbiol.* **3**, 16-22.

Jach, G., Goernhardt, B., Mundy, J., Logemann, J., Pinsdorf, E., Leah, R., Schell, J., and Maas, C. (1995) Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. *Plant J.* **8**, 97-109.

Jack, R. W. and Jung, G. (2000) Lantibiotics and microcins: polypeptides with unusual chemical diversity. *Curr. Opin. Chem. Biol.* **4**, 310-317.

Jain, M. K., Rogers, J., Berg, O., and Gelb, M. H. (1991) Interfacial catalysis by phospholipase A2: activation by substrate replenishment. *Biochemistry* **30**, 7340-7348.

Javadpour, M. M., Juban, M. M., Lo, W.-C., Bishop, S. M., Alberty, J. B., Cowell, S. M., Becker, C. L., and McLaughlin, M. L. (1996) *De novo* antimicrobial peptides with low mammalian cell toxicity. *J. Med. Chem.* **39**, 3107-3113.

Jaynes, J. M., Burton, C. A., Barr, S. B., Jeffers, G. W., Julian, G. R., White, K. L., Enright, F. M., Klei, T. R., and Laine, R. A. (1988) In vitro cytocidal effect of novel lytic peptides on *Plasmodium falciparum*. *FASEB J.* **2**, 2878–2883.

Jones, E. M., Smart, A., Bloomberg, G., Burgess, L., Millar, M. R. (1994) Lactoferricin, a new antimicrobial peptide. *J. Applied Bacteriol.* **77**, 208-214.

Kagan, B. L., Selsted, M. E., Ganz, T., and Lehrer, R. I. (1990) Antimicrobial defensin peptides form voltage-dependent ion-permeable channels in planar lipid bilayer membranes. *Proc. Natl. Acad. Sci. USA* **87**, 210-214.

Keller, H., Pamboukdjian, N., Ponchet, M., Poupet, A., Delon, R., Verrier, J. L., Roby, D., and Ricci, P. (1999) Pathogen-induced elicitin production in transgenic tobacco generates a hypersensitive response and nonspecific disease resistance. *Plant Cell* **11**, 223-235.

Ketchem, R, Hu, W., and Cross, T. A. (1993) High-resolution conformation of gramicidin A in a lipid bilayer by solid-state NMR. *Science* **261**, 1457-1460.

Khush, R. S., Leulier, F., and Lemaitre, B. (2001) Drosophila immunity: two paths to NF-kappaB. *Trends Immunol*. **22**, 260-264.

Kieffer, A.-E., Goumon, Y., Ruh, O., Chasserot-Golaz, S., Nullans, G., Gasnier, C., Aunis, D., and Metz-Boutigue, M.-H. (2003) The N- and C-terminal fragments of ubiquitin are important for the antimicrobial activities. *FASEB J.* **17**, 776-778.

Kikukawa, T. and Araiso, T. (2002) Changes in lipid mobility associated with alamethicin incorporation into membranes. *Arch. Biochem. Biophys.* **405**, 214-222.

Kinnunen, P. K. J. (1991) On the principles of functional ordering in biological membranes. *Chem. Phys. lipids* **57**, 375-399.

Kiyota, T., Lee, S., and Sugihara, G. (1996) Design and synthesis of amphiphilic alpha-helical model peptides with systematically varied hydrophobic-hydrophilic balance and their interaction with lipid- and bio-membranes. *Biochemistry* **35**, 13196-13204.

Kobayashi, S., Hirakura, Y., and Matsuzaki, K. (2001) Bacteria-selective synergism between the antimicrobial peptides alpha-helical magainin 2 and cyclic beta-sheet tachyplesin I: toward cocktail therapy. *Biochemistry* **40**, 14330-14335.

Kondejewski, L. H., Jelokhani-Niaraki, M., Farmer, S. W., Lix, B., Kay, C. M., Sykes, B. D., Hancock, R. E., and Hodges, R. S. (1999) Dissociation of antimicrobial and hemolytic activities in cyclic peptide diastereomers by systematic alterations in amphipathicity. *J. Biol. Chem.* **274**, 13181-13192.

Kourie, J. I. and Shorthouse, A. A. (2000) Properties of cytotoxic peptide-formed ion channels. *Am. J. Physiol-Cell Physiol.* **278**, C1063-C1087.

Kuchinka, E. and Seelig, J. (1989) Interaction of melittin with phosphatidylcholine membranes. Binding isotherm and lipid head-group conformation. *Biochemistry* **28**, 4216-4221.

Kwon, M.-Y., Hong, S.-Y., Lee, K.-H. (1998) Structure-activity analysis of brevinin 1E amide, an antimicrobial peptide from *Rana esculenta*. *Biochim. Biophys. Acta* **1387**, 239-248.

Ladokhin, A. S., Holloway, P. W., and Kostrzhevska, E. G. (1993) Distribution Analysis of Membrane Penetration of Proteins by Depth-Dependent Fluorescence Quenching. *J. Fluorescence* **3**, 195-197.

Ladokhin, A. S. (1997) Distribution analysis of depth-dependent fluorescence quenching in membranes: a practical guide. *Methods Enzymol*. **278**, 462-473.

Ladokhin, A. S., Selsted, M.. E., and White, S. H. (1997) Bilayer interactions of indolicidin, a small antimicrobial peptide rich in tryptophan, proline, and basic amino acids. *Biophys. J.* **72**, 794-805.

Ladokhin, A. S. (1999) Analysis of protein and peptide penetration into membranes by depth-dependent fluorescence quenching: theoretical considerations. *Biophys. J.* **76**, 946-955.

Ladokhin, A. S., Selsted, M. E., and White, S. H. (1999) CD spectra of indolicidin antimicrobial peptides suggest turns, not polyproline helix. *Biochemistry* **38**, 12313-12319.

Lamberty, M., Caille, A., Landon, C., Tassin-Moindrot, S., Hetru, C., Bulet P., and Vovelle, F. (2001) Solution structures of the antifungal heliomicin and a selected variant with both antibacterial and antifungal activities. *Biochemistry* **40**, 11995-112003.

Lambotte, S., Jasperse, P., and Bechinger, B. (1998) Orientational distribution of alpha-helices in the colicin B and E1 channel domains: a one and two dimensional 15N solid-state NMR investigation in uniaxially aligned phospholipid bilayers. *Biochemistry* **37**, 16-22.

Langosch, D. and Heringa, J. (1998) Interaction of transmembrane helices by a knobs-into-holes packing characteristic of soluble coiled coils. *Proteins* **31**, 150-159.

Latal, A., Degovics, G., Epand, R. F., Epand, R. M., and Lohner, K. (1997) Structural aspects of the interaction of peptidyl-glycylleucine-carboxyamide, a highly potent antimicrobial peptide from frog skin, with lipids. *Eur. J. Biochem.* **248**, 938-946.

Lawyer, C., Pai, S., Watabe, M., Borgia, P., Mashimo, T., Eagleton, L., and Watabe, K. (1996) Antimicrobial activity of a 13 amino acid tryptophan-rich peptide derived from a putative porcine precursor protein of a novel family of antibacterial peptides. *FEBS Lett.* **390**, 95-98.

Lehrer, R. I. and Ganz, T. (1999) Antimicrobial peptides in mammalian and insect host defence. *Curr. Opin. Immunol.* **11**, 23-27.

Lehtonen, J. Y. A. and Kinnunen, P. K. J. (1995) Phospholipase A2 as a mechanosensor. *Biophys. J.* **68**, 1888-1894.

Leistner, L. and Gorris, L. G. M. (1995) Food preservation by hurdle technology. *Trends Food Sci. Technol.* **6**, 41-46.

Leisner, J. J., Greer, G. G., and Stile, M. E. (1996) Control of beef spoilage by a sul®de-producing Lactobacilus sake strain with bacteriocinogenic Leuconostoc gelidum UAL 187 during anaerobic storage at 2 °C. *Appl. Environ. Microbiol.* **62**, 2610-2614.

Linde, C. M. A., Hoffner, S. E., Refai, E., and Andersson, M. (2001) In vitro activity of PR-39, a proline-arginine-rich peptide, against susceptible and multi-drug-resistant Mycobacterium tuberculosis. *J. Antimicrob. Chemother.* **47**, 575-580.

Lindholm, P., Göransson, U., Johansson, S., Claeson, P., Gullbo, J., Larsson, R., Bohlin, L., and Backlund, A. (2002) Cyclotides: a novel type of cytotoxic agents. *Mol. Cancer Ther.* **1**, 365-369.

Liang, J. F. and Kim, S. C. (1999) Not only the nature of peptide but also the characteristics of cell membrane determine the antimicrobial mechanism of a peptide. *J. Peptide Res.* **53**, 518-522.

Lohner, K. and Prenner, E. J. (1999) Differential scanning calorimetry and X-ray diffraction studies of the specificity of theinteraction of antimicrobial peptides with membrane-mimic systems. *Biochim. Biophys. Acta* **1462**, 141-156.

Lohner, K. (2001) The role of membrane lipid composition in cell targeting of antimicrobial peptides. In: Lohner, K. (ed.), Development of novle antimicrobial agents: emerging strategies. Horizon Scientific Press, Wymondham, Norfolk, UK, pp.149-165.

Ludtke, S. J., He, K., Heller, W. T., Harroun, T. A., Yang, L., and Huang, H. W. (1996) Membrane pores induced by magainin. *Biochemistry.* **35**, 13723-13728.

Luisi, P. L. and Walde, P. (2000) Giant vesicles. John Wiley & Sons Ltd, Chichester, England.

Maget-Dana, R., Lelievre, D., and Brack, A. (1999) Surface active properties of amphiphilic sequential isopeptides: Comparison between α-helical and β-sheet conformations. *Biopolymers* **49**, 415-423.

Mai, J. C., Mi, Z., Kim, S. H., Ng, B., and Robbins, P. D. (2001) A proapoptotic peptide for the treatment of solid tumors. *Cancer Res.* **61**, 7709-7712.

Mangoni, M. L., Rinaldi, A. C., Di Giulio, A., Mignogna, G., Bozzi, A., Barra, D., and Simmaco, M. (2000) Structure-function relationships of temporins, small antimicrobial peptides from amphibian skin. *Eur. J. Biochem.* **267**, 1447-1454.

Mattila, K., Kinder, R., and Bechinger, B. (1999) The alignment of a voltage-sensing peptide in dodecylphosphocholine micelles and in oriented lipid bilayers by nuclear magnetic resonance and molecular modeling. *Biophys. J.* **77**, 2102-2113.

Matsuzaki, K., Harada, M., Funakoshi, S., Fujii, N., and Miyajima, M. (1991) Physicochemical determinants for the interactions of magainins 1 and 2 with acidic lipid bilayers. *Biochim. Biophys. Acta* **1063**, 162-170.

Matsuzaki, K., Sugishita, K., Fujii, N., and Miyajima, K. (1995a) Molecular basis for membrane selectivity of an antimicrobial peptide, magainin 2. *Biochemistry* **34**, 3423-3429.

Matsuzaki, K., Murase, O., and Miyajima, M. (1995b) Kinetics of pore formation by an antimicrobial peptide, magainin 2, in phospholipid bilayers. *Biochemistry* **34**, 12553-12559.

Matsuzaki, K., Murase, O., Fujii, N., and Miyajima, M. (1996) An antimicrobial peptide, magainin 2, induced rapid flip-flop of phospholipids coupled with pore formation and peptide translocation. *Biochemistry* **35**, 11361-11368.

Matsuzaki, K., Yoneyama, S., Fujii, N., Miyajima, K., Yamada, K., Kirino, Y., and Anzai, K. (1997) Membrane permeabilization mechanisms of a cyclic antimicrobial peptide, tachyplesin I, and its linear analog. *Biochemistry* **36**, 9799-9806.

Matsuzaki, K. (1998) Magainins as paradigm for the mode of action of pore forming polypeptides. *Biochim. Biophys. Acta* **1376**, 391-400.

Matsuzaki, K., Sugishita, K., Ishibe, N., Ueha, M., Nakata, S., Miyajima, K., and Epand, R. M. (1998) Relationship of membrane curvature to the formation of pores by magainin 2. *Biochemistry* **37**, 11856-11863.

Matsuzaki, K. (1999) Why and how are peptide-lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes. *Biochim. Biophys. Acta* **1462**, 1-10.

Matsuzaki, K., Sugishita, K., Miyajima, K., McIntosh, T. J., and Holloway, P. W. (1999) Interactions of an antimicrobial peptide, magainin 2, with lipopolysaccharide-containing liposomes as a model for outer membranes of gram-negative bacteria. *FEBS Lett.* **4492**, 221-224.

McCafferty, D. G., Cudic, P., Yu, M. K., Behenna, D. C., and Kruger, R. (1999) Synergy and duality in peptide antibiotic mechanisms. *Curr. Opin. Chem. Biol.* **3**, 672-680.

McIntosh, T. J. and Holloway, P. W. (1987) Determination of the depth of bromine atoms in bilayers formed from bromolipid probes. *Biochemistry* 26, 1783-1788.

Medzhitov, R. (2001) Toll-like receptors and innate immunity. *Nature Rev. Immunol*. **1**, 135-145.

Meister, M., Lemaitre, B., and Hoffmann, J.A. (1997) Antimicrobial peptide defense in *Drosophil*a. *BioEssays* **19**, 1019-1026.

Menger, F. M. (1998) Giant vesicles: imitating the cytological processes of cell membranes. *Acc. Chem. Res.* **31**, 789-797.

Miyasaki, K. T. and Lehrer, R. I. (1998) β-Sheet antibiotic peptides as potential dental therapeutics. *Int. J. Antimicrob. Agents* **9**, 269-280.

Moffitt, M. C. and Neilan, B. A. (2000) The expansion of mechanistic and organismic diversity associated with non-ribosomal peptides. *FEMS Microbiol. Lett.* **191**, 159-167.

Monaco, V., Formaggio, F., Crisma, M., Toniolo, C., Hanson, P., and Millhauser, G. L. (1999) Orientation and immersion depth of a helical lipopeptaibol in membranes using TOAC as an ESR probe. *Biopolymers* **50**, 239-253.

Montville, T. J. and Winkowski. K. (1997) Biologically-based preservation systems and probiotic bacteria. In: Doyle MP, Beuchat LR, Montville TJ (eds) Food microbiology: fundamentals and frontiers. American Society for Microbiology Press, Washington D.C., pp 557-577.

Montville, T. J. and Chen, Y. (1998) Mechanistic action of pediocin and nisin: recent progress and unresolved questions. *Appl. Microbiol. Biotechnol.* **50**, 511-519.

Moore, A. J., Devine, D. A., and Bibby, M. C. (1994) Preliminary experimental anticancer activity of cecropins. *Pept. Res.* **7**, 265-269.

Mor, A. and Nicolas, P. (1994) The NH2-terminal α-helical domain 1-18 of dermaseptin is responsible for antimicrobial activity. *J. Biol. Chem.* **269**, 1934-1939.

Morassutti, C., De Amicis, F., Skerlavaj, B., Zanetti, M., and Marchetti, S. (2002) Production of a recombinant antimicrobial peptide in transgenic plants using a modified VMA intein expression system. *FEBS Lett.* **519**, 141-146.

Mosca, D. A., Hurst, M. A., So, W., Viajar, B. S. C., Fujii, C. A., and Falla, T. J. (2000) IB-367, a protegrin peptide with in vitro and in vivo activities against the microflora associated with oral mucositis. *Antimicrob. Agents Chemother.* **44**, 1803-1808.

Mustonen, P. and Kinnunen, P. K. J. (1991) Activation of phospholipase A2 by adriamycin in vitro. *J. Biol. Chem.* **266**, 6302-6307.

Mustonen, P. and Kinnunen, P. K. J. (1992) Substrate level modulation of the activity of phospholipase A2 in vitro by 12-O-tetradecanoylphorbol-13-acetate. *Biochem. Biophys. Res. Commun.* **185**, 185-190.

Nes, I. F. and Holo, H. (2000) Class II antimicrobial peptides from lactic acid bacteria. *Biopolymers* **55**, 50-61.

Nevalainen, T. J., Haapamäki, M. M., and Grönroos, J. M. (2000) Roles of secretory phospholipases A_2 in inflammatory diseases and trauma. *Biochim. Biophys. Acta* **1488,** 83-90.

New, R. R. C. (ed.) (1990) Liposomes. A practical Approach. IRL Press, New York.

Nurminen, T. A., Holopainen, J. M., Zhao, H., and Kinnunen, P. K. J. (2002) Observation of topical catalysis by sphingomyelinase coupled to microspeheres. *J. Am. Chem. Soc*. **124**, 12129-12134.

Nürnberger, T., Nennstiel, D., Jabs, T., Sacks, W. R., Hahlbrock, K., and Scheel, D. (1994) High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multiple defense responses. *Cell* **78**, 449-460.

Oh, J. E., Hong, S. Y., and Lee, K.-H. (1999) Structure-activity relationship study: short antimicrobial peptides. *J. Pept. Res.* **53**, 41-46.

Oh, J., Cajal, Y., Skowronska, E. M., Belkin, S., Chen, J., Van Dyk, T. K., Sasse, R. M., and Jain, M. K. (2000) Cationic peptide antimicrobials induce selective transcription of *micF* and *osmY* in *Escherichia coli*. *Biochim. Biophys. Acta* **1463**, 43-54.

O'Leary, W. M. and Wilkinson, S. G. (1988) Gram-positive bacteria. In: Ratledge, C., Wilkinson, S. G. (eds.), Microbial lipids, vol. 1. Academy Press, London, pp. 117-201.

Oren, Z., Hong, J., and Shai, Y. (1997) A repertoire of novel antibacterial diastereomeric peptides with selective cytolytic activity. *J. Biol. Chem.* **272**, 14643-14649.

Oren, Z. and Shai, Y. (1998) Mode of action of linear amphipathic α-helical antimicrobial peptides. *Biopolymers (Peptide Sci.)* **47**, 451-463.

Oren, Z., Lerman, J. C., Gudmundsson, G. H., Agerberth, B., and Shai, Y. (1999a) Structure and organization of the human antimicrobial peptide LL-37 in phospholipid membranes: relevance to the molecular basis for its non-cell-selective activity. *Biochem. J.* **341**, 501-513.

Oren, Z., Hong, J., and Shai, Y. (1999b) A comparative study on the structure and function of a cytolytic alpha-helical peptide and its antimicrobial beta-sheet diastereomer. *Eur. J. Biochem.* **259**, 360-369.

Oren Z. and Shai Y. (2000) Cyclization of a cytolytic amphipathic alpha-helical peptide and its diastereomer: effect on structure, interaction with model membranes, and biological function. *Biochemistry* **39**, 6103-6114.

Park, C. B, Kim, H. S., and Kim, S. C. (1998) Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. *Biochem. Biophys. Res. Commun.* **244**, 253-257.

Perez-Paya, E., Houghten, R. A., and Blondelle, S. E. (1994) Determination of the secondary structure of selected melittin analogs with different hemolytic activities. *Biochem. J.*, **299**, 587-591.

Persson, S., Killian, J. A., and Lindblom, G. (1998) Molecular ordering of interfacially localized tryptophan analogs in ester- and ether-lipid bilayers studied by 2H-NMR. *Biophys. J.* **75**, 1365-1371.

Peschel, A. (2000) How do bacteria resist human antimicrobial peptides? *Trends Microbiol.* **10**, 179-186.

Pieterse, C. M. and Van Loon, L. C. (1999) Salicylic acid-independent plant defence pathways. *Trends Plant Sci.* **4**, 52-58.

Prenner, E. J., Lewis, R. N. A. H., Neuman, K. C., Gruner, S. M., Kondejewski, L. H., Hodges, R. S., and McElhaney, R. N. (1997) Nonlamellar phases induced by the interaction of gramicidin S with lipid bilayers. A possible relationship to membrane-disrupting activity. *Biochemistry* **36**, 7906-7916.

Prenner, E. J., Lewis, R. N., and McElhaney, R. N. (1999) The interaction of the antimicrobial peptide gramicidin S with lipid bilayer model and biological membranes. *Biochim. Biophys. Acta* **1462**, 201-221.

Pouny, Y., Rapaport, D., Mor, A., Nicolas, P., and Shai, Y. (1992) Interaction of antimicrobial dermaseptin and its fluorescently labeled analogues with phospholipid membranes. *Biochemistry* **31**, 12416-12423.

Raj, P. A. and Dentino, A. R. (2002) Current status of defensins and their role in innate and adaptive immunity. *FEMS Microbiol. Lett.* **206**, 9-18.

Rana, F. R., Macias, E. A., Sultany, C. M., Modzrakowski, M. C., and Blazyk, J. (1991) Interactions between magainin 2 and Salmonella typhimurium outer membranes: effect of lipopolysaccharide structure. *Biochemistry* **30**, 5858-5866.

Rao, A. G. (1999) Conformation and antimicrobial activity of linear derivatives of tachyplesin lacking disulfide bonds. *Arch. Biochem. Biophys.* **361**, 127-134.

Rapaport, D. and Shai, Y. (1991) Interaction of fluorescently labeled pardaxin and its analogues with lipid bilayers. *J. Biol. Chem.* **266**, 23769-23775.

Resnick, N. M., Maloy, W. L., Guy, H. R., and Zasloff, M. (1991) A novel endopeptidase from Xenopus that recognizes alpha-helical secondary structure. *Cell* **66**, 541-554.

Rietveld, A. and Simons, K. (1998) The differential miscibility of lipids as the basis for the formation of functional membrane rafts. *Biochim. Biophys. Acta* **1376**, 467-479.

Rinaldi, A. C., Di Giulio, A., Liberi, M., Gualtieri, G., Simmaco, M., Barra, D. and Bozzi, A. (2001) Effects of temporins on molecular dynamics and membrane permeabilization in lipid vesicles. *J. Pept. Res.* **58**, 213-220.

Rinaldi, A. C., Mangoni, M. I., Rufo, A., Luzi, C., Simmaco, M., Barra, D., Zhao, H., Kinnunen, P. K. J., Bozzi, A., and Di Giulio, A. (2002) Temporin L: antimicrobial, cytotoxic activities and effects on membrane permeabilization in lipid vesicles. *Biochem. J.* **368,** 91-100.

Robertson, C. N., Roberson, K. M., Pinero, A., Jaynes, J. M., and Paulson, D. F. (1998) Peptidyl membrane-interactive molecules are cytotoxic to prostatic cancer cells in vitro. *World J. Urol.* **16**, 405-409.

Robinson, W. E. Jr., McDougall, B., Tran, D., and Selsted, M. E. (1998) Anti-HIV-1 activity of indolicidin, an antimicrobial peptide from neutrophils. *J. Leukoc. Biol.* **63**, 94-100.

Rozek, A., Friedrich, C. L., and Hancock, R. E. (2000) Structure of the bovine antimicrobial peptide indolicidin bound to dodecylphosphocholine and sodium dodecyl sulfate micelles. *Biochemistry* **39***,* 15765-15774.

Ruissen. A. L. A., Van der Reijden, W. A., Van ‘t Hof, W., Veerman, E. C. I., and Nieuw Amerongen, A. V. (1999) Anti-fungal activity of a cationic peptide, analogous to salivary histatin 5, mixed with and covalently linked to xanthan. *J. Controlled Rel.* **60**, 49-56.

Ryan, L. K., Rhodes, J., Bhat, M., and Diamond, G. (1998) Expression of beta-defensin gene in bovine alveolar macrophages. *Infec. Immun.* **66**, 878-881.

Sablon, E., Contreras, B., and Vandamme, E. (2000) Antimicrobial peptides of lactic acid bacteria: mode of action, genetics and biosynthesis. *Adv. Biochem. Eng. Biotechnol.* **68**, 21-60.

Sajjan, U. S., Tran, L. T., Sole, N., Rovaldi, C., Akiyama, A., Friden, P. M., Forstner, J. F., and Rothstein, D. M. (2001) P-113D, an antimicrobial peptide active against Pseudomonas aeruginosa, retains activity in the presence of sputum from cystic fibrosis patients. *Antimicrob. Agents Chemother.* **45**, 3437-3444.

Saiman, L., Tabibi, S., Starner, T. D., San Gabriel, P., Winokur, P. L., Jia, H. P., McCray, P. B. Jr., Tack, B. F. (2001) Cathelicidin peptides inhibit multiply antibiotic-resistant pathogens from patients with cystic fibrosis. *Antimicrob. Agents Chemother.* **45**, 2838-2844.

Samakovlis, C., Kylsten, P., Kimbrell, D. A., Engstrom, A., and Hultmark, D. (1991) The andropin gene and its product, a male-specific antibacterial peptide in *Drosophila melanogaste*r. *EMBO J.* **10**, 163-169.

Sansom, M. S. P. (1993) Alamethicin and related peptaibols-model ion channels. *Eur. Biophys. J.* **22**, 105-124.

Sansom, M. S. P. (1998) Peptides and lipid bilayers: Dynamic interactions. *Curr. Opin. Colloid Interface Sci.* **3**, 518-524.

Schneider, M. F., Marsh, D., John, W., Kloesgen, B., and Heimburg, T. (1999) Network formation of lipid membranes: triggering structural transitions by chain melting. *Proc. Natl. Acad. Sci. USA* **96**, 14312-14317.

Schutte, B. C. and McCray, P. B. Jr. (2002) β-defensins in lung host defense. *Ann. Rev. Physiol.* **64**, 709-748.

Scocchi, M., Wang, S., and Zanetti, M. (1997) Structural organization of the bovine cathelicidin gene family and identification of a novel member. *FEBS Lett.* **417**, 311-315.

Selsted, M. E., Novotny, M. J., Morris, W. L., Tang, Y. Q., Smith, W., and Cullen, J. S. (1992) Indolicidin, a novel bactericidal tridecapeptide amide from neutrophils. *J. Biol. Chem.* **267**, 4292-4295.

Shai, Y. (1999) Mechanism of the binding, insertion, and destabilization of phospholipid bilayer membranes by α-helical antimicrobial and cell non-selective membrane-lytic peptides. *Biochim. Biophys. Acta* **1462**, 55-70.

Shai, Y. and Oren, Z. (1996) Diastereoisomers of cytolysins, a novel class of potent antibacterial peptides. *J. Biol. Chem.* **271**, 7305-7308.

Shai, Y. and Oren, Z. (2001) From "carpet" mechanism to de-novo designed diastereomeric cell-selective antimicrobial peptides. *Peptides* **22**, 1629-1641.

Sharon, M., Oren, Z., Shai, Y., and Anglister, J. (1999) 2D-NMR and ATR-FTIR study of the structure of a cell-selective diastereomer of melittin and its orientation in phospholipids. *Biochemistry* **38**, 15305-15316.

Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., and Kimoto, M. (1999) MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J. Exp. Med.* **189**, 1777-1782.

Silvestro, L., Gupta, K., Weiser, J. N., and Axelsen, P. H. (1997) The concentration-dependent membrane activity of cecropin A. *Biochemistry* **36**, 11452-11460.

Simmaco, M., Mignogna, G., Canofeni, S., Miele, R., Mangoni, M. L., and Barra, D. (1996) Temporins, antimicrobial peptides from the European red *frog Rana temporaria*. *Eur. J. Biochem*. **242**, 788-792.

Simmaco, M., Mignogna, G. and Barra, D. (1998) Antimicrobial peptides from amphibian skin: what do they tell us? *Biopolymers* **47**, 435-450.

Skerlavaj, B., Gennaro, R., Bagella, L., Merluzzi, L., Risso, A., and Zanetti, M. (1996). Biological characterization of two novel cathelicidin-derived peptides and identification of

structural requirements for their antimicrobial and cell-lytic actvities. *J. Biol. Chem.* **271**, 28375-28381.

Steinberg, D. A., Hurst, M. A., Fukii, C. A., Kung, A. H. C., Ho, J. F., Cheng, F.-C., Loury, D. J., and Fiddes, J. C. (1997) Protegrin-1: a broad-spectrum, rapidly microbicidal peptide with in vivo activity. *Antimicrob. Agents Chemother.* **41**, 1738-1742.

Stumpe, S., Schmid, R., Stephens, D. L., Georgiou, G., and Bakker, E. P. (1998) Identification of OmpT as the protease that hydrolyzes the antimicrobial peptide protamine before it enters growing cells of Escherichia coli. *J. Bacteriol.* **180**, 4002-4006.

Su, S. B., Gong, W. H., Gao, J. L., Shen, W. P., Grimm, M. C., Deng, X., Murphy, P. M., Oppenheim, J. J., and Wang, J. M. (1999) T20/DP178, an ectodomain peptide of human immunodeficiency virus type-1 gp41, is an activator of human phagocyte N-formyl peptide receptor. *Blood* **93**, 3885-3892.

Subbalakshmi, C., Krishnakumari, V., Nagaraj, R., and Sitaram, N. (1996) Requirements for antibacterial and hemolytic activities in the bovine neutrophil derived 13-residue peptide indolicidin. *FEBS Lett.* **395**, 48-52.

Subbalakshmi, C. and Sitaram, N. (1998) Mechanism of antimicrobial action of indolicidin. *FEMS Microbiol. Lett.* **160**, 91-96.

Subbalakshmi, C., Nagaraj, R., and Sitaram, N. (1999) Biological activities of C-terminal 15-residue synthetic fragment of melittin: design of an analog with improved antibacterial activity. *FEBS Lett.* **448**, 62-66.

Tailor, R. H., Acland, D. P., Attenborough, S., Cammue, B. P., Evans, I. J., Osborn, R. W., Ray, J. A., Rees, S. B., and Broekaert, W. F. (1997) A novel family of small cysteine-rich antimicrobial peptides from seeds of *Impatiens balsamina* is derived from a single precursor protein. *J. Biol. Chem.* **272**, 24480 – 24487.

Takeuchi, O. and Akira, S. (2001) Toll-like receptors; their physiological role and signal transduction system. *Int. Immunopharmacol*. **1**, 625-635.

Tam, J. P., Lu, Y. A., Yang, J, L., and Chiu, K. W. (1999) An unusual structural motif of antimicrobial peptides containing end-to-end macrocycle and cystine-knot disulfides. *Proc. Natl. Acad. Sci. USA* **96**, 8913-8918.

Tamamura, H., Ishihara, T., Otaka, A., Murakami, T., Ibuka, T., Waki, M., Matsumoto, A., Yamamoto, N., and Fujii, N. (1996) Analysis of the interaction of an anti-HIV peptide, T22 ([Tyr5, 12, Lys7]-polyphemusin II), with gp120 and CD4 by surface plasmon resonance. *Biochim. Biophys. Acta* **1298**, 37-44.

Tamamura, H., Waki, M., Imai, M., Otaka, A., Ibuka, T., Waki, K., Miyamoto, K., Matsumoto, A., Murakami, T., Nakashima, H., Yamamoto, N., and Fujii, N. (1998) Downsizing of an HIV-cell fusion inhibitor, T22 ([Tyr5,12, Lys7]-polyphemusin II), with the maintenance of anti-HIV activity and solution structure. *Bioorganic. Med. Chem.* **6**, 473-479.

Tasiemski, A., Hammad, H., Vandenbulcke, F., Breton, C., Bilfinger, T. J., Pestel, J., and Salzet, M. (2002) Presence of chromogranin-derived antimicrobial peptides in plasma during coronary artery bypass surgery and evidence of an immune origin of these peptides. *Blood* **100**, 553-559.

Tauszig, S., Jouanguy, E., Hoffmann, J. A., and Imler, J.-L. (2000) Toll-related receptors and the control of antimicrobial peptide expression in Drosophila. *Proc. Natl. Acad. Sci. USA* **97**, 10520-10525.

Tenenholz, T. C., Klenk, K. C., Matteson, D. R., Blaustein, M. P., and Weber, D. J. (2000) Structural determinants of scorpion toxin affinity: the charybdotoxin (α-KTX) family of K+-channel blocking peptides. *Rev. Physiol. Biochem. Pharmacol.* **140**, 135-185.

Thomma, B. P. H. J, Eggermont, K., Penninckx, I. A. M. A., Mauch-Mani, B., Vogelsang, R., Cammue, B. P. A., and Broekaert, W. F. (1998) Separate jasmonate-dependent and salicylate-dependent defense response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. *Proc .Natl. Acad. Sci. USA* **95**, 15107-15111.

Thuren, T., Virtanen, J. A., and Kinnunen, P. K. J. (1986) Polyamine-phospholipid interaction probed by the accessibility of the phospholipid sn-2 ester bond to the action of phospholipase A2. *J. Membr. Biol.* **92**, 1-7.

Thuren, T., Virtanen, J. A., Somerharju, P. J., and Kinnunen, P. K. J. (1988) Phospholipase A2 assay using an intramolecularly quenched pyrene-labeled phospholipid analog as a substrate. *Anal. Biochem.* **170,** 248-255.

Tossi, A., Sandri, L., and Giangaspero, A. (2000) Amphipathic, alpha-helical antimicrobial peptides. *Biopolymers* **55**, 4-30.

Tsai, H. and Bobek, L. A. (1998) Human salivary histatins: promising anti-fungal therapeutic agents. *Crit. Rev. Oral Biol. Med.* **9**, 480-497.

Tytler, E. M., Anantharamaiah, G. M., Walker, D. E., Mishra, V. K., Palgunachari, M. N., and Segrest, J. P. (1995) Molecular basis for prokaryotic specificity of magainin-induced lysis. *Biochemistry* **34**, 4393-4401.

Uematsu, N. and Matsuzaki, K. (2000) Polar angle as a determinant of amphipathic α-helix-lipid interaction: a model peptide study. *Biophys. J.* **79**, 2075-2083.

Utsugi, T., Schroit, A. J., Connor, J., Bucana, C. D., and Fidler, I. J. (1991) Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes. *Cancer Res.* **51**, 3062-3066.

Vandenbergh, P. A., Pucci, M. J., Kunka, B. S., and Vedamuthu, E. R. (1989) Method for inhibiting Listeria monocytogenes using a bacteriocin. European Patent Application 89101125.6.

van't Hof, W., Veerman, E. C. I., Helmerhorst, E. J., and Amerongen, A. V. N. (2001) Antimicrobial peptides: properties and applicability. *Biol. Chem.* **382**, 597-619.

Vogel, H. J., Schibli, D. J., Jing, W., Lohmeier-Vogel, E. M., Epand, R. F., and Epand, R. M. (2002) Towards a structure-function analysis of bovine lactoferricin and related tryptophan- and arginine-containing peptides. *Biochem. Cell Biol*. **80**, 49-63.

Vogt, T. C. B. and Bechinger, B. (1999) The interactions of histidine-containing amphipathic helical peptide antibiotics with lipid bilayers. The effects of charges and pH. *J. Biol. Chem.* **274**, 29115-29121.

Vunnam, S., Juvvadi, P., and Merrifield, R. B. (1997) Synthesis and antibacterial action of cecropin and proline-arginine-rich peptides from pig intestine. *J. Pept. Res.* 49, 59-66.

Wachinger, M., Kleinschmidt, A., Winder, D., Von Pechmann, N., Ludvigsen, A., Neumann, M., Holle, R., Salmons, B., Erfle, V., and Brack-Werner, R. (1998) Antimicrobial peptides melittin and cecropin inhibit replication of human immunodeficiency virus 1 by suppressing viral gene expression. *J. Gen. Virol.* **79**, 731-740.

Wei, X., Decker, J. M., Liu, H., Zhang, Z., Arani, R. B., Kilby, J. M., Saag, M. S., Wu, X., Shaw, G. M., and Kappes, J. C. (2002) Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. *Antimicrob. Agents Chemother.* **46**, 1896-1905.

Welling, M. M., Hiemstra, P. S., Van den Barselaar, M. T., Paulusma-Annema, A., Nibbering, P. H., Pauwels, E. K., and Calame, W. (1998) Antibacterial activity of human neutrophil defensins in experimental infections in mice is accompanied by increased leukocyte accumulation. *J. Clin. Invest.* **102**, 1583-1590.

Wenk, M. R. and Seelig, J. (1998) Magainin 2 Amide Interaction with Lipid Membranes: Calorimetric Detection of Peptide Binding and Pore Formation. *Biochemistry* **37**, 3909-3916.

Wiedmer, S. K., Hautala, J., Holopainen, J. M., Kinnunen, P. K. J., and Riekkola, M.-L. (2001) Study on liposomes by capillary electrophoresis. *Electrophoresis* **22,** 1305-1313.

Wieprecht, T., Dathe, M., Krause, E., Beyermann, M., Maloy, W. L., MacDonald, D. L., and Bienert, M. (1997a) Modulation of membrane activity of amphipathic, antibacterial peptides by slight modifications of the hydrophobic moment. *FEBS Lett.* **417**, 135-140.

Wieprecht, T., Dathe, M., Epand, R. M., Beyermann, M., Krause, E., Maloy, W. L., MacDonald, D. L., and Bienert, M. (1997b) Influence of the angle subtended by the positively charged helix face on the membrane activity of amphipathic, antibacterial peptides. *Biochemistry* **36**, 12869-12880.

Wieprecht, T., Beyermann, M. and Seelig, J. (1999) Binding of antibacterial magainin peptides to electrically neutral membranes: thermodynamics and structure. *Biochemistry* **38**, 10377-10387.

Williams, M. J., Rodriguez, A., Kimbrell, D. A., and Eldon, E. D. (1997) The 18-wheeler mutation reveals complex antibacterial gene regulation in Drosophila host defense. *EMBO J.* **16**, 6120-6130.

Wong, H., Bowie, J. H., and Carver, J. A. (1997) The solution structure and activity of caerin 1.1, an antimicrobial peptide from the Australian green tree frog, *Litoria splendida*. *Eur. J. Biochem.* **247**, 545-557.

Wu, M., Maier, E., Benz, R., and Hancock, R. E. W. (1999) Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of *Escherichia coli. Biochemistry* **38**, 7235-7242.

Wösten, M. M. and Groisman, E. A. (1999) Molecular characterization of the PmrA regulon. *J. Biol. Chem.* **274**, 27185-27190.

Yang, D., Biragyn, A., Kwak, L. W., and Oppenheim, J. J. (2002) Mammalian defensins in immunity: more than just microbicidal. *Trends Immunol*. **23**, 291-296.

Yang, L., Weiss, T. M., Lehrer, R. I., and Huang, H. W. (2000) Crystallization of antimicrobial pores in membranes: magainin and protegrin. *Biophys. J.* **79**, 2002-2009.

Yang, L,. Harroun, T. A., Weiss, T. M., Ding, L., and Huang, H. W. (2001) Barrel-stave model or toroidal model? A case study on melittin pores. *Biophys. J.* **81**, 1475-1485.

Yaniv, M., Dabbi, D., Amir, H., Cohen, S., Mozes, M., Tsuberi, H., Frietkin, M., Dekel, S., and Ofek, I. (1999) Prolonged leaching time of peptide antibiotics from acrylic bone cement. *Clin. Orthop.* **363**, 232-239.

Yau, W.-M., Wimley, W. C., Gawrich, K., and White, S. H. (1998) The preference of tryptophan for membrane interfaces. *Biochemistry* **37**, 14713-14718.

Yomogida, S., Nagaoka, I., and Yamashita, T. (1997) Comparative studies on the extracellular release and biological activity of guinea pig neutrophil cationic antibacterial polypeptide of 11 kDa (CAP11) and defensins. *Comp. Biochem. Physiol.* **116B**, 99-107.

Yoo, Y. C., Watanabe, S., Watanabe, R., Hata, K., Shimazaki, K., and Azuma, I. (1998) Bovine lactoferrin and Lactoferricin inhibit tumor metastasis in mice. *Adv. Exp. Med. Biol.* **443**, 285-291.

Yoshida, M., Kimura, T., Kitaichi, K., Suzuki, R., Baba, K., Matsushima, M., Tatsumi, Y., Shibata, E., Takagi, K., Hasegawa, T., and Takagi, K. (2001) Induction of histamine release from rat peritoneal mast cells by histatins. *Biol. Pharm. Bull.* **24**, 1267-1270.

Zaltash, S., Palmblad, M., Curstedt, T., Johansson, J., and Persson, B. (2000) Pulmonary surfactant protein B: a structural model and a functional analogue. *Biochim. Biophys. Acta* **1466**, 179-186.

Zanetti, M., Gennaro, R., Scocchi, M., and Skerlavaj, B. (2000) Structure and biology of cathelicins. *Adv. Exp. Med. Biol.* **479***,* 203-218.

Zasloff, M. (1987) Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc. Natl. Acad. Sci. USA.* **84,** 5449-5453.

Zasloff, M. (2000) Reconstructing one of nature's designs. *Trends Pharmacol. Sci.* **21**, 236-238.

Zasloff, M. (2002) Antimicrobial peptides of muticellular organisms. *Nature* **415**, 389-395.

Zhang, L., Benz, R., and Hancock, R. E. W. (1999) Influence of proline residues on the antibacterial and synergistic activities of alpha-helical peptides. *Biochemistry* **38**, 8102-8111.

Zhang, X. L., Selsted, M. E., and Pardi, A. (1992) NMR studies of defensin antimicrobial peptides. 1. Resonance assignment and secondary structure determination of rabbit NP-2 and human HNP-1 *Biochemistry* **31**, 11348-11356.

Zhao, C., Ganz, T., and Lehrer, R. I. (1995) Structures of genes for two cathelin-associated antimicrobial peptides: prophenin-2 and PR-39. *FEBS Lett.* **376**, 130-134.

Zhao, H., Rinaldi, A. C., Rufo, A., Bozzi, A., Kinnunen, P. K. J. and Di Giulio, A. (2002) Structural and charge requirements for antimicrobial peptide insertion into biological and model membranes. In Pore-Forming Peptides and Protein Toxins (Menestrina, G., Dalla Serra, M. and Lazarovici, P., eds.), pp. 151-177, Harwood Academic Publishers, Reading, UK.