J. Membrane Biol. 156, 197–211 (1997)

The Journal of
Membrane
Biology

Topical Review

Structure and Functions of Channel-Forming Peptides: Magainins, Cecropins, Melittin and Alamethicin

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Received: 25 November 1996/Revised: 6 December 1996

Introduction

Membrane-active peptides exhibit many interesting biological and pharmacological activities. These peptides can be synthesized and purified by chemical or biochemical means in sufficient quantities to allow for their study by biophysical techniques. Whereas the characteristics and biological activities of some of these peptides are highly interesting in themselves, they also provide model systems for large membrane proteins. The study of these peptides therefore increases our understanding of many biological processes, such as nerve conduction, mass and information transport, energy conversion or cellular signaling.

Many organisms, including fungi, insects, amphibians and humans, produce hydrophobic and amphipathic peptides which exhibit antibiotic, fungicidal, hemolytic, virucidal and tumoricidal activities by interaction with the membranes of living cells. The systems of particular interest for pharmacological applications are those which manifest immunological or tumoricidal activity, but under the same conditions, do not show hemolytic or cytotoxic activity against healthy vertebrate cells [123]. Membrane-active peptides also exhibit channel-like conductivities across planar lipid bilayer systems as well as bilayer disruption. These bilayer openings deprive the affected organisms of their transmembrane electrochemical gradients, result in increased water flow concomitant with cell swelling, osmolysis and cell death. A detailed understanding of the mechanisms of channel-formation will, therefore, help in the design of improved antibiotics.

Whereas in electrophysiological experiments a single pore can be observed, other techniques, in particular those that deal with intact cells or large unilamellar vesicle systems, are orders of magnitude less sensitive. It is not always clear which of the properties measured by physico-chemical methods are responsible for the peptides' activities *in vivo* and how different characteristics are related to each other. As a consequence the correlations between *in vitro* and *in vivo* activity often remain a matter of speculation.

This article presents a review of the structures and activities of magainins and cecropins which are shortly compared to the characteristics of melittin and alamethicin. The appropriate sections make reference to some of the earlier reviews on selected topics concerning these peptides which are recommended to the interested reader for further details.

Magainins

Magainin antibiotics are a family of immunogenic peptides which are expressed in the skin and intestines of

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Key words: α-helical bundle — Protein-lipid interactions — Pore formation — Peptide antibiotics — Bilayer disruption — Hemolysis

Abbreviations: BLM: black lipid membranes; CD: circular dichroism; DMPC: 1-2-dimyristoyl-*sn*-glycero-3-phosphocholine; DLPG: 1-2-dilaureoyl *sn*-glycero-3-phosphoglycerol; DMPG: 1-2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DOPG: 1-2-dioleoyl-*sn*-glycero-3-phosphoglycerol; ESR: electron spin resonance; FTIR: Fourier transform infra red spectroscopy; HFIP: hexafluoroisopropanol; MW: molecular weight; NMR: nuclear magnetic resonance; PC: 1-2-diacyl-*sn*-glycerol-3-phosphocholine; PE: 1,2-diacyl-*sn*-glycerol-3-phosphoethanolamine; PS: 1,2-diacyl-*sn*-glycerol-3-phosphoserine

Table 1. Amino acid sequences of channel-forming peptides

GIGAV LKVLT TGLPA LISWI KRKRQ Q-$CONH_2$	Melittin
GIGKF LHSAK KFGKA FVGEI MNS-$CONH_2$	Magainin 2
KWKLF KKIEK VGQNI RDGII KAGPA VAVVG QATQI AK-$CONH_2$	Cecropin A
AC-UPUAU AQUVU GLUPV UUEQF-OH	Alamethicin

U: α-methylalanine; O: hydroxyproline; $CONH_2$: C-terminal carboxyamine; OH: C-terminal amino alcohol; AC-: N-terminal acetyl

frogs [31, 92, 111, 186, 254]. These peptides exhibit bacteriocidal, fungicidal and virucidal activities and thereby provide an immediate response to infections [1, 30, 38, 102, 167]. More recently, magainins were also shown to selectively lyse tumor cells without killing healthy vertebrate cells [57, 173, 210]. The peptides, however, do not selectively kill all transformed cell lines, therefore, their tumor specific activity remains to be studied in further detail [100]. Interestingly, some of the naturally occurring combinations of members of this peptide family, such as PGLa and magainin 2, are more potent than the equivalent amounts of either of these peptides alone, indicating a synergistic enhancement of activity [242].

All-D-magainins as well cause channel formation and cell lysis of the same organisms sensitive to the L-enantiomer [29, 237]. These results, the lack of primary sequence homology within the magainin family as well as a strong correlation between antibiotic activity and peptide amphiphilicity [178] indicate that the structural and physico-chemical properties of magainins, rather than specific receptor-ligand interactions are responsible for their biological activity.

Structures in Membranes

Magainin polypeptides consist of 21 to 26 amino acid residues and are strongly basic in character (Table). Although they dissolve well in aqueous solution where they assume a random coil conformation [146], they also show a strong affinity for phospholipid membranes.

CD [140, 150], Raman [244], and FTIR [122], as well as multidimensional solution (B. Bechinger, J. Gesell, M. Zasloff and S. Opella, *unpublished*) and solid-state NMR spectroscopy [22] all indicate that magainins assume right-handed α-helical conformations in the presence of phospholipid bilayers or organic solvents [146]. As optical experiments are performed with dilute samples, the presence of negatively charged lipids is required to enhance the membrane affinity of the basic peptides by electrostatic attraction [150, 151, 256]. The apparent degree of helicity in these samples is usually low as conformational averaging of peptide dissolved in the water phase (random coil) and peptide associated with the membrane (highly α-helical) occurs. Additional averaging over all residues is present and thus a structural analysis of the polypeptide at high resolution is difficult, if not impossible. In contrast, the oriented samples used in solid-state NMR experiments are characterized by a reduced bulk water content and the experimental setup allows a focus on the immobilized polypeptide sample by using a cross-polarization pulse sequence. ^{15}N solid-state NMR spectroscopy of magainins which have been incorporated into oriented phospholipid membranes indicate that the magainin helix extends at least from residue 2 to 20 in phospholipid model membranes and that the helix axis of the peptide is oriented parallel to the bilayer surface when the concentrations of magainin 2 are 0.8–3 mole % (Figs. 1*A* and 2*A*) [21, 22, 24]. Similar conclusions are obtained from intra- and intermolecular distance measurements by means of magic angle spinning solid-state NMR spectroscopy [110]. CD spectroscopy on double-D-amino acid derivatives of magainin indicate that the N-terminal part of this extended helix is less stable than the structure formed between residues 9 and 21 [243]. Fluorescence quenching experiments of magainin derivatives were performed, in which the three phenylalanines of magainin 2 are independently replaced by tryptophanes [154]. These experiments indicate that all three fluorescent probes are localized approximately 10 Å equidistant from the bilayer center in agreement with an in-plane orientation found by solid-state NMR spectroscopy. This orientation parallel to the membrane surface allows the lysine side chains to remain in the aqueous phase or to interact with the lipid head groups (Fig. 1*A*). The solid-state NMR results were taken as an input to optimize computer programs which use molecular dynamics calculations to determine the incorporation, structure and topology of bilayer-associated peptides and at the same time visualize the fluctuations of peptide structure and orientation [164].

Helical wheel projection shows that such a helix is amphipathic with the polar and hydrophobic amino acid side chains neatly separated on opposite faces of the helix (Fig. 1*A*). Molecular modeling shows that the helical diameter of 10–12 Å is insufficient to completely fill the whole depth of a lipid monolayer, even when a possible 'snorkeling' mechanism of the extended lysine side chains is taken into consideration. Due to this configuration the magainin molecule creates a distortion of the bilayer which extends over a diameter of up to 100 Å and causes a decreased average bilayer thickness [141]. ^{31}P solid-state NMR spectroscopy shows that magainins possess a propensity to induce nonbilayer structures in phosphatidylcholine membranes at concentrations where these peptides exhibit biological activity and where fluorescent dyes are released out of phospholipid vesicles ([128, 151], B. Bechinger, *unpublished*). This observation agrees well with the classification of [204] where

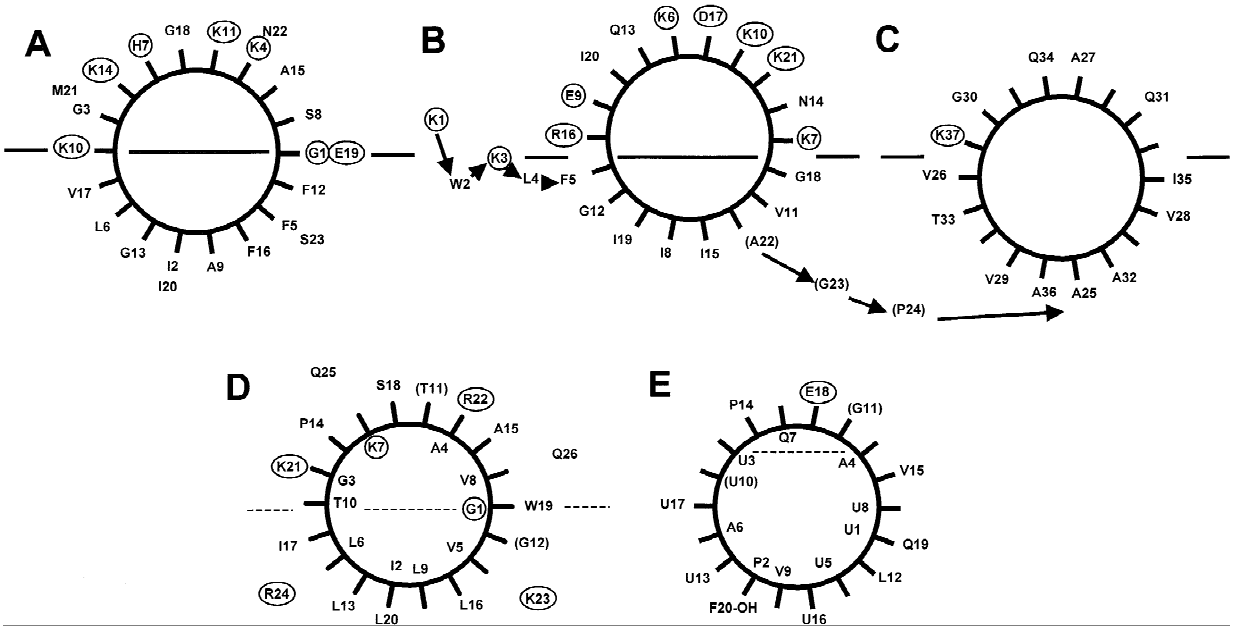

Fig. 1. Helical wheel diagrams. The potentially charge-carrying amino acids are circled. (*A*) Magainin 2 according to [22]. (*B*) and (*C*) CecropinA, residues 5 to 21 and residues 25 to 37, respectively, according to [112]. The hydrophobic faces in *A–C* are shown below and the hydrophilic faces above the line. (*D*) Melittin, according to [64, 119, 220]. The brackets indicate residues where the helix is distorted. The N-terminal residues are shown inside the wheel, the C-terminal ones along its outer diameter. The majority of hydrophobic residues of melittin are located below the interrupted line. (*E*) Alamethicin according to [84]. The narrow polar face is above the interrupted line.

magainin belongs to the class L(ytic) amphipathic peptides.

At magainin concentrations > 3.3 mole % (*ca.* 10 wt %) oriented CD spectroscopy [140] indicates contributions of peptide molecules aligned parallel to the normal of DMPC/DMPG (3:1) membranes. At similar concentrations large water-filled cavities are observed in the bilayer [142]. In addition at peptide-to-lipid molar ratios of 1:10 a mixture of α-helical and β-sheet conformations are detected by means of FTIR and rotational echo solid-state NMR spectroscopy in the presence of gel state, frozen or lyophilized DPPG and DPPC/DPPG 1:1 membranes [110]. More detailed NMR-structural studies will become possible due to the bacterial expression of selectively and uniformly labelled antibiotic peptides [257].

Increase in Bilayer Conductivity (Pore Formation)

The electrophysiological investigation of model membranes indicates that the conductivity for ions increases due to the presence of magainins. Some authors describe a stepwise increase in conductivity during electrophysiological experiments which was taken to suggest that magainin forms well defined porelike structures. These events are rare and short lived, however [58, 68] (L. Bühler and M. Montal, *personal communication*). In addition, a large range of conductivities of the pores, starting at 1.8 pS, has been detected. These vary within each electrophysiological recording as well as from experiment to experiment. The putative 'channels' were described early on to be anion-selective (ratio P_K/P_{Cl} = 1:3) [56, 68], however more recent investigations indicate a selectivity ratio of cations over anions of 5:1 in mixed PE/PS 1:1 membranes [58]. Selectivity within the respective class of monovalent ions was not detected in any of the published electrophysiological experiments. More typical for magainin-induced electrophysiological events are 'erratic currents' (L. Bühler, M. Zasloff and M. Montal, *personal communication*) or 'occasional brief multilevel fluctuations' (at 0.1 mg/ml), which at higher peptide concentrations resemble 'melittin-like bilayer disruptions' [100]. The large variety of different electrophysiological recordings obtained in the presence of magainins suggests that a multitude of different ion-conducting structures are formed by these peptides and that the characteristics of these bilayer openings are dependent on details of the experimental setup and the sample preparation.

The presence of 0.6 to 3 mole % magainin was shown to result in the half-maximal decoupling of the respiratory free-energy transduction of bacterial or spermatozoal cells, isolated mitochondria, or reconstituted cytochrome oxydase liposomes [60, 128, 240, 241]. This effect on the cellular energy metabolism suggests that these peptides exhibit their cytotoxic activity by disrupting the electrochemical gradient across free-energy transducing membranes.

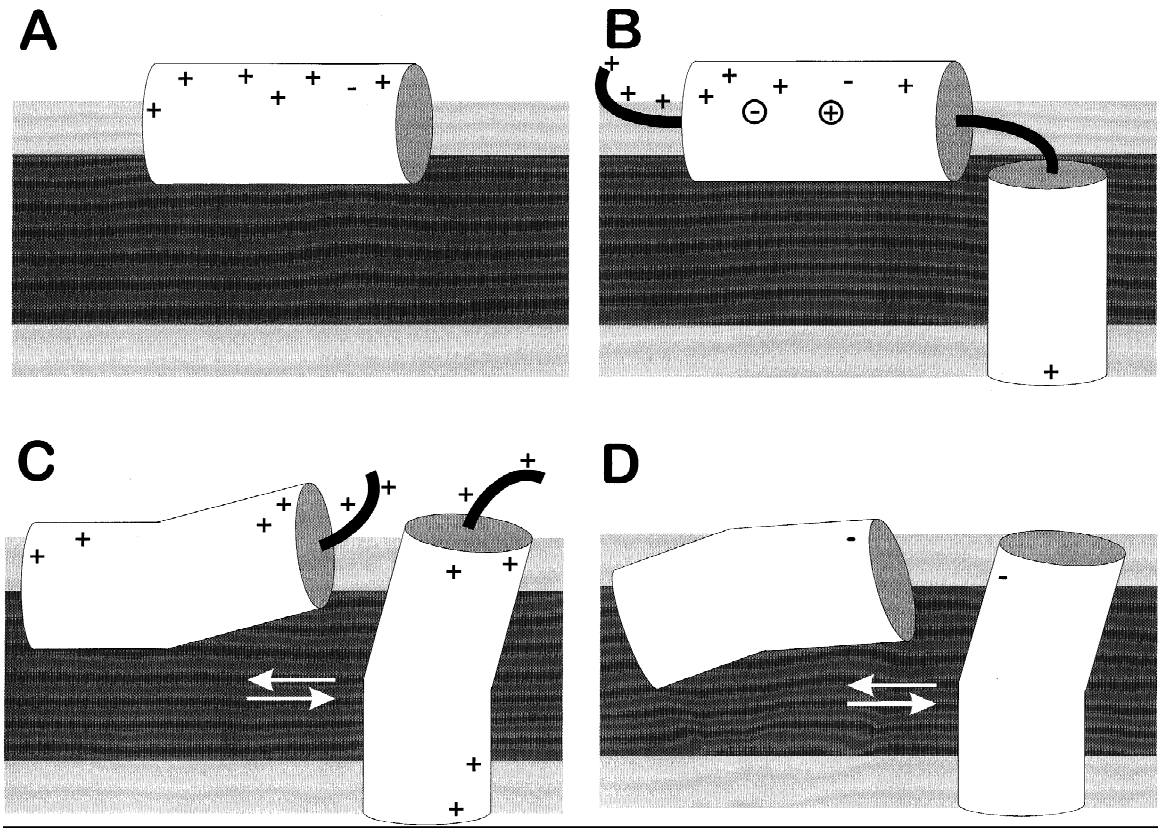

Fig. 2. Models for the topology of monomeric α-helical polypeptides. (*A*) Magainins [22], (*B*) Cecropins. No experimental data are published for the cecropin helices. Although a transmembrane configuration has been modeled for the C-terminal helix [72], the amphipathic distribution of polar and hydrophobic residues suggests the possibility of an in-plane orientation of this helix domain (Fig. 1*C*). The Glu-9 and Arg-16 are circled. (*C*) Melittin. (*D*) Alamethicin.

Although transmembrane electrical potentials increase the electrophysiological activity of magainin peptides [58, 68] this is not a requirement for the expression of their membrane permeabilizing properties. In the absence of electric fields, a chemical gradient and the presence of magainin are sufficient conditions to allow for the leakage of the large fluorescence dyes calcein (MW 623) or 6-carboxyfluorescein [97, 150]. A biphasic dye release kinetics was taken to suggest that the initial asymmetric association only at the outer surface of the vesicle causes the membrane instability and hence bilayer openings [152]. Deactivation occurs as the peptide slowly equilibrates into the inner leaflet.

Many functional studies of magainin peptides are characterized by a sigmoidal dependence of activity on concentration indicating that the magainin molecules act in a cooperative manner [58, 150, 153, 231]. Analysis of the steepness of this function allows one to extract the cooperativity parameter. The basic unit itself may be a monomer, dimer or any higher order aggregate and the size of the functional oligomer is of concomitantly increased size. Whereas a cooperativity parameters between 1.7 and 6 have been described from electrophysiological experiments [58, 68], the dissipation of the proton electrochemical gradient across liposomal membranes exhibits cooperativity of order between 1 and 5 [128, 231]. This cooperativity analysis, however, does not provide information about the detailed structure of the channel, the involvement of other molecules nor the composition of the oligomeric complex.

Cecropins

Immediate defense mechanisms are also established in insects by cecropins, and in humans by defensins [38, 117, 144]. Cecropins are induced upon infection and act in a manner that is probably related to the mechanisms of magainin activity. Cecropins A, B and D are close homologues which consist of 35–39 residues and have been found in the pupae of the cecropia moth (Table) [41, 98, 116]. Cecropinlike proteins, named lepodopteran [221], bactericidin [71], moricin [106] or sarcotoxin [169] have been identified in other insects. Cecropin-melittin hybrid peptides exhibit an up to 100-fold increased antibi-

otic activity relative to native cecropins but retain the lack in hemolytic activity [7, 163, 180, 238]. As a consequence these mixed sequences have been studied almost as extensively as cecropins themselves (e.g., [8, 39, 80]). A mammalian analogue, the 31 amino acid cecropin P was isolated from pig intestines and was shown to inhibit bacterial growth, probably by bacteriolysis [2, 40, 50, 138].

Structures in Organic Solvents

Whereas cecropin A assumes a predominantly random coil conformation in water [6] it adopts a highly ordered structure in 15% (v/v) hexafluoroisopropanol [112]. The conformation of cecropin A is characterized by two amphipathic helical regions extending from residues 5 to 21 and 24 to 37 that are connected by a flexible hinge region (Figs. 2*B* and *C*). The length and the continuous distribution of basic residues along one face of the amphipathic N-terminal helix closely resembles the amino acid distribution of magainins (Figs. 1*A* and *B*). In contrast, the central part of the C-terminal helix (25–33) is much more hydrophobic (Fig. 1*C*). A mixed peptide consisting of the first 13 N-terminal amino acids of cecropin followed by the 13 N-terminal residues of melittin also assumes a helix-hinge-helix conformation in 30% (v/v) HFIP [208]. $^{1}H-^{2}H$ exchange data indicate that the first helix is less stable than the C-terminal one.

Bilayer Interactions

Spectrofluorometric studies show that fluorophore labeled cecropin B or P bind to lipid membranes in a noncooperative manner suggesting that they associate with membranes in a monomeric form [67, 90]. The same studies also indicate a localization of the N-terminus along the bilayer surface. More than 100 peptides per vesicle are required to induce initial ion leakage which seems to indicate that these highly charged peptides disrupt the lipid bilayer packing. This contrasts the computer-modeled oligomeric, barrel stave channel structures in which either the C- or the N-terminal helices form a pore with antibiotic activity [72].

ESR spectroscopy indicates that the binding of cecropin AD spin-labeled at the Cys-33 residue is enhanced by electrostatic interactions [158]. Small membrane-associated aggregates, probably dimers, form in DOPG but not in DLPG vesicles. This dependence of oligomerization on the lipid hydrophobic length was taken to suggest that the length of the apolar C-terminal helix determines the penetration depth of the polypeptide into the bilayer (Figs. 1*B* and 2*B*). When the hydrophobic thickness of the bilayer exceeds the hydrophobic span of the peptide, the Glu-9 and Arg-16 residues become positioned in the membrane interior. To avoid unfavorable exposure of these charged amino acid residues, salt bridges supposedly form in an antiparallel peptide dimer.

Cecropins interact with lipid membranes to form channels largely varying in size (7 pS to 2.5 nS). These peptides exhibit low cation selectivity in PC/PS planar lipid membranes [53]. Synthetic cecropin-melittin hybrids, some as short as 15 amino acids, also show channel activity in black lipid membranes when the bilayers have been formed from squalene but not when they have been applied from decane solutions [161]. To explain the antibiotic activity of these short peptides [238], channels consisting of tail-to-tail dimers supposedly associate to form barrel structures in the solvent-free membranes of biological organisms.

Natural or synthetic insect cecropins show strong antibiotic activity against a variety of Gram-negative and Gram-positive bacteria without lysing mammalian cell lines or yeast [41, 117, 162]. For most sensitive organisms all-D-cecropins, cecropins with inverted sequences (retro) or inversed D-cecropins (retro-enantio), all possess the high antibiotic activity of the parent L-enantiomer [29, 161, 163, 237]. Whereas these findings suggest that the cell killing activity of cecropins is not mediated through specific, chiral receptor interactions, the cell lytic activity of these peptides and its single-site 'mutants' correlates with their ability to form α-helical secondary structures in membrane environments as well as with their binding affinity to liposomes [81].

The first eleven N-terminal residues have been shown to be particularly important for the high antibiotic activity of cecropins, although the short peptide consisting of just these residues is inactive [6, 83, 216]. Among several bacterial organisms tested, only the susceptibility of *E. coli* to cecropins seems unaffected by mutations in this N-terminal region [6, 139]. Insertion of a proline into the N-terminal helix or the exchange of Trp-2 with a charged amino acid results in a marked decrease in binding and bacteriocidal activity. The presence of a phenylalanine at position 2, however, restores the antibiotic efficiency [216] suggesting two possible explanations: either an acidic amino acid at this position interferes with binding due to electrostatic repulsion, or an interfacial positioning of an aromate at this site is important for the biological activity of the molecule [124]. Furthermore the flexibility of the linker region between the two helical regions has been shown to be important for antibiotic activity as well as for the voltage-sensitivity of these channels [53, 83].

At LD50 concentrations the amount of radioactively-labeled cecropins bound to cells has been estimated to be sufficient to cover the bacterial membrane surface with a polypeptide monolayer [90, 216]. Interestingly, other bacteria, although resistant to the antibiotic activity of cecropins, are loaded with cecropins to a

similar extent whereas nonpermissive erythrocytes are not. Therefore, factors other than the binding affinity must also play an important role in determining the susceptibility to this toxin [215, 216]. It was shown e.g., that the incorporation of cholesterol into unilamellar vesicles of *E. coli* phospholipid decreases the cecropin-related conductivity across phospholipid membranes [53].

The destruction of the integrity of lipid bilayers has been suggested to be the main reason for the cytotoxic effect of these polypeptides [89]. The hybrid cecropinA-melittin also permeabilizes mitochondrial inner membranes for charged or noncharged solutes even in the absence of transmembrane potentials [67]. Cecropins and other related peptides have been shown to release respiratory control, to inhibit protein import, and at higher concentrations also to inhibit respiration. When comparing the concentration-dependent effects of cecropins and its biosynthetic precursors, however, no simple correlation can be established between antibacterial activity and the uncoupling of the respiratory phosphorylation in mitochondria [115]. Preliminary experiments indicate that cecropins also exhibit anticancer activity [166].

To access the plasma membrane of Gram-negative bacteria polycationic peptides, including magainins [185] and cecropin-melittin hybrids [148, 180], have to cross the outer membrane of Gram-negative bacteria. A self-promoting uptake pathway [103, 230] has been suggested to allow passage of polypeptides as big as lysozyme (14 kD).

Melittin

It is instructive to compare the structure and function of the cecropin-magainin class amphipathic helices with those of other well-characterized channel peptides. Melittin, for example, is the main polypeptide component of the venom of the European honey bee *Apis mellifera* (reviewed e.g., in [63, 193]). This peptide is characterized by six positive charges, four of which accumulate in the sequence Lys^{21}-Arg^{22}-Lys^{23}-Arg^{24}, i.e., close to the C-terminus (Fig. 1*D*).

X-ray analysis [220] as well as NMR-spectroscopy in methanol [19, 62], micellar solutions [48, 118], or in the presence of multilamellar vesicles [175] all indicate that the peptide assumes an extended α-helical conformation which is interrupted at positions 10 to 12. The resulting two helix axes therefore arrange in a bent conformation where the hydrophobic residues sequester at the inside of the concave surface. In solution the angle between the two helical domains has been found to be less well defined and larger than in the crystal structure (> 160°). This conformation allows the optimal face-to-face packing of the hydrophobic side chains in the crystal tetramer while simultaneously the polar residues T10, T11, S18 and the carbonyl oxygen of residue 10 are exposed to water (Fig. 1*D*). Due to the charge distribution, however, a major contribution to the hydrophobic moment of melittin runs parallel to its long axis. This contrasts the magainin helix whose hydrophobic moment is oriented approximately perpendicular to its long axis.

Melittin partitions into phosphatidylcholine membranes ($K_p \approx 10^4$ M^{-1}) [27, 28, 131, 199], where it assumes a predominantly α-helical conformation up to residue 21 [64, 134, 234, 239]. The combination of a wide variety of spectroscopic techniques indicates that the peptide helix orientation with respect to the bilayer normal follows a dynamic equilibrium and is dependent on the physical state of the membrane (Fig. 2*C*) [5, 44, 45, 86, 155, 209].

Whereas in solution the interplay between electrostatic repulsion of the positive charges and the hydrophobic interactions determine the peptide conformation as well as the monomer-tetramer equilibrium of melittin (e.g., [47, 96, 183, 235]), aggregation has not been observed in hydrophobic solvents [192]. The lipid bilayer configuration therefore is not likely to be the same. This conclusion is confirmed by fluorescence energy transfer experiments which indicate that melittin remains monomeric in liquid crystalline bilayers at peptide-to-lipid ratios < 1:200 [126].

Membrane Activity

Whereas the bilayer morphology of liquid crystalline phosphatidylcholine is retained unaltered in the presence of up to 5 mole % melittin, the presence of intermediate amounts of melittin results in reversible micellization concurrent with magnetic orientation of the structures at temperatures below the liquid crystalline-to-gel state phase transition of the pure lipid [65, 66, 69]. Freeze fracture electron microscopy, light scattering and gel filtration indicate that disk-shaped micelles with an approximate diameter of 235 Å are formed [70, 79]. At higher peptide concentrations both gel and liquid crystalline bilayers are transformed into small objects [165, 172, 181]. In contrast, the peptide exhibits bilayer-stabilizing effects when mixed with phosphatidylethanolamine membranes under conditions where the pure lipid arranges in a hexagonal phase (H_{II}) [18].

The molecular shape concept provides a common explanation for the polymorphism observed in lipid membranes [59]. In aqueous environments, the molecules composing biological membranes form tight structures of reduced permeability due to strong hydrophobic, van der Waals and electrostatic intermolecular interactions. As a result the geometrical space of the molecule is an important factor that determines the macroscopic structure of the resulting aggregate. To form

lipid bilayers the average geometrical shape of the individual molecules has to be cylindrical. In contrast, molecular geometries that resemble inverted cones result in micellar phases. Melittin, for example, combines a highly cationic C-terminal surface anchor with a short hydrophobic N-terminal helix of 11 residues [239]. Melittin is therefore expected to partition into the lipid bilayer interfacial region without filling the fatty acyl chain region equally well. It has been suggested that such a configuration increases the bilayer curvature thereby exerting the experimentally observed bilayer disruption of zwitterionic membranes [220]. In the presence of negatively charged phospholipids the phase preferences and the fluorescence emission spectra of the melittin W19 suggest a localization of the peptide more deeply in the bilayer, i.e., closer to the fatty acyl chains [14–16, 133].

Increase in Ion Permeability

In the presence of *trans*-negative membrane potentials conductance changes are observed [226] which, under specific conditions (e.g., 5 M NaCl), exhibit discrete multilevel conductances ranging over three orders of magnitude [104, 223]. More common, however, are erratic, less well defined currents. The permeability increase that is caused by melittin is characterized by a fourth power dependence on melittin concentration and an apparent gating charge of 0.95 [78, 213, 225, 227]. The pores show selectivity of anions over cations, probably due to the accumulation of positive charges on the peptide C-terminus [179, 226]. The increase in ion permeability in the presence of melittin also provides a plausible explanation for the colloid osmotic mechanisms of melittin hemolytic activity [61, 108, 218, 222].

Alamethicin

Alamethicin, is a 20 amino acid peptide that has been isolated from the fungus *Trichoderma viride* (reviewed e.g., in [51, 193, 194, 247]). In contrast to melittin or magainin the peptide is rich in hydrophobic amino acids, in particular α-methylalanine (Fig. 1*E*, Table). The presence of proline-14 results in a backbone conformation of alamethicin that resembles the flexible helix-bend-helix arrangement of melittin [42, 77, 84, 193, 251, 260]. The effect of aqueous paramagnetic reagents on the ^{1}H resonances indicates that alamethicin is buried in the micelle interior [85]. The nonbonded U10 and G11 residues as well as peptide-associated water molecules enhance the inherently polar character of the peptide backbone [130] and together with the side chains of Q7, E/Q18, Q19 and the N-terminus create a hydrophilic convex surface on the helix (Fig. 2*E*). In contrast the concave face remains hydrophobic [194].

Interactions with Lipid Membranes

Monomeric alamethicin strongly binds to lipid bilayers (partition coefficients of about 10^{-3} M) [189, 214] exhibiting a cooperativity parameter of 5.5 [200]. In lipid bilayers the degree of helicity is dependent on the physical state of the lipid [234], the lipid-peptide ratio [52] or the presence of transmembrane potentials [49]. The localization and conformation of bilayer-associated alamethicin have been determined by CD, Raman and ^{15}N solid-state NMR spectroscopies. The orientational distribution is a function of peptide concentration and the bilayer hydration level [114, 170, 234]. ESR spectroscopy indicates that in the absence of potentials alamethicin is monomeric [12] and that the N-terminus remains 16 Å distant from the headgroup phosphates of the opposing bilayer leaflet in the 'transmembrane' configuration [13].

Electrophysiology

Alamethicin exhibits a voltage-dependent conductance increase when added to phospholipid bilayers (reviewed by [36, 135, 247]). The macroscopic I–V relationship is highly asymmetric, i.e., a positive voltage has to be applied at the *cis*-side to which the peptide has been added [232, 233]. The concentration dependent *I–V* functions allow one to extract an alamethicin gating charge of 0.59 and a cooperativity factor of 2–11, the latter being dependent on the bilayer hydrophobic thickness [74, 101].

Single-channel conductance measurements indicate that the alamethicin channels are characterized by multilevel bursts interrupted by prolonged periods of silence [34, 94, 95, 194]. Once an initial small channel has formed the conductance increases fast and in a stepwise manner. Analysis of the alamethicin multiple channel kinetics indicates respective activation energies of 120 and 50 kJ/mole [37, 176].

Whereas reasonable agreement exists that the alamethicin channels are formed of helical bundles (Fig. 3, *cf.* discussion), various models have been suggested for the molecular mechanism of alamethicin voltage-gating [46, 51, 135, 149, 160, 193, 247]. All of the proposed models suggest an interaction of the alamethicin helix dipole with the transmembrane electric field. The dipole moment of alamethicin was determined to be 60–79 D[1], corresponding to a net +½ charge at the N- and a −½ charge at the C-terminus of the helix [201, 250].

[1] 1 eÅ = 4.8 D (Debye)

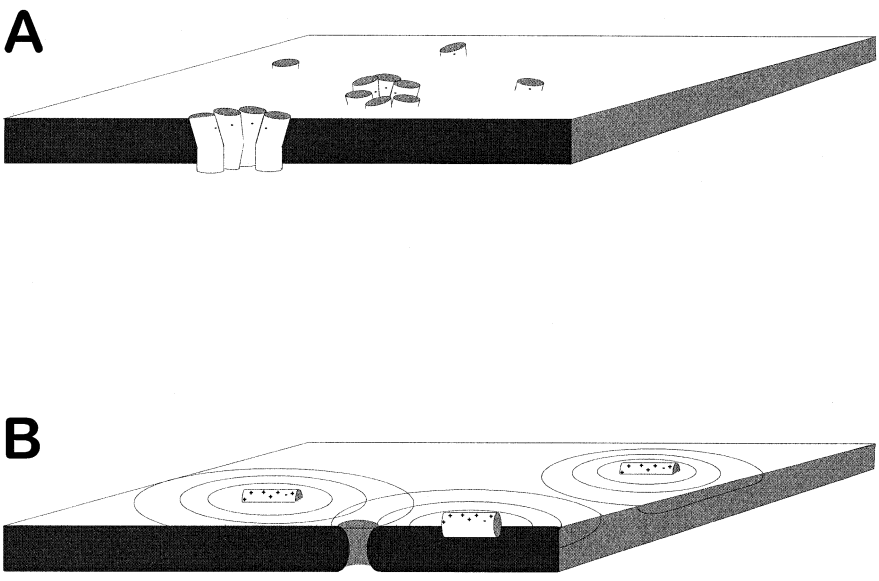

Fig. 3. Models of the mechanisms of membrane conductivity increase. (*A*) Transmembrane helical bundles of polypeptides. (*B*) Diffusion of disturbances along the surface causes transient openings when zones of metastability overlap (*see text for details*).

Models for Membrane Permeability Increase

Only a selection of channel-forming peptides has been presented in this review article. There are of course many more substances that share the capability to increase the conductivity across lipid bilayers in a stepwise manner with magainins, cecropins, melittin and alamethicin. These include other amphipathic polypeptides such as pardaxin [205], helical fragments from channel proteins [82, 91, 171, 174, 207] or designed amphipathic helical peptides [3, 121, 147, 211, 224]. In addition detergents [4, 198], pure lipid membranes [9, 129, 252], or small unilamellar phospholipid vesicles when added to planar lipid bilayers [245] exhibit channel-like behavior in experimental setups designed for single-channel measurements.

The α-Helical Bundle Model

The formation of bundles of amphipathic polypeptide helices provides the most commonly accepted explanation for the increase in conductivity in the presence of membrane polypeptides (Fig. 3*A*). This model consists of a water-filled pore formed by the hydrophilic faces of several helices. At the same time, the hydrophobic side chains interact wtih the fatty acyl chains of the lipids [109]. The structure of the channel forming nicotinic acetylcholine receptor (290 kDa) was determined by cryoelectron microscopy at 9 Å resolution and provides some evidence for such a structure [228]. The electron density suggests that a pentamere is formed where five central pore-lining helices are surrounded by a β-barrel.

The channel–forming peptides discussed in this paper exhibit helical conformations in membranes. This structure, however, is only one of several prerequisites for the α-helical bundle model. The well defined subconductance states of alamethicin in conjunction with its cooperative membrane partitioning have therefore been taken as the strongest argument in support of a 'transmembrane helical bundle' or 'barrel staves' which consist of 3–11 helical rods arranged around a water-filled pore (Fig. 3*A*) [35, 84, 101, 143, 246, 253]. The channel diameters calculated from geometrical considerations and structural measurements agree well with the observed conductivities and ion selectivities [105, 107, 136, 194].

More recently, however, the alamethicin channel was measured to be impermeable to polyelectrolytes of a size that would be expected to pass the large channel diameters underlying the assumption of circular oligomeric bundles [32, 33, 188]. Therefore, the formation of a cluster of helices in which ions pass through a large number of small holes has been suggested.

In contrast, the openings formed by melittin or magainins are less well defined. The concentration dependence of many functional measurements, however, indicate cooperative interactions of these polypeptides when associated with the membrane [58, 128].

Energetics of Membrane Polypeptide Interactions

A detailed evaluation of the energies of the interactions that are involved during pore formation is complicated by the anisotropic nature of the lipid bilayer environment and the highly dynamic structure. Peptide-bilayer association can be accompanied by both conformational changes of the polypeptide and modifications of the membrane macroscopic phase. Even without knowing the details necessary for a full understanding of these

interactions, it is helpful to consider the energetic contributions involved during the formation of transmembrane helical bundles or other structures. In the following section, different contributions to the Gibbs free energy of channel formation shall be discussed and, where applicable, numerical values will be estimated for magainins.

The experimentally observed in-plane orientation of the magainin helices [22] allows for both hydrophobic and electrostatic interactions to be satisfied (Fig. 1*A*). From a physico-chemical viewpoint, this orientation is energetically highly favorable and therefore provides a good reference state.

In contrast, the channel structure which is observed in electrophysiological experiments possibly consists of a relatively unstable higher energy structure. To observe a channel event in a BLM experiment, it is sufficient (and desired) that only one channel forms within an area of typically 10^{-8}–10^{-10} m^2. At a bilayer concentration of 1 mole% peptide about $3 \cdot 10^8$ peptide molecules are present in a patch of this size. For a single hexameric channel, P_n, to be observed the equilibrium constant, K, of the reaction $n \cdot P \leftrightarrow P_n$ should, therefore, not exceed $1/[P]^n$. From the cooperativity observed in *I–V* functions it has been suggested that magainins form hexameric pores [68], therefore $K \leqslant 10^{51}$ and the Gibbs free energy of channel formation should be smaller than

$$\Delta G \leqslant -RT \ln K = 300 \text{ kJ/(mole hexamer)}.$$

Whereas hydrophobic interactions are of main importance during the initial membrane association of hydrophobic or amphipathic polypeptides, a first order approximation suggests that the reorientation of the magainin helix from an in-plane alignment to a transmembrane water-filled pore does not cause large changes in the hydrophobicity of the environment of single amino acids. By pointing into the water-filled lumen, the hydrophilic side chains will remain in an aqueous environment before and after such a transition. On the other hand, the surroundings of some of the hydrophobic side chains are expected to change as reorientation causes movement from a positioning deep in the membrane interior towards the interface, and vice versa. Several tables that list the transfer energies from water to oil [76, 132, 236], or from the interface into the membrane interior [164] have been published. Whereas most of the former tables are based on a hydrophobic area energy of 20–30 (cal/mole $\cdot$ A^2), other suggestions for this value range from 16 to 60 (cal/mole $\cdot$ A^2) that significantly modifies the generally agreed-on values of transfer energies [25, 219].

During the aggregation of magainins, the many identical charges will come into close contact and thereby strongly repel each other. These interactions can be approximated by the electrostatic energy to move (z·e) charges from a random distribution in the plane of the membrane to the surface of a cylinder of length l and radius R_o. This yields: $W = [(ze)^2/2l\pi\varepsilon_o\varepsilon_r] \cdot ln\ R_{eq}/R_o$, where ϵ_o, ϵ_r have their usual meaning and R_{eq} is the average distance between peptides when being uniformly distributed along the bilayer surface. This number, therefore, is related to the molar ratio of membrane-associated protein. Geometrical considerations indicate that the walls of a hexameric channel create a cylinder about 30 Å in height and 4–5 Å in radius. In the case of magainin 2, such a channel is lined by 30 positive and 6 negative charges, assuming that all 4 lysines and the N-terminus remain charged and the histidines are in their neutral state. Using this approach the calculated repulsive energy ranges in the order of magnitude of 1000 kJ/(mole hexamer) when assuming an equilibrium charge density of one elementary charge per 1350 $Å^2$ (1 mole% peptide) and $\epsilon_r = 80$.

A much lower electrostatic energy results when the amino acid side chains (*aa*) are discharged during assembly into aggregates. From the thermodynamic equilibrium $aa^+ \leftrightarrow aa + H^+$ it follows that the energy of discharge is $\Delta G^d = n_i \cdot RT \cdot ln\ r + 2.3\ RT \sum_i (pK_i - pH)$, where r is the minimal ratio of charged side chains that is acceptable for a location in the bilayer interior (usually a value of 99 is assumed), n_i is the number of chargeable side chains of type i, and RT have their usual meaning [23]. The energy of discharge is a linear function of the difference between the actual pH and the pK value of the amino acid, and at neutral pH amounts to about 30 kJ/mole for a lysine (pK = 10) or 20 kJ/mole for the N-terminus (pK = 8.5) [255]. The energy of discharge of 15 lysines, 5 histidines and 5 N-termini (assuming that one magainin molecule remains completely charged) therefore reaches only about 60% of the electrostatic repulsion energy.

The dipolar repulsion between parallel helical dipoles contributes another few kJ/mole which are unfavorable for aggregation [73, 75, 93]. In the case of some peptide configurations the side chain exposure to the bilayer interior can be prevented by salt bridge formation [72] which reduces the unfavorable energy to +42 to 67 kJ/(mole salt bridge) [113]. Favorable energy terms can arise when intermolecular hydrogen bonding interactions occur between helices (−25 kJ/mole) [76, 195]. However, the net effect is close to zero when side chain-side chain hydrogen bonds merely replace existing side chain-water hydrogen bond interactions. Based on crystal structures and molecular modeling studies such interactions have been suggested to stabilize helical bundles of alamethicin by intermolecular hydrogen bonds between Glx residues [36, 149, 194].

On the other hand the interaction of helical dipoles, μ, with the transmembrane electric field, E, provides a driving force for reorientation and incorporation into the

membrane. This results in the energy

$$\Delta G = N_A \cdot \mu \cdot E = N_A \cdot 15e\text{Å} \cdot 200\text{mV}/30\text{Å} = 10\text{kJ/mole},$$

where N_A is Avagadro's constant and $\mu = 15e$Å, a typical value of channel-forming polypeptides.

Unfavorable energy terms arise from the loss in entropy during oligomerization. So far these contributions were too difficult to be included in molecular modeling calculations [72] but they can be estimated from the molecular partition functions. The mobility of a molecule bound to the membrane surface is restricted, nevertheless one rotational and two translational degrees of freedom remain fully operational. These are lost when n molecules of MW 3000 associate and cause an approximately $(n-1) \cdot 100$ kJ/mole increase in entropic energy.

Favorable contributions arise from the van der Waals interactions, in particular when a close ridges-into-grooves packing occurs. Molecular modeling calculations indicate that in case of tightly intertwined alamethicin dimers this energy amounts to about −100 kJ/(mole dimer) [46, 248], or for an Ala_{20}-helix to about −160 kJ/(mole dimer) [87, 88, 182].

Changes in order parameter as well as van der Waals interactions between the lipids have also to be considered when peptides interact with membranes. This first interaction is an entropic effect and has therefore been called 'lipophobic,' in analogy to the hydrophobic effect in aqueous environments [125]. It has been shown that the changes in van der Waals interactions and of the lipophobic effect almost cancel each other, therefore no major contributions supporting aggregation are expected from these terms.

A driving force for aggregation can be derived from the mismatch between the peptide hydrophobic thickness and the lipid bilayer. A too large difference has been shown to result in aggregation and domain formation; this effect should largely be dependent on the type of lipid used. The energies involved range in the order of magnitude of a few kJ/mole [23, 141, 168]. The contribution from cooperative interactions in the alamethicin binding isotherm are unaffected by the lipid composition or the bilayer thickness, however, pleading against the hydrophobic mismatch energy as a driving force for aggregation [214].

In summary, the electrostatic and entropic terms for a magainin 2 hexamer results in an unfavorable energy of about 1100 kJ/mole. This seems much too high for channel formation to be observable. Unfortunately, no data are available on the possible magnitude of the van der Waals interactions when magainins or melittin associate. This term can be quite high as has been shown for isolated alamethicin dimers. In a lipid bilayer, however, only the difference between peptide-peptide and peptide-lipid van der Waals interactions [202] will contribute to aggregation. Assuming that the van der Waals terms modeled for tightly intertwined alamethicin dimers are equally high for other peptide oligomers the contribution to hexamer formation therefore is <600 kJ/(mole hexamer). Without having modeled such a structure the assumption that this energy is released is, at best, optimistic. On the other hand, considering that we are only able to obtain crude estimates of the interactions, the possibility cannot be completely eliminated that the sum of a transmembrane potential and other small energy terms (hydrophobic, hydrophobic mismatch, lipophobic effect etc.) shift the equilibrium sufficiently for a hexameric bundle to form transiently and in a dynamic manner. A better understanding of the interactions is necessary to evaluate the validity of the transmembrane helical bundle model in particular for highly charged peptides.

Bilayer Disruption

The bilayer disruptive properties of amphiphiles provide an explanation for their cytotoxic activity as the resulting dissipation of the transmembrane electrochemical gradient interferes with the energy metabolism of living cells. In a similar manner, the release of fluorescence dyes is probably a consequence of the peptide-induced disintegration of vesicular membranes [26]. The onset of fluorescence dye leakage takes place at magainin concentrations of approximately 3 mole % which is equivalent to 81 g/mole lipid, a based-on-weight-value very similar to those observed for the permeability increases in the presence of the detergents Triton-X100 and octyl glucoside [97, 198]. The use of magainins as 'peptidergents' for protein crystallography further emphasizes the close biophysical relationship between amphipathic peptides and detergents [197]. Therefore, it is not surprising that the addition of high magainin concentrations to phospholipid bilayers results in the appearance of large water-filled bilayer disruptions [142] as well as the optical clearing of dense suspensions and the formation of isotropic phases as recorded by ^{31}P solid-state NMR spectroscopy (B. Bechinger, *unpublished results*).

The membrane-disruptive properties of magainins or melittin [65, 70] are not unique, but have also been observed in the presence of other amphipathic helical peptides, such as apolipoproteins [203], myelin basic protein [190], glucagon [127], signal sequences [17, 73], basic amphiphilic model peptides [187], and after addition of lysolipids [120] or detergents [191] to phospholipid bilayers. Specially designed amphipathic helices consisting of ⩾12 leucines and lysines also exhibit an in-plane orientation [24] and show even stronger antibiotic activity ([55]; B. Vogt and B. Bechinger, *unpublished results*). Some of these peptides are too short to cross the membrane and, in addition, they carry even higher charge densities than the natural peptides discussed in this review.

Interference with the cell-killing activity of these

peptides and regulation of selectivity [258, 261, 262] can occur at any stage during the multistep process of membrane association and pore formation (water soluble ↔ surface associated ↔ bilayer inserted ↔ closed pore (?) ↔ open pore (?)). Tumor cells, for example, have lost part of their lipid asymmetry and therefore exhibit a more anionic character at the outer leaflet of their plasma membrane when compared to healthy vertebrate cells [229]. Cationic peptides exhibit an up to two orders of magnitude increased affinity for acidic membranes (e.g. [28, 150]), which at least partly explains the selective tumorcidal activity.

Channels

The channel-like properties of charged amphipathic peptides, which are measured in electrophysiological experiments, are more difficult to explain as the assumption of helical bundles remains questionable (*cf. above*). In an alternative model it has been suggested that the channel-forming properties of amphipathic polypeptides are due to extended planar 'rafts' of antiparallel peptide aggregates that displace the lipids of one bilayer leaflet [99, 184]. When two of these rafts meet end-on they fold in a way that does not expose the hydrophilic side chains to the bilayer interior. The structure occurring during the transition towards the open cylindrical channel is called a 'boat'. The final structure of this model corresponds to a large α-helical bundle and therefore assumes the presence of strong favorable, so far unidentified interactions that compensate for the electrostatic and entropic terms.

Alternatively, channels that are composed of ordered arrays of phospholipids and magainins were modeled [58, 142]. The presence of negatively charged phospholipids thus reduces the electrostatic repulsion terms and could explain why magainin-induced channels have shown cation selectivity in some electrophysiological experiments. In this model, however, the immobilization of lipids is concomitant with additional unfavorable entropic energy terms.

The unfavorable interactions are reduced when the charged peptide molecules cover the surface equally in a 'carpetlike' manner [206]. In the case of magainins these peptide helices were shown to assume an orientation along the membrane surface [20, 22, 154]. The membrane interactions of amphipathic helices result in significant disturbances of the pure lipid bilayer properties within a 100 Å diameter as well as thinning of the average bilayer thickness [43, 141, 249]. In a similar manner mastoparan (14 residues) [159], short analogues of alamethicin [11, 193, 196], and short synthetic peptides (12 or 14 residues), [10, 137] are also capable of exhibiting channel-like activity. These peptides are too short to reach through the membrane, however. Charged amphipathic peptide helices have been demonstrated to induce nonbilayer phases at a range of intermediate to high peptide concentrations. The curvature strain exerted on the bilayer suggests that a metastable state exists in the vicinity of these peptides also at lower amphiphile concentrations [54, 145].

These properties allow one to outline an alternative model for channel formation that does not require the formation of peptide aggregates (Fig. 3*B*). In agreement with fluorescence energy transfer measurements [260] the molecules diffuse in the membrane continuously, changing their distance and orientation with respect to each other. When these diffusive units approach each other within a 'critical distance', the destabilizing properties add up locally and result in a transient opening of the bilayer. Changes in the number of monomers as well as the angular distribution between them explain the different conductivities. The experimentally observed cooperativity of magainins is in agreement with the participation of several diffusive units in pore formation [128, 231].

Dipolar and charged peptides are expected to respond to transmembrane potentials because of the favorable energy contributions arising from their positioning along the steep electric gradient, which exists in the hydrophobic membrane interior. In analogy to a model suggested for alamethicin [212, 214] the observed voltage gating of magainins or melittin (μ = 115 D, [192]) at high voltages might reflect a voltage-dependent partitioning of these peptides as well as changes in bilayer penetration depth. Furthermore, macroscopic membrane phase transitions have been shown to occur due to the influence of strong electric fields [156, 157, 177, 217]. The propensity of membranes for the formation of nonbilayer macroscopic structures, which already exists either due to their lipid composition or due to the presence of peptides, is therefore enhanced due to transmembrane electric fields.

To better correlate the structural findings with the peptide functional characteristics a quantitative understanding of the polypeptide-bilayer interactions is necessary. Specifically designed model peptides [23] allow one to study selected energetic contributions in more detail and to understand the complex interactions of naturally occurring channel peptides. A detailed evaluation of the different models for polypeptide channels will then also become feasible.

I wish to take this opportunity to express my grateful thanks to Joachim Seelig and Stanley J. Opella, who taught me many fine details of membrane biophysics and solid-state NMR spectroscopy. I also like to acknowledge the valuable contributions of Gabriele Weingärtner and Susan Möller in managing the many publications that have contributed to this text. Felicity Strang helped in editing the final versions of the manuscript. Last, but not least, I want to thank all the members of my

group who do the real work, and, who often make the laboratory a fun-filled place.

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