Properties of cytotoxic peptide-formed ion channels

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Cytotoxic peptides are small proteins that interact with lipid bilayers, resulting in an alteration of the cell’s membrane permeability that leads to cell death. The peptides are thought to contribute to this process through the formation of new ion channels in the cell membrane and/or by changing the activity of existing channels. Many cytotoxic peptides have been shown to act against bacterial cells, e.g., magainin I and II (24, 31), Cyprinus carpio peptides (55), a 9-kDa polypeptide originally extracted from porcine lymphocytes (NK-lysin) (1–5), melittin (reviewed in Ref. 13), lytocoxins (87), insect defensin (20), and scorpion defensin (21). Dermaseptin acts against fungal cells and pilosulin 1 attacks mammalian cells (67, 86). Mammalian defensins are active against all enveloped viruses (43).

The characterization of cytotoxic channel-forming peptides, which modify the cellular signal transduction, forms the basis of understanding how they function in the living organism. This is of great significance to the fundamental science needed for the design of new pharmaceutical agents and for insight into cytotoxic diseases such as Creutzfeldt-Jacob disease or “mad cow” disease. Aside from the benefits flowing from improvement to health and pharmaceutical development, the investigations of cytotoxic peptides could have a marked impact on the meat industries. As shown in the United Kingdom, the meat industry needs to understand and combat cytotoxic disease, which can have devastating economic consequences, while efficiently minimizing the more common pathologies that increase production costs and reduce market acceptance.

Because the molecular mechanisms of interactions between proteins and membranes are an important frontier of cell biology, the investigations of these peptides could make a significant contribution by clarifying the means of interaction between small peptides and cell membranes and by indicating how this process can be modulated. The new information about the membrane-binding properties of peptides, which underlie intracellular signaling, is of fundamental benefit to this increasingly vital field of cytotoxic peptide research. The understanding of the properties of cytotoxic peptides
could allow us to 1) formulate models of bilayer insertion and conditions of lipid composition, voltage, pH and Ca\(^{2+}\), which may be required for peptide interaction and channel formation; 2) model how highly charged, water-soluble peptides come together and interact with the membrane to form channels; 3) determine the roles of peptide domains in the biophysical and pharmacological properties of the channel functions by locating configurations, regions, and polar and nonpolar amino acids that are involved in determining the conductance levels, ionic permeability and selectivity, voltage dependency, gating, and kinetics of the channel; 4) clarify the domain structure of cytotoxic pore-forming peptides, e.g., prion and amyloid β-protein (AβP), an apparently disparate group of cytotoxins that may possess some common structural features; and 5) provide models of how these peptides can modify cellular physiological functions and cause pathologies. Cytotoxic peptides have been isolated from a variety of natural sources, including venoms and antimicrobial secretions, and are involved in the mammalian immune response, being present in a range of cells of the immune system. In addition, there is some evidence that molecules of similar activity may be involved in "autocytotoxic" conditions, where the body produces peptides that act against its own cells, causing disease. Possible examples include the role of amylin in type II diabetes and apoptosis (11, 65), the prion peptide in the mad cow disease (58), and AβP in Alzheimer's disease (6–9, 66, 73). It is thought that these cytotoxic-formed ion channels cause these diseases by modifying a second messenger system, e.g., Ca\(^{2+}\) homeostasis (see Fig. 7B).

The structure, biological activity, and electrical activity of some cytotoxic peptides found in toxins and antimicrobial secretions have been investigated extensively. This information can be used to characterize molecules with cytotoxic activity and may aid in the identification of other, naturally occurring, cytotoxic peptides. Interest in the identification and characterization of such molecules stems from their potential as pharmacological agents, either as antimicrobial or tumoricidal compounds. Knowledge of the mechanisms of their action also provides insight into conditions caused by similar molecules and possible treatments for such conditions. This review examines a selection of not-well-characterized cytotoxic molecules found in venoms, antimicrobial secretions, and the mammalian immune system. We have surveyed and summarized our current understanding of the properties of these cytotoxic-peptide-formed ion channels and provided a framework for future research. These peptides are grouped according to their having several common features. They are small molecules, often cationic and amphipathic in nature. Their amino acid sequences vary, but certain motifs and secondary structures are common. Few of the ion channels formed by cytotoxic peptides have been characterized extensively, but those that have show very diverse characteristics and include anion-selective, cation-selective, and nonselective channels. Some cytotoxic peptides have been well characterized and reviewed previously (see Ref. 13). The natriuretic peptides, some of which are present in venoms and toxins, have been reviewed elsewhere (46–49).

**TYPES OF CYTOTOXIC PEPTIDES**

The cytotoxic peptides discussed here can be divided into three main groups based on the origin of the molecule. Perhaps the most well-studied group is that of the cytotoxic peptides found in various venoms. Melittin, found in bee venom, is particularly well characterized, providing a model for other cytotoxic molecules (see review in Ref. 13). Cytotoxic peptides are also found in scorpion venom (long- and short-chain scorpion toxins), jumper ant (Myrmeleotettix pilosula) venom (pilosulin 1), and the venom of the wolf spider (Lycosa carolinensis; lycotoxin I and II). The second group of cytotoxic peptides is found in antimicrobial secretions and cells involved in immunity. Magainin I and II secreted from Xenopus laevis skin are the most extensively characterized (also reviewed in Ref. 13). Derma- septin is another peptide isolated from frog skin. Antimicrobials have been identified in skin secretions of carp (C. carpio (55)) and in cells of the immune system (e.g., insect defensins, scorpion defensins, mammalian defensins, and cryptdins). The third group of cytotoxic peptides is involved in pathological conditions and is believed to be produced by the organism itself (autocytotoxicity). Amylin, which is cytotoxic to pancreatic β-cells and implicated in the pathogenesis of type II diabetes, is thought to act in this way (65). Also, fragments of the prion peptide are believed to form or alter ion channels causing cytotoxicity, and these can be considered in this group, because the prion peptide is a naturally produced peptide in mammalian systems. Last, synthetic peptides based on the sequences and properties of naturally occurring cytotoxic peptides have been demonstrated to retain cytotoxic activity. These can be distributed within the above three groups according to the cytotoxic peptide from which they are derived (e.g., see Refs. 19, 22).

**STRUCTURE OF CYTOTOXIC PEPTIDES**

**Overall Characteristics**

The minimum length of cytotoxic peptides found naturally is 23 amino acids, i.e., the magainins (24, 31), whereas shorter synthetic peptides with similar characteristics have also been demonstrated to have cytotoxic activity, i.e., prion fragment (58). These lengths correspond to the approximate minimum of 20 amino acids that is required for α-helices to span lipid bilayers. However, much larger peptides, such as NK-lysin, which is 78 residues in length, can also show cytotoxicity (3, 17, 74). Most cytotoxic peptides are positively charged as a result of lysine and arginine residues present in the sequence. The net charge of such molecules ranges from +1, e.g., a peptide found in the rabbit kidney (RK-1) and that is related to the corticostatin/defensins (12) to +8, e.g., NK-lysin (1, 4). Charge...
can vary with pH as a result of ionization of residues such as histidines, e.g., the prion peptide fragment 106–126 [PrP-(106–126)] (58).

Sequence and Structure

The amino acid sequences vary considerably among the cytotoxic peptides, but structurally related subgroups of peptides can be identified (see Table 5). One group contains positive residues distributed along the length of the molecule. This creates a molecule that is amphipathic, with hydrophobic and hydrophilic regions present. These molecules are predicted (and in some cases demonstrated) to form amphipathic \( \alpha \)-helices. However, it is possible that other molecules that have been predicted to form an \( \alpha \)-helix are actually in \( \beta \)-sheet conformation when active. Molecules of another group are cysteine rich and contain \( \alpha \)-helix and \( \beta \)-sheet domains. The consensual cysteine motif, consisting of six to eight cysteine residues forming three to four disulfide bridges, stabilizes a structure composed of \( \alpha \)-helices and \( \beta \)-sheets. A third group of compounds is composed primarily of \( \beta \)-sheets. Additionally, some molecules either do not show any sequence or structural homology with other cytotoxic peptides or their structures have not been determined.

Jones (42) made prediction of the tertiary structure of NK-lysin using multiple sequences and recognized supersecondary structural motifs. Similarly, Liepinsh et al. (57) used the NMR structure of NK-lysin to reveal folding in saposin. NK-lysin is viewed as the first representative of a family of sequence-related proteins, such as saposins, surfactant-associated protein B, pore-forming amoeba proteins, and domains of acid sphingomyelinase, acyloxyacylhydrolase, and plant aspartic proteinases, for which a four-\( \alpha \)-helix bundle motif of cytolytic peptides is suggested (27).

Cationic molecules with evenly distributed positive residues. Many of the cytotoxic peptides are thought to form structures composed primarily of \( \alpha \)-helices when in a hydrophobic environment such as that encountered in the lipid membrane. They are often compared with melittin, one of the first cytotoxic molecules whose structure was determined. Vogel and Jahnig (84) used Raman spectroscopy to determine the three-dimensional shape of melittin in lipid membranes, finding that the molecule forms a bent \( \alpha \)-helix from residues 1 to 21. The helix is divided into regions 1–11 and 12–21 that are bent at an angle of 60° relative to each other. The COOH-terminal residues, 22–26, form a nonhelical segment of residues 22–26 being nonhelical. The convex side of the helix is hydrophilic, the concave side hydrophobic. Circles denote positively charged residues. [From Vogel and Jahnig (84), reprinted with permission from the Biophysical Society.]

Fig. 1. Schematic model of the conformation of water-dissolved and membrane-bound melittin. Two \( \alpha \)-helical segments of residues 1–11 and 12–21 are bent by 60° relative to each other, the COOH-terminal segment of residues 22–26 being nonhelical. The convex side of the bent helix is hydrophilic, the concave side hydrophobic. Circles denote positively charged residues. [From Vogel and Jahnig (84), reprinted with permission from the Biophysical Society.]

Magainin I from X. laevis is 23 amino acids long (31). It is positively charged (+2 at pH = 7, +3 at pH = 6.5), containing three lysine residues distributed along the length of the molecule. Duclohier et al. (31), using circular dichroism, were unable to determine a definitive conformational structure for magainin I in the lipid bilayer. Magainin I was found to exhibit \( \sim 10\% \) \( \alpha \)-helix, 70% random coil, and 30% \( \beta \)-sheet when lecithin was added. Their results suggested that there was minimal \( \alpha \)-helical structure, but these results were determined in the absence of a membrane voltage, thought to be necessary for incorporation of the ion channel protein into the membrane. They do not, therefore, exclude the possibility of the active conductance state of magainin I being \( \alpha \)-helical in structure (31). Another cytotoxic peptide isolated from frog skin is dermaseptin isolated from Phyllomedusa sauvagii. Dermaseptin is a 34-residue peptide that has antifungal activity at low concentrations. It is nonhemolytic and does not have antibacterial activity. The peptide was predicted by various methods to be composed of up to 79% \( \alpha \)-helix and was demonstrated to exhibit \( 77\% \ \alpha \)-helix structure by circular dichroism when in hydrophobic conditions (67). The \( \alpha \)-helix spans the residues 1–27, whereas residues 27–34 are negatively charged and hydrophilic. Pilosulin 1, composed of 56 amino acid residues, is the largest allergenic peptide in the venom of M. pilosula, the jumping ant (86). The basic amino acids, lysine and
arginine, are found at regular intervals in the peptide, with the remainder being composed of hydrophobic residues. This arrangement of residues is similar to those described above and allows the peptide to form an amphipathic secondary structure capable of membrane interaction. When analyzed using circular dichroism, pilosulin 1 is shown to form random coils and have little secondary structure. However, in increasingly hydrophobic conditions, a percentage forms \( \alpha \)-helix structures (32% in 50% trifluoroethanol) (86).

Thus this structural group of cytotoxic peptides is composed of molecules that are thought to form amphipathic \( \alpha \)-helices. These peptides often contain lysine and arginine residues evenly distributed throughout the sequence. To demonstrate that the amphipathic \( \alpha \)-helix structure in such peptides provides the active structure, Cornut et al. (22) designed synthetic molecules composed solely of amphipathic \( \alpha \)-helix. These were between 12 and 22 residues in length and composed of leucine and lysine in, approximately, a 2:1 ratio. The lysine residues were spread evenly along each molecule (see Table 5). All had a positive charge at pH = 7, with the exact value varying between +4 and +8, depending on the length (and therefore lysine content) of the peptide. They were shown to have hemolytic activity up to 5–10 times that of melittin and to illustrate the importance of the \( \alpha \)-helix structure in cytotoxicity and pore formation. The hemolytic activity of these synthetic peptides decreased with their size, probably reflecting the minimum length required for an \( \alpha \)-helix to span a membrane. Similar work emphasizing the importance of amphipathic \( \alpha \)-helices in the cytotoxic activity of these peptides has been done with the magainin II molecule, involving the modification of its sequence to maximize its \( \alpha \)-helical content. Residues with a low propensity to form \( \alpha \)-helices were replaced with alanine, expected to be more likely to form \( \alpha \)-helical structure. The modified magainin II molecule was, as predicted, shown to have an increased \( \alpha \)-helical content. It also was shown to have increased antimicrobial activity compared with native magainin II (19). In addition, an analog was modified to consist of fewer \( \alpha \)-helices and shown to have reduced activity (19). Recently, Chaloin et al. (18) designed an amphipathic peptide, SP-NLS (a cysteamide group Met-Gly-Leu-Gly-Leu-His-Leu-Leu-Leu-Ala\(^{16}\)-Ala-Ala-Leu-Gln-Gly-Ala-Lys-Lys-Lys-Arg\(^{20}\)-Lys-Val-H-CH\(_2\)-CH\(_2\)-SH) containing a hydrophobic signal sequence (SP, Met-1 to Ala-16) followed by a polycationic nuclear localization sequence (NLS, Lys-17 to Val-22) and terminated by a cysteamide group, to act as drug carrier, which proved cytotoxic and bactericidal by inducing the formation of ion channels. The SP-NLS exhibits a voltage-independent pore-forming activity when incorporated into planar lipid bilayers and X. laevis oocyte plasma membranes, with conductance values of 25 pS in 0.1 M NaCl. The SP-NLS ion channels were cation selective (\( K^+ > Na^+ > Li^+ > TEA^+ > \text{choline}^+ \)).

Oh et al. (68) investigated the NMR structure of cecropin A-(1–8)/magainin 2-(1–12) and cecropin A-(1–8)/melittin-(1–12) hybrid peptides. They found that both cecropin A-(1–8)/magainin-(1–12) and cecropin A-(1–8)/melittin-(1–12) have strong antibacterial activity but only cecropin A-(1–8)/melittin-(1–12) has hemolytic activity against human erythrocytes. Cecropin A-(1–8)/magainin-(1–12) has a hydrophobic \( \alpha \)-helix of only two turns combined with one short helix in the NH\(_2\) terminus, with a flexible hinge section in between. Cecropin A-(1–8)/magainin-(1–12) has a severely bent structure in the middle of the peptide. These structural features, as well as the low hydrophobicity of cecropin A-(1–8)/magainin-(1–12), seem to be crucial for the selective lysis against the membrane of prokaryotic cells. They argued that, in cecropin A-(1–8)/melittin-(1–12), an \( \alpha \)-helical structure of about three turns in the melittin domain and a flexible structure with one turn in the cecropin domain connected with a flexible hinge section in between might be the structural features required for membrane disruption against prokaryotic and eukaryotic cells. They further suggest the formation of an ion channel as a mechanism of action of these peptides. This is because the central hinge region (Gly\(^4\)-Ile\(^{10}\)-Gly\(^{13}\)) in an amphipathic antibacterial peptide is considered to play an important role in providing the conformational flexibility required for ion channel formation.

Cysteine-rich molecules composed of both \( \alpha \)-helix and \( \beta \)-sheet domains. Scorpion toxins, scorpion defensins, and insect defensins form a second, structurally similar, group of cytotoxic molecules. Another related peptide group are the thionins (50). The molecules have poor sequence homology but are all cysteine rich and share a similar cysteine motif. These cysteine molecules form disulfide bridges that stabilize a common secondary structure composed of an antiparallel triple-stranded \( \beta \)-sheet linked by two disulfide bridges to an \( \alpha \)-helix; the \( \beta \)-sheet is linked via a third disulfide bridge to the NH\(_2\)-terminal region of the molecule (Fig. 2; see also Refs. 15–16, 50). This structure forms the core of the molecules in this group. There have been three groups of scorpion toxins identified.

First, long-chain toxins, which affect Na\(^+\) channels, are 60–70 amino acid residues in length and contain a conserved sequence consisting of four disulfide bridges (50). This sequence corresponds to the conserved structural motif, composed of an \( \alpha \)-helix packed against a three-stranded antiparallel \( \beta \)-sheet as described above. An example is toxin III from the scorpion, Leiurus quinquestriatus, a 64-amino acid residue peptide with four disulfide bridges (50). Through the use of two-dimensional \( ^1\)H-NMR spectroscopy, toxin III was found to contain the structural motif common to this group (Fig. 3). This region forms the core of the molecule. The hydrophobic residues of the peptide are arranged in small clusters on the surface of the molecule.

Second, the short-chain scorpion toxins are 31–38 amino acids in length and contain three to four disulfide bridges, which stabilize a structural motif similar to that found in the long-chain toxins. These toxins are active against a wide range of channels, blocking K\(^+\) channels.
channels that are either voltage gated or Ca$^{2+}$ dependent (52).

A third group of toxins was proposed with the discovery of Tityus toxin Kβ, a toxin found in the venom of the scorpion Tityus serrulatus and a similar toxin from Androctonus australis (52). Tityus toxin Kβ is active against a voltage-gated, noninactivating K$^+$ channel. The 60-amino-acid-long molecule exhibits some homology with the insect defensins and scorpion defensins. In particular, the six cysteine residues that it contains are consensual between insect defensins, scorpion defensins, and these scorpion toxins (see Ref. 52).

A number of insect families have been found to produce antimicrobial peptides, collectively known as insect defensins. The peptides were first isolated from Phormia terranovae and were initially found to exhibit sequence homology with mammalian defensins (53). The peptides are 4-kDa in size and are cysteine rich, with six cysteine residues forming three disulfide bridges that stabilize the molecule’s three-dimensional structure (14). Insect defensins have a conserved structure, consisting of an NH$_2$-terminal loop, an α-helical domain, and a COOH-terminal domain composed of an antiparallel β-sheet (14). More recent NMR analysis of the three-dimensional structure of the insect defensins indicated that they are probably evolutionarily distinct from the mammalian defensins, since these are composed primarily of β-sheet (70).

Another member of this group is scorpion defensin, which has been found to exhibit homology with insect defensins (20). In particular, it was found to be very similar in sequence to a defensin isolated from the older insect order Odonata (Fig. 4) (see Ref. 20). These two defensins are shorter than defensins from the more recent insect orders and have a shorter NH$_2$-terminal loop than those molecules. The isolated scorpion defensin was also compared with the scorpion toxins and showed to have the characteristic secondary structure of this class of molecules (21). It is noteworthy that the same species of scorpion (L. quinquestriatus) was found to produce both toxins and defensin. Coianci et al. (20, 21) state that these results support the idea that the molecules may derive from a common ancestral antibacterial peptide.

More recently, three defensin-like peptides have been also isolated from platypus venom and sequenced (82). The main NMR three-dimensional structural elements of a 42-residue peptide, synthesized form of one of these peptides, were antiparallel β-sheets comprising residues 15–18 and 37–40 and a small 3(10) helix spanning residues 10–12. It appears that the general three-dimensional fold, with exception of the side chains, resembles that of β-defensin-12 and of the Na$^+$ channel neurotoxin Shl (Stichodactyla helianthus neurotoxin I). However, this peptide has no antimicrobial properties and does not modify Na$^+$ channel currents.

Molecules composed predominantly of β-sheet structures. Yet another group of peptides with cytotoxic activity are those composed predominantly of β-sheet structures. These include mammalian defensins and cryptdins, human amylase, and PrP-(106–126). Of particular interest are those that were predicted on the basis of their amino acid sequence to form α-helices but that have been demonstrated to be composed of β-sheet when active. This raises the question of whether other peptides predicted to form α-helices in fact do so. Investigations of ion channel-forming molecules in other areas has revealed that those forming β-sheets represent an important structural class of ion channel-forming molecules (6–9, 73).

Rabbit protein neuropilin-1 (NP-1) is a mammalian defensin found in the phagocytic cells of rabbits. As is characteristic of this family of molecules, it is a small, positively charged peptide. Mammalian defensins are also cysteine rich (43) but do not form helices in solution or in membranes. Human defensin has a region of antiparallel β-sheet extending for 10 residues (see Ref. 43). The mammalian defensins were initially thought to be related to the insect defensins because of the similarities of high cysteine content and biological activity of the two groups of molecules. However, more recent structural analysis has shown that the mammalian defensins do not have any regions that form α-helix domains. The insect defensins have thus been grouped with the scorpion toxins and defensins, which are more closely related in structure.

The cryptdins, also known as the enteric defensins, have structures and functions similar to those of the
mammalian defensins (56). Mouse cryptdins 2 and 3, which are capable of inducing a Cl⁻ secretory response in human intestinal T84 cells, are extremely similar in amino acid sequence to cryptdins 1, 4, 5, and 6 but do not illicit the secretory response. This allows amino acids important to the activities of cryptdin 2 and 3 to be identified. The biologically active peptides have an arginine at residue 15, and this may be the important...
residue (56). The secondary structure of the cryptdins has not been determined.

Another recently discovered peptide, RK-1, is believed to be related to the corticostatin/mammalian defensin molecules (corticostatin/defensins). RK-1 is found in the kidney and contains 32 amino acid residues (one of which is an arginine residue). It is calculated to have a net charge at pH 7 of +1. There are six cysteine residues, and these form the cysteine motif common to corticostatin/defensins (12).

PrP-(106–126) has been found to retain many of the properties of the total prion protein. The sequence of 21 amino acids is largely hydrophobic but contains two lysine residues, resulting in a net charge of +2 at pH > 7. Also present is a histidine residue, which is ionized at pH 6.5, resulting in a net charge of +3 at pH < 6 (58). PrP-(106–126) corresponds to a predicted α-helical domain of the prion protein (see Table 5). However, there is evidence that the region in fact forms a β-sheet in vitro. De Gioia et al. (29) demonstrated that the β-sheet conformation was the predominant secondary structure when the peptide fragment was incubated with liposomes. Liposomes composed of neutral, negatively charged and positively charged lipids were found to induce this secondary structure. In addition, the region is thought to change conformation to a β-sheet when the normal cellular prion protein (not pathogenic; PrPc) is converted to the strain of altered prion protein (pathogenic; PrPsc) (10, 36). In solutions of acidic pH, PrP-(106–126) exhibits predominantly a β-sheet conformation (29). Human amylin is a 37-amino acid peptide with an overall charge of +5 (65). Human amylin forms a β-sheet structure when it interacts with phosphatidylcholine membranes (62). There is agreement that such structures are capable of forming ion transport pathways, e.g., they include the neurotoxic and channel-forming peptide AβP (39, 78–81), the bacterial porin (23, 76), and the voltage-dependent anion channel (see Ref. 61).

Unknown sequence and/or structure. The amino acid sequence and/or secondary structure of a number of cytotoxic peptides has not been determined. Investigation of the structures of such molecules is needed if we are to gain a more comprehensive picture of the patterns of channel formation by cytotoxic peptides. Two peptides with antibacterial activity were isolated from the skin secretions of C. carpio (55). The 27-kDa protein isolated was found to be glycosylated, and its first 19 amino acid residues were determined. It did not show any similarity to known protein sequences. The 31-kDa peptide was nonglycosylated and able to form ion channels. It could not be sequenced because it was blocked at its NH₂-terminus. NK-lysin, isolated from pig small intestines and believed to originate from cytotoxic T cells and NK cells, is 78 amino acid residues in length. It contains six cysteine residues, which form three disulfide bridges (3, 5, 17, 54). The molecule is much larger than the mammalian defensins and, despite the disulfide bridges, shows no sequence similarity to these molecules (3, 5).

Tertiary Structure Models of Channel Formation

Many amphipathic molecules are commonly thought to form multimeric pores in membranes, with their hydrophilic faces forming the inside of the pore and their hydrophobic region interacting with the hydrophobic lipid membrane. This can be illustrated by a model for the formation of melittin channels. Vogel and Jah (84) proposed that melittin aggregated in tetramers, forming hydrophilic pores in membranes. Fluorescence quenching experiments were used to determine the orientation of melittin in the membrane. These experiments demonstrated that the COOH terminals of melittin molecules were located on the side of the membrane to which melittin was added (i.e., the outside of a bacterial cell membrane in vivo). The helical region of melittin has a hydrophilic and a hydrophobic face. Thus, to form an energetically favorable model of pore formation, melittin is required to form multimeric channels. In this model the hydrophilic faces of the molecules are aligned to form a hydrophilic pore while the hydrophobic faces interact with the hydrophobic regions of the lipid bilayer (Fig. 5 (see Ref. 84)).

Cruciani et al. (24) proposed three structural models for a magainin II-formed ion channel (Fig. 6). In the models, the magainin II molecules are arranged in dimers of α-helices aligned to form antiparallel amphipathic units. Type 1 channels were composed solely of peptide molecules arranged so that the polar side chains of amino acids extended into the lumen, while the hydrophobic portions of the molecules formed the wall of the channel. Large numbers of monomers were required to form channels of this type. The central pore of the channel was calculated to be electropositive, making the channel selective for anions. These channels are similar to those described for melittin above. Type 2 channels were composed of both peptides and

Fig. 4. Alignment of the sequence of the scorpion defensin with the sequences of insect defensins (20). The defensins were characterized from species belonging to three insect orders: Diptera (a), Coleoptera (b), and Odonata (c). The scorpion defensin is closely related to the Aeschna defensin (c). Dashes indicate gaps to optimize the alignment. Identical amino acids are boxed. Boxes in bold represent the identical residues between Aeschna and scorpion residues. [From Cocián C et al. (21), reprinted with permission from Academic Press, Inc.]
lipids, with some of the channel wall being formed by the hydrophilic heads of lipid molecules from the surrounding lipid bilayer. The alkyl tails of lipid molecules involved in channel formation extended into the hydrophobic region of the membrane and were stabilized by aggregates of magainin II peptide on the surface of the membrane, where hydrophobic lipid tails would otherwise come into contact with the aqueous environment. The central pore of the channel thus formed was calculated to be electronegative, consistent with cationic selectivity. Type 3 channels also involved both lipid and peptide molecules. In this case head groups from lipid molecules formed the lining of the ion channel while the peptide molecules remained on the surfaces of the membrane stabilizing the structure. Magainin II is required on both sides of the membrane for this type of channel to form. In this model the channel lumen was calculated to be electronegative, consistent with cationic selectivity. The channel models were dependent on the lipid composition of the membrane in which the channel was formed. Cruciani et al. (24) based their models on membranes composed of a combination of negatively and positively charged lipids. Type 2 structural models assumed that 24 out of the 27 lipid molecules involved in forming the pore were palmitoyl-oleyl-phosphatidylserine (PS), while type 3 models were constructed using palmitoyl-oleyl-phosphatidylethanolamine (PE) to line the channel and PS to form the bilayer regions.

Cruciani et al. (24) were then able to speculate on the type of channel that was formed by magainin II in their experiments. Because the channels that they found were cationic selective, type 1 channels were not probable. They speculated that type 3 channels accounted for the majority of conductance. However, because of the requirement for peptide on both sides of the membrane for the formation of type 3 channels, these were not the initial channels formed. They proposed that type 2 channels were formed first and then, as they broke down, released some peptide on the other side of the membrane, allowing type 3 channels to form.

Extending their work to other literature, Cruciani et al. (24) speculated that the magainin I anionic selective channels, found by Dudloher et al. (31), may have been type 1 channels. However, this work was done using neutrally charged lipid membranes, and these models of channel formation were formed on the basis of some negatively charged lipids in the membrane. They acknowledged that determining the likely channel model was not straightforward. More complex models involving lipids from the membrane-forming sections of the channel have been suggested for the formation of some channels, in particular to explain how cationic peptides could form a cationic-selective channel. Models of channel formation for cytotoxic peptides not composed solely of amphipathic α-helices have not been investigated in such detail. The family of scorpion toxins and scorpion defensin toxins are proposed to interact with preexisting ion channels in the membrane. However, the related peptide, insect defensin, itself forms ion channels (20), suggesting that peptides from this structural group can form ion channels. Further investigation will help to clarify this.

BIOLGICAL ACTIVITY OF CYTOTOXIC PEPTIDES: EVIDENCE FOR CYTOTOXIC ACTIVITY

By definition cytotoxic peptides have cytotoxic activity, mostly antimicrobial activity or hemolytic activity. This activity is demonstrated in assays of cytotoxicity against various cell types (bacterial, fungal, and mammalian cells, especially blood cells). The specific biological activity of each peptide depends on the environment in which it is found. Some peptides found in venom function as toxins and are used by organisms for defense or to subdue prey. Cytotoxic peptides found in antimicrobial secretions function primarily by providing defense against pathogens such as colonizing bacteria. Those produced by immune system cells are also involved in host defense. There is some evidence that molecules with a similar molecular activity are also involved in autocytophobic conditions.

Lycotoxins I and II, from wolf spider venom, were shown to have antimicrobial activity against both prokaryotic and eukaryotic cells. Various microbes were incubated with the peptides to determine the specificity of their antimicrobial activity. Lycotoxin I was inhibitory at concentrations as low as 5 µM for one gram-positive species (Bacillus thuringiensis israelensis), whereas lycotoxin II was most active against gram-negative strains (Escherichia coli strain DH5 minimal inhibitory concentration 40 µM). Both peptides were active against yeast (Candida albicans) at a concentration of 40 µM. Magainin II was also assayed to provide comparison: in most instances the inhibitory activity exhibited by the lycotoxins was stronger than that exhibited by magainin II (87). Inhibition of growth occurred at a critical concentration, indicating self-aggregation of the peptide. Hemolytic activity was also assayed for lycotoxin I. Lycotoxin I was found to cause lysis of erythrocytes at concentrations >100 µM and was more active than magainin II in this respect (87).
Pilosulin 1 causes cell lysis via disruption of membrane integrity in both dividing and nondividing blood cells (86). Lysis of erythrocytes was complete at a concentration of 40 µM, with partial lysis occurring at concentrations as low as 1.25 µM. White blood cells were differentially affected by pilosulin 1, with mononuclear cells being more susceptible to lysis than granulocytes. Lysis was rapid and usually complete, but results (for white

Fig. 6. Examples of the channel models formed as viewed looking down the channel axis. A: type I. The channel is formed by 12 antiparallel magainin α-helices. Hydrophilic side chains extend inward into the pore, and hydrophobic side chains extend outward into the lipid. The magainin helices are displayed as α-carbon traces with the NH2- and COOH-terminal amine and carboxylate groups included. The lysine and phenylalanine residues are also included to indicate the polarity of the helical faces. B: type II. A combination of magainin 2 antiparallel dimers on the transmembrane bound to the lipid head groups (a) and membrane surface (b). The lipid molecules are displayed as circles for the head groups and straight lines for the alkyl chains, parallel to the membrane plane. C: type III. All of the magainin 2 dimers on the lipid head groups completely forming the channel lining (a) and the surface (b). The type III model is illustrated as part of a hexagonal lattice. Squiggy lines represent alkyl chains perpendicular to the membrane plane. [Reprinted from Eur J Pharmacol, vol. 226, Cruciani RA, Barker JL, Durell SR, Raghunathan G, Guy HR, Zasloff M, and Stanley EF. Magainin 2, a natural antibiotic from frog skin, forms ion channels in lipid bilayer membranes, p. 287–296, copyright 1992, with permission from Elsevier Science (24).]
blood cells) differed in normal individuals by up to fivefold. Pilosulin 1 also had a cytotoxic effect on Epstein-Barr virus (EBV)-transformed B lymphocytes [dose of toxin that will kill 50% of test subjects in standard time (LD₅₀) = 0.4 µM] (86).

Lemaître et al. (55) incubated various strains of bacteria with two ion-channel-forming peptides (27 and 31 kDa) isolated from carp (C. carpio) skin mucus. Both compounds were found to have antibacterial activity. Inhibition of bacterial growth occurred at concentrations of 5 µg/ml (0.16–18 µM). Gram-positive and gram-negative bacterial strains were equally affected. However, Micrococcus luteus (gram positive) showed great sensitivity when exposed to the 27-kDa peptide at a concentration of only 0.5 µg/ml (0.018 µM) (55).

NK-lysin was shown to have antimicrobial activity against several strains of gram-negative and gram-positive bacteria, the highest activity being against E. coli and Bacillus megaterium. In assays for hemolytic activity against sheep red blood cells, no hemolysis was detected at concentrations of NK-lysin of 170 µM. The peptide also exhibited antitumor activity in assays against YAC-1 tumor cells (lymphoma cells from the lung) (4). In addition, it has been shown that NK-lysin (1–100 nM) potently and reversibly stimulates insulin secretion in rat pancreatic islets and in the β cell line HIT T15 (89). However, the effect of NK-lysin was not accompanied by changes in Ca²⁺ concentration. Furthermore, the stimulatory activity of NK-lysin on insulin release was observed in permeabilized islets under Ca²⁺-clamped conditions. These findings indicate that NK-lysin action, although it may interact with the membrane, is unlikely to be due to NK-lysin-formed Ca²⁺-permeable channels.

RK-1, a novel agent related to the corticostatins/defensins and isolated from the kidney, was found to have antimicrobial activity. It was active against E. coli cultures at concentrations between 15 and 150 µg/ml (12). Magainin I and II show antibacterial activity against a range of microbes, including both gram-positive and gram-negative bacteria as well as fungi (90). Magainin II has between 5 and 10 times the antimicrobial activity of magainin I and is more abundant in the secretions of X. laevis. Magainins also have anti-protozoan activity, with lysis of the protozoan Paramecium caudatum occurring on exposure to magainin II at concentrations of 10 µg/ml (90).

Selected magainin analogs with amino acid substitutions aimed at increasing the amphipathic α-helical nature of the molecule have been shown to have increased antimicrobial activity (19). The synthetic peptides had the same spectrum of activity as the natural peptide but were more active by one to two orders of magnitude. Whereas magainin I does not cause hemolysis of erythrocytes at concentrations of 250 µg/ml, the altered analogs (except for analog H) were capable of causing hemolysis at concentrations of 100 µg/ml. Dermaseptin was tested for cytotoxic activity against fungal species (Aspergillus fumigatus and Arthrodema simii) known to be pathogenic to the frog P. sauvagii. The fungal species were inhibited at a dermaseptin concentration of 10 µg/ml. Assays against a gram-positive bacterial species (Bacillus subtilis) showed no inhibition (67). The synthetic peptides that were constructed by Cornut et al. (22) were assayed for hemolytic activity with the use of human red blood cells. Hemolytic activity varied according to the length of the peptide, with the longer peptides being more active. Activity was recorded even at very low concentrations (LD₅₀ = 4–6 × 10⁻⁸ µM). This corresponded to an activity 6–10 times higher than that found for melittin (22). On the basis of this cytotoxic activity, such peptides are suspected to cause alterations in membrane permeability. Generally, it appears that some peptides do this via the formation of ion channels, whereas others may interact with ion channels already present in the membrane.

**CYTOTOXINS AS FORMERS OF ION TRANSPORT PATHWAYS: EVIDENCE FOR ION CHANNEL FORMATION**

**Ion Flux Experiments**

Ion flux experiments can be used to help characterize the cytotoxic activity of peptides. These experiments can illustrate the interaction of these molecules with cell membranes and the consequent changes in membrane permeability and ion homeostasis. Cocianch et al. (20) demonstrated that the insect defensin A had antimicrobial activity and that this was mediated through the efflux of K⁺ from cells. They tested a synthetic peptide, corresponding to the sequence of defensin A from P. terranovae, for antimicrobial activity against M. luteus. Cells exposed to defensin A had immediately disrupted membrane permeability, resulting in loss of cytoplasmic K⁺. The effect of defensin was decreased in growth media of high ionic strength and maximal at pH 7.5. The cells maintained a membrane potential of 110 mV, indicating the peptide did not completely disrupt the membrane. Instead, Cocianch et al. (20) suggested, the defensin formed an ion channel that resulted in K⁺ transport.

Lycotoxin I was found to cause an efflux of Ca²⁺ from rat brain synaptosomes and to dissipate the membrane potentials of insect muscle cells (87). Addition of lyctoxin I to Ca²⁺-loaded rat synaptosomes resulted in dissipation of the Ca²⁺ gradient, mediated through an efflux of Ca²⁺ from the synaptosome, and the prevention of Ca²⁺ sequestration. Similarly, the membrane potentials of insect muscle cells were dissipated when the cells were exposed to lycotoxin I at a concentration of 3 µM. In combination with the antimicrobial data, these factors constitute indirect evidence that lycotoxins act as pore-forming peptides (87). However, detailed characterization of these potential pores, including ion channel kinetics, selectivity, and conductance, was not completed.

Florio et al. (36) report that the PrP-(106–126) fragment induces apoptosis in GH3 cells via dose-dependent inactivation of the L-type voltage-sensitive
Ca\textsuperscript{2+} channels. The blockage of these channels resulted in blockage of the increase in cytosolic Ca\textsuperscript{2+} following depolarization and resulted in cell death with features of apoptosis. They speculated that alterations in Ca\textsuperscript{2+} homeostasis may be the trigger for apoptosis. In contrast, Lin et al. (58) found that PrP-(106–126) in planar lipid bilayers induced the formation of ion channels that were permeable to common physiological ions. It was proposed that the channels would cause leakage of ions across the membrane of cells, resulting in disturbance of ion balance. This could lead to a large metabolic demand being placed on the cells with attempts to correct the ion balance. Disturbance of ion balance could also lead to ionic toxicity and might trigger apoptosis. Discharge of membrane potential, due to ion imbalance, could alter cellular Ca\textsuperscript{2+} levels (via voltage-dependent Ca\textsuperscript{2+} channels, N-methyl-D-aspartate receptors, or alteration in Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange mechanisms), which may trigger apoptosis (58, 69).

More recently, Lin et al. (58) used a combined immunofluorescence labeling with an antibody raised against the NH\textsubscript{2}-terminal domain, and atomic force microscopy, to image the structure of AβP-reconstituted phospholipid vesicles, whose \textsuperscript{45}Ca\textsuperscript{2+} uptake was measured to assess the Ca\textsuperscript{2+} permeability across the vesicular membrane. Their findings suggest that globular and fresh AβP-(1–40) forms Ca\textsuperscript{2+}-permeable channels. For example, \textsuperscript{45}Ca\textsuperscript{2+} uptake was inhibited by a monoclonal antibody raised against the NH\textsubscript{2}-terminal region of AβP and by Zn\textsuperscript{2+}, a known blocker of AβP-formed channels (58).

Villus Enterocyte Volume Assay

The villus enterocyte volume assay is used to assess the activity of peptides on villus enterocytes. Because these cells use movement of ions across the cell membrane to regulate their volume, cell volume can be used as an indicator of ion movement (59, 60). The movement of water in response to ion flux contributes to any volume change. A change in cell volume on incubation with a peptide suggests that the peptide has altered the cell membrane permeability and ion balance. This assay has been used to demonstrate membrane permeabilization effects of peptides belonging to the corticostatin/mammalian defensin family of molecules (cryptdins, RK-1, corticostatic peptides/defensins).

MacLeod et al. (60) demonstrated the effects of the family of corticostatic defensin peptides and defensins on villus enterocytes. The peptides, when incubated in isosmotic conditions with enterocytes from guinea pig jejunums, caused a reduction in cell volume. They suggested that the reduction in volume was due to activation of L-type Ca\textsuperscript{2+} channels (60). By using the villus enterocyte volume assay, it was shown that RK-1, like the corticostatin/defensins, causes enterocyte shrinkage of epithelial cells in isotonic media. This shrinkage occurred in both the presence and absence of Na\textsuperscript{+} and was prevented by the use of Ca\textsuperscript{2+}-free media. Niguldipine prevented the volume reduction caused by RK-1. Activation of dihydropyridine-sensitive Ca\textsuperscript{2+} channels is implicated in the shrinkage of the cells (12).

The cryptdins, or enteric defensins, are secreted by cells lining the mammalian gut and have been found to form anion-conductive channels in human intestinal T84 cells in vitro (56). Use of the villus enterocyte volume assay to assess ion movement across the cell membrane showed that the cryptdins elicited a Cl\textsuperscript{−} secretory response. The secretory response was not attributable to receptors or ion channels already present in the cells and was correlated with the formation of new anion-permeable channels in the cell membrane. The pores formed were permeable to carboxyfluorescein.

Electrical Properties

The short-circuit current (I\textsubscript{sc}) method was used to investigate the actions of cryptdins. Cryptdins 2 and 3 caused Cl\textsuperscript{−} secretion when applied to the apical membranes of T84 cells. Both caused an I\textsubscript{sc} response at concentrations of 40 µg/ml, which was reversible by washing off the peptides from the apical reservoir (56). The literature indicates that the respective COOH terminals, NH\textsubscript{2} terminals, and centers of several peptides could all be capable of forming channels. Information regarding the interaction of such peptides with biological membranes is very extensive (25). However, because biological membranes are heterogeneous and dynamic structures, evaluation of the interaction between peptides’ domains and these membranes is very difficult. For example, it is not always clear whether the effects of a peptide on membrane physiology are due to interaction with receptors, ion transport mechanisms, the formation of ion channels, or to some combination of these. For these reasons the strategy of studying the interaction of these peptides with membranes is to use bilayer membranes that, in contrast to biological membranes, lack any receptors or ion transport proteins and permit both control of the phospholipid composition of the membrane and determination of a peptide’s ability to form or induce ion channel activity. However, the artificial lipid bilayer technique has the usual limitations of in vitro approaches, and the findings must ultimately be verified in vivo.

Most experiments to characterize ion channels formed by cytotoxic peptides have used the patch-clamp or lipid bilayer techniques (Table 1). Ion channel experiments directly illustrate the capacity of peptides to form ion-permeable channels. Relatively few peptides known to have cytotoxic activity have been proven to form ion channels in such experiments. Those that do form ion channels include insect defensin (20), melittin (71, 72), C. carpio peptides (55), the magainins (24, 31), mammalian defensin rabbit NP-1 (43), AβP-(1–40) (9), amylin (65), and PrP-(106–126) (58). Some of these channels have also been further characterized to determine their conductance, current-voltage relationships, and ion selectivities.

The characteristics of ion channels formed by cytotoxic peptides vary considerably. For example, magainin I forms anion-selective channels (31) and ma-
<table>
<thead>
<tr>
<th>Cytotoxin Type</th>
<th>Channel Type</th>
<th>Method</th>
<th>[Ca(^{2+})](_{cis/\text{trans or Bath/Pipette}}) (in mM)</th>
<th>Single Channel Conductance (pS)</th>
<th>Current-Voltage and Slope Conductance</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Toxins</strong></td>
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<td>Melittin</td>
<td>Anion selective</td>
<td>Lipid bilayer technique, DOPC lipid bilayers</td>
<td>1.8 M NaCl (10 mM Tris, pH 7)</td>
<td>-110 pS</td>
<td>Linear, frequency of opening and lifetime of pore increased with voltage</td>
<td>Pawlak et al. (72)</td>
</tr>
<tr>
<td>Rabbit neutrophil peptide (NP-1)</td>
<td>Permeable to Cl(^{-}), Na(^{+}), and K(^{+})</td>
<td>Planar lipid bilayer technique PC:PE:PS 2:1 wt/wt/wt</td>
<td>50 mM KCl, 1 mM MgCl(_2) pH 7.0</td>
<td>Heterogeneous 10–1,000 pS with membranes held at –80, –90, and –100 mV</td>
<td>Steady-state conductance increases exponentially at negative voltage</td>
<td>Kagan et al. (43)</td>
</tr>
<tr>
<td>Human cryptdin 2</td>
<td>Cl(^{-})</td>
<td>Short-circuit current</td>
<td>1 M KCl</td>
<td>Heterogeneous values in the range of 100–200 pS</td>
<td>Active at high absolute voltage; not active at low absolute voltages</td>
<td>Lenmaitre et al. (55)</td>
</tr>
<tr>
<td>Human cryptdin 3 27-kDa antibacterial toxin skin mucosa of carp (Cyprinus carpio)</td>
<td>Cl(^{-})</td>
<td>Lipid bilayer technique 7:3 PC:PE</td>
<td>1 M KCl</td>
<td>Heterogeneous values, 1–300 pS, unitary single-channel value given as 1–2 pS</td>
<td>Active at high absolute voltage; not active at low absolute voltages</td>
<td>Cruciani et al. (24)</td>
</tr>
<tr>
<td>Human cryptdin 3 31-kDa antibacterial toxin skin mucosa of carp</td>
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<tr>
<td>Xenopus laevis Magainin I</td>
<td>Anion selective</td>
<td>Artificial planar lipid bilayers 7:3 POPC:DOPE, patch-clamp pipettes</td>
<td>Various solutions, single-channel data in symmetrical 600 KCl</td>
<td>Heterogeneous values, 1–300 pS, unitary single-channel value given as 1–2 pS</td>
<td>Active at high absolute voltage; not active at low absolute voltages</td>
<td>Duclohier et al. (31)</td>
</tr>
<tr>
<td>Magainin II</td>
<td>Cation selective</td>
<td>Planar lipid bilayer technique 1:1 PS:PC</td>
<td>Various solutions, single-channel data in symmetrical 600 KCl</td>
<td>Heterogeneous values, 1–300 pS, unitary single-channel value given as 1–2 pS</td>
<td>Active at high absolute voltage; not active at low absolute voltages</td>
<td>Cruciani et al. (24)</td>
</tr>
<tr>
<td>Insect defensin (Phormia terranovae)</td>
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<td><strong>Autocytotoxins</strong></td>
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<tr>
<td>PrP-(106–126)</td>
<td>Permeable to physiological ions</td>
<td>Planar lipid bilayer technique PC, 45% final concentration of PrP-(106–126)= 20 µM PrP-(106–126) = 1 µM Aged PrP-(106–126) (9 days) = 0.1 µM</td>
<td>100 NaCl, 1 MgCl(_2), 5 HEPES adjusted to pH 7.5</td>
<td>Heterogeneous channels: single-channel conductance 20, 40, and 60 pS; voltage held at +50 mV</td>
<td>Current-voltage relationship is linear</td>
<td>Lin et al. (58)</td>
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<tr>
<td>PrP-(106–126)</td>
<td>Blockade of L-type voltage-sensitive Ca(^{2+}) channels</td>
<td>Wholecell patch clamp technique using GH3 cells</td>
<td>Extracellular: 120 tetraethylammonium chloride, 20 CaCl(_2), 5 MgCl(_2), 10 glucose, 10 HEPES, 4.4-aminopyridine, pH adjusted to 7.4; Intracellular: 124 CsCl, 10 Tris·HCl, 5 BaCl(_2), 3 ATP, 10 HEPES, pH adjusted to 7.3</td>
<td>Heterogeneous channels: single-channel conductance peaks at 10, 20, 30, 40, and 60 pS; voltage held at –50 mV</td>
<td>Channel activity increased; single-channel conductance increases at 10, 20, 30, 40, and 60 pS; voltage held at –50 mV</td>
<td>Florio et al. (36)</td>
</tr>
<tr>
<td>Human amylin</td>
<td>Permeable to Na(^{+}), K(^{+}), Ca(^{2+}), and Cl(^{-})</td>
<td>Planar lipid bilayer technique, bilayers of varying compositions</td>
<td>10 KCl, 3 Tris·HCl pH 7.4</td>
<td>7–8 pS</td>
<td>Linear for the open state</td>
<td>Mirzabekov et al. (65)</td>
</tr>
</tbody>
</table>

[Ca\(^{2+}\)], Ca\(^{2+}\) concentration; DOPC, dioleoylphosphatidylcholine; PC, palmitoyloleoylphosphatidylcholine; PE, palmitoyloleyo/phosphatidylethanolamine; PS, palmitoyl/oleoylphosphatidylserine; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; DOPE, dioleoylphosphatidylethanolamine; PrP-(106–126), prion protein fragment 106–126.
gainin II forms cation-selective channels (24). Rabbit NP-1, a mammalian defensin, forms voltage-dependent, weakly anion-selective channels (43). AβP formed cation-selective channels (6, 9), and human amylin was also found to form voltage-dependent channels that are permeable to Na\(^{+}\), K\(^{+}\), Ca\(^{2+}\), and Cl\(^{-}\) (65). Prion peptide segment PrP-(106–126), which was reported to block L-type voltage-sensitive Ca\(^{2+}\) channels (36), was found to form nonselective ion channels (58). Insect defensin was shown to form cation-selective voltage-dependent channels in giant liposomes (20), and C. carpio peptides form ion channels of differing selectivities (55). The details of these investigations and the characterization of the ion channels appear below and in Tables 1 and 2.

**ION CHANNEL CHARACTERISTICS OF CYTOTOXIC PEPTIDES**

Conditions Influencing the Interaction of Cytotoxic Peptides With Membranes

Various factors, e.g., lipid composition, voltage, pH, and Ca\(^{2+}\), influence the interaction of cytotoxic peptides with cell membranes (e.g., see Ref. 40). Most of the cytotoxic peptides are positively charged, and thus it is expected that the negative phospholipids may affect any interaction between them. Specifically, the effects of different levels (or even the absence) of the negatively charged phospholipid (PS) in bilayers on the channel activity need to be examined. The studies of the interaction of cytotoxic peptides with membranes could both reveal the conditions that may make the cell membranes susceptible to enhanced interaction of cytotoxic peptides and indicate how this susceptibility can be overcome. It is already known that voltage and acidic conditions are required for the domains of some peptides to dock onto and interact with the bilayer to form ion channels, as suggested for antimicrobial colicin E1 (63) and Bcl-2-formed channels (64). The role of acidic solutions in peptide aggregation and β-sheet formation underlying ion channel formation has also been invoked for the PrP-(106–126)-formed channel (58) and AβP-formed channels (40). It appears that the aggregation of this peptide can be disrupted with Congo Red treatment and therefore prevent formation of the channel (40).

The conductance properties and the experimental conditions for the cytotoxic peptide-formed channels are shown in Table 1. The phospholipid composition (Table 1) of bilayers used to investigate ion channel formation by cytotoxic peptides can be important. Although some peptides (e.g., NP-1) have been shown to form ion channels in membranes composed of a wide range of lipids and to have no specific lipid requirements, many peptides require a certain content of negatively charged lipids in the membrane. In some instances the characteristics of the channels formed in membranes of different composition are different (e.g., magainin I).

Kagan et al. (43) used membranes composed of PE, phosphatidylcholine (PC), and PS in a ratio of 2:2:1 (wt/wt/wt) when testing the ability of NP-1 to form ion channels. In addition to forming ion channels in this type of membrane, they found that NP-1 was able to form ion channels in membranes composed of different mixtures of these lipids, indicating that membrane composition did not affect activity (43).

For ion channels formed by magainin I, the channel amplitude was altered by membrane composition. Membranes composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)/1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (7:3) favored conductance levels of 80 pS, considerably lower than the conductance levels determined in membranes composed of POPC/1,2-dioleoyl-3-phosphatidylethanolamine (7:3; 366 and 683 pS) (31). Magainin II ion channel formation requires the presence of negatively charged lipids in the membrane (24). Ion channel formation, as measured by increased membrane conductance, was demonstrated in membranes composed of PS/PC or PS/PE, but not in those containing only PE or PE/PC combinations.Crudiani et al. (24) speculated that the type of lipids present in the membrane helped determine the ion selectivity of the magainin II ion channel. Thus when magainin II interacts with membranes containing negatively charged lipids, ion channels selective for cations are generated. Conversely, membranes that do not contain negatively charged lipids are unable to support ion channel formation. Ion channel formation by magainin I was similarly dependent on the lipid composition of the membrane: membranes composed of PE/PS supported channel formation, whereas those composed of PE alone or PE/PC did not induce channel formation, indicating a requirement for negatively charged lipids in target membranes. Membrane composition altered the channel-forming activity of amylin. Membranes that had a high net negative surface charge induced the highest channel-forming activity. Membranes that contained 40% negatively charged phospholipids were approximately six times more sensitive to amylin channel formation than those containing only 20% negatively charged amino acids (65). Increasing membrane rigidity by including cholesterol causes a reduction in the membrane's sensitivity to amylin.

In some investigations, the reversibility of ion channel formation has been tested. The association of amylin with the membrane appears to be irreversible: extensive washing of the side of the membrane that contained the amylin failed to reduce or eliminate the current (65). Similarly, washing of the compartment containing the prion peptide fragment did not affect conductance, indicating that the channels formed were irreversibly associated with the membrane (58).

For several peptides, channel formation occurs only when the membrane is held at a negative voltage. This reflects the state of the membrane as it would “appear” to such peptides in vivo. For example, ion channels formed from magainin I appeared only when the membrane was held at a negative voltage (31). Similarly, Kagan et al. (43) found that the steady-state conductance could be induced after negative voltages (−70 to −90 mV) were applied to the membrane for 15–30 min, and increased exponentially with increasingly negative voltage. The channels formed by AβP-(25–35) are volt-
Table 2. Summary of AβP-formed ion channels

<table>
<thead>
<tr>
<th>Peptide/Channel Type, and Ionic Selectivity</th>
<th>Method</th>
<th>Characteristics, Single-Channel Conductance (pS), Substrates, K_{m} (mM), and γ_{max} (pS)</th>
<th>Current-Voltage and Slope Conductance</th>
<th>P_{o}</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AβP-(25–35) cation selective</td>
<td></td>
<td></td>
<td>Voltage independent; linear and slope conductance of 1 nS</td>
<td></td>
<td>Hirakura et al. (40)</td>
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<tr>
<td>AβP-(1–40)</td>
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<tr>
<td>AβP-(1–42) slightly cation selective</td>
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<tr>
<td>AβP-(1–40) cation-selective channels</td>
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<tr>
<td>AβP-(1–40) single multiconductance channel</td>
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<tr>
<td>AβP-(1–40) cation selective</td>
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<td>AβP-(1–40)</td>
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</table>

AβP, amyloid β-protein; γ_{max} maximal single-channel conductance (s); P_{o}, probability of the channel being open; E_{rev}, reversal potential; K_{m}, Michaelis-Menten constant; DGPC, deoxyguanidinoprocolavaminic acid; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine.
age dependent, opening when the opposite side of the membrane is made negative with respect to the AβP-(25–35)-containing side and closing when the polarity of the voltage is reversed (66). This is taken to indicate that AβP-(25–35) channels would open at the resting membrane potential of neurons if the peptides were located in an extracellular or endosomal/lysosomal compartment.

The variations in conductance and kinetic properties of cytotoxic peptide-formed channels, e.g., AβP-formed channels (6, 9, 40) reflect the differences in 1) peptide properties, e.g., isoforms, the ratio of α-helices to β-sheets, homogeneity, and aggregation; 2) bilayer properties, e.g., phospholipids’ composition, presence of solvent, bilayer capacitance, and stability; 3) experimental solutions, e.g., ionic composition, ionic concentrations and gradients, buffers, pH, and Ca²⁺ levels; and 4) recording conditions, e.g., voltage, gain, recording duration, and sampling rates.

Conductance and Current-Voltage Relations

The ability of a peptide to form ion channels can be further investigated by the demonstration of an increase in membrane conductance. In addition, the current-voltage relationship for the peptide-associated membranes can be determined (Table 1). Lemaitre et al. (55) isolated two hydrophobic peptides with channel-forming properties from the skin mucus of carp (C. carpio). The two peptides induced strong current fluctuations in lipid bilayers (7:3 PC/PE). The 27-kDa peptide gave a conductance value of 900 pS in 1 M KCl (at +50 mV) and the 31-kDa peptide gave a conductance value of 500 pS (1 M KCl, and at +40 mV). Lemaitre et al. (55) state that these conductances were higher than those found previously for toxins or outer membrane proteins from bacteria (41) and higher than those of insect defensins (72); conductances previously determined for antimicrobial peptides from amphibians are closer in value (31).

Kagan et al. (43) found that single-channel conductance values were heterogeneous, ranging between 10 and 1,000 pS. Conductance increased with NP-1 concentration, suggesting that a multimer of NP-1 may form the ion channel. Conductance also consistently increased with time, which may be due to the formation of larger channels with an increased number of the NP-1 molecules per channel. Magainin I ion channels were shown to exhibit two possible conductance levels (366 and 683 pS). Each occurred at approximately equal rates but tended not to occur in the same experiment (31). Conductance was dependent on peptide concentration, with increased macroscopic conductance occurring with increased peptide concentration. Greater concentrations of peptide resulted in increased current amplitude. This is very likely to be due to the recruitment of more subunits of the channel-forming peptide. The current-voltage relationships for channels of both conductance levels were nonsaturable and ohmic (31).

Cociancich et al. (20) demonstrated that insect defensin formed ion channels in giant liposomes. Peptide samples were incorporated into giant liposomes composed of asolectin, and patch-clamp techniques were used to determine the single-channel conductance of ion channels thus formed. Insect defensin was found to form channels of heterogeneous conductance values (ranging from 100 to 200 pS). The channels also had varying open and closed times. The current through the insect defensin-formed channels increased at high absolute membrane voltages. At potentials more positive than +27 mV and more negative than −45 mV, the frequency of current transitions between the main open state and the closed state also increased.

The channels formed by PrP-(106–126) (58) were found to have heterogeneous conductance values, with the most common single-channel conductance values being 20, 40, and 60 pS (membrane voltage held at +50 mV). The channels were voltage dependent and the current-voltage relationship was linear. Lin et al. (58) stated that the concentration at which ion channels formed (>20 µM) was similar to that which causes neurotoxicity (37) and also comparable to the concentrations required for channel formation by other ion-channel-forming peptides (58). Channel activity was increased at acidic pH and also after aging of the peptide under adverse conditions (detailed below in CYTOTOXIC CHANNELS UNDER ADVERSE CONDITIONS). Amylin was shown to cause an increase in membrane conductance, with single-channel conductance determined as 7–8 pS. The current-voltage relationship for the open state of the channel was ohmic (65).

The properties of AβP-formed channels are summarized in Table 2. The conductance of the AβP-formed channels can be as high as 5 nS (9), with multiconductance properties (Fig. 7) (see also Refs. 7, 44, 73). AβP-(1–40) channel activity resulting from the incorporation of the peptide from the solution into an inside-out excised patch showed spontaneous transitions between different current levels, which is characteristic of multiconductance channels.

The steady-state conductance of the cell membrane of bullfrog sympathetic neurons after the application of AβP-(25–35) was examined (77). The current was linear between −110 and +50 mV, and the reversal potential was close to 0 mV. There was a time lag between the time of application of AβP-(25–35) and the observed increase in conductance. The time lag was
shorter when higher concentrations of AβP-(25–35) were used. The magnitude of the conductance increase, however, was not dependent on the concentration of AβP-(25–35).

Recently, Hirakura et al. (40) reported that AβP-(1–42) incorporation into anionic planar bilayer membrane also forms slightly cation-selective, voltage-independent ion channels with multiple conductance levels at neurotoxic concentrations in acidic solutions. The channels show substantial irregularity of activity, and the size of conductances and the length of open lifetimes depend on solvent history. These properties are in agreement with the suggestion that these channels are very likely to be formed by aggregates of AβP-(1–42). The pharmacology of AβP-(1–42)-formed channels is in agreement with other AβP-formed channels, in that the channels are reversibly blocked by Zn$^{2+}$ in a voltage-independent manner.

**Cation/Anion Permeability and Selectivity Sequence**

Cytotoxic peptides and other toxins often exert their effects by inducing nonselective or weakly selective anion or cation channels that depolarize membrane potential and dissipate ionic and water gradients, leading to cell lysis and death. Some of these peptides may also function by allowing the movement of Ca$^{2+}$ across the membrane, leading to changes in the Ca$^{2+}$ homeostasis that underlies muscle contraction and relaxation, cell volume regulation, and salt secretion of epithelial cells and neuron synapses.
The channels formed from cytotoxic peptides may be expected to be selective for anions over cations because of the cationic nature of most of these peptides. However, both anionic and cationic channels, as well as nonselective channels, have been reported (Table 3). Interestingly, more molecules have been shown to form cation-selective channels than either nonselective or anion-selective channels. This has implications for the models of channel formation proposed, suggesting that the more simplistic models that predict anion selectivity may not reflect the actual structure of the channels that form.

Channels formed from rabbit NP-1 showed weak anion selectivity, favoring Cl⁻ over Na⁺ and K⁺, but this was not exclusive (P_{Cl}/P_{Na} = 2.4:1) (43). Similarly, as expected from its overall positive charge, the magainin I molecule forms anion-selective channels in artificial planar membranes (P_{Cl}/P_{K} = 3) (31). In contrast to the results obtained for magainin I by Duclo­hier et al. (31), magainin II, also a positively charged peptide, was found to form cation selective channels (P_{Na}, P_{K}/P_{Cl} = 5:1) (24). The ion channel formed by magainin II showed no difference in conductance for different monovalent cations (Na⁺, K⁺, Li⁺) or monovalent anions (e.g., Cl⁻, I⁻) tested. Cruciani et al. (24) also reported results for magainin I, which indicated that it also formed cation selective channels, contradicting previous findings (31).

Cociancich et al. (21) showed that the antimicrobial activity of insect defensins was caused by efflux of K⁺ from the cytoplasm of cells. In addition, they demonstrated that the peptide formed ion channels in giant liposomes. These results imply that the channels formed by the insect defensin were responsible for the K⁺ movement and thus selective for cations, in particular K⁺. They do not, however, report data that directly demonstrate the cationic selectivity and sequence.

Channels formed by PrP-(106–126) exhibited cationic selectivity (P_{Na}/P_{Cl} = 2.5) and were permeable to most physiological ions. The sequence of ion permeability was Ca²⁺ > Na⁺ > K⁺ > Li⁺ > Rb⁺ > Cs⁺ > Cl⁻. Lin et al. (58) state that the channels were relatively nonselective and sufficiently large to cause cell death via membrane disruption. This leads to the loss of the membrane potential and to changes in membrane permeability that result in altered ion balance and, in particular, altered Ca²⁺ balance, which is a potential trigger for apoptosis.

The two peptides isolated by Lemaitre et al. (55) from the secretions of C. carpio skin showed different ion selectivities. The 27-kDa peptide showed weak cationic selectivity (P_{Cl}/P_{K} ratio = 0.6), whereas the 31-kDa peptide was nonselective (P_{Cl}/P_{K} = 1.0). In addition to the nonselective channel formed by the 31-kDa peptide isolated by Lemaitre et al. (55), channels formed by amylin were relatively nonselective. These channels were demonstrated to be permeable to Na⁺, K⁺, Ca²⁺, and Cl⁻ (65). The AβP channel is permeable to K⁺, Cs⁺, Na⁺, and Li⁺ in the presence of 1 mM Ca²⁺. The permeability sequence of amyloid channels, P_{Cl} > P_{Li} > P_{Ca} > P_{Na}, is thought to be typical of Ca²⁺ channels (9, 73). Also, as expected, Ca²⁺ was able to block the conduction of Cs⁺ through the AβP-(1–40) channels. The AβP-(25–35) channel showed a slight preference for the transport of cations over anions. The permeability ratio was Ca²⁺:K⁺:Na⁺:Cl⁻ = 5.4: 1.6: 1.4: 1 (66).

### Kinetics

Very little is known about the kinetics of channels formed by cytotoxic peptides. The channels formed by insect defensin in giant liposomes (20) showed variable kinetics, with heterogeneous values for the open and closed times as well as the single-channel conductance values. Single events of channels formed by mamma­lian defensin NP-1 were also heterogeneous, but this was not investigated further (43). The channels formed by magainin I were rare and short lived. Duclo­hier et al. (31) report data for one channel only, indicating that the open probability was 0.08 for the channel opening.

### Table 3. Selectivity of cytotoxic peptide-formed channels

<table>
<thead>
<tr>
<th>Cytotoxin Type</th>
<th>Permeability Ratio</th>
<th>Selectivity Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Toxins and antimicrobials</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit NP-1 27-kDa antibacterial toxin skin mucosa of carp</td>
<td>P_{Cl}/P_{Na} = 2.4:1.0</td>
<td>Cl⁻ &gt; Na⁺ but not exclusively so</td>
<td>Kagan et al. (43)</td>
</tr>
<tr>
<td>31-kDa antibacterial toxin skin mucosa of carp</td>
<td>P_{Cl}/P_{K} = 0.6</td>
<td>Weak cation selectivity</td>
<td>Lemaitre et al. (55)</td>
</tr>
<tr>
<td>X. laevis magainin I</td>
<td>P_{Cl}/P_{K} = 3</td>
<td>Nonselective</td>
<td>Duclohier et al. (31)</td>
</tr>
<tr>
<td>X. laevis magainin II</td>
<td>P_{Cl}/P_{K}/P_{Cl} = 5:1</td>
<td>No conductance change with different anions (e.g., Cl⁻, I⁻) and cations (Na⁺, K⁺, Li⁺) tested</td>
<td>Cruciani et al. (24)</td>
</tr>
<tr>
<td><strong>Insect defensin</strong></td>
<td></td>
<td>Data indirectly supports cation selectivity, in particular K⁺ selectivity</td>
<td>Cociancich et al. (21)</td>
</tr>
<tr>
<td><strong>Autocytotoxins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PrP-(106–126)</td>
<td>P_{Na}/P_{Cl} = 2.5</td>
<td>Ca²⁺ : Na⁺ : K⁺ : Li⁺ : Rb⁺ : Cs⁺ : Cl⁻</td>
<td>Lin et al. (58)</td>
</tr>
<tr>
<td>AβP-(1–40)</td>
<td>P_{Cl}/P_{Cl} &lt; 11</td>
<td>Cs⁺ : Li⁺ : Ca²⁺ : K⁺ : Na⁺</td>
<td>Arispe et al. (6)</td>
</tr>
<tr>
<td>Human amylin</td>
<td>Poor selectivity, favoring cations</td>
<td></td>
<td>Mirzabekov et al. (65)</td>
</tr>
</tbody>
</table>
at the 360-pS level. The mean lifetime of the open state of this channel was 100 ms. These data are rather incomplete, given that they refer to a single-channel experiment only. The kinetics of channels formed by melittin have not been extensively investigated. However, it has been shown that frequency of opening and lifetime of the pore increases with voltage (72). Kawahara et al. (44) found that the probability of the channel being open (P_o) of an AβP-(1–40) channel incorporated from solution into an inside-out excised membrane patch was unaffected by a change in the transmembrane potential from −40 to +40 mV, remaining at ~0.8. The frequency of transitions between current levels changed at different transmembrane potentials. For instance, the P_o at the lowest level (~0.69 pA) decreased from 0.7 at +40 mV to 0.4 at +10 mV.

Pharmacology

Another area in which little research has been undertaken is that of the pharmacology of the channels formed by cytotoxic peptides. Further research would obviously be required, since knowledge of organic and inorganic blockers and inhibitors of channels would not only help to further characterize the channels but could possibly lead to treatments for conditions caused by such peptides. The efflux of K^+ caused by the action of defensin on M. luteus cells, was blocked by prior treatment of the cells with divalent cations. In addition, exposure of cells already affected by defensin to divalent cations resulted in cessation of the K^+ efflux (20). This suggests that divalent cations block channels already formed in addition to preventing new channels from forming. Cociancich et al. propose that the formation of ion channels is blocked by preventing the initial interaction of the peptide with the membrane. This occurs via the interaction of divalent cations with membrane lipids, which causes the negative charge of the membrane to be masked. They propose that inactivation of already formed channels occurs either via a direct interaction of divalent cations with channels or via changes in the interactions of the lipids with the peptide (mediated by the presence of divalent cations) that result in changes in the ion channel structure (20).

High ionic concentration has been shown to reduce the channel-forming activity of amylin (65) and insect defensins (20). High ionic concentrations may mask the negative charge on membranes in a manner similar to that reported for divalent cations and insect defensin. For peptides thought to interact with ion channels already in the target membrane, the action of pharmaceutical agents can help to identify the specific ion channels involved (28, 75). This possibility has not been fully investigated in this report, which instead emphasizes peptides that form ion channels. It is important to note that the channel hypothesis for a peptide-induced cytotoxicity is not inconsistent with other cytotoxic effects via modification of intrinsic ion channels (51), changes in Ca^{2+} homeostasis (38), and oxidative stress (83, 88). However, two examples are of note because of differing ideas about their actions in the literature.

Florio et al. (35) showed that the action of PrP-(106-126) was a result of its actions on L-type voltage-sensitive Ca^{2+} channels. The prevention of its action by nicardipine, which blocks L-type voltage-sensitive Ca^{2+} channels, gave support, as did the prevention of its action by the use of Ca^{2+}-free conditions (35, 36). In light of this finding, it would be interesting to investigate the action of nicardipine on prion peptide-formed channels (58). Mammalian defensins were found to form ion channels by Kagan et al. (43). RK-1, a molecule thought to be related to the mammalian defensins, was found to cause a decrease in cell volume in the villus enterocyte volume assay. This action was prevented by treatment of the cells with nifuldin, which blocks dihydropyridine-sensitive L-type Ca^{2+} channels, therefore implicating the L-type Ca^{2+} channels in the action of this peptide (12). Other defensins have been shown to act in a similar manner in this assay (60), but the two actions (cell volume reduction via actions on preexisting channels and channel formation) do not seem to have been reconciled in the literature. Ion channel blockers, organic and inorganic, have also been used to examine the inhibition of the AβP-formed channels (Table 4). The weak base Tris is able to block the AβP-(1–40) channel (9). Although 1–2 mM Tris completely blocked the large channels (<400 pS), it had very little effect on the giant channels (>400 pS). The L-type Ca^{2+}-channel-blocking drug nitrindipine is ineffective in blocking AβP-(1–40) channels. Similarly, the compound Cognex (tetrahydroacridine), an inhibitor of acetylcholinesterase, was ineffective (at 100 µM) in blocking Cs^{2+} permeation of AβP-(1–40) channels (73). Unlike Tris, aluminum inhibits AβP channel current irreversibly (micromolar range). The blockade of the AβP channel current by aluminum and Tris depends on transmembrane potential and the dose of aluminum (9). Similarly, the blockade of the AβP channel (<400 pS) is dependent on Zn^{2+} concentration. The block is characterized by an increase in the frequency of flickering. However, Zn^{2+}-induced channel block is reversible by the Zn^{2+} chelator o-phenanthroline (8). It is suggested that the uniform frequency of the transitions between conductance levels indicates that the Zn^{2+}-AβP interactions occur at the mouth of the AβP channel where the electric field is thought to be relatively constant (8). The AβP-(25–35) channel is also blocked reversibly with micromolar concentrations of Cu^{2+} or Cd^{2+} (66).

**SPECIALIZED REGIONS OF PROTEIN STRUCTURE AND PROPERTIES OF FORMED ION TRANSPORT PATHWAYS**

Only a small amount of research has been completed on the regions of the cytotoxic peptides that are important for channel formation. The NH₂ terminus was found to be the important region of pilosulin 1 for cytotoxic activity. This was determined by incubation of pilosulin 1 fragments with EBV-transformed cells. Results indicated that partial peptides, corresponding to amino acid residues 1–22 and 11–56, retained some cytotoxic activity, although this was reduced compared with
pilosulin 1. Other peptide fragments (23–56, 37–56, and 47–56) had no cytotoxic activity, indicating that pilosulin 1 requires the NH$_2$ terminus for cytotoxicity. Partial peptide 1–22 was shown to interact with unilamellar membranes, whereas peptide 23–56 did not, suggesting that the NH$_2$ terminus is required for membrane association rather than lytic activity (86). Because fragment 1–22 had reduced cytotoxic activity, the domain responsible for cell lysis is likely to extend further than residue 22.

Cuervo et al. (26) determined that the first 12 residues of the magainin molecules were necessary for antimicrobial activity. Adding to this work, Zasloff et al. (90) used truncated forms of the magainin II molecule to determine the amino acids necessary for antimicrobial activity. They found that removing amino acids from the NH$_2$ terminus of the peptide reduced antimicrobial activity. The first three amino acids could be removed with minimal effect on activity, but shortening the peptide to 19 amino acids in length, by removing the lysine at position 4, resulted in markedly reduced antimicrobial activity. Further truncation of magainin II resulted in virtually inactive molecules. They suggested that the loss of activity correlated with an inability of the truncated peptide to span the membrane. In addition, Zasloff et al. (90) investigated the activity of a peptide that had a single amino acid (serine) removed from the COOH terminal.

### Table 4. Blockers of AβP-formed ion channels

<table>
<thead>
<tr>
<th>AβP Type</th>
<th>Pharmacological Agents</th>
<th>Inorganic Blockers (in mM)</th>
<th>Mechanism of Inhibition by Inorganic Blockers: Single-Channel Conductance, Kineti</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AβP-(1–42)</td>
<td>14 µM [Congo Red]$_{cis}$, blocked channel formation</td>
<td>250 µM [Zn$^{2+}$]$_{cis}$, block is voltage independent</td>
<td>Hirakura et al. (40)</td>
<td></td>
</tr>
<tr>
<td>AβP-(1–40)</td>
<td>No channel block with Tris</td>
<td>50–500 µM [Zn$^{2+}$]$<em>{cis}$ or [Zn$^{2+}$]$</em>{trans}$ blocked AβP-(1–40) channel</td>
<td>Kawahara et al. (44)</td>
<td></td>
</tr>
<tr>
<td>AβP-(1–40)</td>
<td>Gating and conductance modulated by 250–500 µM Zn$^{2+}$ at −40 mV (EC$_{50}$ 300)</td>
<td>At low conductance &lt;400 pS increased F$<em>{o}$ to &gt;88 pS; decreased P$</em>{o}$ for the 225-pS conductance (0.5 control = 0.5 and Zn$^{2+} = 0.14$)</td>
<td>Arispe et al. (8)</td>
<td></td>
</tr>
<tr>
<td>AβP-(1–40)</td>
<td>Tris</td>
<td>10 µM [Al$^{3+}$] blocked conductance</td>
<td>Pollard et al. (73)</td>
<td></td>
</tr>
<tr>
<td>AβP-(1–40)</td>
<td>Large channels (&lt;400 pS) blocked completely by 1–2 mM Tris; giant channels (&gt;400 pS) minimally affected</td>
<td>Increasing [Ca$^{2+}$] from 1 to 10 mM in cis blocks Cs$^{+}$ flow</td>
<td>Durell et al. (32)</td>
<td></td>
</tr>
<tr>
<td>AβP-(1–40)</td>
<td>Blocked by 25 mM Tris when conductance &lt;400 pS; less effective in ns range</td>
<td>Increasing [Ca$^{2+}$] from 1 to 10 mM in cis blocks Cs$^{+}$ flow (voltage-dependent blockade of channel activity)</td>
<td>Arispe et al. (6, 7, 9)</td>
<td></td>
</tr>
<tr>
<td>AβP-(25–35)</td>
<td>Reversibly blocked by micromolar concentrations of Cu$^{2+}$ or Cd$^{2+}$ added to either side of membrane</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
cated form also exhibited reduced antimicrobial activity. It would be interesting to determine whether the reduced activity of the truncated peptides was due to the nature of the particular amino acids removed or solely to the reduced length of the peptide.

In the cytotoxic peptides discussed, the important amino acids that have been identified are essentially involved in the secondary structure. In particular, positively charged residues (lysine, arginine) are important in providing the overall cationic charge of the peptide (Table 5). They also play a significant role in the amphipathic nature of many of the cytotoxic peptides, a feature thought to be important in channel formation. In particular, as mentioned previously, the arginine residue at position 15 in cryptidins 2 and 3 is thought to be important for the biological activity of these molecules; position 15 is not occupied by an arginine in the inactive peptides cryptidin 1 and 6 (56). There appears to be little information on Ca$^{2+}$ binding sites that could be important for formation and regulation of cytotoxic peptide-formed channels (see Ref. 48). However, there are findings that may indicate that the AβP(1–40) channel gating could become sensitive to the transmembrane potential in the presence of divalent cations (44).

As noted in relation to the structure of cytotoxic peptides, the disulfide bridge plays an important role in the stabilization of the secondary structures of cytotoxic peptides. In particular, disulfide bridges associated with particular consensual structural motifs are found in insect defensins, scorpion defensins, and scorpion toxins (Table 5). Similarly, the mammalian defensins are characterized by a structural motif stabilized by disulfide bonds. Experiments involving the alteration of amino acid residues and consequent peptide features, including channel formation, have been largely limited to investigating the features of truncated peptides. In addition, a small amount of work has been completed that involves the alteration of residues to increase the $\alpha$-helical content of the peptides. This work is described above (19) in Sequence and Structure.

**CYTOTOXIC CHANNELS UNDER ADVERSE CONDITIONS**

Few of the channels formed by cytotoxic peptides have been investigated under varying conditions. This is another area of interest, as the environmental conditions required for activity and the limits placed on the peptides by naturally occurring environments can help us understand the biological activity of these peptides. Environmental parameters that may be of interest and that have been investigated for some peptides include low oxygen levels and low pH. Another interesting parameter, perhaps worth further investigation with other peptides, is the effect of age on the peptides, e.g., AβP and prions (see below). In conditions of high salt concentration, amylin has reduced channel-forming activity (channel-forming activity is 100 times higher in 10 mM KCl than in 1 M KCl) (65). Similarly for insect defensin, high ionic concentration seems to reduce channel-forming activity. As in conditions of high ionic concentration, the K$^+$ efflux, thought to result from channel formation, was reduced (20). As mentioned above, high ionic concentration may act to “mask” the charge of the membrane, reducing the interaction of the peptide with the lipid bilayer and hence reducing channel formation.

Amylin exhibits no change in activity with changes in pH (65). In contrast, changes in pH did affect channel formation by PrP-(106–126): acidic conditions (pH 4.5) increased ion-channel-forming activity. At this pH, ion channels were formed at concentrations as low as 1 µM, with characteristics different from those of channels formed at higher pH values. The most common single-channel conductances recorded at this pH were 20, 100, and 120 pS (58). For insect defensin, the maximal K$^+$ efflux from cells treated with the peptide was recorded when pH = 7.5 (20). Aging of the PrP-(106–126) fragment increased its ion-channel-forming activity (58). Fragments were aged by incubation at room temperature in 100 mM NaCl for up to 9 days. Nine-day-old fragments showed ion channel formation at concentrations of 0.1 µM, a 200-fold increase in activity. The distribution of single-channel conductance values for such samples were different from those of nonaged samples with peaks at 10, 20, 30, 40, 50, and 60 pS. Three-day-old fragments showed a 20-fold increase in channel activity (58).

**PHYSIOPATHOLOGICAL SIGNIFICANCE OF CYTOTOXIC PEPTIDES AS ION-CHANNEL-FORMING PROTEINS**

Little is known about the ion channels formed by these peptides to determine their role in biological systems. However, it is known that these peptides can disrupt ion homeostasis across the membrane and that this leads to cell death. The formation of large nonselective pores could result in a complete loss of ion gradients across the membrane, and it can be envisaged that this would lead to cell death via a combination of pathways, including loss of gradients for ATP production. However, ion channels that are selective for either anions or cations must have a more specific result, which could involve the subsequent activation of other channels in the membrane and loss of ion homeostasis as above, or a more specific action such as a triggering of apoptosis.

The properties of the significant domains and individual residues of the channels can be used as criteria to investigate how these channels function under adverse conditions. Pathological conditions often lead to depolarization of cell membranes and subsequently cause cell death in vivo. Voltage-depolarizing protocols (mimicking membrane depolarization under pathological conditions) can be used to indicate how the cytotoxic peptide-formed channel may function under pathological conditions e.g., hypoxia, low pH, and Ca$^{2+}$ overload. Modulation of the cytotoxic peptide-formed channels, containing cysteine residues, under hypoxia-reperfusion is based on the fact that SH groups and S-S bridges of several ion channels are regulated by oxidation and reduction, oxygen-reactive species, and low oxygen (45). Effects of solutions with different oxygen levels, H$_2$O$_2$, and oxidizing and reducing agents on the function of ion channels can be examined by perfusing the solution in the cis and trans chambers (cytoplasmic and
<table>
<thead>
<tr>
<th>Cytotoxin Type</th>
<th>Length</th>
<th>Amino Acid Sequence</th>
<th>Overall Charge</th>
<th>Secondary Structure</th>
<th>Important Amino Acids</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pilosulin 1</td>
<td>56</td>
<td>GLGSVFKRAGRLGRLKTVKAKRLPKVYKVLRLKKLKLKLK</td>
<td>+7</td>
<td>Random coils, little secondary structure; in hydrophobic conditions some form α-helices</td>
<td>Lysine and arginine found at regular intervals; the other residues are hydrophobic; 22 NH2-terminal amino acids necessary for cytotoxicity</td>
<td>Wu et al. (86)</td>
</tr>
<tr>
<td>Tityus serrulatus tityustoxin</td>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lyctin I</td>
<td>25</td>
<td>Lyctin I: GIGKFLHSAKKFGKAFVGEIMNS</td>
<td>+5</td>
<td>Predicted secondary structure is an α-helix</td>
<td>Residues 1–27 exhibit α-helicity; 27–34 are hydrophilic</td>
<td>Ducloisier et al. (31)</td>
</tr>
<tr>
<td>Lyctin II</td>
<td>27</td>
<td>Lyctin II: GIGKFLHSAKKFGKAFVGEIMNS</td>
<td>+6</td>
<td>α-Helix aligned to antiparallel 3-stranded β-sheet, stabilized by disulfide bridges</td>
<td>Conserved region consisting of 4 disulfide bridges</td>
<td>Yan and Adams (87)</td>
</tr>
<tr>
<td>Melittin</td>
<td>23</td>
<td>GIGAVLKVLTTGLPALISWIKRKRQQ</td>
<td>+4</td>
<td>Antimicrobial</td>
<td>Arginine residue at position 15</td>
<td>Yan and Adams (87)</td>
</tr>
<tr>
<td>X. laevis magainin 1</td>
<td>23</td>
<td>GIGKFLHSAKKFGKAFVGEIMNS</td>
<td>+2</td>
<td></td>
<td></td>
<td>Ducloisier et al. (31)</td>
</tr>
<tr>
<td>Magainin II</td>
<td>23</td>
<td>GIGKFLHSAKKFGKAFVGEIMNS</td>
<td>+3</td>
<td></td>
<td></td>
<td>Legros et al. (52)</td>
</tr>
<tr>
<td>Dermaneptin</td>
<td>34</td>
<td>Dermaneptin: GIGKFLHSAKKFGKAFVGEIMNS</td>
<td>+3</td>
<td></td>
<td></td>
<td>Yan and Adams (87)</td>
</tr>
<tr>
<td>C. carpio 27 kDa</td>
<td>31</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenoregulin</td>
<td>41</td>
<td>Adenoregulin: GIGKFLHSAKKFGKAFVGEIMNS</td>
<td>+3</td>
<td></td>
<td></td>
<td>Yan and Adams (87)</td>
</tr>
<tr>
<td>NK-lysin</td>
<td>78</td>
<td>NK-lysin: GIGKFLHSAKKFGKAFVGEIMNS</td>
<td>+8</td>
<td></td>
<td></td>
<td>Yan and Adams (87)</td>
</tr>
<tr>
<td>Cryptidins</td>
<td>2</td>
<td>Cryptidins: GIGKFLHSAKKFGKAFVGEIMNS</td>
<td></td>
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<tr>
<td></td>
<td>3</td>
<td>Cryptidins: GIGKFLHSAKKFGKAFVGEIMNS</td>
<td></td>
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<tr>
<td>Mammalian</td>
<td></td>
<td>Mammalian: GIGKFLHSAKKFGKAFVGEIMNS</td>
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<tr>
<td>defense: rabbit N-P-1</td>
<td>small</td>
<td>N-P-1: GIGKFLHSAKKFGKAFVGEIMNS</td>
<td>Positively charged +1 at pH = 7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RK-1</td>
<td>32</td>
<td>RK-1: GIGKFLHSAKKFGKAFVGEIMNS</td>
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<tr>
<td>Insect defense (P. terranovae)</td>
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<tr>
<td>Insect defense (P. terranovae)</td>
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<tr>
<td>Insect defense (P. terranovae)</td>
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<tr>
<td>Scorpion defense (L. quinquestriatus)</td>
<td>38</td>
<td>Scorpion defense: GIGKFLHSAKKFGKAFVGEIMNS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PrP-(106–126)</td>
<td>21</td>
<td>PrP-(106–126): GIGKFLHSAKKFGKAFVGEIMNS</td>
<td>+2 at pH &gt; 7, +3 at pH &lt; 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aβ-(1–40)</td>
<td>40</td>
<td>Aβ-(1–40): GIGKFLHSAKKFGKAFVGEIMNS</td>
<td>+5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human amylin</td>
<td>37</td>
<td>Human amylin: GIGKFLHSAKKFGKAFVGEIMNS</td>
<td>+5</td>
<td></td>
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<tr>
<td>Cornut et al.</td>
<td>12–20</td>
<td>Cornut et al.: GIGKFLHSAKKFGKAFVGEIMNS</td>
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<tr>
<td>Modified analogs of magainin 2, designed to increase helical content (see Table 1 of Chen et al. (19) for sequence details)</td>
<td>23</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
luminal sides of channel proteins) with solutions of different concentrations of O2 and N2, redox agents. This could indicate the role of S-S in the function of different cytotoxic peptide-formed channels and its physiological regulation under the reduced cell environment or increased levels of reactive oxygen species (ROS), providing insight into the role of these peptides in cell function under ischaemic conditions of the heart, kidney, and intestines.

One of the early cytosolic changes occurring under hypoxia of epithelial and muscle cells is the lowering of cytoplasmic pH, which depresses force output in muscle (30, 34) and electrolyte regulation in epithelial cells (85), probably via modifications in the mechanism(s) involved in Ca2+ homeostasis. Whether Ca2+ is involved in conferring cytotoxicity on a cytotoxic peptide-formed channel is not known and it needs be determined. This is because of the possibility that these channels may become functionally active only under physiological conditions where Ca2+ levels are high, e.g., during cardiac and skeletal muscle contractions, volume regulation, and pathological conditions that lead to a Ca2+ overload, e.g., ischemic heart and muscle fatigue. Cytotoxic peptide-formed channels that could be regulated with Ca2+ may modify a cascade of physiological functions that are dependent on the Ca2+-activated channels, e.g., vasodilation and salt secretion. Such accumulating knowledge can indicate the likely conditions for channel formation in vivo and under pathological conditions. Knowledge of the biophysical and regulatory properties of the cytotoxic-peptide formed channels allows the evaluation of the physiological significance and possible pathological functions of these peptides. These can be verified by testing the in vitro and in vivo toxicity of these peptides, e.g., measuring muscle contraction and relaxation, cell volume regulation of cultured epithelial cells, and/or liposomes. Pharmacological agents used to modulate these channels can then be used to rectify the cytotoxic effects of these peptides in vivo.

CONCLUSIONS

The peptides discussed here have several characteristics in common: they are small, generally cationic, often amphipathic peptides. They have cytotoxic activity, which is often manifested as antibacterial activity but which can also be antifungal or anti-mammalian cell activity. These peptides can mediate their effects by interacting with cell membranes and forming ion channels, which are examinable with electrophysiological techniques. The channels can then be characterized in terms of their biophysical, pharmacological, and functional properties. The biophysical and pharmacological investigations of these small peptide-formed channels could 1) clarify conditions needed for peptide-bilayer interaction and ion channel formation (bilayer properties and conditions required for peptide interaction and incorporation, differences in the incorporation of these peptides, and structural characteristics to identify the domains that interact with lipid membrane); 2) characterize the biophysical properties (channel type and its ion concentration dependency, the channel’s selectivity and conductance properties, and the voltage dependency of the channel’s conductance and kinetic properties); and 3) characterize the pharmacological properties (fundamental mechanisms of agonist-induced activation and antagonist-induced channel block, channel regulation by cytokotic factors and second messengers, the symmetry or asymmetry of the transmembrane channel-forming peptide, and the domains important in channel formation and regulation). Although these small ion-channel-forming peptides could provide relatively simple structural models, it is still difficult to predict the structural requirements for ion channel formation. There are no models for the sequence of events leading to channel formation, e.g., if a multimer forms the channel, when is this multimer itself formed: before the peptide is incorporated into the membrane or once the peptide molecules are within the membrane (see Ref. 20)? In conclusion, the investigations of these peptides could enhance our understanding of their structure-function relationship and elucidate the molecular events underlying their interactions with biological membranes and the role of such interactions both in major physiological mechanisms and in cytotoxic and pathological conditions.

We thank Dr. W. Armarego and H. Wood for numerous discussions, suggestions, and critical reading of the manuscript. The assistance of P. Farrelly, S. Pradhananga, C. Henry, and A. Culverson is greatly appreciated.

J. I. Kourie is supported by National Health and Medical Research Council Project Grant 970122 and The Australian Research Council Grant F99123.

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REFERENCES


