Mode of Action of the Antimicrobial Peptide Indolicidin*

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Indolicidin is a cationic antimicrobial peptide isolated from bovine neutrophils. It consists of only 13 amino acids, has the highest tryptophan content of any known protein, and is amidated at the carboxyl terminus in nature. By circular dichroism spectroscopy a weak poly-$\alpha$-proline II extended helix structure was observed that became substantially more pronounced upon interaction with liposomes. Indolicidin bound purified surface lipopolysaccharide with high affinity and permeabilized the outer membrane of Escherichia coli to the small hydrophobic molecule 1-N-phenylnaphthalamine (M, 200), results consistent with indolicidin crossing the outer membrane via the self-promoted uptake pathway. The methyl esterification of indolicidin's carboxyl terminus increased its activity for Gram-negative and Gram-positive bacteria. In Gram-negative bacteria this was associated with an increased binding to lipopolysaccharide and increased permeabilization of the outer membrane. The cytoplasmic membrane was the site of action of indolicidin as assayed in E. coli by the unmasking of cytoplasmic $\beta$-galactosidase due to membrane permeabilization. The mechanism for this activity was shown to be the ability of the peptide to cause an increase in the transmembrane current of planar lipid bilayers. This current increase was activated by transmembrane potentials in excess of $\approx 70$ to $\approx 80$ mV. Consistent with this, there was a substantial decrease in indolicidin-mediated bacterial killing and permeabilization of the cytoplasmic membrane of E. coli that had been pretreated with the uncoupler carbonyl cyanide-$m$-chlorophenyl hydrazone. In planar bilayers, indolicidin induced the formation of discrete channels, which ranged in conductance from 0.05–0.15 nS. Thus despite the small size and unique composition of indolicidin, it was capable of killing Gram-negative bacteria by crossing the outer membrane and causing disruption of the cytoplasmic membrane by channel formation.

The third group comprises those peptides forming looped structures containing one or more disulfide bonds such as bactacene (5). The fourth group involves peptides that contain a high percentage of specific amino acids such as the proline/arginine-rich bovine peptides, Bac5 and Bac7 (6), and the porcine peptide PR-39 (7). The majority of these well characterized antimicrobial peptides range in size from 30 to well over 100 residues in length and consist of a wide range of different amino acids. The mode of action of the majority of these peptides has been demonstrated to be the permeabilization of the inner membrane. Such a mechanism has been demonstrated for proline-$\alpha$-helical, $\beta$-structured, and proline/arginine-rich peptides either directly by in vivo assay or indirectly by their effect upon lipid bilayers (8–11).

Indolicidin is a 13-amino acid antimicrobial peptide present in the cytoplasmic granules of bovine neutrophils (12). As a naturally occurring peptide, indolicidin has a unique composition consisting of 39% tryptophan and 23% proline (ILPWKWPWPWW), and in nature the peptide is amidated at the carboxyl terminus. Indolicidin is the smallest of the known naturally occurring linear antimicrobial peptides, contains the highest percentage tryptophan of any known protein, and consists of only six different amino acids. Due to the distribution of proline and tryptophan residues throughout the indolicidin sequence, it may assume a structure distinct from the well described $\alpha$-helical and $\beta$-structured peptides. Indolicidin has activity against Gram-negative and -positive bacteria (12), fungi (13), and protozoa (14). In addition the peptide is cytotoxic to rat and human T lymphocytes (15) and lyses red blood cells (13). However, despite this broad range of activity, little is known of indolicidin's structure or mode of action.

Because of its broad spectrum of activity, indolicidin may prove a good candidate for therapeutic use as was recently demonstrated in an in vivo antifungal study using liposome-entrapped peptide (13). However, because of its small size, unique composition, and potentially different secondary structure, it may have a specific mode of action distinct from the other natural antimicrobial peptides described above. Here we investigated the mechanism of action of indolicidin and the effect of carboxyl-terminal modification of the peptide by examining its ability to bind and permeabilize Gram-negative bacterial membranes and its effect on planar lipid bilayers.

EXPERIMENTAL PROCEDURES

Materials and Bacterial Strains—Indolicidin was synthesized by Fmoc (N-(9-fluorenylmethoxycarbonyl) chemistry at the University of Victoria (BC, Canada). Polymyxin B, gentamicin, lysozyme, 1-N-phenylnaphthalamine (NPN), carbonyl cyanide-m-chlorophenyl hydrazone (CCCP), and o-nitrophenyl-\(\beta\)-o-galactoside (ONPG) were purchased from Boehringer Mannheim (L.P. 170 70 to 170 15 nS). Thus despite this wide range of activity, little is known of indolicidin's structure or mode of action. Because of its broad spectrum of activity, indolicidin may prove a good candidate for therapeutic use as was recently demonstrated in an in vivo antifungal study using liposome-entrapped peptide (13). However, because of its small size, unique composition, and potentially different secondary structure, it may have a specific mode of action distinct from the other natural antimicrobial peptides described above. Here we investigated the mechanism of action of indolicidin and the effect of carboxyl-terminal modification of the peptide by examining its ability to bind and permeabilize Gram-negative bacterial membranes and its effect on planar lipid bilayers.

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1 The abbreviations used are: NPN, 1-N-phenylnaphthalamine; CCCP, carbonyl cyanide-m-chlorophenyl hydrazone; ONPG, o-nitrophenyl-\(\beta\)-o-galactoside; POPC, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoglycerol; MRC, minimal inhibitory concentration(s); LPS, lipopolysaccharide; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

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from Sigma. Phosphatidylcholine and phosphatidylserine were purchased from Avanti Polar Lipids Inc. (Birmingham, AL), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) were purchased from Northern Lipids Inc. (Vancouver, BC, Canada). Dansyl polymyxin B was prepared as described previously (16).

Bacterial strains used for antimicrobial activity testing included Escherichia coli UB1005 and its antibiotic supersusceptible derivative DC2 (17), Pseudomonas aeruginosa PA01 strain H103 (18), P. aeruginosa K799 and its antibiotic supersusceptible derivative Z61 (19), Salmonella typhimurium 14028s and its defensin-supersusceptible derivative MS7953 (20), Staphylococcus aureus RN4220 (21), and a clinical isolate of Staphylococcus epidermidis. Relevant phenotypic descriptions are listed in Table I. E. coli ML-35, a lactose permease-deficient strain with constitutive cytoplasmic β-galactosidase activity (lac+ , lacZ− ), was obtained from E. Ruby, University of Southern California.

Membrane Permeabilization Assays—The outer membrane permeabilization activity of the peptides was determined by the NPN assay of Moore et al. (25). Dansyl polymyxin B displacement assay—The relative binding affinity of each peptide for LPS was determined using the dansyl polymyxin B displacement assay of Moore et al. (25). Dansyl polymyxin B (2.5 μM) and P. aeruginosa H103 LPS (3 μg/ml) were mixed in 1 ml of 50 mM HEPES (pH 7.2). This resulted in >90% maximum fluorescence as measured by a fluorescence spectrophotometer. The decrease in fluorescence due to partitioning of NPN into the outer membrane was measured using a fluorescence spectrophotometer. The increase in fluorescence due to partitioning of NPN into the outer membrane was measured using the addition of various concentrations of antibiotic or peptide. For the lysozyme lysis assay, 600 μl of cells were mixed with 50 μg/ml chicken egg white lysozyme and varying concentrations of antibiotic or peptide. Cell lysis, due to permeabilization of the outer membranes to lysozyme, was measured as a decrease in the A550. For each assay controls were done as described in the original papers to ensure that actual uptake of NPN and lysozyme was being assessed.

Inner membrane permeability was determined by measurement in E. coli ML-35 of β-galactosidase activity using ONPG as substrate (8, 9). Logarithmic phase bacteria were washed in 10 mM sodium phosphate buffer (pH 7.0) in the presence or the absence of POPC/POPG (7:3) liposomes. Unilamellar liposomes (0.1 mg/ml POPC and POPG, 1.5 mM NPN were mixed, and fluorescence was measured using a fluorescence spectrophotometer. The increase in fluorescence due to partitioning of NPN into the outer membrane was measured using a fluorescence spectrophotometer. The decrease in fluorescence due to partitioning of NPN into the outer membrane was measured using a fluorescence spectrophotometer.
Antimicrobial Activity—The MIC of indolicidin to a range for Gram-negative bacteria and their respective antibiotic sensitive mutants are shown in Table I. The MIC was taken as the lowest antibiotic concentration at which growth was inhibited. The carboxyl-terminal methyl ester of indolicidin, indolicidin-C, showed increased activity against Gram-negative and Gram-positive bacteria, notably the wild type *E. coli*, the defensin supersensitive *S. typhimurium* and *Staphylococcus sp*. However, there was no such improvement in activity against any of the *P. aeruginosa* strains tested. Indolicidin exhibited 4–16-fold greater activity against the outer-membrane-altered, antibiotic-supersusceptible mutants *E. coli* DC2 and *P. aeruginosa* Z61 compared with their respective parent strains. Against the *S. typhimurium* defensin supersusceptible, phoP/phoQ mutant, indolicidin was 8-fold more active as compared with the parent strain. The killing of Gram-negative bacteria due to indolicidin was immediate resulting in several log orders of death within minutes of adding peptide at a concentration equivalent to the MIC (Fig. 2). This killing was significantly reduced in *E. coli* cells pretreated with 100 μM of the uncoupler CCCP (Fig. 2).

Binding of Indolicidin to *P. aeruginosa* H103 LPS—The mechanism of uptake across the outer membrane of the studied α-helical (cecropin/melittin hybrids) and β-sheet (rabbit defensin) cationic peptides in Gram-negative bacteria has been demonstrated to be via the self-promoted uptake pathway (32, 33). This is initiated by binding of the peptide to divalent cation binding sites on LPS and displacing the divalent cations that stabilize the adjacent LPS molecules. Peptides such as defensins (32), magainins (34), and melittin (35) have also been shown to interact directly with LPS. The ability of positively charged compounds to bind LPS and displace divalent cations that stabilize the adjacent LPS molecules. Peptides such as defensins (32), magainins (34), and melittin (35) have also been shown to interact directly with LPS. The ability of positively charged compounds to bind LPS and displace bound dansyl polymyxin B has been demonstrated to result in decreased fluorescence of the dansyl group (25). The concentration of peptide resulting in 50% maximal displacement of dansyl polymyxin (I\text{so} value) can be used as an indicator of relative binding affinity. The I\text{so} values for each of the compounds tested are listed in Table II. Maximal displacement of LPS was expressed as a percentage where 100% displacement of dansyl polymyxin was taken as that obtained with polymyxin B. The avidity of indolicidin and indolicidin-C for LPS was comparable with that of polymyxin B, whereas Mg\textsuperscript{2+}, the native divalent cation normally bound to LPS, showed a substantially lower affinity.

Membrane Permeabilization by Indolicidin—Gram-negative bacteria such as *E. coli* have two cell envelope membranes. We examined the ability of indolicidin to interact with both the outer and cytoplasmic membranes. The displacement of divalent cations from surface LPS leads to destabilization of the

![Fig. 2. Killing of *E. coli* by indolicidin in the presence and the absence of CCCP](image-url)

**TABLE I**

| Species and strain | Relevant phenotype | MIC | Gentamicin | Polymyxin B | Indolicidin | Indolicidin-C |
|-------------------|--------------------|-----|------------|-------------|-------------|---------------|
| *E. coli*          |                    |     |            |             |             |               |
| UB1005            | Parent of DC2      | 1   | 0.5        | 16          | 4           |               |
| DC2               | Polymyxin sensitive| 0.5 | 0.1        | 4           | 4           |               |
| *P. aeruginosa*   |                    |     |            |             |             |               |
| H103              | PAO1 strain        | 1   | 0.5        | 64          | 64          |               |
| K799              | Parent of Z61      | 1   | 0.5        | 64          | 64          |               |
| Z61               | Antibiotic supersusceptible | 0.25 | 0.1 | 4 | 4 |               |
| *S. typhimurium*  |                    |     |            |             |             |               |
| 14028s            | Parent of MS7953s  | 4   | 1          | 64          | 32          |               |
| MS7953s           | Defensin supersusceptible | 2 | 0.25 | 8 | 2 |               |
| *S. aureus*       |                    |     |            |             |             |               |
| RN4220            | Wild type          | 32  | 64         | 8           | 4           |               |
| C621              | Clinical isolate   | 0.5 | 64         | 4           | 1           |               |

**TABLE II**

| Compound         | I\text{so} | I\text{max} |
|------------------|------------|-------------|
|                  | μM         | %           |
| Indolicidin      | 8.5        | 70          |
| Indolicidin-C    | 1.2        | 75          |
| Polymyxin B      | 2          | 100         |
| MgCl\textsubscript{2} | 620      | 56          |

Gram-negative outer membrane and subsequent uptake of the destabilizing compound as demonstrated for defensins and cecropin/melittin hybrids (32, 33). In addition, Bac5 and Bac7 (9) and the defensins HNP-1 to HNP-3 (8) have been shown to permeabilize the outer membrane of *E. coli*. However, the outer membrane can prove much more of a barrier to other cationic peptides such as PR-39, which is initially ineffective against Gram-negative bacteria (36). This lag time was absent when
outer membrane mutants, permeable to the peptide, were used.

In the present study, the ability to permeabilize the outer membrane of wild type E. coli to NPN and lysozyme was determined for each of the antibiotics. NPN is a hydrophobic fluorescent probe that fluoresces weakly in aqueous environment and strongly when it enters a hydrophobic environment such as the interior of a membrane. Normally NPN is excluded from Gram-negative bacterial cells. However, as previously reported polymyxin B facilitated the uptake of NPN into the hydrophobic interior of the outer membrane.

The ability of each compound to facilitate the uptake of the larger (14,000 Da) protein lysozyme, which is also normally excluded from accessing its peptidoglycan substrate, was assayed. The degree of cell lysis due to lysozyme uptake is represented as the decrease in \( A_{600} \) plotted as a function of antibiotic concentration. As with the NPN assay, polymyxin B facilitated the uptake of lysozyme at concentrations as low as 0.2 \( \mu \)g/ml (Fig. 3). Indolicidin and its methyl ester permeabilized E. coli to NPN at minimum concentrations of 3–5 and 0.6–0.9 \( \mu \)g/ml respectively. Therefore, indolicidin was capable of disrupting the outer membrane of E. coli at modest concentrations.

The target of many cationic peptides is the cytoplasmic membrane, and the depolarization of this membrane by such peptides leads to dissolution of the electrical potential gradient (\( \Delta \psi \)) and results in cell death, presumably through loss of membrane integrity. This has been demonstrated for a range of cationic peptides including magainins (37), defensins (38), and lantibiotics such as Pep5 (39). The loss of cytoplasmic membrane integrity has been followed by the unmasking of cytoplasmic \( \beta \)-galactosidase in E. coli. When cells were treated with defensins this only occurs at high (50 \( \mu \)g/ml) peptide concentrations and revealed considerable lag times (8). However, for the bactenecins Bac5 and Bac7, lag times have been demonstrated to be in the order of a few minutes and permeabilization of the cytoplasmic membrane occurs at peptide concentrations as low as 10 \( \mu \)g/ml (9). Indolicidin permeabilized the inner membrane of E. coli ML-35 as determined by unmasking of cytoplasmic \( \beta \)-galactosidase in this permease negative mutant (Fig. 4). Permeabilization occurred after a lag of less than 1 min, and the rate of permeabilization was dependent on peptide concentration. The activity of indolicidin against the inner membrane occurred at concentrations as low as 4 \( \mu \)g/ml. As seen in Fig. 4, inner membrane permeabilization of bacteria pretreated with 100 \( \mu \)M CCCP was significantly reduced. This is consistent with a reduction in killing of E. coli UB1005 by indolicidin when the cells were likewise pretreated with 100 \( \mu \)M CCCP (Fig. 2).

Conductance Measurements with Planar Bilayer Membranes—The mechanism of membrane depolarization has been demonstrated in planar lipid bilayer experiments in which \( \beta \)-structured defensins and \( \alpha \)-helical peptides have been shown to form voltage-dependent multistate channels (10, 11). In the majority of cases such activity increases exponentially with voltage. However, in the case of the cationic lantibiotic Pep5 there is a threshold potential of -100 mV above which activity occurs (39). The interaction of indolicidin with lipid membranes might be expected due to its high tryptophan content. Indolicidin is at the lower end of peptide sizes that could potentially span a membrane, and its ability to form channels in lipid was uncertain.

Voltage was increased in steps of 10 mV and maintained at the set voltage for 2 min. Between each increase the bilayer...
was kept at 0 mV for 2 min. Once at −80 mV, the voltage was reduced step-wise to 0 mV, in 10-mV steps at 2-min intervals but not returning the voltage to 0 mV between each reduction. Current readings were taken following the 2 min at each voltage increment.

Fig. 5A shows the current-voltage characteristic of indolicidin. At less than −70 mV, the increase in voltage had only a minor effect on current, but at and above −70 mV there was a dramatic increase in current. As voltages were subsequently decreased, the current decline was linear or superlinear. When the voltage was subsequently reincreased to −80 mV from 0 mV, returning the voltage to 0 mV between each increment again, the increase was linear following the same path as the descending line in Fig. 5A. Increases and decreases in current were characterized by gradual delayed increases or decreases that stabilized at a steady state. There was no increase in current with trans-positive potentials. However, as seen in Fig. 5B, the rapid change from −70 to +70 mV resulted in an initial equal and opposite current reading, followed by a temporary rapid increase in this current and subsequent decrease to a reading of approximately one-tenth the initial current at −70 mV, which was maintained for up to 20 min. Reversal of the potential back to −70 mV again created an equal current of opposite sign, but this gradually returned to the initial current reading at time 0 (Fig. 5B).

The increase in membrane current caused by indolicidin, characterized in the macroscopic experiments, was due to the formation of single channels (Fig. 6). Single-channel conductances varied from 0.05–0.15 nS. However, channels of similar size were repeatedly observed (e.g. 0.13 nS). The lifetimes of individual channels also varied, but as a general observation, smaller channels were more stable. The characteristics of indolicidin-formed channels were consistent with each channel being formed by a number of peptide molecules resulting in a limited heterogeneous population of channel conductance levels.

**DISCUSSION**

The target for the majority of α-helical and β-structured antimicrobial cationic peptides is the cytoplasmic membrane. We have demonstrated here that this is the case for the unique proline-tryptophan-rich peptide indolicidin, a peptide that appeared to be unrelated to the above-defined groups of peptides by composition and, as indicated in CD spectral analyses, by secondary structure. To reach the target cytoplasmic membrane, antimicrobial cationic peptides must overcome the barrier of the Gram-negative outer membrane, which they apparently do by utilizing the self-promoted uptake pathway (32, 33). This involves displacement of divalent cations from their binding sites on surface LPS and the consequent permeabilization of the outer membrane. Indolicidin was shown to have both of these properties. Firstly, it was demonstrated to bind to outer membrane LPS with similar affinity to polymyxin B, defensins, and α-helical cecropin-melittin hybrids (32, 33) and significantly better than Mg²⁺. Secondly, indolicidin permeabilized outer membranes rapidly and at modest concentrations facilitating the uptake of the small (M, 200) hydrophobic probe NPN. However, in contrast to other cationic peptides and polymyxin B, indolicidin did not permeabilize the outer membrane to the larger (M, 14,000) compound lysozyme, even at concentrations 3-fold the MIC. This may be a reflection of the small size of indolicidin, resulting in a more subtle outer membrane perturbation than produced by the larger cationic peptides. Nevertheless, the outer membrane does provide a barrier to indolicidin because the antibiotic supersusceptible strain P. aeruginosa Z61 was significantly more susceptible to indolicidin than its parent strain.

The carboxyl-terminal modification of indolicidin increased...
its antimicrobial activity against both Gram-negative and Gram-positive bacteria presumably by increasing its net positive charge. This was consistent in part with the demonstrated increase in LPS binding and subsequent outer membrane permeabilization by the carbonyl-terminal modification. In addition its improved activity against Gram-positive bacteria, which do not have an outer membrane, must have reflected an improved ability to insert into the cytoplasmic membrane. These enhanced activities could explain why indolicidin in nature has an amidated carboxyl terminus.

Inner membrane permeabilization by indolicidin as assayed by unmasking of cytoplasmic β-galactosidase occurred with minimal lag, suggesting that inner membrane permeabilization followed on rapidly after outer membrane permeabilization. The maximal rate of permeabilization was five times that observed for the cationic defensins HNP-1 to HNP-3 (8) and the bactenecins Bac5 and Bac7 (9). The ability of indolicidin to cause a voltage-dependent current increase across planar lipid bilayers provided a potential mechanism for its action on the inner membrane of E. coli. A potential of between ~70 and ~80 mV was required for activity, a phenomenon previously observed with the cationic peptide lantibiotic Pep5, although in the case of Pep5 the threshold potential was in excess of ~90 to ~100 mV (39). Nevertheless, growing bacteria carry membrane potentials in excess of ~140 mV at neutral pH. This potential was demonstrated in vivo to be necessary for the antibacterial activity of indolicidin, because cells, treated with the uncoupler CCCP, were more resistant to inner membrane permeabilization and killing.

The almost linear decrease in current with decreasing negative potential indicated that the threshold potential may only be required to draw the peptide into the membrane to form channels but that once this has occurred, the channels remain open and relatively stable, i.e. channels were voltage-induced rather than voltage-gated. This disruption of planar lipid bilayers was due to discrete channel-forming events with single-channel conductances of 0.05–0.15 nS. This is consistent with certain other cationic peptides that form multistate channels of varying size from 0.01 to 2 nS (11, 39), a result interpreted as reflecting a barrel-stave mechanism of channel formation in which the number of peptide molecules (staves) contributing to the formation of the channel (barrel) determines the channel size (41). However, a far more restricted variety of channel sizes were observed for indolicidin. Thus these different conductance increments may reflect dimers and trimers of single channels or even substrates of a given channel. Indolicidin is only 13 amino acids long, and as such would be expected to be too small to span the bilayer as an α-helix or β-strand. Thus it probably adopts an extended structure as suggested by the CD measurements and either spans the membrane as an aggregate or stacks in the membrane as demonstrated for gramicidin (42).

The unique composition of indolicidin distinguishes it from the well studied α-helical and β-structured cationic peptides, and it is by far the smallest of the natural, linear cationic peptides examined to date. Nevertheless although its mode of action against bacteria shows certain distinct features, overall it demonstrates a variation on the themes provided by other antimicrobial cationic peptides. These peptides are found in all forms of life (1) and come in all shapes and sizes, but indolicidin certainly represents one extreme. Nevertheless the indications that many natural cationic peptides act in a similar way, despite substantive compositional and structural dissimilarities, is a powerful argument that such peptides are a product of convergent evolution. Given that liposomal indolicidin has been shown to be an effective antifungal in animal model infections, we feel that cationic peptides offer a potentially fruitful subject for sequence modification to design novel antimicrobials.