Molecular electroporation: a unifying concept for the description of membrane pore formation by antibacterial peptides, exemplified with NK-lysin

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Abstract The antibacterial activity of many small, positively charged peptides and proteins is based on pore formation in lipid bilayers. It is here proposed to arise from an electroporation effect. This hypothesis is supported by calculations of the electrostatic potential of NK-lysin associated to a membrane. For a significant area of the protein-membrane interface, the electrostatic potential is found to be above the minimum threshold for electroporation. A single highly charged α-helical segment of NK-lysin is mainly responsible for this effect. It is experimentally demonstrated that a peptide comprising this helix has antibacterial activity. We propose that superficial association to membranes suffices to trigger electroporation, provided the peptide is sufficiently charged. The effect is referred to as molecular electroporation.

Key words: Protein-membrane interaction; Electroporation; NK-lysin; Electrostatic interaction

1. Introduction

More than 100 natural cationic bactericidal peptides are known and many are toxic also to eukaryotic cells [1]. In general, they effect cell death through pore formation and, at higher concentrations, complete disintegration of the cell membrane. Ion channels formed by cationic antibacterial peptides are heterogeneous in conductance and opening and closing duration and it is not clear how many peptide molecules, if any, participate in the lining of a channel [2–4]. There is indeed evidence that antibacterial peptides can be active without significant penetration into the lipid bilayer. For example, pores are achieved by peptides and amphipathic α-helices which bind parallel to the membrane surface [5,6]. Maximum membrane-permeating activities of defensins and other peptides have been reported for peptide concentrations at which a single monomolecular layer of peptide could be formed on the surface of the membrane [2,7,8]. Very simple sequences exclusively composed of Leu and Lys and capable of forming amphipathic α-helices can be active against bacteria, with maximum activity for peptides of 11–15 residues [9]. Yet, antibacterial activity does not depend on helicity, as peptides composed of L-Leu and L-Lys residues interspersed with four D-Leu residues also show high antibacterial activity [10]. Similarly, defensins have a globular three-dimensional fold, stabilised by disulfide bridges, which is not α-helical [11].

NK-lysin is a small basic protein of 78 residues secreted by porcine natural killer cells. It displays cytolytic activity towards a broad range of bacteria and tumour cells [12] and seems to act via a very similar mechanism as small antibacterial peptide. Pore formation in lipid bilayers has been demonstrated [13]. It contains three disulfide bridges, is extremely heat-stable [14] and has a well-defined globular structure composed of five α-helices [15]. Infra-red spectroscopy experiments suggest that its three-dimensional fold is maintained in the lipid-bound form. Because of its many charged residues, both positive and negative, NK-lysin probably binds only superficially to a membrane surface. Ion conductivity measurements across a planar lipid bilayer reveal a pronounced destabilisation of the lipid bilayer, apparently without formation of any well-defined ion channels [13].

In the present contribution, we argue for molecular electroporation as a unifying principle for antibacterial activity. Electroporation is the formation of pores in membranes due to the presence of an external electric field. When short pulses are used, a voltage of about 1 V across the membrane is required for pore formation [16]. The threshold falls to about 0.2 V for electric fields applied over long time periods (>0.1 ms) [17]. By the term 'molecular electroporation', we denote electroporation caused by the electric field produced by a charged molecule binding to the surface of a membrane. Molecular electroporation has been proposed earlier to explain membrane pore formation by annexin V [18]. In the following, we exemplify the idea with NK-lysin as a representative of an antibacterial peptide and argue for its general applicability to a broad range of antibacterial peptides and detergents.

2. Materials and methods

2.1. Calculation of electrostatic potentials

The electrostatic interactions were calculated with a continuum dielectric model similar to that used in the analysis of annexin V-membrane interactions [18]. In this model, the protein and the water phase were represented as homogenous materials with a low ($\varepsilon=4$) and high ($\varepsilon=80$) dielectric constant ε, respectively. Two dielectric regions were defined for the membrane: a central region, corresponding to the aliphatic moiety, with a dielectric constant of $\varepsilon=4$, and two adjacent regions, corresponding to the polar lipid head groups [19], with a dielectric constant of $\varepsilon=20$ [20,21]. The thickness of the membrane was 40 Å, including both polar sublayers with a thickness of 6 Å. The Poisson-Boltzmann equation was solved by means of a finite difference algorithm [22–24].

The electrostatic potentials were calculated in two steps. First, the pK_a values of titratable groups were calculated using the parameters and the procedure described previously [25,26], but partial atomic charges taken from the CHARMM parameter set 19 [27]. In the

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second step, the electrostatic potential of the molecule was calculated, taking into account the degree of ionisation of the titratable groups at a given pH. Both steps were executed in the absence and presence of the membrane. Calculations were carried out for all 20 NMR structures of NK-lysin [15] (PDB [28] accession code 1NKL).

2.2. Peptide synthesis

The protected peptide with the amino acid sequence LRGLCKKIMRSFL-NH_2 (corresponding to residues 41–53 of NK-lysin) was assembled by standard solid-phase methodology on a ABI 430 A peptide synthesiser using Boc-amino acids and *p*-methyl-BHA-resin. The deprotected peptide was treated with dithioerythritol and subsequently purified by analytical high performance liquid chromatography with a Vydac C4 column (4.6×250 mm) using a linear gradient of acetonitrile/0.1% trifluoroacetic acid. The purity was checked by quantitative amino acid analysis and mass spectrometry.

2.3. Antibacterial assay

Thin plates were poured with LB broth (pH 7.0) including medium E, 1% agarose and *Escherichia coli* (D21) bacteria. Sample wells were punched into the agarose and 3 μl of peptide dissolved in water was applied. After overnight incubation at 30°C, the diameters of the inhibition zones were recorded and lethal concentrations (LC values) were calculated from the zones of a dilution series of the peptide [29].

3. Results

3.1. Electrostatic potentials of NK-lysin in water

Electrostatic potentials were calculated for the ionisation state at pH 7.0. The pK_a calculations confirmed that all arginine and lysine side chains are protonated at this pH, while all but one of the carboxylic acid groups are deprotonated. The exception is Asp-59, which was found to be protonated in agreement with NMR data [15]. At pH 7.0, virtually identical ionisation states of the titratable groups were determined for each of the 20 NMR conformers of NK-lysin, resulting in very similar electrostatic potentials around the molecule.

The electrostatic potential of NK-lysin at pH 7.0 is shown in Fig. 1A. Since NK-lysin is a basic molecule, its overall charge is positive and the areas of negative potential are limited, as illustrated by the zero isopotential surface. Only helix 1 and parts of helix 2 (residues 20–24) and helix 5 (residues 57–61) are in a negative potential.

3.2. Model of membrane-bound NK-lysin

The initial approach of a positively charged NK-lysin molecule to a negatively charged membrane was assumed to be guided by the asymmetry of the electrostatic potential, so that the region with the most positive potential contacts the membrane first. This region is located near the N-terminal end of helix 3 (Fig. 1B). The contact area between the positive potential of the protein molecule and the membrane was subsequently maximised by a reorientation of the protein molecule with respect to the membrane and the depth of protein penetration into the membrane was adjusted so that all regions of negative potential were outside the membrane. This resulted in a protein-membrane complex with an interface determined by helix 3, in an orientation parallel to its surface, as well as by the hydrophobic patch, comprising residues Phe-3, Phe-52 and residues nearby are immersed into the membrane (Fig. 1C).

3.3. Electrostatic potential of membrane-bound NK-lysin

Before calculating the electrostatic potential of membrane-bound NK-lysin, the p*K* values of the titratable groups were recalculated in the presence of the membrane. Because of the reduced dielectric constant at the protein-membrane interface, Lys-47 was predicted to be uncharged. Yet, the overall appearance of the electrostatic potential (Fig. 1C) was similar as in water solution (Fig. 1A). An area of 450 $Å^2$ or about half of the protein surface in contact with the membrane was found to be in an electrostatic potential equal or greater than 0.2 V and 184 $Å^2$ was exposed to more than 0.4 V. The main contribution to the high electrostatic potential, about two thirds of the area, is provided by helix 3.

3.4. Antibacterial activity of helix 3

To test the possibility that helix 3 alone could have antibacterial activity, the fragment Leu-41–Leu-53 was synthesised as a C-terminally amidated peptide and tested for activity against *E. coli*. The calculated LC value was 0.79 μM, which is about 3-fold lower than for NK-lysin (0.25 μM). The dilution curves were parallel (Fig. 2), suggesting a similar killing mechanism. To exclude artefacts by oxidative dimerisation via the single cysteine residue in the peptide, the assay was repeated with peptide treated for 2 h in dithioerythritol and subsequent loading in dithioerythritol solution. The results obtained were identical to that obtained in water.

4. Discussion

4.1. Molecular electroporation

The concept of molecular electroporation assumes that electroporation can be achieved not only by externally applied electric fields, but also by highly charged molecules binding to the membrane surface. Molecular electroporation is expected, if a reagent can bind to a lipid bilayer and if it carries a sufficient charge density to provide an electrostatic potential of at least 0.2 V across the bilayer. Our calculations indicate that electric field strengths of this magnitude are achieved by NK-lysin as well as by a peptide fragment thereof. Both NK-lysin and the peptide exhibit pronounced antibacterial activity.

Molecular electroporation would explain why the search for specific transmembrane ion channels has remained elusive for most antibacterial peptides. It explains how NK-lysin can form membrane pores without changing its conformation, how antibacterial peptides can act without forming transmembrane helices, why ion channels in artificial lipid bilayers appear heterogeneous when induced by NK-lysin [13] or other antibacterial peptides and why maximum pore formation activity is reached for liposomes at concentrations at which the liposome would be covered by a monomolecular peptide layer.

Pore sizes of 2–4 nm diameter have been reported for pores induced by conventional electroporation [30] and by at least two cationic peptides, polymyxin B [31] and melittin [9]. Such pore sizes are comparable to the NK-lysin-membrane interface area with a potential greater 0.2 V. The minimum requirement for antibacterial activity in amphiphilic peptides is about 12 residues and an overall charge of about +4 [1,9,10], in accord with the size and calculated charge of helix 3 of NK-lysin.

4.2. Membrane fusion

The analogy between molecular and conventional electroporation extends to cell fusion which can be effected both by antibacterial peptides [32] and electric fields [33]. Positively

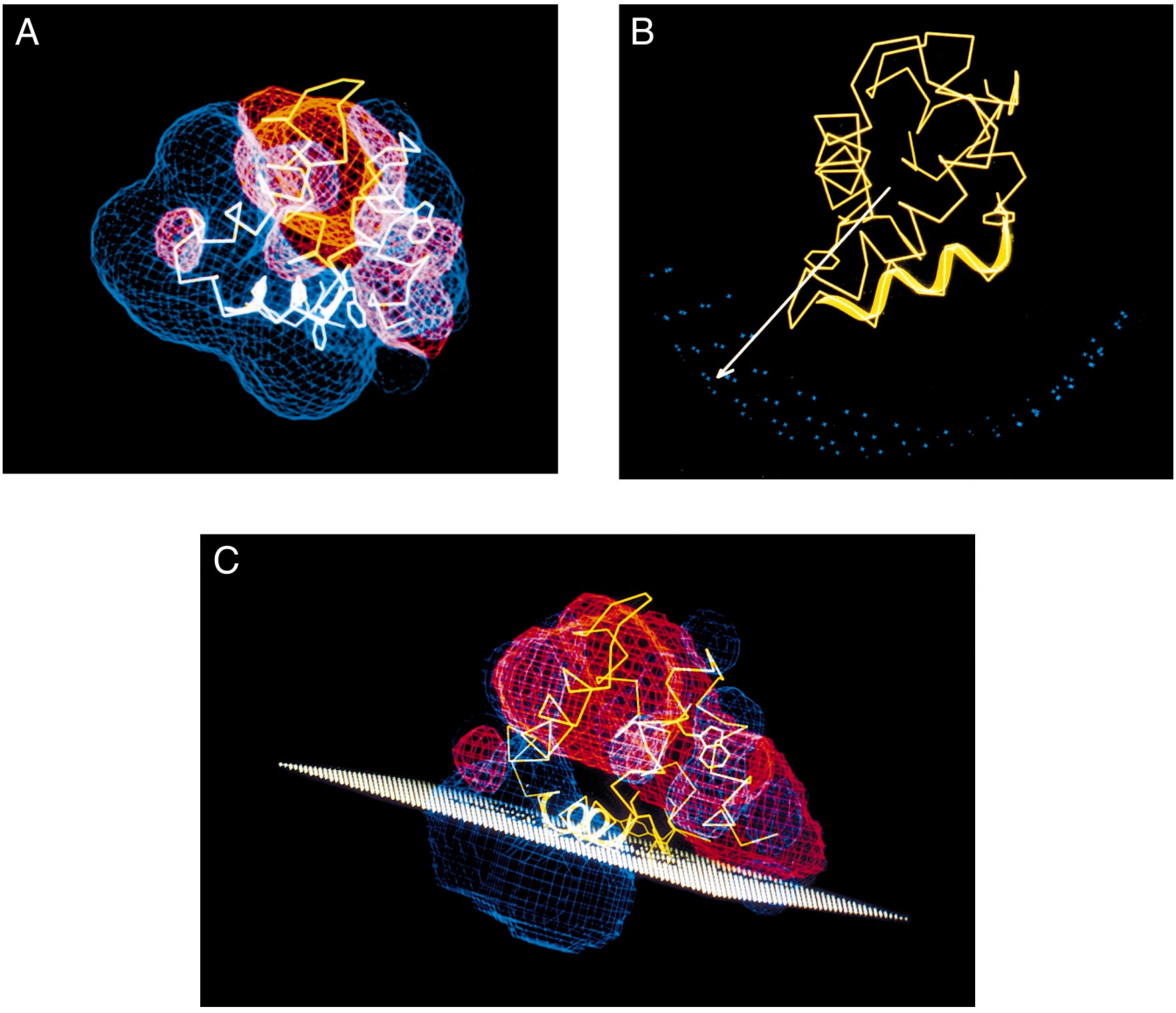

Fig. 1. (A) Electrostatic potential around NK-lysin in aqueous solution contoured at 0.1 V (blue) and 0 V (red). Note that blue contours overlapping red contours appear magenta. The protein is presented with the C^{α}-backbone in yellow together with selected side chains (Phe-3, Phe-52, Trp-58). Helix 3 (residues 42–51) which is believed to be important for membrane binding is highlighted by a ribbon. The region of positive potential forms a nearly closed ring around the molecule as expected from the distribution of basic amino acid side chains. The highest values of positive potential are in the vicinity of helix 3 (see also B). (B) Area of favourable electrostatic interaction energy ($\leq -3kT$) between the electrostatic potential of NK-lysin and a hypothetical probe of unity negative charge, represented as points (blue) on a sphere with a radius of 25 Å around the protein molecule. The arrow points at the region of highest positive potential, where the interaction energy is between −2.1 and −2.6 kcal/mol. While the highest positive potential is located near the N-terminus of helix 3 (indicated by a ribbon), significant interaction energies are measured all along helix 3. The initial approach of the protein to a negatively charged membrane could be guided by the positive electrostatic potential as shown by the arrow. (C) Model of a NK-lysin-membrane complex. The protein is presented with the C^{α}-backbone in yellow and helix 3 by a ribbon. The membrane surface is represented by a dotted plane. The electrostatic potential, contoured at 0.2 V (blue) and 0 V (red), was calculated in the presence of the membrane. In this model, about half of the intersection area between the protein and the membrane is in a potential equal or greater than 0.2 V. Phe-3, Phe-52 and further hydrophobic side chains nearby which are solvent-exposed in solution are buried at the protein-membrane interface.

charged protein domains in concert with Ca^{2+} have recently been suggested to initiate cell fusion through the formation of transient, non-specific 'fusion pores' [34].

4.3. Charge and antibacterial activity

The importance of charge in antibacterial activity is well-established. Thus, simple charge compensation provides a natural way to avoid autocytotoxicity in the cells synthesising antibacterial peptides. For example, defensins, cecropins, dermaseptins and magainins are synthesised with negatively charged peptide segments in the prepropeptides [35]. After maturation, defensins are stored in granules together with anionic mucopolysaccharides [36]. Internal charge compensation would also resolve the apparent paradox that NK-lysin looses its antibacterial activity upon selective reduction of one of its disulfide bridges [37], presumably leading to a collapse of its three-dimensional structure, while the native protein and a short helical fragment thereof are active.

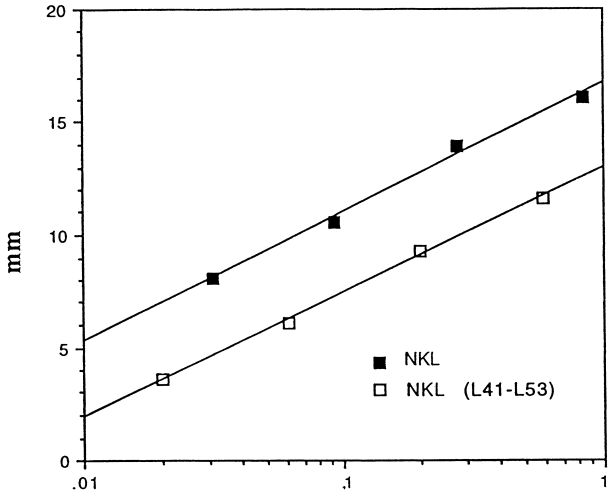

Fig. 2. Antibacterial activity of NK-lysin (filled symbols) and the fragment Leu-41–Leu-53 containing helix 3 of NK-lysin (open symbols). The diameters of the lysed *E. coli* cell cultures on agarose plates were plotted against the peptide concentrations. The parallel curves suggest a similar mechanism of cell lysis.

5. Concluding remarks

Molecular electroporation provides a simple explanation for membrane pore formation by a large class of small, cationic peptides, even though the detailed aspects of antibacterial activity can be more complex (see e.g. [31,38–40]). The strength of the concept lies in its predictive value, as electrostatic calculations can be used to address the question of pore formation, as compared to qualitative descriptions of antibacterial peptides as 'detergent'-like [8,31,35,41]. In the case of NK-lysin, even highly simplified calculations of the electrostatics of free and membrane-associated NK-lysin resulted in a plausible model predicting electroporation. Although the model did not deal with details (fluorescence quenching experiments indicated reduced solvent accessibility of Trp-58 in membrane-associated NK-lysin [13], suggesting deeper penetration into the membrane than in the model of Fig. 1C), antibacterial activity of a peptide comprising only helix 3 was accurately predicted. It should be possible to apply the concept of molecular electroporation to the design of new non-peptidic molecules with high antibacterial activity. This will be particularly important as antibiotic peptides are too expensive for large scale production [42].

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