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Generation of analogs having potent antimicrobial and hemolytic activities with minimal changes from an inactive 16-residue peptide corresponding to the helical region of *Staphylococcus aureus* δ-toxin

V.M.Dhople and R.Nagaraj[1]

Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

[1]To whom correspondence should be addressed

The δ-toxin is a 26-residue peptide from *Staphylococcus aureus* with the sequence formyl-MAQDIISTIGDLVKWII-DTVNKFTKK. NMR studies indicate that the segment IISTIGDLVKWIIDTV occurs in an α-helical conformation in the toxin. A synthetic peptide corresponding to this segment, although helical, did not exhibit hemolytic activity. Since charged residues like D and K are likely to modulate cytolytic activity, analogs of the 16-residue peptide were synthesized where D was systematically replaced by K. Analogs in which the first D and both Ds were replaced by K showed potent antimicrobial and hemolytic activities. The analog in which the second D was replaced by K was relatively less active. However, all the peptides showed an α-helical structure with similar helical content. The activities of the peptides were found to correlate directly with their ability to permeabilize model membranes. Thus, by minimal judicious replacement of charged amino acids, it should be possible to generate cytolytic peptides from short segments of peptide toxins.

Key words: antimicrobial activity/helical segment/δ-hemolysin/hemolytic activity/16-residue peptides

Introduction

Peptides form a part of the host defense mechanism in insects (Boman, 1991; Hoffman and Hetru, 1992; Hultmark, 1993), amphibians (Bevins and Zasloff, 1990) and mammals, including humans (Lehrer *et al.*, 1991). While peptides δ-toxin (Fitton *et al.*, 1980) and bombolitin (Argiolas and Pisano, 1985) are hemolytic, others like cecropins (Boman, 1991) and magainins (Bevins and Zasloff, 1990) exhibit only antimicrobial activities. Melittin exhibits potent hemolytic (Habermann and Jentisch, 1967) and antibacterial activities (Boman *et al.*, 1989). There has been considerable interest in recent years on the structure, function and biology of antimicrobial peptides due to the serious problem of resistance to conventional antibiotics that are used therapeutically (Neu, 1992; Silver and Bostian, 1993). Considering the fact that peptides are used by organisms across the evolutionary scale to combat bacteria and also the observation that these peptides exert their activity by permeabilizing the bacterial plasma membrane (Christensen *et al.*, 1988; Duclhier *et al.*, 1989; Lehrer *et al.*, 1989; Ohta *et al.*, 1992), it is conceivable that resistance may not be developed easily by them. Although cecropins, magainins and defensins exhibit potent, broad-spectrum antimicrobial activity (Saberwal and Nagaraj, 1994), they may be of limited use in therapies because they are composed of 25–40 residues which would be expensive to synthesize. Hence, it would be pertinent to explore the possibility of generating shorter peptides (~15 residues) with antimicrobial activity, especially ones which are parts of longer toxins. Structure–function correlations with shorter segments of peptide toxins would also help in determining the minimal structural requirements for biological activity. In this paper we describe the antimicrobial and hemolytic activities of peptides derived from a 16-residue peptide corresponding to the helical region of the hemolytic toxin δ-hemolysin.

Materials and methods

Synthesis of peptides

Peptides IISTIGDLVKWIIDTV (1), IISTIG**K**LVKWIIDTV (2), IISTIGDLVKWII**K**TV (3) and IISTIG**K**LVKWII**K**TV (4), were synthesized by solid-phase methods using fluorenylmethoxycarbonyl (Fmoc) chemistry protocols (Atherton and Sheppard, 1989). The bold amino acids indicate changes from δ-toxin. Peptides 1 and 2 were synthesized manually using *p*-hydroxymethyl phenoxymethyl polystyrene resin (HMP resin; Applied Biosystems, Foster City, CA). Peptides 3 and 4 were synthesized using KA resin (Nova Biochem, UK) on a Pharmacia LKB Biolynx 4175 peptide synthesizer. Peptide chains were assembled using Fmoc amino acid and 1-hydroxy benzotriazole (HOBT) active esters. Fmoc amino acids were obtained from Nova Biochem (UK). Peptides were deprotected from resin after synthesis by acidolytic cleavage using trifluoroacetic acid (TFA), thioanisole, meta-cresol and ethanedithiol (10:1:1:0.5). Purification of peptides was achieved by fast performance liquid chromatography (FPLC) using solvents A (0.1% TFA in water) and B (0.1% TFA in CH_3CN) on Pep RPC HR (5/5) reverse-phase (C_{18}) columns purchased from Pharmacia. The purified peptides were characterized by amino acid analysis on a Pharmacia LKB 4151 Alpha Plus amino acid analyzer and sequencing on an Applied Biosystems 473A automated protein sequencer. The data were fully compatible with the expected structures.

CD studies

CD spectra were recorded in 5 mM HEPES buffer at pH 7.4 containing 10 mM SDS on a Jobin Yvon Dichrograph V spectropolarimeter at 25°C using a quartz cell of 1 mm path length. Data are represented as mean residue ellipticities.

Permeabilization of lipid vesicles

The ability of peptides to cause release of carboxyfluorescein (CF) was checked by monitoring the fluorescence intensity of CF encapsulated in dioleoyl phosphatidylcholine (DOPC) vesicles at self-quench concentrations. Lipid film was dispersed in 5 mM HEPES, pH 7.4, 75 mM NaCl and 50 mM CF, and was sonicated in a Branson sonifier. Liposomes were separated from non-encapsulated CF by gel filtration on a Sephadex G-75 column (elution buffer: 5 mM HEPES, pH 7.4, 125 mM NaCl and 1 mM EDTA). The excitation monochromator was set at 493 nm and the emission at 520 nm was continuously monitored after the addition of peptide from a stock solution to the lipid vesicles. The fluorescence of CF is enhanced

considerably on dilution; hence the increase in fluorescence is a measure of vesicle permeabilization. Complete release of CF was obtained by the addition of Triton X-100 (1% v/v). All experiments were carried out at 25°C in a Hitachi 650-10S spectrofluorimeter.

Hemolytic activity

It has been observed that natural δ-toxin causes the lysis of guinea pig erythrocytes to a slightly greater extent than erythrocytes from other mammalian species (Alouf *et al.*, 1989). Hence, we have examined the ability of peptides 1–4 to cause lysis of guinea pig erythrocytes. Freshly collected blood from a guinea pig was centrifuged and washed with 5 mM HEPES buffer (pH 7.4) containing 150 mM NaCl to remove the buffy coat. Erythrocytes (0.4% v/v) were incubated at 37°C in the same buffer with different concentrations of peptide in duplicate. Time course measurements indicated that lysis was complete in 20 min. Lysis at the end of incubation for 30 min was measured for all the peptides. Samples were centrifuged and the A_{540} of the supernatant was measured. The absorbance obtained by the treatment of erythrocytes with 1% Triton X-100 was taken as 100%.

Antimicrobial activity

The antimicrobial activities were assayed by adding different concentrations of peptides to 1 ml of synthetic medium (Miller, 1972) containing ~10^6 c.f.u./ml of the test organisms which were *Escherichia coli* W160-37, and *Staphylococcus aureus* in duplicate. Microbial growth was determined by increase in OD_{600}, after incubation at 37°C for 6–9 h depending upon growth rate. The lowest concentration of peptide that completely inhibited growth was recorded as the minimal inhibitory concentration (MIC).

Results

The structure of δ-toxin has been determined in methanol and micelles by ^{1}H NMR. Based upon observed nuclear Overhauser enhancements (NOE) and restrained molecular dynamics analysis, Tappin *et al.* (1988) have suggested that residues 2–20 form a stable helix in methanol at pH 3.0. Lee *et al.* (1987) have argued, based on their NMR data, that residues 5–23 are in a helical conformation in micelles. An examination of the NMR data of Tappin *et al.* (1988) indicated that J_{NH}–$C^{\alpha}H$ is uniformly low between residues 6 and 20. Also, the conformation of δ-toxin in micelles would be more relevant to membrane interactions. Hence, we chose the segment IISTIGDLVKWII-DTV to assess its biological activity as well as for 'engineering'. The sequence IISTIGDLVKWIIDTV has one lysine and two aspartic acid residues. Since positively charged amino acids like lysine play an important role in determining the activity of antibacterial and hemolytic peptides like cecropins, magainins and melittin (Bernheimer and Rudy, 1986; Saberwal and Nagaraj, 1994), variants of this 16-residue peptide, where D(7) and D(14) were replaced by lysines, i.e. peptides 2–4 (sequences indicated in Materials and methods), were synthesized.

Conformation of peptides

Since the ability to adopt helical conformation is an important requirement for binding to membranes and permeabilizing them in the case of membrane-interacting peptides (Kaiser and Kezdy, 1987), the conformation of peptides 1–4 was examined by CD spectroscopy particularly in the hydrophobic environment. The CD spectra of the peptides are shown in Figure 1. The spectra are characteristic of peptides in a helical conformation. An estimation of the secondary structural parameters using the program LINCOMB (Perczel *et al.*, 1992) with the template CD curves for polylysine (Yang *et al.*, 1986) yielded helix and random conformation with no β-sheet. The helical content for the four peptides was between 65 and 75%. The spectrum of peptide 1 indicates that the segment which is helical in the entire toxin has the tendency to be helical even in isolation. The spectra of peptides 2–4 indicate that the replacement of Asp by Lys does not affect structure.

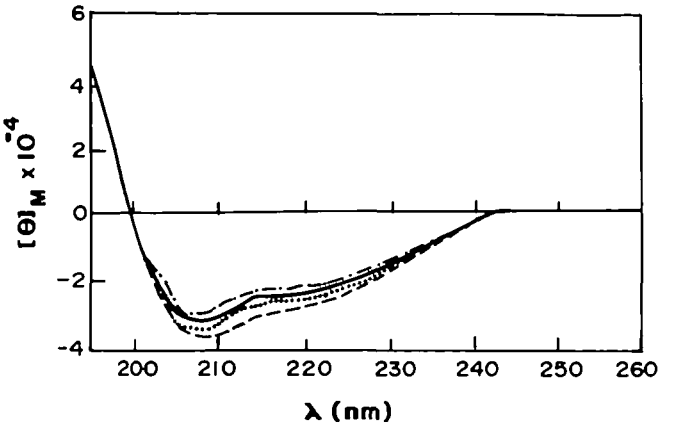

Fig. 1. CD spectra of peptides in micelles of SDS: peptide 1 (—), peptide 2 (– –), peptide 3 (– · –) and peptide 4 (· · ·). Peptide concentration = 10 nmol.

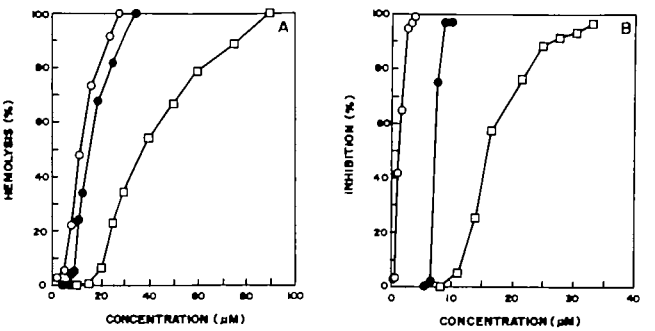

Fig. 2. Hemolytic and antimicrobial activity of peptides. (**A**) Hemolytic activity of peptides 2 (●), 3 (□) and 4 (○). Data points correspond to the mean of two independent observations. The variation was 30% in the low concentration range, i.e. up to 10 μM for peptides 2 and 4, and up to 20 μM for peptide 3. Above these concentrations the variation was 15%. (**B**) Antimicrobial activity against *E.coli*: peptides 2 (●), 3 (□) and 4 (○). Data points correspond to the mean of two independent observations. The variation was ~5%.

Hemolytic activity

The hemolytic activity of peptides 1–4 was determined against guinea pig erythrocytes. Peptide 1, corresponding to the helical region of δ-toxin, did not exhibit hemolytic activity even at a concentration of 100 μM. However, peptides 2–4 have the ability to lyse erythrocytes, as shown in Figure 2A. Peptides 2 and 4 cause 50% lysis at concentrations of ~15 and 11 μM, whereas peptide 3 is relatively inactive, with 50% lysis occurring at ~40 μM.

Antimicrobial activity

The antimicrobial activity of the peptides against *E.coli* and *S.aureus* was examined. Peptide 1 did not exhibit antimicrobial

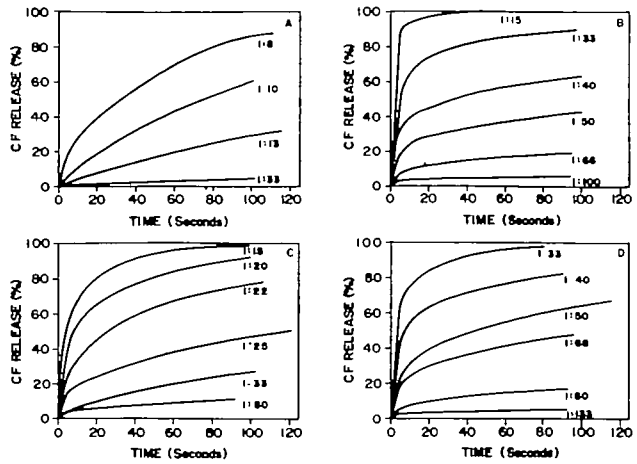

Fig. 3. Release of CF from small unilamellar vesicles of dioleoylphosphatidylcholine by peptides 1 (**A**), 2 (**B**), 3 (**C**) and 4 (**D**). Ratios indicated in the figure are peptide:lipid molar ratios. Lipid = 200 μM.

activity even at concentrations >100 μg/ml. Peptides 2–4 exhibited activity against both micro-organisms. The growth inhibition of *E.coli* in the presence of peptides 2–4 is shown in Figure 2B. The most potent peptide is peptide 4, with a MIC of 3 μM. Peptide 2 has a MIC of 8 μM, while peptide 3 is less active with a MIC of ~35 μM. The MIC against *S.aureus* was 16–22 μM for peptide 2 and 3–4 μM for peptide 4. No inhibition of growth was observed for peptide 3, even at a concentration of ~75 μM. The extents of hemolysis at MIC for peptides 2, 3 and 4 were 5, 50 and ~3%, respectively. The values indicate percentage lysis as compared with that obtained by Triton X-100. At MIC against *E.coli*, peptides 2 and 4 exhibit very little hemolytic activity. Thus, by changing the location of charged amino acids in helical peptides (like 1–4), peptides with varying antimicrobial and hemolytic activities can be generated.

Membrane-permeabilizing properties

Antimicrobial and hemolytic peptides, as well as several peptide toxins which form channels in membranes under the influence of a potential (Sansom, 1991), also permeabilize lipid vesicles which results in the efflux of trapped solutes like CF and calcein (Kayalar and Duzgunes, 1986; Menestrina, 1988; Schwarz and Robert, 1990; Shai *et al.*, 1990; Vaz Gomes *et al.*, 1993; Saberwal and Nagaraj, 1994). The extent of permeabilization was found to correlate with antimicrobial and/or hemolytic activities. Since the extents of antimicrobial and hemolytic activities of peptides 1–4 are different, with peptide 1 being inactive, the membrane-permeabilizing abilities of the peptides were examined in model membranes and the data are presented in Figure 3. Peptide 1 is not effective in permeabilizing lipid vesicles as only at a peptide:lipid ratio of 1:10 is 60% release observed at 100 s. At peptide:lipid ratios of 1:13 and 1:10 complete release of CF was observed after 20 min. Peptides 2–4 permeabilize lipid vesicles effectively. The results indicate that peptide 4 permeabilizes lipid vesicles most effectively, followed by peptide 2. No decrease in 90° scatter of lipid vesicles was observed at lipid:peptide ratios where maximal CF release occurred, indicating that the lysis of lipid vesicles did not occur. The model membrane-permeabilizing abilities of peptides 1–4 clearly correlate well with their antimicrobial and hemolytic activities, indicating that their association with lipid components of membranes and their ability to permeabilize them is an important determinant of cytolytic activity.

Discussion

The δ-toxin is a 26-residue peptide isolated from *S.aureus* with hemolytic activity against a variety of red blood cells (RBCs; Yianni *et al.*, 1986). Unlike melittin, the peptide does not inhibit bacterial growth (Dhople and Nagaraj, 1993). This specificity has been proposed to arise due to differences in its interaction with the bacterial cell surfaces and RBC surface (Dhople and Nagaraj, 1993). Structural analysis of the toxin by ^{1}H NMR indicates a helical stretch between residues 2 and 22 with a 'frayed' C-terminal region (Tappin *et al.*, 1988). The stretch between residues 5 and 20 gives NOE effects characteristic of an ordered helical conformation and slow exchange rates for amide hydrogens (Tappin *et al.*, 1988). This region is thus likely to be important for the structure and function of the toxin. Hence, we chose this stretch to evaluate its importance in determining the biological activity of the toxin. We also wished to examine how charged residues like aspartic acid and lysine would modulate cytolytic activity. Thus, analogs where Asp residues were systematically replaced by lysine were also synthesized and their antimicrobial and hemolytic activities examined.

Our studies indicate that the peptide IISTIGDLVKWITDTV, corresponding to the α-helical region of δ-toxin, can adopt helical conformation in isolation. However, the peptide does not possess hemolytic or antimicrobial activity. One reason for this lack of activity could be the net charge the peptide would have at neutral pH. The two D and K residues would impart a net charge of −1 at neutral pH. A net negative charge would not favor association with either erythrocytes or bacteria as both cells have negatively charged molecules on their surface (Lughtenberg and Van Alphen, 1983; Viitala and Jarnefelt, 1985). Replacement of either of the D residues with K results in peptides which would have a net positive charge at neutral pH. Both such peptides, i.e. peptides 2 and 3, exhibit antimicrobial and hemolytic activities. However, peptide 2 (where the first D has been replaced by a K) is more active than peptide 3 (where the second D has been replaced by a K), although both peptides have similar helical contents. Peptide 2 is less active against *S.aureus* and peptide 3 did not inhibit the growth of *S.aureus* at concentrations where the growth of *E.coli* was inhibited completely. However, in the case of peptide 4, the MICs against *E.coli* and *S.aureus* are almost identical. It is conceivable that proteases are secreted by *S.aureus* and peptides 2 and 3 are more susceptible to the action of these proteases as compared with peptide 4. In peptide 3, D is at the *i* and K is at the *i* + 3 position. Extensive analyses of protein structures (Presta and Rose, 1988; Richardson and Richardson, 1988) and studies on model peptides (Marqusee and Baldwin, 1987) indicate that such an arrangement would favor side-chain interactions like salt-bridge formation. It is thus conceivable that in peptide 3 there is salt-bridge formation between the side chains of D at the *i* and K at the *i* + 3 positions, leaving only the side chain of K at position 14 for interaction with erythrocyte and bacterial cell surfaces. However, in peptide 2, where salt-bridge formation would not be favored, the side chain of the lysines would be available for interaction. Hence, peptide 2 is more active than peptide 3. Peptide 4, where both D residues have been

replaced by K, is clearly more active than peptides 2 and 3. In this peptide, at MIC hemolysis is only ~5%, indicating that increased positive charges favor antimicrobial activity.

The biological activities of the peptides correlate well with their model membrane-permeabilizing ability. Peptides as well as protein toxins that form channels under the influence of a membrane potential also permeabilize small unilamellar vesicles like the ones used in this study, to cause efflux of entrapped solutes (Kayalar and Duzgunes, 1986; Menestrina, 1988; Schwarz and Robert, 1990; Shai *et al.*, 1990; Vaz Gomes *et al.*, 1993). Analysis of the efflux rates suggests that these peptides permeabilize lipid vesicles by forming channels (Schwarz and Robert, 1990; Shai *et al.*, 1990; Vaz Gomes *et al.*, 1993). Since peptides 1–4 do not appear to lyse the lipid vesicles, it is likely that they permeabilize lipid vesicles by forming channels. Although peptide 1 does permeabilize lipid vesicles, albeit inefficiently, it does not cause the lysis of RBCs. In almost all cases, the lysis of RBCs by membrane-active peptides proceeds through a colloid osmotic lysis mechanism, and it is likely that in the case of peptide 1 lysis does not occur because the volume-regulating ability of the Na^+ pump is not overcome (Bashford *et al.*, 1986). Model membrane permeabilization studies suggest that the motif DXXKXXXD observed in peptide 1 does not favor channel formation in lipid vesicles. Peptides 1–4 have an average hydrophobicity ($<H>$) of ~0.45 and hydrophobic moment ($<\mu>$) of ~0.63. This indicates that they would associate with membranes as they fall in the surface-active region of the hydrophobic moment plot (Eisenberg, 1984). It is likely that all four peptides, although exhibiting varying channel-forming abilities, do bind to membranes. Hence, $<H>$ and $<\mu>$ values, although a useful index to judge the ability of a peptide to associate with membranes, may not indicate its channel-forming and consequently membrane-permeabilizing ability.

Studies directed towards structure–function correlations in antibacterial and hemolytic peptides have involved the replacement of amino acids and the generation of deletion peptides (Habermann and Kowallek, 1970; Merrifield *et al.*, 1982; Chen *et al.*, 1988; Zasloff *et al.*, 1988; Boman *et al.*, 1989). While peptides where amino acid replacements were carried out did result in improved antimicrobial activity, the deletion peptides were often inactive, suggesting a minimal length requirement for activity. Peptides corresponding to the helical segment of δ-toxin, IISTIGDLVKWIIDTV, do not appear to have hemolytic activities exhibited by the parent toxin. However, by judicious replacement of D by K in the above sequence, we have been able to generate peptides with antimicrobial and hemolytic activities. By adopting a similar approach, it should be possible to derive short peptides of ~15–16 residues with potency essentially comparable with or even greater than the parent peptides. Such an approach would be necessary for cecropins and defensins because although they are potent antibacterial agents (Bevins and Zasloff, 1990; Lehrer *et al.*, 1991), they are composed of ~36 residues and would be of limited use as therapeutic agents. Short peptides would be more appropriate for therapeutic consideration. Our approach reported here, as well as that of Andreu *et al.* (1992) which involves cecropin A–melittin hybrids, indicate that it should be possible to develop ~15–16-residue peptides with considerable potency. Our approach would also help in determining the requirements for the antibacterial and hemolytic activities of the parent peptides and should help in developing analogs with increased potency as compared with the parent peptides.

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