A conserved tryptophan in pneumolysin is a determinant of the characteristics of channels formed by pneumolysin in cells and planar lipid bilayers

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Pneumolysin is one of the family of thiol-activatable, cytolytic toxins. Within these toxins the amino acid sequence Trp-Glu-Trp-Trp is conserved. Mutations made in this region of pneumolysin, residues 433–436 inclusive, did not affect cell binding or the formation of toxin oligomers in the target cell membrane. However, the mutations did affect haemolysis, leakage of low-molecular-mass metabolites from Lettre cells and the induction of conductance channels across planar lipid bilayers. Of eight modified pneumolysins examined, Trp-433 → Phe showed the smallest amount of haemolysis or leakage (less than 5% of wild type). Pneumolysin-induced leakage from Lettre cells was sensitive to inhibition by bivalent cations but the extent of inhibition varied depending on the modification. Leakage by the mutant Trp-433 → Phe was least sensitive to cation inhibition. The ion-conducting channels formed across planar lipid bilayers exhibit small (less than 30 pS), medium (30 pS–1 nS) and large (more than 1 nS) conductance steps. Small- and medium-sized channels were preferentially closed by bivalent cations. In contrast with wild-type toxin, which formed predominantly small channels, the modified toxin Trp-433 → Phe formed large channels that were insensitive to cation-induced closure. Polysaccharides of molecular mass more than 15 kDa inhibited haemolysis by wild-type toxin, but polysaccharide of up to 40 kDa did not prevent haemolysis by Trp-433 → Phe. Electron microscopy revealed that Trp-433 → Phe formed oligomeric arc and ring structures with dimensions identical with those of wild-type toxin, and that the ratio of arcs to rings formed was the same for wild-type toxin and the Trp-433 → Phe variant. We conclude that the change Trp-433 → Phe affects channel formation at a point subsequent to binding to the cell membrane and the formation of oligomers, and that the size of arc and ring structures revealed by electron microscopy does not reflect the functional state of the channels.

INTRODUCTION

Bacterial thiol-activatable toxins interfere with the function of eukaryotic cells by generating channels in the membrane of target cells. One of these toxins, pneumolysin, is known to contribute to the virulence of the important human pathogen *Streptococcus pneumoniae* [1]. Pneumolysin is a multifunctional protein. Not only can it lyse all eukaryotic cells but at sublytic concentrations it will interfere with the activity of cells of the immune system [2]. These anticellular effects are thought to result from damage to the membrane of target cells brought about by the formation of channels by toxin oligomers [3]. Furthermore pneumolysin can activate the classical complement pathway independently of anti-pneumolysin antibodies [4].

In view of the role pneumolysin has in the virulence of *S. pneumoniae* and the possibility that it might serve in a future human vaccine [5], there is interest in relating the structure of the toxin to its function. By using site-directed mutagenesis we had previously investigated [6] the role of the single cysteine residue at position 428. Although the residue was not found to be essential for activity, the nature of the amino acid present at this site did influence activity. The cysteine residue lies in a region of 11 residues that forms the largest region of homology within the family of thiol-activatable toxins [3]. Within this region in pneumolysin there is the amino acid sequence Trp^{433}-Glu-Trp-Trp^{436}. We used-site directed mutagenesis to change these residues. Here we present results showing how these changes affect toxin activity against erythrocytes, pneumolysin-induced leakage of low-molecular-mass metabolites from a nucleated cell and the behaviour of the toxin within planar lipid bilayers.

MATERIALS AND METHODS

Bacteria

Escherichia coli JM109 was used in this study. Bacteria were grown in Luria broth, containing 100 μg/ml ampicillin when appropriate.

Pneumolysin

Site-directed mutagenesis of the pneumolysin gene was done by the oligonucleotide-mediated method of Kunkel et al. [7], as described previously [6]. M13 phages containing presumed mutant pneumolysin genes were plaque-purified twice; the nucleotide sequence of the insert was then determined [8]. Wild-type and mutated pneumolysins were purified from lysates of *E. coli* harbouring pKK233-2 carrying the wild-type or mutated pneumolysin gene, on a hydrophobic interaction chromatography column, as described previously [9]. Purity was assessed by SDS/PAGE [10] and protein was assayed by the method of Bradford [11], with BSA as the standard.

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Haemolytic activity

Haemolytic activity was measured on the basis of the method described previously [12]. Human, rabbit or sheep erythrocytes were washed three times in Hepes-buffered saline [HBS; 0.15 M NaCl/5 mM KCl/1 mM $MgSO_4$ in 5 mM Hepes (pH 7.4)] and resuspended in HBS. Washed erythrocytes at 0.1–1 % (v/v) were incubated with pneumolysin at 37 °C in round-bottomed, 96-well tissue culture plates for 20–60 min. Haemolysis was either estimated by eye or by the amount of haemoglobin present in the supernatant as measured by absorbance at 543 nm. Haemolytic activity is given as the weight (in μg) of toxin required to cause 50 % lysis of the sheep erythrocytes. The apparent size of pneumolysin-induced channels in human erythrocytes was estimated from the molecular mass of 10 mM dextran sufficient to prevent colloid osmotic lysis [13,14].

Assay of cell binding

Binding of toxin to erythrocytes was assayed by Western blot analysis of erythrocyte membranes exposed to toxin. Wild-type or modified toxin (10 ng–500 μg) was incubated with 0.2 % (v/v) packed sheep erythrocytes in Hanks balanced salt solution (Gibco) for 30 min in an ice–water bath. The cells were washed three times in ice-cold Hanks balanced salt solution and then lysed in water. The membranes were harvested and washed twice in water by centrifugation at 9500 ***g*** in a Microfuge and then resuspended in SDS/PAGE loading buffer [10]. SDS/PAGE was performed [10], after which proteins were transferred to nitrocellulose membranes (Sartorius) [15]. The membranes were incubated in 5 % (w/v) skimmed milk powder in PBS for 60 min, washed in PBS and incubated with rabbit anti-pneumolysin antiserum, diluted 1:1000 in PBS, for 90 min. The membranes were incubated for 60 min in 1:2000-diluted goat anti-(rabbit immunoglobulin) conjugated with horseradish peroxidase (Sigma). The proteins recognized by the anti-pneumolysin antiserum were detected by enhanced chemiluminescence, in accordance with the manufacturer's (Amersham) instructions. The endpoint of the titration of toxin binding was estimated by eye.

Complement activation

Activation was measured by two-dimensional immunoelectrophoresis as described previously [4], with human serum. The complement-activating activity of 10 μg/ml of the mutants was estimated by comparison of area of the peak formed with that formed by 10 μg/ml wild-type pneumolysin.

Oligomer formation

The formation of oligomers of pneumolysin in sheep erythrocytes was assessed with a combination of sucrose density gradient centrifugation and SDS/PAGE, as described previously [6].

Electron microscopy

Pneumolysin oligomers were observed after treatment of erythrocytes or liposomes with pneumolysin. Liposomes were made, as described previously [16], with a mixture of 10 μmol of phosphatidylcholine, 10 μmol of cholesterol and 1 μmol of dicetyl phosphate dissolved in chloroform/methanol (1:1, v/v) before drying under nitrogen. Lipids were resuspended in PBS, pH 7.3 (8 mM Na_2HPO_4/1.5 mM KH_2PO_4/137 mM NaCl/2.5 mM KCl) and dispersed by sonication in a water-bath for 90 s. Suspensions were filtered through a 0.45 μm filter before use. Erythrocytes and liposomes were treated with pneumolysin as previously [16,17]. Samples were negatively stained with 0.8 % sodium phosphotungstate, pH 6.8 [17]. Magnification was calibrated with a diffraction-grating replica and electron micrographs were measured on an Apple digitizer. At least 30 measurements were made of each dimension.

Leakage from cells

The murine ascites tumour line, Lettre, was maintained as described previously [18]. Pneumolysin-induced leakage from Lettre cells was assayed by following the release of phospho-[^{3}H]choline from cells previously labelled by incubation in [^{3}H]choline [18,19]. Cation leakage was estimated from the Na^+ plus K^+ content of cells pelleted through oil [20]. Leakage, expressed as a percentage, was calculated relative to control cells not treated with pneumolysin. Membrane depolarization was monitored with oxonol-V and expressed relative to depolarization (100 %) induced by gramicidin [21,22].

Conductance across planar lipid bilayers

Planar bilayers of a 1:2 molar mixture of dioleoyl phosphatidylcholine and ergosterol were prepared as described previously [23]. Lipids were obtained from Sigma. Conductance was measured with Ag/AgCl electrodes as described previously [23].

RESULTS

Effect of altered pneumolysins on cell binding, oligomer formation and complement activation

None of the modified toxins seemed to be defective in their ability to bind to erythrocytes or to form oligomers on the membranes of these cells; neither did any of the modified toxins show complement activating activity that could be distinguished from that of wild-type toxin (Table 1).

Effect of altered pneumolysin on erythrocytes

Table 1 shows the haemolytic activity of pneumolysins against sheep erythrocytes. The effect against rabbit or human erythrocytes was similar. These results confirm our previous observation [6] that modifications Cys-428 → Gly or Cys-428 → Ser result in a large decrease in activity but that Cys-428 → Ala retains full potency. Further mutagenesis of the pneumolysin

Table 1 Activity of mutated pneumolysins against erythrocytes and Lettre cells

Numbers in parentheses are μg of pneumolysin required to cause 50% lysis of erythrocytes or leakage of ^{3}H from Lettre cells. Abbreviation: n.d., not done.

Variant	Haemolytic activity (% of wild-type toxin)	Leakage from Lettre cells (% of wild-type toxin)	Concentration (M) of bivalent cation causing 50% inhibition of [^{3}H]choline from Lettre cells			Complement activation (% of wild-type)
			Zn^{2+}	Ca^{2+}	Mg^{2+}	
Wild-type	100 (0.1)	100 (0.05)	3×10^{-5}	10^{-3}	$>10^{-2}$	100
Cys-428 → Gly	3 (3.3)	6 (0.8)	6×10^{-5}	$>10^{-2}$	$>10^{-2}$	100
Cys-428 → Ser	25 (0.4)	62 (0.08)	3×10^{-5}	10^{-3}	$>10^{-2}$	100
Cys-428 → Ala	100 (0.1)	25 (0.2)	10^{-4}	$>10^{-2}$	$>10^{-2}$	100
TRP-433 → Phe	1 (9.0)	3 (1.5)	6×10^{-4}	$>10^{-2}$	$>10^{-2}$	n.d.
Glu-434 → Gln	20 (0.5)	12 (0.4)	8×10^{-5}	2×10^{-3}	$>10^{-2}$	100
Glu-434 → Asp	50 (0.2)	6 (0.8)	10^{-4}	10^{-3}	$>10^{-2}$	100
Trp-435 → Phe	20 (0.5)	5 (1.1)	6×10^{-5}	$>10^{-2}$	$>10^{-2}$	n.d.
Trp-436 → Phe	50 (0.2)	10 (0.5)	6×10^{-5}	$>10^{-2}$	$>10^{-2}$	n.d.

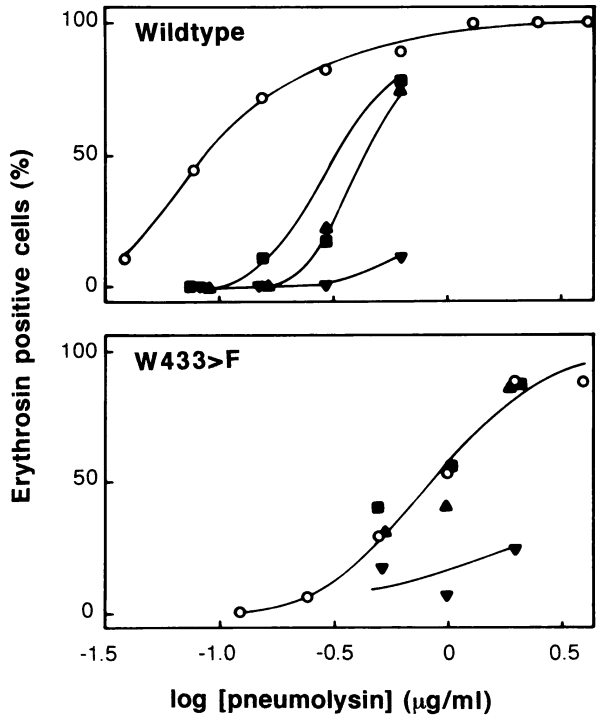

Figure 1 Effect of Zn^{2+} on preformed pneumolysin channels in Lettre cells

Cells attached to concanavalin A-coated dishes were incubated with wild-type pneumolysin (Cys-428) or Trp-433 → Phe (W433 F) in Hanks balanced salt solution at 37 °C for 20 min. Erythrosin uptake by washed cells was assessed without (○) or with 0.1 (■), 0.3 (▲) or 1 mM (**) Zn^{2+}.

gene showed that other changes within the conserved 11 amino acid residues surrounding this cysteine had profound affects on the haemolytic activity of pneumolysin. The modification Trp-433 → Phe had a particularly marked affect, resulting in a 99% loss in haemolytic activity.

Effect of altered pneumolysins on Lettre cells

When leakage of phospho-[^{3}H]choline from [^{3}H]choline-labelled cells induced by pneumolysin mutants was assessed, the relative order of efficacy of the modified toxins was similar to that for haemolysis of sheep or human erythrocytes. When the sensitivity of leakage to inhibition by bivalent cations was measured, it was found that order of potency was $Zn^{2+} > Ca^{2+} > Mg^{2+}$ in every case (Table 1). Cells on which toxin had been adsorbed at 0 °C and from which excess toxin had then been washed away revealed the same degree of inhibition by Zn^{2+} and Ca^{2+} as when the bivalent cations were present throughout, showing that the effect of the cations is not on the binding of toxin to cells. Trp-433 → Phe consistently showed the biggest difference from wild-type toxin in cation sensitivity. This also was true for the inhibition of haemolysis of sheep or human erythrocytes (results not shown). Thus this mutant was chosen for further studies of bivalent cation sensitivity.

To distinguish between an effect on the induction of channels (subsequent to binding) and the closure of preformed channels, the following experiment was performed. Lettre cells, prelabelled with [^{3}H]choline, were attached to concanavalin A-coated dishes and treated with pneumolysin in buffer for 20 min at 37 °C. The supernatant was removed and its ^{3}H content was assayed to confirm that cells on all the pneumolysin-treated dishes had been permeabilized. These dishes were then exposed to fresh buffer with or without Zn^{2+} for 10 min at room temperature. Erythrosin (0.05%, w/v) was then added and dishes were scored for red (erythrosin-positive) cells (Figure 1). It was consistently found that erythrosin uptake by wild-type-treated cells was inhibited by approx. 50% by 0.1–0.3 mM Zn^{2+}, in conditions under which a similar degree of inhibition was observed if Zn^{2+} and pneumolysin were added together before permeabilization. A comparison of the effect of Zn^{2+} on wild-type- and Trp-433 → Phe-treated cells shows that Trp-433 → Phe was approx. one-third to one-tenth as sensitive to Zn^{2+}. Similar data were obtained when Trypan Blue (0.05%, w/v) was used in place of erythrosin (results not shown).

The above experiments demonstrate a differential cation sensitivity between wild-type and Trp-433 → Phe pneumolysin with regard to closure of channels through which phospho-[^{3}H]choline (183 Da), erythrosin (836 Da) or Trypan Blue (961 Da) leak. To test whether the leakage of smaller molecules, namely monovalent cations, was similarly affected, an experiment was performed in which membrane depolarization, cation leakage and phospho-[^{3}H]choline leakage were assessed simultaneously.

Table 2 Effect of Ca^{2+} and Zn^{2+} on pneumolysin-induced damage of Lettre cells

Results are means ± S.E.M. of *n* (in parentheses) experiments. In each case cells were incubated with pneumolysin with or without Ca^{2+} or Zn^{2+}; depolarization was monitored continuously. At the endpoint, cells were pelleted and a sample of the cell pellet and medium was analysed for content of Na^+, K^+ and ^{3}H. Percentage damage is defined as follows. Depolarization induced by pneumolysin in cells incubated with oxonol-V was calculated from $A_{630} - A_{590}$ immediately before (0% depolarization) and after the addition of pneumolysin, once a steady level had been reached; 100% depolarization is defined as the endpoint induced by the addition of 10 μM gramicidin [21]. Under the conditions of these experiments the membrane potential before the addition of pneumolysin was −57 ± 3 mV. Cation leakage induced by pneumolysin was calculated from the Na^+ content of the cells {expressed as $[Na^+]/([Na^+]+[K^+])$ in the cell pellet} immediately before (0% leakage) and after the addition of pneumolysin for the time taken to achieve a steady state of depolarization; 100% cation leakage is defined as complete equilibration between internal and external cations. Under the conditions of these experiments $[Na^+]/([Na^+]+[K^+])$ in the cell pellet before pneumolysin addition was in the range 0.25–0.35. [^{3}H]Phosphocholine leakage induced by pneumolysin was calculated from the ^{3}H in the medium {expressed as $[^3H]_{supernatant}/([^3H]_{supernatant}+[^3H]_{pellet})$} immediately before (0% leakage) and after the addition of pneumolysin, for the time taken to achieve a steady state of depolarization; 100% [^{3}H]phosphocholine leakage is defined as complete equilibration between internal and external ^{3}H. Under the conditions of these experiments $[^3H]_{supernatant}/([^3H]_{supernatant}+[^3H]_{pellet})$ before pneumolysin addition was always less than 0.25. The concentration of wild-type pneumolysin was 0.35 μg/ml; the concentration of Trp-433 → Phe toxin was 6.0 μg/ml. Results that were difficult to assess independently of the damaging action of 1 mM Zn^{2+} are indicated with a dash. Abbreviation: n.d., not done.

	Damage (%)		
Pneumolysin	Depolarization	Cation leakage	Phosphocholine leakage
Wild-type	80 ± 6 (4)	75 ± 9 (7)	60 ± 10 (9)
+ 0.3 mM Ca^{2+}	67 ± 4 (2)	45 ± 13 (5)	23 ± 7 (7)
+ 1 mM Ca^{2+}	37 ± 7 (2)	34 ± 11 (5)	27 ± 5 (7)
+ 10 mM Ca^{2+}	24 ± 11 (2)	0 ± 5 (5)	2 ± 4 (7)
+ 0.1 mM Zn^{2+}	80 (1)	60 ± 5 (10)	14 ± 7 (10)
+ 0.3 mM Zn^{2+}	72 (1)	41 ± 5 (10)	7 ± 4 (10)
+ 1 mM Zn^{2+}	–	–	1 ± 2 (4)
Trp-433 → Phe	83 ± 17 (3)	74 ± 12 (6)	58 ± 11 (8)
+ 0.3 mM Ca^{2+}	62 ± 4 (2)	74 ± 8 (5)	46 ± 7 (7)
+ 1 mM Ca^{2+}	50 ± 4 (2)	63 ± 4 (5)	34 ± 4 (7)
+ 10 mM Ca^{2+}	21 ± 12 (2)	28 ± 5 (5)	10 ± 6 (7)
+ 0.1 mM Zn^{2+}	n.d.	90 ± 13 (6)	74 ± 10 (6)
+ 0.3 mM Zn^{2+}	83 (1)	83 ± 7 (10)	31 ± 3 (10)
+ 1 mM Zn^{2+}	–	–	3 ± 1 (4)

The results (Table 2) are essentially the same as before. First, more (17-fold) Trp-433 → Phe was required to elicit the same degree of depolarization and cation leakage as wild-type toxin. Next, for a given amount of depolarization or leakage induced by wild-type or Trp-433 → Phe toxin, inhibition by Ca^{2+} and Zn^{2+} was approx. one-third to one-tenth as effective with Trp-433 → Phe.

A further feature of damage that emerged from these experiments was that at low concentrations of pneumolysin, or in the presence of inhibitory concentrations of Ca^{2+}, Lettre cells recovered spontaneously as far as the restoration of membrane potential and cation asymmetry were concerned (results not shown). Such recovery has also previously been observed in haemolytic Sendai virus [24,25], cytolysin [26] and *Staphylococcus aureus* α toxin [27].

Effect of altered pneumolysins on planar lipid bilayers

The differential sensitivity of wild-type toxin and Trp-433 → Phe to bivalent cations was further explored by measuring conductivity changes induced across planar bilayers composed of purified lipids. In a previous paper, Korchev et al. [23] showed that wild-type toxin, like the related tetanolysin [28], forms channels having a wide spectrum of conductances. Channels with small conductance (less than 30 pS) are highly selective for cations over anions (transfer number, t_+, 0.9 or 1.0), whereas channels with large conductance (more than 1 nS) are unselective (t_+ 0.5–0.6); channels of intermediate size (30 pS–1 nS) show intermediate selectivity (t_+ approx. 0.85). All types of channel tend to remain open unless bivalent cations are present. In that case small and intermediate-sized channels start to close, in a voltage-dependent manner; bringing the voltage to zero reopens the channels.

All these properties were found when Trp-433 → Phe was added to planar lipid bilayers. Figure 2, for example, which is typical of ten bilayer experiments, shows large channels (approx. 15–30 nS) appearing and remaining open in the absence (Figure 2, upper panel, trace i) or presence (Figure 2, lower panel, trace i) of 10 mM Ca^{2+}. Superimposed on some records (Figure 2, upper panel, trace i) are flickerings that indicate the opening and closing of smaller channels. Subsequent addition of 10 mM Ca^{2+} to the membrane from which trace i of Figure 2 (upper panel) is derived caused voltage-dependent closing (Figure 2, upper panel, trace ii) at +200 or +150 mV (but not at +100 mV) with re-opening at −200 or −150 mV. Individual channel events are not resolved in trace ii, but they are clearly at most 1 nS and not 15 nS as in trace i. Analysis of some single channels in the presence of 10 mM Ca^{2+} is possible in the membrane from which the records presented in Figure 2 (lower panel) are derived. Trace ii illustrates a section of the record where two or three intermediate-sized channels (approx. 150–200 pS) were active. At +50 and +75 mV fewer channels were open than at −50 and −75 mV; at +100 mV channel closure was more extensive than at +50 and +75 mV and although the channels re-opened on switching to −100 mV they subsequently tended to close. Thus the Ca^{2+}-dependent closure of intermediate channels induced by Trp-433 → Phe toxin (Figure 2, lower panel, trace ii) was much more voltage-sensitive than the closure of larger channels (Figure 2, upper panel, trace ii).

It should be noted that the decreased activity of Trp-433 → Phe compared with wild-type toxin that was apparent in all cellular assays was not seen with bilayers. On the contrary, although there was no clear-cut proportionality between the amount of pneumolysin added to bilayers and the number or type of channels that were induced, it was consistently found that

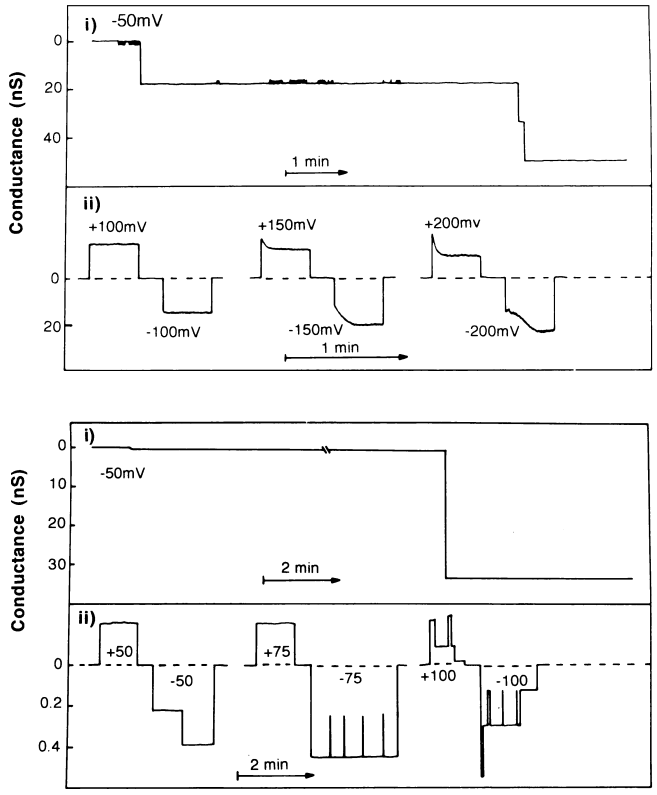

Figure 2 Pneumolysin-induced channels in planar lipid bilayers

Dioleoyl phosphatidylcholine/ergosterol (1:2, mol/mol) bilayers were bathed with 100 mM KCl/5 mM Hepes/0.1 mM EDTA (pH 7.4) at 25 °C with 50 μg/ml Trp-433 → Phe toxin in the absence (upper panel, trace i) or presence (upper panel, trace ii; lower panel) of 10 mM Ca^{2+} on the *cis* side. The applied potential was −50 mV except where otherwise indicated.

less, not more, Trp-433 → Phe compared with wild-type toxin was required to induce a significant number of channels.

If the bivalent cation was present on only one side of the bilayer (that to which toxin is added), small and medium-sized channels exhibited rectification: there was more current at negative voltages and less current at positive voltages, than in the absence of bivalent cations (Figure 3). Rectification might reflect the opening (or insertion) of channels at negative voltage and the closing (or removal) of channels at positive voltage. Bivalent cations do not induce closure of or rectification by large, unselective channels. The effect of Ca^{2+} on rectification by medium-sized, partly selective, channels but not by large unselective channels is most evident when results from many different experiments are pooled. Figure 3 shows the results for Trp-433 → Phe (left panel) in comparison with those for wild-type toxin (right panel). For each toxin there is no rectification for any type of channel in the absence of Ca^{2+} (panels A). In the presence of 10 mM Ca^{2+}, analysis of intermediate-sized channels (t_+ 0.85) shows voltage–dependent rectification (panels B); at higher voltage the scatter becomes more marked because of occasional closures at negative voltage of the type depicted in Figure 2 (lower panel). Analysis of large channels (t_+ 0.58) shows no evidence of rectification for Trp-433 → Phe or wild-type toxin (Figure 3, panels C). The effects of Zn^{2+} on channels induced by Trp-433 → Phe toxin are, like those of Ca^{2+}, broadly similar to those channels induced by wild-type toxin (results not shown). Thus no striking difference between Trp-433 → Phe and wild-type toxin was detected.

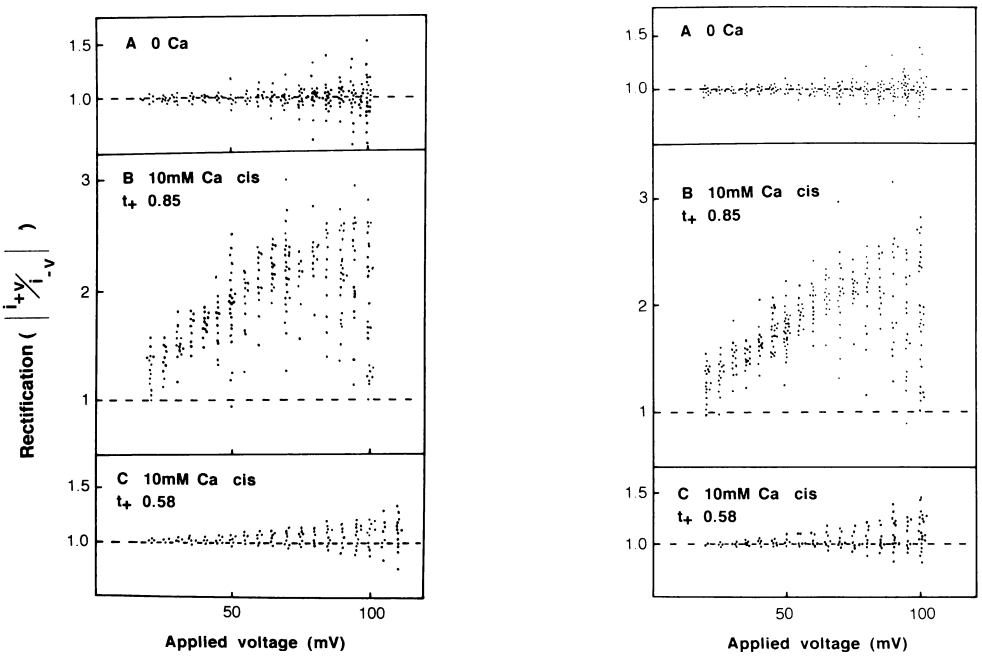

Figure 3 Rectification of pneumolysin-induced currents in planar lipid bilayers

Dioleoyl phosphatidylcholine/ergosterol (1:2, mol/mol) bilayers were bathed with 100 mM KCl (*cis* side) and 10 mM KCl (*trans* side) each with 5 mM Hepes/0.1mM EDTA (pH 7.4) at 25 °C with 50 μg/ml Trp-433 → Phe toxin (left panels) or Cys-428 → Gly toxin (right panels) in the absence (panels **A**) or presence (panel **B**, **C**) of 10 mM Ca^{2+} on the *cis* side. Panels **A** and **B** show rectification of intermediate-sized channels (t_+ 0.85); panels **C** show rectification of large channels (t_+ 0.58). Rectification is expressed as the modulus of the ratio of the current at positive potential (i_{+v}) to that observed immediately after reversing the polarity (i_{-v}).

Table 3 Probability of finding ion-conducting channels in pneumolysin-treated planar bilayers

All channels from a series of 18 separate experiments (wild-type) or 22 separate experiments (Trp-433 → Phe) were categorized by their conductance in 0.1 M KCl.

		Probability		
Pneumolysin	Channel type ...	Small (< 30 pS)	Medium (30 pS–1 nS)	Large (> 1 nS)
Wild-type		0.73	0.58	0.22
Trp-433 → Phe		0.38	0.59	0.67

When the results from all the bilayer experiments were pooled, it was seen that Trp-433 → Phe induced more large channels than small channels, whereas the reverse was seen with wild-type toxin (Table 3). Note that the sum of the probabilities of finding small, medium or large channels in pneumolysin-treated bilayer membranes exceeds 1 because many membranes were found to contain more than one type of channel.

Estimation of the size of pneumolysin lesions in erythrocytes

Protection by high-molecular-mass molecules against lysis has been used to estimate the size of channels in membranes [14,29]. To assess whether Trp-433 → Phe generated physically larger

Table 4 Effect of neutral solutes on pneumolysin-induced haemolysis

Wild-type and Trp-433 → Phe pneumolysin were incubated with 1% (v/v) rabbit erythrocytes in Hanks balanced salt solution at 37 °C without (control) and with either 5–10 mM stachyose or dextran 1500 (D1500), dextran 6000 (D6000), dextran 15000 (D15000) or dextran 40000 (D40000) for 20–60 min. Data are presented as percentage haemolysis [means ± S.D. of *n* (in parenthesis) experiments].

	Haemolysis (%)					
Pneumolysin	Control	Stachyose	D1500	D6000	D15000	D40000
Wild-type						
0.025 μg/ml	48 ± 10 (3)	50 (1)	20 (1)	3 ± 21 (3)	10 ± 10 (3)	2 ± 3 (3)
0.05 μg/ml	83 ± 6 (3)	70 ± 17 (3)	60 ± 35 (3)	53 ± 31 (3)	27 ± 31 (3)	2 ± 3 (3)
0.1 μg/ml	93 ± 5 (4)	87 ± 6 (3)	87 ± 6 (3)	80 ± 18 (4)	63 ± 15 (3)	27 ± 12 (3)
0.2 μg/ml	93 ± 7 (8)	95 ± 5 (3)	88 ± 8 (3)	90 ± 10 (3)	64 ± 14 (8)	54 ± 5 (5)
Trp-433 → Phe						
2.0 μg/ml	90 ± 15 (6)	80 ± 35 (3)	90 ± 10 (3)	93 ± 12 (3)	87 ± 17 (8)	97 ± 8 (6)

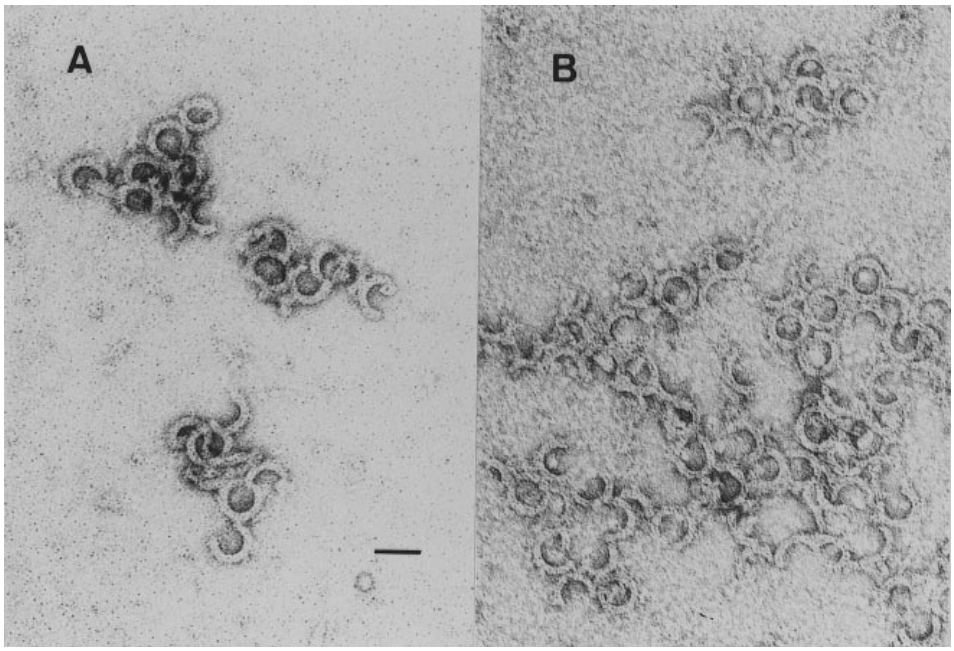

Figure 4 Negatively stained oligomers of Trp-433 → Phe (A) and wild-type (B) pneumolysin formed in liposomes

Scale bar, 40 nm.

channels in cells, the effect of high-molecular-mass osmolytes (polysaccharides) on the pneumolysin-induced haemolysis of rabbit erythrocytes was investigated. Table 4 shows that haemolysis induced by wild-type toxin was ameliorated by including 5–10 mM polysaccharide of molecular mass greater than 15 kDa. At low levels of wild-type toxin, i.e. 25 and 50 ng/ml, polysaccharides of less than 15 kDa also inhibited haemolysis. In contrast, haemolysis induced by Trp-433 → Phe toxin was unaffected by any of the polysaccharides used, including the largest (40 kDa). Similar results were obtained with human erythrocytes (results not shown).

Electron microscopy

Electron microscopy of negatively stained Trp-433 → Phe oligomers revealed large numbers of arc and ring structures on erythrocytes or liposomes. Figure 4(A) shows oligomers formed on liposomes. The dimensions of rings formed by wild-type (Figure 4B) and Trp-433 → Phe toxin were within the same range and were the same for rings on liposomes or erythrocytes. The dimensions measured in this study confirm the measurements previously reported for wild-type toxin in liposomes [16] and erythrocytes [17]. The ring width was measured as 6.7 ± 0.4 nm (mean $\pm$ S.D.). The inner diameter of the rings varied from 23 to 38 nm and the radius of curvature of the arcs was the same as that of the rings. The ratio of arcs to rings formed was approx. 0.5 with both forms of the toxin.

DISCUSSION

Previous studies [6] had shown that the residue at position 428 of pneumolysin was important for haemolytic activity. Here we show that other residues in the conserved region surrounding Cys-428 also have a part in inducing the formation of membrane pores (measured as haemolysis or leakage from Lettre cells). All three tryptophan residues in this region seem to contribute to haemolytic activity but it was at Trp-433 that the effect of mutagenesis was most pronounced. Michel et al. [30] had previously reported that tryptophan residues in this region were required for haemolytic activity of the related toxin listeriolysin and found that a change in Trp-492 (equivalent to Trp-436 in pneumolysin) almost abolished haemolytic activity. However, they did not report mutagenesis at Trp-489 (equivalent to Trp-433). Collectively these results and ours suggest that the whole of the conserved cysteine-containing region has a role in haemolysis. The results showing that Glu-434 also has a role supports this contention.

What are the reasons for the lower haemolytic activity of the altered toxins? Toxin action against target membranes can be divided into relatively discrete stages: (1) initial binding of the toxin; (2) oligomerization of bound monomers; and (3) induction of membrane pores. Our results suggest, as do previous studies with listeriolysin [30] and streptolysin [31], that it is in the properties of induced membrane pores that differences between the modified toxins occur and not in binding or oligomer formation. The cysteine region might correctly align pneumolysin [32] and other thiol-activated toxins in cell membranes. In that case the tryptophan residues, like those in gramicidin [33], might interact specifically with cholesterol.

Pneumolysin-induced damage, in common with that induced by other cytotoxins [18,34], is ameliorated by bivalent cations, especially Zn^{2+} and Ca^{2+}. In addition to their differences in potency, the various pneumolysin mutants also induce pores with differential sensitivities to Zn^{2+} and Ca^{2+}. The effect of these cations is not on the binding of pneumolysin, because inhibition occurs when cations are added to cells pretreated with toxin, as also occurs with the related toxin perfringolysin [35,36].

The explanation for the differential cation sensitivity of channels formed by Trp-433 → Phe and wild-type toxin might lie in the effective size of the pore generated. In planar lipid bilayers, Trp-433 → Phe forms more large than small channels, whereas

the opposite is true for wild-type toxin. Strictly speaking, it is more correct to refer to large compared with small conductance changes (i.e. openings and closings), because the nature of channels has not been established, and large channels might be multiples of smaller channels. As small channels are more sensitive to bivalent cations than large ones [23], Trp-433 → Phe-induced lesions will be less sensitive to such cations than wild-type-induced lesions. Although evidence that Trp-433 → Phe toxin generates larger channels than wild-type toxin in cell membranes is indirect, the protective action of high-molecular-mass polysaccharides in the haemolysis assay (Table 4) is consistent with the notion.

The electron microscopy results, however, do not reveal any significant differences between Trp-433 → Phe and wild-type toxin structures in membranes. The dimensions of the arc and ring structures seen with Trp-433 → Phe and wild-type pneumolysin are similar and identical with those previously reported for wild-type toxin in erythrocyte membranes [17]. Hence the structures seen on membranes, which reflect the degree of oligomerization, do not reflect their function as pores. An alternative explanation for the effect of polymers on channels is that the polymers cause closing via osmotic stress [37,38], in which case the channels induced by Trp-433 → Phe toxin would be less susceptible to changes in internal water activity, which suggests a different internal structure of the channel. There is an indication that the formation of the 'polymer-resistant' channels in cells occurs only at high concentrations of pneumolysin. Thus the proportion of channels formed by wild-type toxin in cells that are not closed by 40 kDa dextran increases with increasing concentration. Trp-433 → Phe toxin forms channels only at high concentrations and therefore these might be of the 'polymer-resistant' type. We propose that pneumolysin can form different types of channel with different activities. Low conductance channels in bilayers and 'polymer-sensitive' pores in erythrocytes require the presence of this tryptophan, but high conductance channels or 'polymer-resistant' pores do not.

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