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Functional modifications of alamethicin ion channels by substitution of glutamine 7, glycine 11 and proline 14

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Abstract

Alamethicin is a 20 amino acid, potentially helical peptaibol which forms voltage-dependent ion channels in bilayer systems. Two aspects of alamethicin structure have been suggested to be of particular functional significance for stabilization of alamethicin channels. (i) Proline 14 inducing a helix kink is together with glycine at position 11 responsible for an appropriate orientation of the molecules in the conducting associates. (ii) Glutamine 7 lining the channel interior is assumed to stabilize the channel structure by forming inter-helix hydrogen bonds. The functional importance of these residues was probed in macroscopic and single-channel experiments with alamethicin analogs containing polar, side chain bearing residues at position 11 (glutamine, asparagine) or at position 14 (glutamine). In order to investigate the crucial role of glutamine 7 for the stabilization of channel aggregates, this residue was substituted by alanine. The conformation of the lipid bound peptides was determined by circular dichroism spectroscopy. The results show that glutamine 7, glycine 11 and proline 14 are not essential for channel formation but substitution of any residue reduced the number of conductance levels and significantly reduced their lifetimes. Channel stabilization by the introduction of residues with potential hydrogen bonding capacity at positions 11 and 14 was not observed. Differences in the conformation of the lipid bound peptides, their orientation in the bilayer and their affinity for the lipid membrane appear thus to contribute to the modulation of functional properties.

Keywords: Alamethicin analog; Voltage-dependent ion channel; Conductance; Lipid bilayer; Peptaibol; Conformation

1. Introduction

Alamethicin is a member of the peptaibol family of channel-forming peptides. The linear 20 amino acid containing peptide which was originally isolated from the fungus *Trichoderma viride* [1] is characterized by a high proportion of α-aminoisobutyric acid (Aib), an acetylated N terminus and an α-amino alcohol at the C terminus. Its conformation, interaction with lipid bilayers, biological properties and ion channel-forming behavior have been intensively investigated (for review see [2,3]). While the alamethicin chain is very flexible in aqueous solution, the tendency of Aib to restrict the possible conformers of peptides is confirmed in the case of alamethicin by its predominantly helical crystal structure [4] and its largely helical conformation in methanol, in the associated state and when bound to lipids [5–8]. The N-terminal helix is highly hydrophobic, while the C terminus is more polar and bent away from the helix axis by the proline residue at position 14. Thus the peptide appears to be

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ideally suited for the N-terminal insertion into the membrane interior.

The voltage-dependent formation of alamethicin ion channels has been described by different models [9]. Evidence for the 'barrel-stave' model, which explains the conductivity inducing property of the peptaibol by the formation of water filled, transmembrane bundles of helical monomers, derives from the interpretation of single-channel investigations [10,11]. Association of up to 12 monomers has been suggested to determine the different conductance levels, and the asymmetry of the electrophysiological properties point to a parallel orientation of the monomers in the conducting states.

Two aspects of the alamethicin conformation have been found to be of particular functional significance: (i) proline (Pro) at position 14 inducing a helix kink and (ii) glutamine (Gln) at position 7 lining the channel interior. Remarkably, these residues are highly conserved in many channel-forming peptides [2]. The Pro induced break in the hydrogen bonding pattern results in the funnel shaped appearance of the channel. The formation of the 'mouth' structure at the C terminus is supported by the conformationally flexible glycine 11 (Gly) [12]. Both the lack of hydrogen bond constraints between Pro-14 and Aib-10 and the absence of a restricting side chain at position 11 have been suggested as contributing to an optimal orientation of the monomers in the gating alamethicin pores. The functional importance of Pro-14 of alamethicin has been described in several investigations [13,14]. The residue was shown to be not essential for the voltage induced ion flow through lipid bilayers. Any substitution at this particular position, however, was found to be connected with distinct conformational changes and substantial loss of single-channel activity reflected by a reduced number and lifetime of conductance levels.

Like Pro-14 and Gly-11, the polar side chain of Gln-7 is presumed to be directed towards the center of the channel lumen. Early investigations indicated that the residue might play a role in bundle stabilization by inter-helix hydrogen bonding between the side chains [4]. Molle et al. [15] made an attempt to verify the role of Gln-7 in channel formation by alamethicin by investigating non-Aib-containing synthetic analogs with substitution of Gln at position 7 by alanine (Ala), serine (Ser) or asparagine (Asn). The Gln^7-Ala^7 substitution abolished channel-forming activity which was, however, restored in the Ser analog and quite well developed in the Asn containing sequence, thus supporting the concept of a stabilizing role for a polar residue at this particular position.

Our study tested the significance of Pro-14, Gly-11 and Glu-7 using analogs derived from the native alamethicin sequence. In order to confirm the crucial role of Gln-7 for the stabilization of channels the residue was substituted by Ala. The importance of the conformational flexibility in the central chain region was probed with alamethicin analogs containing side chain bearing residues at position 11 (Gln, Asn). Additionally, the substitution of Gly by polar residues with amide side chain functionality and different lengths was directed towards the question as to whether steric hindrance could contribute to channel stabilization via an intermolecular hydrogen-bond annulus at this position. Furthermore, the tendency of a polar residue with potential hydrogen-bonding capacity to contribute to channel stabilization at position 14 was studied by substitution of Pro-14 by Gln.

The functional properties of the alamethicin analogs were compared in both macroscopic and single-channel conductance experiments after incorporation in a planar lipid bilayer and are discussed in relation to structural parameters and the conformation of the lipid bound peptides as determined by circular dichroism.

2. Materials and methods

2.1. Synthesis of peptides

The alamethicin peptides were synthesized by the solid phase technique using Fmoc protected amino acid fluorides which were prepared via (diethylamino)sulfur trifluoride [16]. The peptide assembly was carried out on a MPS synthesizer ACT 348 (Advanced ChemTech, Louisville, KY, USA) as described [17]. After completion of the synthesis and acetylation of the N terminus, the peptides were released from the resin by treatment with 2% triisopropylsilane and 5% water in 50% trifluoroacetic acid (TFA)/dichloromethane (DCM) for 45 min to give

Table 1
Amino acid sequences of alamethicin F30 (Alam-P14) and the alamethicin analogs

	1	7	11	14	20
Alam-P14:	Ac-Aib-Pro-Aib-Ala-Aib-Ala	Gln-Aib-Val-Aib-	Gly-Leu-Aib-	**Pro**-Val-Aib-Aib-Glu-Gln-	Pheol
Alam-Q14:	Ac-Aib-Pro-Aib-Ala-Aib-Ala-	Gln-Aib-Val-Aib-	Gly-Leu-Aib-	**Gln**-Val-Aib-Aib-Glu-Gln-	Pheol
Alam-A7:	Ac-Aib-Pro-Aib-Ala-Aib-Ala	**Ala**-Aib-Val-Aib-	Gly-Leu-Aib-	Pro-Val-Aib-Aib-Glu-Gln-	Pheol
Alam-Q11:	Ac-Aib-Pro-Aib-Ala-Aib-Ala	Gln-Aib-Val-Aib-	**Gln**-Leu-Aib-	Pro-Val-Aib-Aib-Glu-Gln-	Pheol
Alam-N11:	Ac-Aib-Pro-Aib-Ala-Aib-Ala	Gln-Aib-Val-Aib-	**Asn**-Leu-Aib-	Pro-Val-Aib-Aib-Glu-Gln-	Pheol

the *N*-acetylated free peptide. The peptides were purified by reverse phase HPLC and characterized by electrospray ionization mass spectrometry (ESI-MS).

2.2. Circular dichroism experiments

2.2.1. Vesicle preparation

Small unilamellar vesicles (SUVs) for CD measurements were prepared by suspending and vortexing the dried 1-palmitoyl-2-oleoylphosphadidylcholine (POPC) (Avanti Polar Lipids, Alabaster, AL, USA) in buffer (10 mM Tris, 154 mM NaF, 0.1 mM EDTA, pH 7.4) to give final lipid concentrations between 20 and 40 mM. The suspensions were sonicated (under nitrogen in an ice bath) for 25 min using a titanium tip ultrasonicator. Titanium debris was removed by centrifugation. Dynamic light scattering experiments (N4 Plus, Coulter Corporation, Miami, FL, USA) confirmed the existence of a major population of vesicles ($>95\%$ mass content) with mean diameter of 46 ± 1 nm (polydispersity index 0.3).

2.2.2. CD measurements

Stock peptide solutions were prepared by dissolving the samples in methanol. The solutions were mixed with SUV suspensions to the desired peptide concentration and solvent composition. CD measurements were carried out on a J 720 spectrometer (Jasco, Japan) between 200 and 250 nm at room temperature. Usually six CD scans were accumulated for each sample and at least two independent preparations of each type of sample were measured, smoothed and averaged. Circular dichroism and differential scattering of the SUVs were eliminated by subtracting the spectra of the corresponding peptide-free suspensions. The helicity was determined from the mean residue ellipticity [Θ] at 222 nm according to the relation $[\Theta]_{222} = -30\,300\ [\alpha]+2340$ ([α] = amount of helix) [18]. The error was 5% helicity.

2.3. Macroscopic and single-channel conductance experiments

Macroscopic and single-channel conductance experiments were performed at planar lipid bilayers formed across either a 175 μm or 125 μm diameter hole in a 25 μm thick Teflon film (PTFE; Goodfellow, Cambridge, UK). The Teflon film was sandwiched between two glass half-cells and pretreated with hexane/hexadecane (40:1 v/v) (Fluka, spectroscopic grade). For both macroscopic and single-channel conductance experiments the lipid mixture used was 1-palmitoyl-2-oleoylphosphadidylcholine (POPC)/dioleoylphosphadidylethanolamine (DOPE) (both from Avanti Polar Lipids) (7:3 w/w). The electrolyte solution was 1 M KCl. The lipid dissolved in *n*-hexane was spread on the surface of electrolyte solution in both compartments. Bilayer formation was achieved by lowering and raising the level of one or both reservoirs [19] and was monitored by the capacity response. Before adding the peptide from a methanolic stock solution to the *cis* (positive) side the bare membrane was checked, under an applied potential, for stability and absence of channel-like events.

For the macroscopic conductance experiments,

Table 2
Expected and experimentally determined masses of the peptides

Peptide	$[M+Na]^+$	
	Experimental	Expected
Alam-P14	1987	1986.0
Alam-Q14	2018	2018.4
Alam-A7	1931	1930.3
Alam-Q11	2059	2058.4
Alam-N11	2043	2044.0

Table 3
Helicity of alamethicin peptides in buffer and in the presence of SUVs composed of phosphatidylcholine

Peptide/POPC-SUV (mM)	Helicity (%)				
	Alam-P14	Alam-Q14	Alam-A7	Alam-Q11	Alam-N11
Buffer	7	7	2	9	8
1.3	37	38	n.d.	n.d.	n.d.
2.6	41	44	30	14	14
5.2	46	44	26	19	19
10.4	44	n.d.	31	38	38
15.0	n.d.	n.d.	n.d.	39	36

The peptide concentration was 2.5×10^{-5} M, the solvent comprised 10 mM Tris, 154 mM NaF, 0.1 mM EDTA, pH 7.4 containing 5% (v/v) methanol. n.d., not determined.

the voltage was set and currents were measured using an amplifier and software from Axon Instruments (USA). The current-voltage curves were generated in response to triangular voltage ramps (up to $\pm$200 mV, 40 s/cycle). Three or five such ramps were recorded for each compound and concentration. The characteristic parameters differed by less than 5%. The macroscopic curves were analyzed as described by Hall et al. [20].

Single-channel events were studied using an amplifier (Axon Instruments, USA) with a CV-5-1G headstage (gain: 1 mV/pA). The current fluctuations were filtered at 1 kHz in all cases and stored on a digital tape recorder (DTR 1200, Biologic, Claix, France). Manually selected records were subsequently analyzed using the PCLAMP6 software from Axon Instruments (USA).

3. Results

3.1. Synthesis

Alamethicin F30 and the four analogs (Table 1) synthesized via Fmoc-amino acid fluoride strategy were obtained with at least 92% purity as confirmed by analytical reversed phase HPLC. Electrospray ionization mass spectrometry (ESI-MS) indicated the correct molecular mass for alamethicin and the analogs (Table 2).

3.2. Secondary structures

The helicity of the peptides in the presence of lipid vesicles was calculated on the basis of CD spectra (Table 3). All peptides show little regular structure in aqueous solution. In interactions with phosphatidylcholine (POPC) liposomes the helicity of Alam-P14 is about 45%. The fact that the helical content remained constant with increasing lipid concentration above 2.6 mM reveals a high affinity of Alam-P14 for the lipid membrane. Replacement of Pro-14 by Gln (Alam-Q14) changed neither the conformation of the bound peptide (44% helix) nor had distinct influence on membrane affinity (Table 3). However, amino acid substitution in the N-terminal, potentially heli-

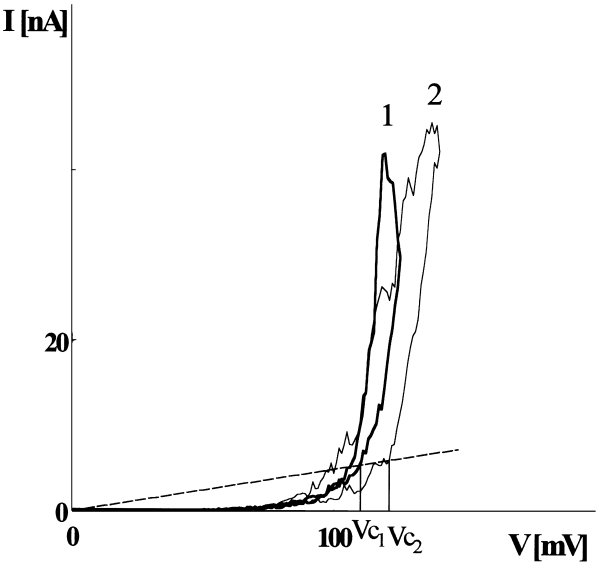

Fig. 1. Macroscopic current-voltage curves at a POPC/DOPE bilayer, 1 M KCl both sides and room temperature. 20×10^{-8} M Alam-Q14 (curve 1) and 4×10^{-8} M Alam-P14 (curve 2). The dotted line represents a reference conductance of 50 nS. V_{c1} = 110 mV; V_{c2} = 121 mV. The panel shows one representative *I-V* curve of three to five records which differed in the characteristic parameter by less than 5%.

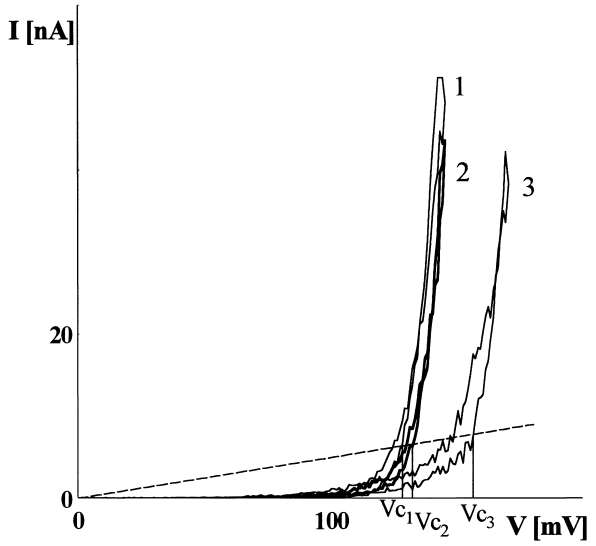

Fig. 2. Macroscopic current-voltage curves at a POPC/DOPE bilayer, 1 M KCl both sides and room temperature. 20×10^{-8} M Alam-Q11 (curve 1), 25×10^{-8} M Alam-A7 (curve 2) and 20×10^{-8} M Alam-N11 (curve 3). The dotted line represents a reference conductance of 50 nS. $V_{c1}=128$ mV; $V_{c2}=132$ mV; $V_{c3}=156$ mV. The panel shows one representative *I-V* curve of three to five records which differed in the characteristic parameter by less than 5%.

cal chain region led to reduced helicity, even after the introduction of more helix promoting Ala residue [21]. Thus, with the Gln^7-Ala^7 exchange the α-helical content declined to about 31%, but the high membrane affinity was preserved as shown by constant helicity of Alam-A7 at different lipid concentrations. The maximal helical content of Alam-Q11 and Alam-N11, both 14% at 2.6 mM POPC, increasing to 38% at a lipid concentration of 10.4 mM reflected lower affinity and slightly reduced helicity in the lipid bound state compared with the native sequence (Table 3).

Table 4
Macroscopic conductance parameters yielding the concentration dependence, the voltage dependence, and the apparent mean number of peptide molecules forming the channel

Peptide	Concentration dependence V_a (mV)	Voltage dependence V_e (mV)	Apparent mean number of monomers $\langle N\rangle$
Alam-P14	60	6	10
Alam-Q14	50	6/7	8/7
Alam-A7	40	8	5
Alam-Q11	48	9	5
Alam-N11	40	10	4

The data were derived by analyzing the macroscopic *I-V* curves as described by Hall et al. [20] and are the means of the data from three to five experiments which differed by less than 5%.

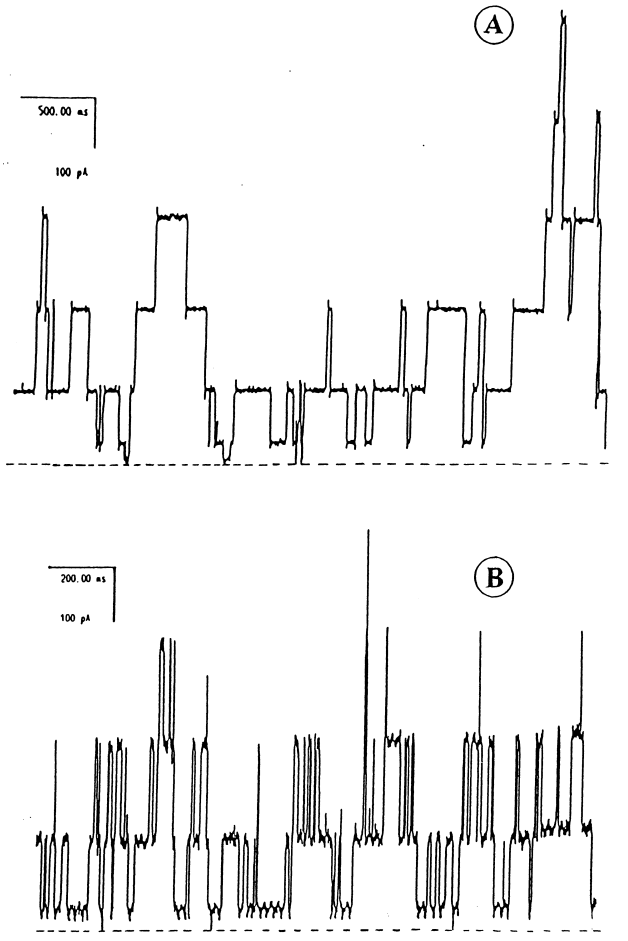

Fig. 3. Representative single-channel traces induced by (A) 5×10^{-9} M Alam-P14 at 120 mV; (B) 5×10^{-8} M Alam-Q14 at 150 mV. The electrolyte solution on both sides of a POPC/DOPE bilayer was 1 M KCl, room temperature.

3.3. Functional properties

All peptides induced voltage-dependent current changes in POPC/DOPE bilayers as shown by the macroscopic current-voltage curves (*I-V* curves) (Figs. 1 and 2). However, compared with the native peptide, the activity of the analogs was reduced. About five times higher concentration (20×10^{-8} M) of Alam-Q14 was required to reach the characteristic voltage V_c of about 120 mV (V_c defined here as the voltage necessary to reach a reference conductance of 50 nS) (Fig. 1) thus indicating a reduced

Table 5
Single-channel conductance levels (pS) of alamethicin analogs in POPC/DOPE membranes at room temperature

Peptide	Level						
	1	2	3	4	5	6	7
Alam-P14	170	400	1300	2500	4000	5600	7200
Alam-Q14	150	380	1250	2500	3800	5200	
Alam-A7	200	390	1300	2700	4400	6700	
Alam-Q11	140	460	1300	2300	3700		
Alam-N11	200	540	1400	2500	4100		

channel-forming ability. The distinct hysteresis of the *I-V* curves of Alam-P14 and Alam-Q14 points to relatively slow channel kinetics. Fig. 2 compares the voltage-dependent macroscopic ion flow recorded for Alam-A7, Alam-Q11 and Alam-N11 at about 20×10^{-8} M peptide concentration. Substitution of Gly at position 11 by Asn is more effective in reducing the activity than Gly^{11}-Gln^{11} exchange. The activation threshold of the latter is lower and the slope of the curve steeper. The hysteresis in the *I-V* curves of the analogs modified at positions 7 and 11 is minimal, suggesting much faster kinetics of single channel events in comparison to Alam-P14 and Alam-Q14. The characteristic parameters illustrating the influence of the individual peptides on the macroscopic electrical properties of the lipid bilayer are summarized in Table 4. The decrease of V_a (the characteristic voltage shift caused by an *e*-fold change in peptide concentration) reveals a reduced concentration dependence of the analogs in comparison to the native alamethicin. Moreover, except Alam-Q14 all analogs possess a reduced voltage dependence, as shown by their higher V_e values which denote the voltage increment producing an *e*-fold change of conductance at a given peptide concentration. As a consequence, the calculated apparent number of monomers $\langle N\rangle = V_a/V_e$ forming the conducting aggregates significantly decreased with amino acid substitutions in Alam-P14 (Table 4). States of less monomers are favored when compared with the native sequence.

Figs. 3 and 4 show typical single-channel current traces induced by the various peptides in POPC/POPE bilayers. The number of detectable conductance levels decreased from seven for Alam-P14 to six for the analogs Alam-Q14 and Alam-A7 and to five for Alam-Q11 and Alam-N11 (Table 5). The

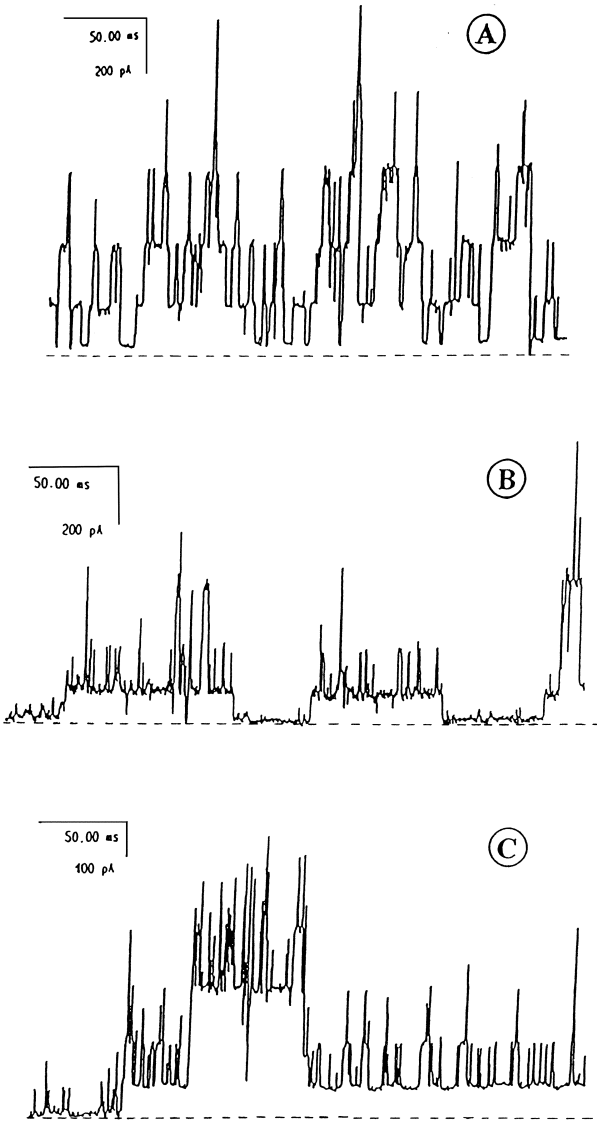

Fig. 4. Representative single-channel traces induced by (A) 5×10^{-8} M Alam-A7 at 150 mV; (B) 10×10^{-8} M Alam-N11 at 160 mV; (C) 15×10^{-8} M Alam-Q11 at 120 mV. The electrolyte solution on both sides of a POPC/DOPE bilayer was 1 M KCl, room temperature.

changes correlate with the reduction of the apparent number of monomers in the channel as determined from macroscopic measurements. The conductance levels found for the different peptides are similar, except those for Alam-A7. The slightly enhanced levels might be associated with the reduced size of the Ala side chain within the channel. The channel lifetimes drastically decreased with amino acid exchange (Table 6). The very short lifetimes, in the range of 1–3 ms determined for Alam-A7, Alam-Q11 and Alam-

Table 6
Mean-channel open lifetimes (ms) of alamethicin analogs

Peptide	Level						
	1	2	3	4	5	6	7
Alam-P14	10	34	59	96	71	45	32
Alam-Q14	7	8	11	9	5.5	1.2	
Alam-A7	3	2	3	2	1.9	1	
Alam-Q11	1.2	1	9.1	0.9	1.1		
Alam-N11	0.6	7.2	1	1.4	0.7		

N11 confirm the low channel stability derived from the minimal hysteresis of the *I-V* curves of these peptides. Remarkably, while Alam-A7 induced channels switch rapidly between the multiple levels, Alam-Q11 and Alam-N11 channels, once induced, appear to be relatively stable in the third (9.1 ms) and second (7.2 ms) levels, respectively, which form the basis for rapid fluctuations to the higher conductance states (Table 5).

4. Discussion

Inspection of the 'barrel stave' channel model of alamethicin reveals that Gln-7, Gly-11 and Pro-14 as well as Glu-18 at the C terminus are directed toward the water filled channel interior. Thus the channel lining may be considered as consisting of polar rings at different positions of the alamethicin bundles. Furthermore, the residues have been suggested to have a crucial role in channel stability. Gln-7 was described in early investigations to contribute to bundle stabilization by formation of interhelical hydrogen bonds [4]. Pro-14 was found to induce a bend in the largely helical alamethicin structure and to provide conformational flexibility in the central chain region promoted by the absence of helix stabilizing hydrogen bonding between Pro-14 and Aib-10 and of restricting side chains at residue 11. This flexibility may be expected to contribute to an optimal architecture of the C-terminal channel mouth by preventing intermolecular repulsive interactions between the negatively charged Glu-18 residues, supporting hydrogen bonding 'ring' formation [15] and anchoring the polar C-terminal side chains in the polar region of the lipid bilayer as suggested for trichosporin B-VI [22].

This study shows that Gln-7, Gly-11 and Pro-14 play a crucial role in the formation and stability of alamethicin channels. Although substitution of the polar amino acid residue Gln-7 by Ala, introduction of side chain bearing residues with amide functionality at position 11 and replacement of Pro-14 by Gln did not affect the ability of the peptides to induce a voltage-dependent ion flow in lipid bilayers, the number of conductance levels was reduced. Furthermore, substantially declined lifetimes point to significantly reduced channel stability. Nevertheless, well resolved multistate channel events apply for the barrel stave mechanism.

Alam-Q14 was found to be less active than alamethicin with six well resolved levels and lifetimes of the most probable states (two, three and four) of between 8 and 11 ms (Fig. 3, Table 6). As reported for different modifications at position 14 of alamethicin and related peptaibols such as Pro-Ala, Pro-Aib exchange [13,14,23], the introduction of Gln had less influence on the size of the channels than on the stability of the individual levels. However, the current traces induced by Alam-Q14 were quite different from those observed recently for the Ala-14 analog [14]. While the Alam-Q14 induced channels switch rapidly between the different levels (Fig. 3), the analog with a more hydrophobic, helix promoting Ala residue at position 14 exhibited distinctly fewer channel events interspersed by extended closed periods [14].

These differences must be associated with differences in the conformation and orientation of the bilayer bound peptides. Ala-14 has been found to substantially enhance helicity by its ability to contribute to the intramolecular hydrogen-bonding pattern of the alamethicin helix [14,15]. However, the straight peptide helix seems to inhibit bundle formation since electrostatic repulsive forces between the charged C termini become effective. Furthermore, introduction of the more hydrophobic residue Ala into a chain region exposed to the water filled channel should favor a reorientation of the peptide C terminus and thus diminish stabilizing peptide-peptide and peptide-lipid interactions in this region [15,22]. In contrast, neither the conformation nor orientation of the alamethicin molecule in the lipid bound state seems to be substantially changed by substitution of Pro-14 with the polar Gln residue. Compared with the native sequence, Alam-Q14 exhibited comparable affinity to the lipid bilayer and the same helicity in the

lipid bound state (Table 3). Obviously, the substitution did not influence the structure inducing and stabilizing peptide-bilayer interactions. Additionally, in contrast to Ala there is no distinct preference of the conformational parameters of Gln for helix, β-structure or turn formation [24].

Evidence for additional channel stabilizing interactions between neighboring Gln side chains with potential hydrogen bonding capacity at position 14 was not found. In contrast, the short lifetimes of the individual levels implicate rapid incorporation of monomers into and release from the channel aggregates.

To elucidate the importance of the Gly-11 mediated conformational flexibility in the central chain region of alamethicin for functional properties, analogs with Gln and Asn at position 11 were studied. Although voltage sensitivity was preserved, the modifications led to a reduced number of conductance levels as well as changes in the lifetimes, thus reflecting a substantial reduction of activity. The poor channel-forming properties of the analogs underline the functional significance of the Gly-11 mediated structural heterogeneity in the alamethicin molecule [3,4]. The structural disorder in this central chain region promoted by the absence of hydrogen bond constraints of the Aib-10 and Gly-11 carbonyl [12,25] and absence of a bulky side chain at position 11 has been confirmed by many structural investigations and molecular modeling. The carbonyl groups of Aib-10 and Gly-11 are solvent-accessible [4,26] and may provide a further means of solvating the channel interior. Additionally, recent modeling of alamethicin channels emphasized that when attempting to understand the forces stabilizing helix bundles, it is essential to take into account hydrogen bond and helix dipole/water dipole interactions, which are about 10 times larger than helix-helix interaction within the channel structure [27]. On the basis of these findings, our functional studies with Alam-Q11 and Alam-N11 lead to the conclusion that introduction of the side chain bearing residues Gln or Asn at position 11 should both impair backbone-solvent interactions and restrict chain flexibility. Thus, the results support the suggested role of conformational flexibility in the central helix region for channel formation.

Furthermore, substitution of Gly-11 with variously sized side chain bearing residues does not only appear to influence either the stability or the dimension of the channels. The distinctly lower membrane affinity of the Alam-Q11 and Alam-N11 analogs compared to the native peptide also reduces the probability of channel formation. This suggestion is in agreement with recent work on synthetic analogs [15].

In early studies the Gln-7 was thought to form hydrogen bonds to the same residue on the neighboring helix in the alamethicin bundle. More recent investigations underline the stabilization of alamethicin channels by a network of hydrogen bonds between Gln-7 and intrapore water molecules [4,27]. Support for this structural hypothesis was provided by investigations of alamethicin analogs containing Leu instead of Aib and bearing Gln, Asn, Ser and Ala at position 7 [15]. Gln-Ala substitution completely abolished single channel activity as well as macroscopic conductance while channel-forming ability was preserved with Asn at position 7. In contrast to these observations the investigation of Alam-A7 clearly shows that an amide side chain functionality at position 7 is not essential for voltage-induced conductivity and multistage single-channel activity. Up to six well resolved conductance levels with values comparable to that of the native peptide were observed (Fig. 4, Table 5), leading to the conclusion that the number of monomers forming the individual levels of the Alam-P14 and Alam-A7 channels are almost identical. However, the low hysteresis of the Alam-A7 induced macroscopic *I-V* curve and extremely low single-channel lifetimes point to a very rapid uptake and release of the molecules and thus underline the importance of a polar side chain at this position. Although the data do not provide direct experimental evidence for the participation of Gln-7 in intermolecular hydrogen bonding, the results implicate a channel stabilizing function for this residue.

The differences in the activity of alamethicin and Alam-A7 are related to conformational changes in the lipid bound peptides. It is striking to note that introduction of the helix promoting Ala residue into position 7 [21] caused a substantial loss of helicity (Table 3). Ala would present a hydrophobic surface to the channel interior. Obviously, the burial of this residue in the alkyl chain region of the bilayer serves as the driving force for conformational changes in the helical N terminus. The results support the sug-

gestion that the N-terminal alamethicin helix is most important for the stability of the channel arrangement [28].

The discrepancy between our findings and the results published by Molle et al. [15] may be due to the reduction of channel lifetime as result of the Aib-Leu substitution [29]. Leu instead of Aib was described to enhance the α-helical conformation and thus to decrease the length of the transmembrane part of the alamethicin analog compared to the 3_{10}-helical parts containing native alamethicin which may be expected to span the bilayer [30]. Obviously, the cooperative lifetime reducing effects caused by Gln^7-Ala^7 and all-Aib-Leu substitutions resulted in a complete loss of activity of the non-Aib-containing Ala^7-alamethicin analog.

In summary, this study supports the key role of the residues Pro-14, Gly-11 and Gln-7 for the optimal function of alamethicin channels. Although not essential for the formation, the experimental data convincingly confirm the contribution of the three residues for ion channel stability.

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