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Influence of the nature of the aromatic side-chain on the conductance of the channel of linear gramicidin: study of a series of 9,11,13,15-Tyr(O-protected) derivatives

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Abstract. This paper describes the single channel properties of a series of synthetic analogues of gramicidin A, where all four tryptophans are replaced either by tyrosine or by several O-protected (benzyl, methyl, ethyl or t-butyl) derivatives. It is shown that, although all analogues bear similar dipole moment on their side-chains, the conductance depends on the hydrophobicity of these protecting groups. An analysis of the conductance data suggests that the conductance is governed by the binding process and a possible explanation, based on conformational considerations, is proposed.

Key words: Linear gramicidins – Analogues – Conductance – Dipole moment – Hydrophobicity

Introduction

The mechanism governing the conductance of linear gramicidin (HCO–Val^1–Gly^2–Ala^3–DLeu^4–Ala^5–DVal^6–Val^7–DVal^8–X^9–DLeu^{10}–X^{11}–DLeu^{12}–X^{13}–DLeu^{14}–X^{15}–NH–C_2H_4–OH, where X = Trp for gramicidin A or GA (Sarges and Witkop 1965)) is still an open question. Some recent results obtained on N- or C-terminal modified GA suggested that the dipoles of the side-chains could play a major role and determine the channel conductance. Indeed, introduction of polar residues in position 1 markedly alters the single channel conductance (Mazet et al. 1984; Russell et al. 1986) and substitution of all or some of the tryptophan residues, which bear a strong dipole moment, by phenylalanine (Bamberg et al. 1976; Heitz et al. 1982, 1986; Becker et al. 1991) or naphthylalanine (Nal) (Ranjalahy-Rasoloarijao et al. 1989; Daumas et al. 1991), which have nearly no dipole moment, leads to a considerable decrease in the channel conductance. These variations can be interpreted on the basis of modifications of the energy profile of the gramicidin channel; for GA the entry barrier is rate determining while for GM (X = Phe) and GN (X = Nal) it is the central barrier. From the experimental point of view, these differences are characterized by a quasi constant conductance for GA whereas it depends strongly on the applied potential for GM and GN. These observations suggested that the presence of a dipole moment on the side-chains of residues 9, 11, 13 and 15 is required to obtain a "high" conducting channel. However, such a conclusion was questioned by the study of gramicidins where the four Trp residues were replaced by either 8- or 4-quinolylalanine (GQ8 and GQ4 respectively) (Daumas et al. 1991). Although these analogues bear aromatic side-chains with dipole moments of similar magnitude to that of Trp, they show a low and voltage-dependent conductance. A preliminary approach based on investigations made on indole N-methylated gramicidin A (Daumas et al. 1991) suggested that the role of the side-chain dipole moments could be influenced by the hydrophobicity and therefore the orientations of these side-chains.

In order to specify the role of these aromatic side-chains, we undertook a study of a series of GA analogues where these side-chains bear very similar dipole moments but with varying hydrophobicities. For chemical reasons, mainly owing to the sensitivity to oxidation of the indole group, we decided to make these investigations using a series of gramicidins where the four tryptophans were replaced by four tyrosine residues with various O-substitions. These were GT and GTBzl (X = Tyr and Tyr(OBzl) respectively) which were synthesized previously (Trudelle and Heitz 1987), and GTMe (X = Tyr(OCH_3)), GTEt (X = Tyr(OC_2H_5)) and GTBu (X = Tyr($OC(CH_3)_3$)). The present paper reports conductance data obtained with each of these analogues, together with a proposal for the role of the dipole moment of the aromatic side-chains.

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Abbreviations used and designation of X in the chemical structure of linear gramicidin: GA, X = tryptophane; GM, X = phenylalanine; GN, X = naphthylalanine, GQ8, X = 8-quinolylalanine; GQ4, X = 4-quinolylalanine; GT, X = tyrosine; GTBzl, X = O-benzyltyrosine, GTMe, X = O-methyltyrosine; GTEt, X = O-ethyltyrosine; GTBu, X = O-*t*-butyltyrosine

Material and methods

Black lipid membranes were formed at 24–25 °C from a 1.25% solution of glycerylmonooleate (GMO) (Sigma) in *n*-decane (Fluka) across a hole (area 3.10^{-4} cm^2) in a teflon cell filled with the unbuffered solution of the alkali chloride. All measurements were made on symmetrical systems using Ag/AgCl electrodes. Final gramicidin concentrations were about 10^{-11}–10^{-12} M starting from solutions in ethanol. A current to voltage convertor (Keithley Model 427) was used as current amplifier and the data were stored on a microcomputor Apple II^+.

GT and GTBzl were prepared as described by Trudelle and Heitz (1987). The other gramicidins were synthesized by a solid phase method in the following way. The syntheses were carried out on an aminated polyacrylic resin (Expansin™, Expansia, BP 6, Aramon, France), by a fluorenemethoxycarbonyl (Fmoc) strategy on a continuous flow synthesizer (9050, Pepsynthesizer, Milligen). The coupling reagent was TBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate). The peptides were prepared using FmocTyr(OMe)OH, FmocTyr(OEt)OH and FmocTyr(OtBu)OH. Among these derivatives, only the latter was commercially available (Novabiochem). Fmoc Me and Et hydroxy protected tyrosines were synthesized starting from BocTyr(OMe)OH and BocTyr(OEt)OH purchased from Peninsula Lab. The Boc groups were first cleaved by 50% trifluoroacetic acid in dichloromethane for 1 h. Trifluoroacetic acid and the solvent were evaporated under vacuum without heating and the white residues dried overnight under high vacuum over P_2O_5. Using a procedure similar to that reported by Paquet (1982), Tyr(OMe)OH and Tyr(OEt)OH (5.5 mM) were then treated overnight with 9-fluorenemethylsuccinimidyl carbonate (6.1 mM) in 50 ml acetone/water (50/50, V/V) containing 2 equivalents of CO_3HNa (pH 9). The Fmoc protected amino acids were extracted with ethyl acetate and used for the syntheses without further purification. The overall yield of these operations was close to 90%.

The C-terminal amino acids (FmocTyr(OMe)OH, FmocTyr(OEt)OH and FmocTyr(OtBu)OH) were linked to the resin with a glycolamidic ester group using the cesium salt method as previously described (Calas et al. 1985). After completion of the syntheses, the peptide–resin adducts were suspended in 50 ml of ethanolamine/dimethylformamide (50/50). After 72 h stirring at room temperature, 200 ml of water was added and the precipitates were collected by centrifugation at 0 °C. The yields in peptides were about 65%.

The crude peptides were formylated and purified as previously described (Trudelle and Heitz 1987) and the chemical structures assessed by NMR (360 MHz) and Fast Atom Bombardment Mass Spectrometry.

Results and discussion

The chemical structures of the various aromatic side chains used in this work are shown in Fig. 1, together with their dipole moments. This figure indicates that both the intensity and the orientation are roughly the same for all these side chains.

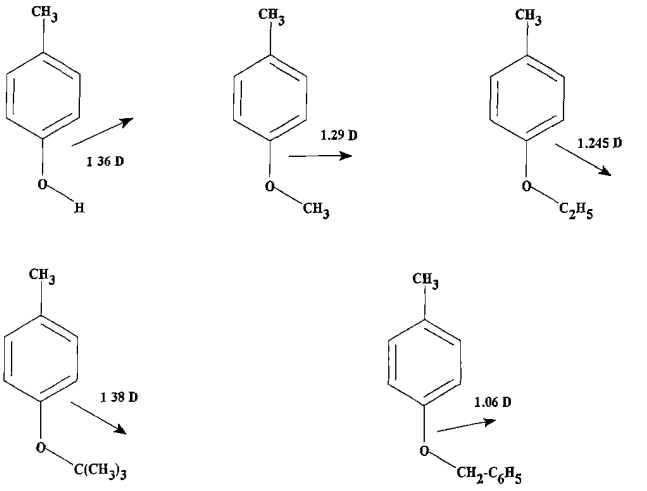

Fig. 1. Chemical structures of the side chains corresponding to the various O-substituted tyrosines in positions 9, 11, 13 and 15. The dipole moments were calculated using MOPAC version 5.0 with AM1 calculation. The calculations were based on all atoms shown in the figure

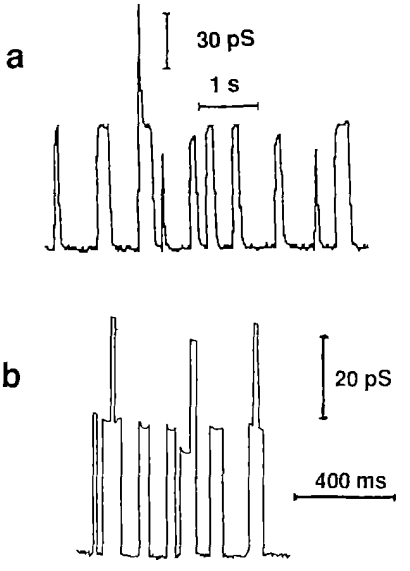

Fig. 2a, b. Transmembrane current fluctuations induced by **a** GT (applied voltage 100 mV) **b** GTBu (applied voltage 300 mV). Data for the other analogues are not shown as they give very similar traces. CsCl 1 M as electrolyte, GMO/decane membranes

Before starting the description and the analysis of the conductance data it must be mentioned that all synthetic GA analogues give rise to single channel events (Fig. 2). The channel function can be attributed to the presence of right handed Π_{DL} helices (Arseniev et al. 1985) as deduced from to the formation of hybrids between GA and the analogue considered. This holds true at least for GT and GTBzl and very probably also for the other analogues, as all these compounds show similar lifetimes. Indeed, a modification of the channel structure, such as formation of a double helix, leads to a considerable increase ($\sim$1 000 fold) in the channel lifetime (Koeppe et al. 1991).

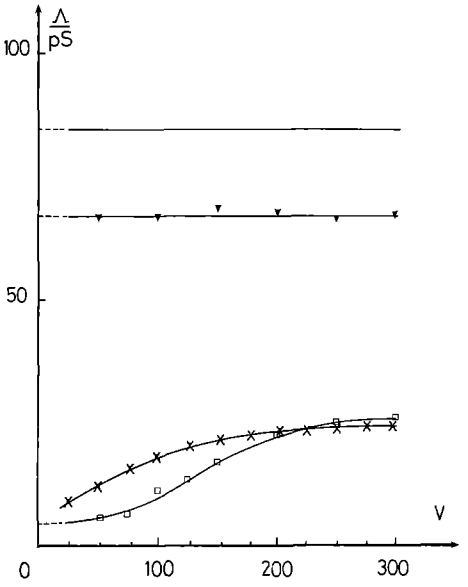

Fig. 3. Variations of the single channel conductance of the various gramicidins with the applied voltage. CsCl 1 M as electrolyte; GMO/*n*-decane membranes. V in mV. ▽, GT; ×, GTMe; □, GTEt. The upper and non-labelled variation corresponds to GA and is given for comparison. The data corresponding to GTtBu and GTBzl are not reported as they are indistinguishable from these of GTEt

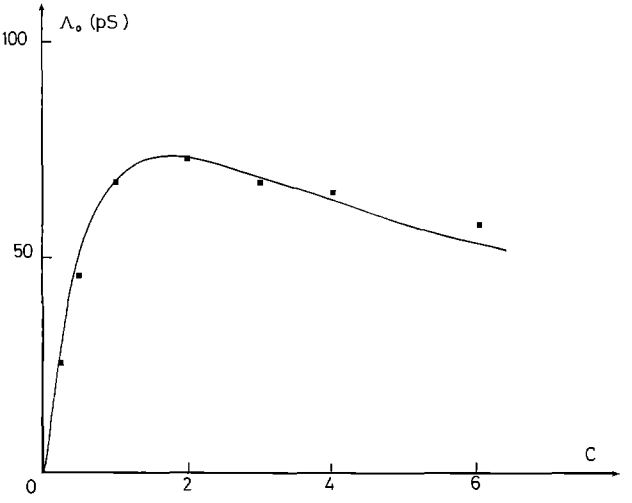

Fig. 4. Variation of the conductance as a function of the CsCl concentration for GT. ■, experimental values; —, calculated using the rate constants reported in Table 1

Examination of the variations of the single channel conductances with the applied voltage at various CsCl concentrations reveals strong differences between the various gramicidins. On the basis of their $\lambda = f(V_{applied})$ relationship (Fig. 3) and of the amplitude of their conductances, they can be divided into three groups. The first one which contains only GT shows a behavior very similar to that of GA. The two other groups are characterized by voltage-dependent conductances with nearly identical conductances for GTBzl, GTEt and GTBu (second group) while GTMe, although it shows a low and voltage-dependent conductance, differs from the members of the second group in its behavior at low applied voltages.

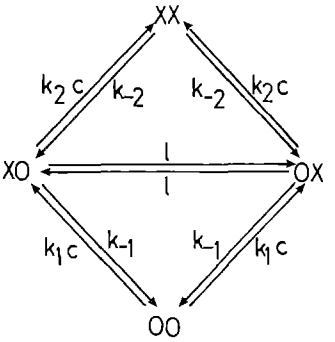

Fig. 5. 3B2S model and attribution of the various rate constants. OO corresponds to an empty channel, XO and OX to a mono-occupied channel and XX to a channel with two ions. k_i, k_{-i} and l are the rate constants between the various occupancy states of the channel. k_1 and k_2 are the association rate constants of the first and second ion respectively, k_{-1} and k_{-2} the dissociation rate constants and l is the translocation constant

1) First group: GT

Owing to the similarity of the $\lambda_0 = f(c)$ variation obtained for GT (Fig. 4) to that of GA for Cs^+ cations (Haldky and Haydon 1972), which shows an increase of the conductance up to about 2 M and then a decline consistent with a multi-occupied channel, the data for GT were analyzed on the same basis as that already reported for the natural molecule (Finkelstein and Andersen 1981). This is supported by the fact that GT and GA are very probably in the same conformational state, as revealed by the possibility of formation of heterodimers (Fonseca et al. 1992). In a model with three barriers and two sites (3B2S) and with one or two ions in the cannel as represented by the scheme of Fig. 5, the conductance λ_0 is given by relation [1]

$$\lambda_0 = \frac{eF^2}{RT} \cdot \frac{2c}{K_1 + 2c + c^2/K_2} \cdot \frac{l(k_1 + k_2 c)}{2(2l + k_{-1} + k_2 c)}$$

where c is the molal activity (m) of the cation, $K_1 = k_{-1}/k_1$, $K_2 = k_{-2}/k_2$, and k_1, k_{-1}, k_2, k_{-2} and l are as shown in Fig. 5.

As the various parameters reported by Finkelstein and Andersen (1981) correspond to the GA–diphytanoylphosphatidylcholine/*n*-decane system and as our experiments were carried out on GMO membranes, all the rate constants were recalculated for GA using the experimental data of Haldky and Haydon (1972). It must be mentioned that, using our experimental set up, we obtained for GA, in 1 M CsCl, the same conductances as reported by these authors, thus allowing the GA–GT comparison. Using relation [1] the calculated rate constants obtained for GA are reported in Table 1 together with these for GT, which were obtained on the same basis and started in both cases with the values reported by Finkelstein and Andersen (1981). All parameters were allowed to vary one after another in each cycle and the rate constant selected was that which led to the minimal deviation from the experimental data. After each cycle the increment between two successive values was lowered (for example from 0.1×10^8 to 0.01×10^8) and the permitted deviation

from the experimental values was lowered. Calculations were stopped when all the deviations were lower than 5%. The choice of the first constant allowed to vary had no influence on the final result. The fits to the experimental data are shown in Fig. 4. Comparison of the rate constants of these two gramicidins indicates that the main difference, on going from GA to GT, lies in a slight lowering of the rate constant corresponding to the binding process, k_1, which is in accordance with the observed lowering of the single channel conductance. Assuming that the binding process is identical for both molecules, this corresponds to an increase in the height of the entry barrier of about 0.4 kcal.

2) Second group: GTBzl, GTEt and GTBu

For these analogues the situation is more complicated than that described above. First, as already shown in Fig. 3, the single channel conductances depend on the applied voltage, suggesting that strong modifications of the energy profile of the channel occur on going from GA or GT to the three analogues discussed here. The voltage dependence of the conductances would also suggest that the rate determining step for these samples corresponds to the translocation process, while it is the binding for GA and GT. The modification of the energy profile is confirmed by examination of the variations of λ_0 with the cation concentrations (Fig. 6) which show the same unusual feature as that found for GM (Heitz et al. 1986) and GN (Daumas et al. 1991). These variations are characterized by an increase of the conductance up to about 1.5 M, a decline between 1.5 and 3 M and then a new increase of the conductance when the salt concentration is increased still further.

Although these data favour the idea that modifications of the channel profiles occur, there is no particular reason to assume that the ion migration processes of GTBzl, GTEt and GTBu differ from that of the GA channel. This holds true, as already described above, at least for GTBzl since it was shown that this analogue, just like at, is able to form hybrid channels with GA (Fonseca et al. 1992). Therefore, it can also be stated that GTBzl adopts the same Π_{DL} helical (Urry 1971) conformation as GA. Hence, it was tempting to analyse the data using relation [1] for a 3B2S profile. As both GA–GA or GT–GT and GTBzl–GTBzl dimers in their channel forms adopt the same conformational state we started the calculations, as described for GT, using the rate constants determined for GA (see Table 1). Unlike the situation of GT, no fit covering the whole experimental range could be obtained, and in the present case it is reduced to the 0–3 M range (Fig. 6). The various rate constants thus determined to get the best fit are reported in Table 2. Close examination of their values reveals large differences from those reported for GM- or GN (see Table 1), although this analogue shows the same behavior as GTBzl (Daumas et al. 1991). The major difference between GT and GA concerns the binding rate constant, while the translocation step remains almost identical. As the values reported here conflict with these of Daumas et al. (1991), we repeated the

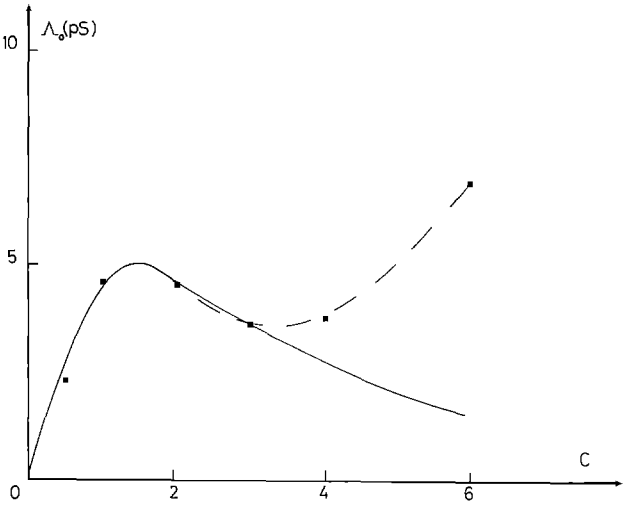

Fig. 6. Variation, in the case of GTBzl, of the conductance extrapolated at zero applied voltage with the CsCl concentration. ■, experimental data; —, calculated using the rate constants reported in Table 1

Table 1. Rate constants for the various gramicidins determined on the basis of the 3B2S model

	GA	GT	GTBzl	GT(tBu)	GN*
$K_1(m^{-1}s^{-1})$	0.95×10^8	0.14×10^8	0.018×10^8	0.014×10^8	0.03×10^8
$k_{-1}(s^{-1})$	0.67×10^8	0.08×10^8	0.16×10^8	0.18×10^8	0.012×10^8
$k_2(m^{-1}s^{-1})$	1.74×10^8	3.8×10^8	1.38×10^8	1.41×10^8	0.11×10^8
$k_{-2}(s^{-1})$	1.60×10^8	5.2×10^8	0.046×10^8	0.037×10^8	0.13×10^8
$l(s^{-1})$	1.10×10^8	0.54×10^8	0.50×10^8	0.72×10^8	0.025×10^8

[a] from Daumas et al. (1991)

calculation but starting from the values reported by those authors. In these calculations the fit between experimental and calculated conductances was again obtained, in the 0–3 M range, when the rate constants were close to these reported in Table 1. Although the fit is probably not unique it does have a tendency to converge toward the values of Table 1 and so it appears from the present results that the main difference on going from GT to GTBzl lies in a major lowering of the rate constant corresponding to the binding process and thus to an increase of the entry energy barrier, while the translocation process remains almost unchanged. The origin of the difference between the present results and the previous ones very probably lies in the fact that the fit covers different experimental ranges. Although the calculation procedure is the same as that used by Daumas et al., we are more confident in the present calculations because we investigated a larger concentration range and because, whatever the starting values, the rate constants are always of the same order of magnitude. The present results are also more satisfying from the point of view of the chemical structures of the various gramicidens which have been studied. Indeed, the chemical modifications deal with the aromatic residues which are located at the entry of the channel and it is therefore not surprising that the major modifications affect the binding process. As to the analysis of the results at higher salt concentrations, at this stage we can

only attempt qualitative explanations. When increasing the salt concentration the properties of the electrolyte are modified and their consequences on the water structure, especially on the double layer at the lipid – water interface are extensive. Furthermore, at very high salt concentrations (>4 M) the solvatation of the cations can be questioned. For example, at 6 M the average number water molecules per Cs^+ cation is 6.70, indicating that the hydration shell of the cation is not complete. Thus, for such a cation, entering the gramicidin channel (which requires further dehydration) has a lower energy cost than for a fully hydrated one. It must be mentioned here that this situation also occurs for GA and GT and may be the origin of the small deviations between calculated and experimental $\lambda_0 = f(c)$ curves, although they appear less clearly probably due to the different scales used (compare Figs. 4 and 6).

3) Third group: GTMe

This gramicidin differs from the others in two different ways. While the main difference from GT lies in the fact that the conductance of GTMe is voltage-dependent, the differences from the gramicidins of the second group are found in the features of the $\lambda = f(V_{applied})$ variations and in the conductance amplitudes at low applied voltages (between 50 and 150 mV) (Fig. 3). It must be noticed that there is a strong similarity between the $\lambda = f(V)$ at 1 M CsCl and that which was reported for indole *N*-methylated GA under the same experimental conditions. Owing to the difficulty of obtaining a confident extrapolation at zero applied voltage, no attempts to analyze the $\lambda_0 = f(c)$ variations were made. Nevertheless, it appears clear that O-methylation of the tyrosine residues induces less drastic modifications of the profile of the gramicidin channel than ethylation, *t*-butylation or benzylation as compared to GA and GT.

4) Possible implications of the role of the dipole moment

Although, all gramicidin analogues described here bear similar dipole moments (see Fig. 1 and Fonseca et al. (1992)) on their aromatic side-chains, they show different conductance properties with a conductance scale in accordance with the hydrophobic scale; an increase of the hyphobicity of the side-chains induces a lowering of the conductance with a saturation effect, as GTBzl, GTEt and GTBu are almost identical. The results reported here corroborate previous observations made on GQ4, GQ8 and GAMe, which suggested that the hydrophobicity of the side-chains could be important and which were the origin of this work. Recalling that, on the basis of heterohybrid formation, all gramicidins are very probably in the right handed Π_{DL} helical conformation, we interpret the lowering of the channel conductance when increasing the hydrophobicity of the polar side-chains as follows. It now appears to be well established by both experimental (Smith et al. 1990) and theoretical (Roux and Karplus 1991) investigations that, owing to its partial or total dehydration, the cation is solvated by the peptide carbonyl groups during its movement in the channel. This was illustrated by Smith et al. (1990) by ^{13}C NMR spectroscopy. The experiments reported by these authors are consistent, in response to ion occupation of the channel, with an inward movement of the carbonyl oxygens of residues Leu^{10}, Leu^{12} and Leu^{14}, which are not hydrogen bonded in a right handed helix (Arseniev et al. 1985). In our opinion a lowering of the channel conductance may be due to an increase of the stability of the intermediate ion–gramicidin complex. How can this stability be modified by slight modifications of the side chain? To answer this question let us consider the three partners which can be involved in the process; namely, the ion, the carbonyl oxygens and the dipole of the side-chains. Two types of interactions can occur; ion–peptide groups and dipole–dipole involving the peptide bonds and the polar side-chains, both these interactions acting in opposite ways. When the aromatic side-chains do not bear a dipole moment, the flexibility of the helix is maximum and thus the ion–carbonyl oxygen interaction is favoured and leads to an increase of the stability of the ion–peptide complex. In contrast, when the aromatic side-chains bear a dipole moment, which is the situation in the case of Trp and Tyr, this dipole can interact with that of the backbone and lead to "stressing" of the helix. Hence, less deformation of the peptide bond can occur, thus lowering the stability of the complex and allowing an increase of the rate of ion transfer. However, when some properties of these side-chains are modified, such as an increase of the hydrophobicity by the introduction of a methylene group, the orientation of the dipole moment will also be modified owing to an increase of the interaction of the side-chain in the organic (lipid) medium. The consequence of this will be a weakening (in the case of GTMe) or the removal of the peptide dipole–side-chain dipole interactions (for GTBzl, GTBu and GTEt). Such an explanation may also account, at least qualitatively, for the voltage dependence of the conductance. In this case the effect of the voltage is to induce the "stress" of the helix or modifications of the dipole orientations. This appears to be an extremely subtle phenomenon as only small changes such as the introduction of a CH_2 (compare quinoleine with indole and ethyl with methyl) can induce such an effect. It is also tempting to relate the above explanation with the problem of the favored conformation of the gramicidin molecule. Does the absence of a dipole moment or the modification of its orientation favor, under particular experimental conditions, the double helical form as already observed for GM and GN? This question is still open.

Conclusion

In the present paper, by the study of a series of gramicidin A analogues bearing aromatic side-chains with similar dipole moments but with varying hydrophobicities, we have confirmed previous observations which suggested that an increase of the hydrophobicity leads to a decrease of the conductance of the gramicidin channel. This

very probably occurs through reorientation of these side-chains which are able to interact with the lipid medium. A mechanism involving the ion which crosses the channel, the peptide group which participates in the solvatation of the ion during this crossing and the side-chain dipole moments is proposed on the basis of the variation of flexibility of the Π_{DL} helix due to modifications of peptide–side-chain dipole interactions.

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