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SINGLE CHANNEL CHARACTERISTICS OF SOME SYNTHETIC GRAMICIDINS

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Summary

The synthesis of two new variants of gramicidin is described. It is shown that the changes in the aromatic side groups do not influence the single channel conductivity. Experiments in which solutions having different molarities on the two sides of the bilayer lipid membrane are described and their results compared with a rate theory analysis. It is concluded that the gramicidin pore contains approximately 10 equal potential maxima.

Introduction

The single channel phenomenon associated with gramicidin A in bilayer lipid membranes was first observed by Hladky and Haydon [1,2] using a technique developed by Bean, Shephard, Chan and Eichner [3]. Subsequently this method has been used by a number of other workers [4—8] who have succeeded in bringing about a situation in which gramicidin is very much the best understood of any membrane ion pore. Nevertheless, many details of its behaviour remain obscure. Even the structure of the dimer, now known to lead to the formation of the pore, is in question, the rival theories of Urry [9] and of Veatch, Fossel and Blout [10] both having certain attractions. For this reason we have carried out a series of investigations in this laboratory using various synthetic gramicidins with a view to clarifying the conduction mechanism of the gramicidin dimer. It was shown by Bamberg, Noda, Gross and Läuger [6] that it is possible to synthesise gramicidins A, B and C and incorporate them in bilayer lipid membranes and observe single channel behaviour. They obtained results which agreed very well with those obtained from naturally occurring materials. This suggested the possibility of producing analogues of gramicidin which do not correspond to the naturally occurring variants and using them to test various hypotheses concerning the way in which the material mediates ion transport through membranes.

Gramicidin is a pentadecapeptide in which residues 9, 11, 13 and 15 have

aromatic side groups, the remaining residues having uncharged aliphatic side groups, the larger side chains residing mainly near the carboxyl acid end of the chain. Alternate residues are in the D and L configuration. The structure of valine gramicidin A is shown below.

HCO-L-Val-L-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-
D-Leu-L-Trp-D-Leu-L-Trp-$NHCH_2CH_2OH$

In gramicidn B residue 11 is phenylalanine. In gramicidin C residue 11 is tyrosine.

It was suggested by Urry [9] that such a structure could form a helix having a length of approx. 1.5 nm and an internal diameter of approx. 0.4 nm. Two such helices, end to end, could provide a tube approx. 3 nm long which would form a pore through the hydrocarbon part of a membrane. Veatch, Fossel and Blout [10] suggested on the other hand that the two molecules would form a double helix. They put forward four possible configurations. However, a study of the interactions of the side groups indicates that an antiparallel double helix represents the only plausible alternative to the structure postulated by Urry [9]. Urry also suggested [11] that, in the structure which he postulated, the electric dipoles associated with the peptide bonds could rotate, producing a radial polarisation and lowering the potential energy of an ion in the tube. This is a particular example of the general polarisation mechanism postulated by Tredgold and Hole [12]. Simple electrostatic considerations show that some such polarisation is required in order to lower the energy of the ion in the pore to correspond roughly to the hydration energy of the ion in water. Without such a polarisation the hydrocarbon part of the membrane would act as a potential barrier having a height in excess of 1 eV. However, the gramicidin dimer contains other groups which possess substantial permanent electric dipole moments. Using the quantum chemical calculation of Song and Kurtin [13] one can show that the indole ring which forms the side groups of tryptophan has a dipole moment of about 2.4 Debye units. This agrees fairly well with the experimental result obtained by Janetzky and Labret [14] (compare 3.7 Debye units for the peptide bond). The gramicidin A dimer contains eight tryptophan residues which compares with six tryptophans and two phenylalanines for gramicidin B and six tryptophans and two tyrosines for gramicidin C. The tyrosine side group also possesses a dipole moment but phenylalanine does not. Now, Bamberg et al. [6] found that the single channel conductivity for gramicidins A and C are almost identical whereas the conductivity for gramicidin B is less, being approx. 0.7 of the conductivity for gramicidin A. This suggests the possibility that the dipole moments of the side groups may influence the electrostatic potential in the pore and, hence, the single channel conductivity.

We thus decided to carry out two experiments each of which should help to clarify an aspect of the behaviour of the gramicidin dimer:

(1) We synthesised two variants of the gramicidin structure which we denote by gramicidin L in which residues 9, 11 and 15 are tryptophan and 13 is phenylalanine and gramicidin M in which residues 9, 11, 13 and 15 are all phenylalanine. Studies were made of their single channel conductivity.

(2) We used pure synthetic gramicidin A in a cell containing different concentrations of electrolyte on the two sides in order to test a particular model for the potential profile in the pore. This model is discussed further below.

Methods

The gramicidin variants were synthesised by the Merrifield [15] technique with chloromethylated cross-linked polystyrene (Sigma Limited, London) as the resin base. L-Amino acids were purchased as the *t*-BOC-L-amino acid (Sigma). D-Amino acids (Koch-Light Limited, Colnbrook, U.K.) were *N*-protected using butylazidoformate (Koch-Light) [16]. A programmable electronic control [17] unit was used to sequence the reaction processes. To limit the degradation of the tryptophan during the deprotection step, 1% mercaptoethanol was added to the acid and solvent solution. Completion of the coupling step was tested by the method of Kaiser, Colescott, Bossinger and Cook [18]. Quantitative determination of the peptide composition was achieved by acid hydrolysis of the peptide from the resin at various stages of the synthesis and subsequent amino acid analysis. Alkali hydrolysis (using tetrahydrofuran, methanol and molar sodium hydroxide in the ratios (7 : 2 : 1) and spectrophotometric estimation of the aromatic amino acids at 280 nm was also used [19]. The completed peptide chain was cleaved from the resin by aminolysis with ethanolamine/methanol and formylated with *p*-nitrophenylformate [20]. The crude product was dissolved in 10% ethanol and passed through Dowex 2 and Dowex 50 ion exchange columns.

The worst discrepancy between expected ratios of residues to measured ratios was 2% which probably represents the inaccuracy of the method of measurement. We thus conclude that, in the worst possible case one completed peptide molecule in twelve may be defective. We return to this point when discussing the results. The completed gramicidins were stored in ethanol in a refrigerator. Single channel measurements were made by adding dilute gramicidin in ethanol to the experimental cell and allowing it to become incorporated in the membrane. The experimental method was largely based on those already employed in this field of study [2,5,8]. We used a polytetrafluoroethylene and glass cell [10] and employed glycerol monooleate in *n*-decane to form membranes. The head amplifier consists of a high grade operational amplifier and 10^9 Ω resistor in parallel with a 25 pF condenser in a conventional negative feedback circuit. The observed voltage fluctuations were recorded on a magnetic tape and subsequently played back at a lower speed onto a pen recorder. We used calomel electrodes connected via saturated KCl bridges to the two halves of the cell. In order to avoid contamination we used expendable bridges formed from 1% agar in saturated KCl. In other respects precautions were similar to those already described in the literature [2,5,8].

In the second series of experiments referred to above we used a 0.5 molar solution of KCl on one side of the membrane and a 0.1 M solution on the other. This situation may be brought about by filling both halves of the cell to just below the hole in the septum and then spreading the lipid-decane solution across the hole. The two halves of the cell were then filled to above the hole and the membrane was allowed to become thin. Very small quantities of

gramicidin in ethanol were introduced and single channel behaviour was subsequently observed.

Results

(1) Measurements were made using a 0.5 M KCl solution and an applied potential of 50 mV. Fig. 1 shows a histogram of relative frequency of channel conductivities for gramicidin M. Similar histograms were obtained from gramicidin L and from our own synthetic gramicidin A. The mean single channel conductivities for these materials (given in Ω^{-1}.) are:

A, Hladky and Haydon [2], $3.06 \cdot 10^{-11}$; A, our material, $3.02 \cdot 10^{-11}$;
L, $3.11 \cdot 10^{-11}$; M, $3.18 \cdot 10^{-11}$.

We estimate a possible error of ±5% and thus, within this margin, all these materials exhibit the same single channel conductivity. The peaks in Fig. 1, corresponding to very much lower values of conductivity, probably arise from defective molecules in which one complete residue is missing.

(2) Fig. 2 illustrates the single channel amplitude plotted as a function of bias across the cell (open circles). We have measured 20 step heights on the record associated with each membrane. The experimental points are each based on the results from several membranes. If one assumes that the pore contains a number of potential barriers having equal height and spacing one arrives at the following expression for the single channel current:

$$J = \frac{NkT\,\Lambda_M\,\mathrm{Sinh}(\phi e/2Nkt)(C'e^{\phi e/2kT} - C''e^{-\phi e/2kT})}{e\,\mathrm{Sinh}(\phi e/2kT)} \qquad (1)$$

(see for example Läuger [21]. Here N is the number of potential barriers, Λ_M is the single channel conductivity for molar KCl, ϕ is the potential across the

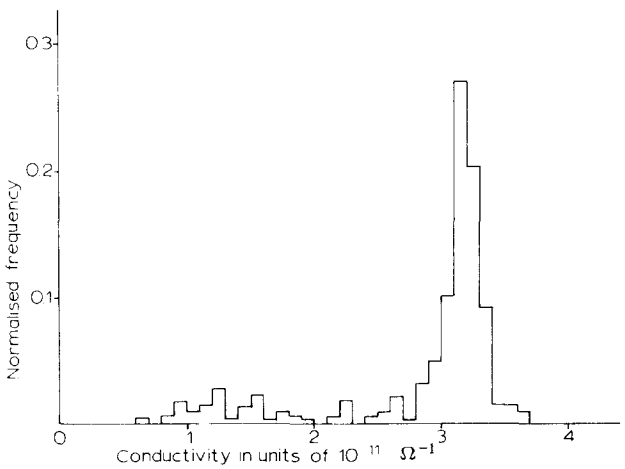

Fig. 1. Normalised histogram of the number of discontinuities in conductivity in an interval of 10^{-12} Ω^{-1} versus conductivity for gramicidin M. The membrane material was glycerol monooleate in *n*-decane. The electrolyte was 0.5 M KCl and the applied potential 50 mV.

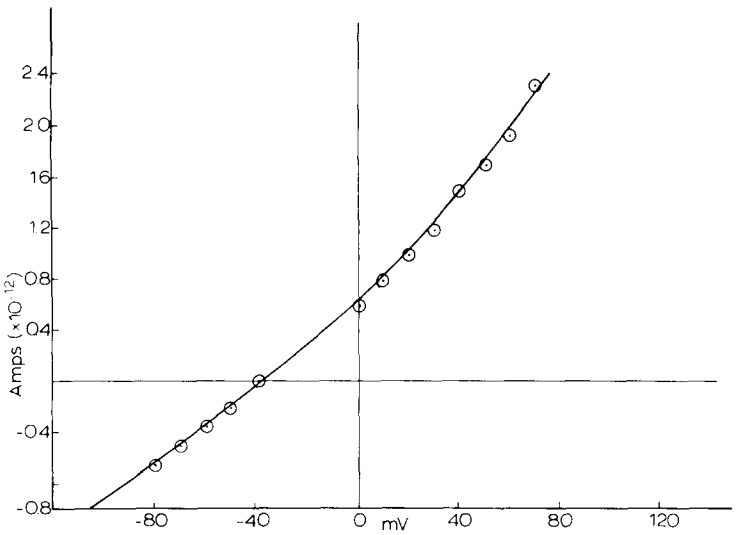

Fig. 2. Gramicidin A single channel amplitudes as a function of bias potential. The membrane is glycerol monooleate in *n*-decane and the solutions on the two sides contain 0.5 and 0.1 M KCl. The open circles are experimental points and the solid line represents the predictions of Eqn. 2.

membrane and C' and C'' are the molar concentrations of KCl on the two sides of the membrane. The other symbols have their usual meanings.

For $2NkT >> \phi e$ this expression becomes

$$J = \frac{\Lambda_M \phi (C' e^{\phi e/2kT} - C'' e^{-\phi e/2kT})}{2 \operatorname{Sinh}(\phi e/2kT)} \quad . \tag{2}$$

This expression is represented by the solid line in Fig. 2. Here the values of Λ are taken from the results of Hladky and Haydon [2] who made measurements of unit channel conductivity for various different molarities of the electrolyte but having the same molarity on either side. For positive currents (i.e., flow from 0.5 M solution to 0.1 M solution) $\Lambda_M/2$ is replaced by $\Lambda_{0.5M} = 3.06 \cdot 10^{-11}\ \Omega$. For negative currents (i.e., flow from 0.1 M to 0.5 M solution) $\Lambda_M/2$ is replaced by $4.6 \cdot 10^{-11}\ \Omega$. It will be seen that provided allowance is made for the influence of molarity on Λ, the agreement between experimental results and the predictions of Eqn. 2 are excellent. It thus seems likely that the model for the pore discussed here is realistic and that $2NkT >> \phi e$ in the range studied. This implies that N must be at least 10. These results give independant support to the rate theory model for this pore originally discussed by Läuger [22]. The unit conductivities used here have been read from the published [2] graphical results and are, thus, only approximate.

Conclusions

We conclude that the nature of the aromatic side groups attached to residues 9, 11, 13 and 15 does not influence the single channel conductivity of the gramicidin dimer to within the error of our experiments in the materials we studied (but see Bamberg et al. [6]) and thus it is only the dipole moments

associated with the peptide bonds which contribute to the radial polarisation needed to mediate ion conduction. We also conclude that the gramicidin pore must contain approximately 10 equal potential maxima.

References

1 Hladky, S.B. and Haydon, D.A. (1970) Nature 225, 451—453
2 Hladky, S.B. and Haydon, D.A. (1972) Biochim. Biophys. Acta 274, 294—312
3 Bean, R.C., Shephard, W.C., Chan, H. and Eichner, J. (1969) J. Gen. Physiol. 53, 741—757
4 Goodall, M.C. (1973) Arch. Biochem. Biophys. 157, 514—519
5 Bamberg, E. and Läuger, P. (1974) Biochim. Biophys. Acta 367, 127—133
6 Bamberg, E. Noda, K., Gross, E. and Läuger, P. (1976) Biochim. Biophys. Acta 419, 223—228
7 Kolb, H.A. and Bamberg, E. (1977) Biochim. Biophys. Acta 464, 127—141
8 Veatch, W.R., Mathias, R., Eisenberg, M. and Stryer, L. (1975) J. Mol. Biol. 99, 75—92
9 Urry, D.W. (1971) Proc. Natl. Acad. Sci. U.S. 68, 672—676
10 Veatch, W.R., Fossel, E.T. and Blout, E.R. (1974) Biochemistry 13, 5249—5256
11 Urry, D.W. (1973) The Jerusalem Symposia on Quantum Chemistry and Biochemistry, V, Jerusalem 1973, pp. 723—736
12 Tredgold, R.H. and Hole, P.N. (1976) Biochim. Biophys. Acta 443, 137—142
13 Song, P, and Kurtin, W.E. (1969) Photochem. and Photobiol. 9, 175—177
14 Janetzky, E.F.J. and Labret, M.C. (1944) Rec. Trav. Chim. 63, 123—126
15 Merrifield, R.B. (1963) J. Am. Chem. Soc. 85, 2149—54
16 Akhtar, A., Falk, F. and Weinstein, B. (1972) Angew. Chem. 11, 289
17 Tredgold, R.H., Griffin, T.J. and Hole, P.N. (1975) J. Phys. E. 8, 656—658
18 Kaiser, E., Colescott, R.L., Bossinger, C.D. and Cook, P.I. (1970) Anal. Biochem. 34, 595—598
19 Fontana, A. and Gross, E. (1973) in Proc. Eur. Pept. Symp. 12th, (Hanson, H. and Jakubke, H.-D., eds.), pp. 229—234 North Holland, Amsterdam
20 Okawa, K. and Hase, S. (1963) Bull. Chem. Soc. Japan 36, 754
21 Läuger, P. (1976) Biochim. Biophys. Acta 455, 493—509
22 Läuger, P. (1973) Biochim. Biophys. Acta 311, 423—441