

## CATION TRANSLOCATING EFFECTS OF ALAMETHICIN AND ITS SYNTHETIC FRAGMENTS IN LIPID MEMBRANES

### Influence of peptide chain length and charge

R. NAGARAJ, M. K. MATHEW and P. BALARAM

*Molecular Biophysics Unit, Indian Institute of Science, Bangalore - 560 012, India*

Received 15 September 1980

#### 1. Introduction

Alamethicin, a 20 residue polypeptide ionophore forms transmembrane channels and has been shown to induce excitability phenomena in artificial lipid membranes [1]. The discovery of a number of related polypeptides, containing  $\alpha$ -aminoisobutyric acid (Aib) [2], has stimulated considerable interest in the conformational analysis of Aib peptides, with a view towards establishing the structural requirements for

*Abbreviations:* Aib,  $\alpha$ -aminoisobutyric acid; CTC, chlortetracycline; Z, benzyloxycarbonyl; Ac, acetyl; OMe, methyl ester; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2 ethane sulfonic acid; TLC, thin-layer chromatography

Address correspondence to P. B.

formation of membrane channels [3-5]. The natural peptides, listed in fig.1, vary in length from 15 residues in emerimicin [6] to 24 residues in suzukacillin [7]. Alamethicin, suzukacillin and hypelcin [8] are negatively charged at neutral pH due to the presence of a glutamic acid residue, whereas the others are neutral. It is therefore relevant to explore the effect of chain length and charge on ionophore properties. Here, we describe studies on the effect of synthetic alamethicin fragments on the divalent cation permeability of phospholipid vesicles, using chlortetracycline (CTC) as fluorescent probe [11-13]. We show that small fragments have no effect, the 1-13 fragment weakly affects membrane permeability and the 1-17 fragment and alamethicin have a pronounced effect.

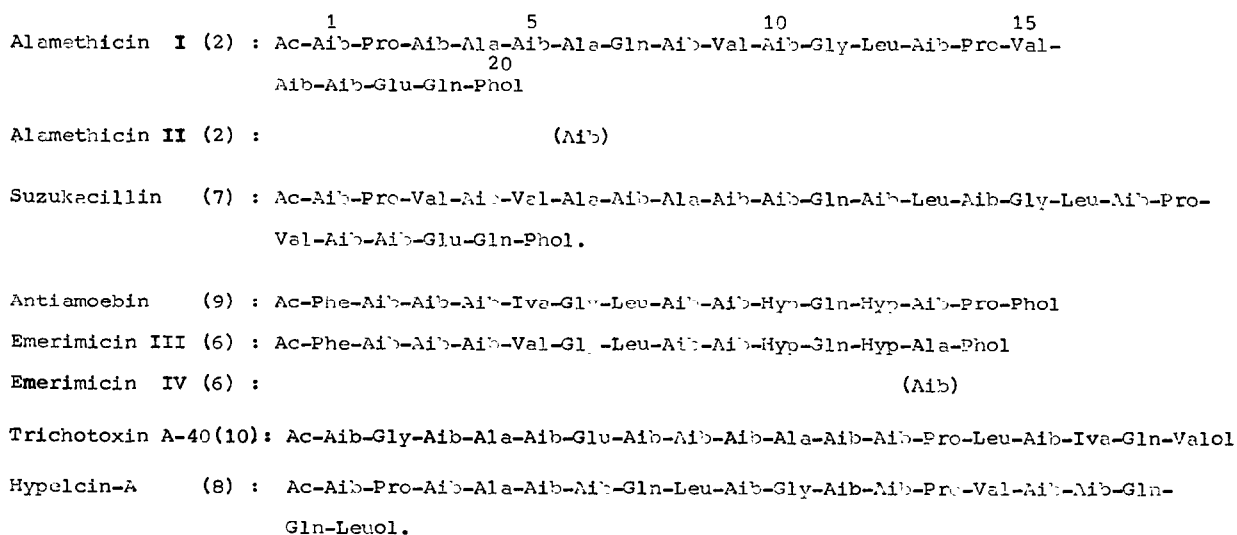


Fig.1. Sequences of alamethicin and related polypeptides.

## 2. Experimental

The protected 13 and 17 residue fragments Z-1-13-OMe, Z-1-13-OH, Ac-1-17-OMe, Z-1-17-OMe and Z-1-17-OH and alamethicin I were synthesized by solution phase procedures in [14]. All peptides were checked for homogeneity by TLC and characterized by 270 MHz  $^1\text{H}$  NMR and amino acid analysis. A sample of natural alamethicin was kindly provided by Dr J. E. Grady, Upjohn Co., Kalamazoo, MI. Egg phosphatidylcholine, Sephadex G-50 and CTC were from Sigma (St Louis, MO).  $\text{LaCl}_3$  was from Ventron. All other chemicals were of analytical grade. All metal salts were chlorides.

Unilamellar vesicles of egg phosphatidylcholine were prepared according to [15] by removal of cholate from mixed phospholipidcholate dispersions, by Sephadex G-50 chromatography. Ion transport was followed using 25  $\mu\text{M}$  CTC, 200  $\mu\text{g}/\text{ml}$  vesicles in 5 mM Hepes, 100 mM NaCl (pH 7.0). After CTC had equilibrated across the membranes, metal was added at 1 mM, following which ionophore was introduced. The fluorescence of the CTC-metal complex was monitored on a Perkin-Elmer MPF-44A spectrometer

operated in the ratio mode with 10 nm excitation and emission band pass;  $\lambda_{\text{ex}} = 390 \text{ nm}$ ,  $\lambda_{\text{em}} = 530 \text{ nm}$ . 1 cm cells were used and solutions were stirred with a magnetic pellet to minimise settling. Vesicle integrity was checked by a flat response after adding metal, followed by a sharp rise on adding X537A [16].

The use of the CTC method as a probe of transmembrane fluxes is based on the ability of CTC to permeate lipid membranes, while the vesicles used remain impermeable to cations, in the absence of ionophore. Attempts to generate vesicles with entrapped CTC, followed by gel filtration were not successful due to equilibration of CTC across the membranes, during chromatography. The presence of external CTC leads to a large basal fluorescence in the presence of metal ions. This was compensated using background suppression. Fig.2 shows the effect of synthetic alamethicin fragments on the time-dependent changes in CTC fluorescence, in the presence of unilamellar lipid vesicles and  $\text{Ca}^{2+}$ . In the absence of peptides there is no change in emission intensity, supporting the view that the membranes are impermeable to cations. This is confirmed by a large increase in fluorescence, reach-

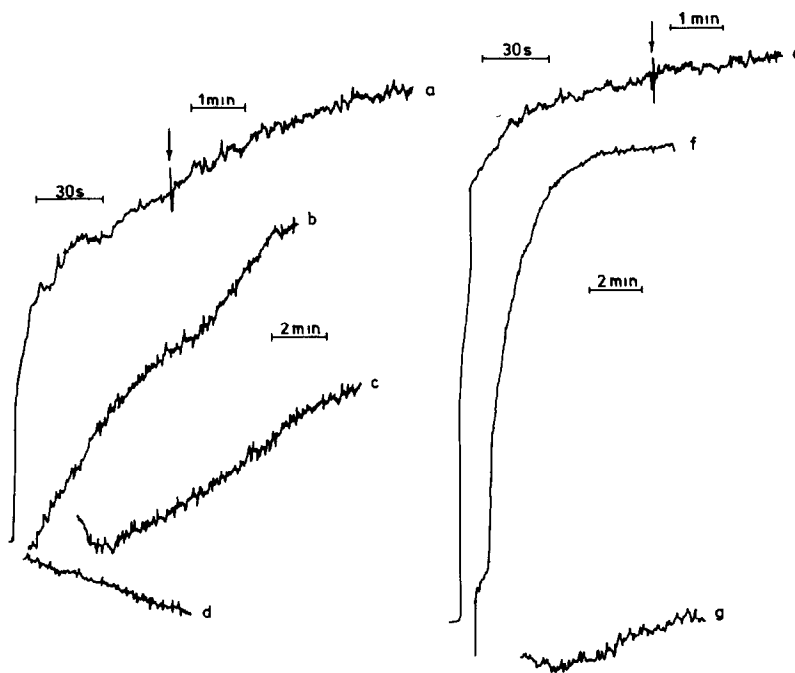


Fig.2. Time-dependent changes in CTC- $\text{Ca}^{2+}$  fluorescence in response to ionophore addition: lipid 200  $\mu\text{g}/\text{ml}$ ; CTC 25  $\mu\text{M}$ ;  $\text{Ca}^{2+}$  1 mM. (a) Natural alamethicin 10  $\mu\text{M}$ ; (b) Ac-1-17-OMe 16  $\mu\text{M}$ ; (c) Z-1-17-OH 10  $\mu\text{M}$ ; (d) Z-1-13-OH 20  $\mu\text{M}$ ; (e) synthetic alamethicin 10  $\mu\text{M}$ ; (f) Z-1-17-OMe 10  $\mu\text{M}$ ; (g) Z-1-13-OMe 20  $\mu\text{M}$ . Time scales indicated at the top refer to traces (a) and (e) with the scale change indicated by an arrow, while the lower scales are for the other traces. Ionophore was added just before the start of the recording.

ing a saturation value in  $\sim 1$  min, on the addition of the divalent cation ionophore X537A [16] (not shown). From fig.1 it is observed that the protected 13 residue peptide acid, Z-1-13-OH does not cause any fluorescence changes even at  $20 \mu\text{M}$ . The corresponding ester, Z-1-13-OMe, however has a long lag time followed by a slow increase in fluorescence, suggesting that there is an increased permeability of the membrane to  $\text{Ca}^{2+}$ . Addition of the 17 residue peptides, Z-1-17-OMe, Ac-1-17-OMe and Z-1-17-OH resulted in considerable increase in CTC emission, as a function of time.

Fig.3 shows the dependence of the ion translocation process on peptide concentration for Ac-1-17-OMe. At  $< 5 \mu\text{M}$ , there does not appear to be any ionophore activity. At  $5\text{--}10 \mu\text{M}$ , the emission intensity changes occur after an initial lag time, which decreases with increasing peptide concentration. At higher concentrations there is a steady increase in fluorescence and no initial lag is observed. Fig.4 shows a plot of the initial slope of the fluorescence vs time curves, as a function of peptide concentration, for the 17 residue fragments. While the peptide ester Z-1-17-OMe appears to act as an ionophore even at  $2 \mu\text{M}$ , a minimum level of  $\sim 14 \mu\text{M}$  is required in the case of Z-1-17-OH and Ac-1-17-OMe. Both synthetic [14] and natural alamethicin (Upjohn) showed high ionophore activity (fig.2). Since the amount of CTC incorporated in the vesicles is small all of it may be complexed before cations attain equilibrium across the membrane. This deprives the plateau values of information content. Further, as the vesicles are reportedly  $< 150 \text{ \AA}$  in radius [15], rapid break down of the concentration gradient is expected even at low cation translocation rates, so that only the initial rate of influx is related to ionophore concentration. The selectivity of the peptides for different ions was also tested. The differential response of CTC to various ions [12] was compensated by dividing the initial slopes of transport by the relative intensities of CTC-metal emission. The rates of translocation by Z-1-17-OMe with respect to that for  $\text{Ca}^{2+}$  are  $\text{Zn}^{2+} = 0.75$  and  $\text{La}^{3+} = 0.95$ . This is in agreement with earlier studies suggesting a lack of specificity in alamethicin channels [17]. It may be noted that the assay used here is not suitable for studying alkali cation transport, as these ions do not significantly alter CTC fluorescence.

The above results suggest that a minimum peptide chain length of  $\sim 13$  residues is necessary before ionophore activity is observed. The results also imply that

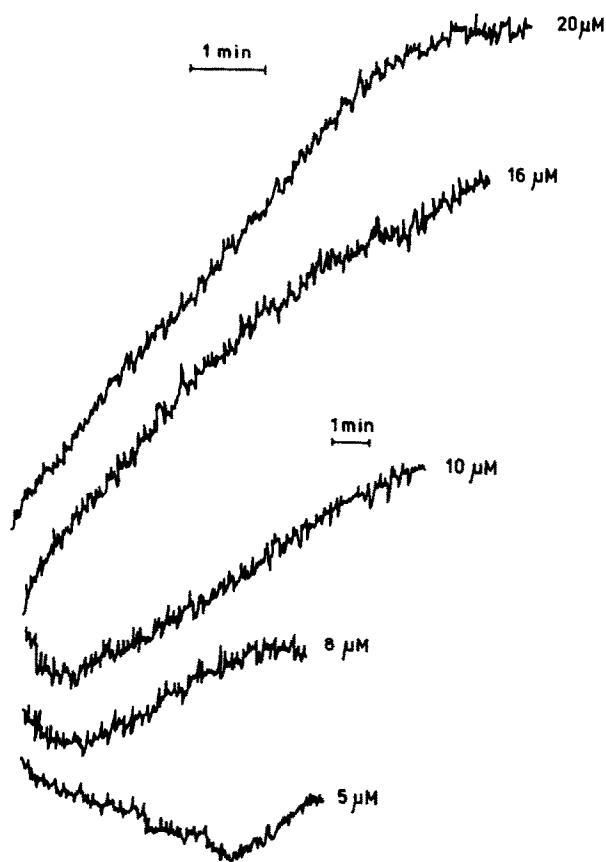


Fig.3. Time-dependent changes in CTC- $\text{Ca}^{2+}$  fluorescence in response to varying concentrations of Ac-1-17-OMe. Conditions as in fig.2. The top time scale is for 20 and  $16 \mu\text{M}$  traces while the lower one is for the others.

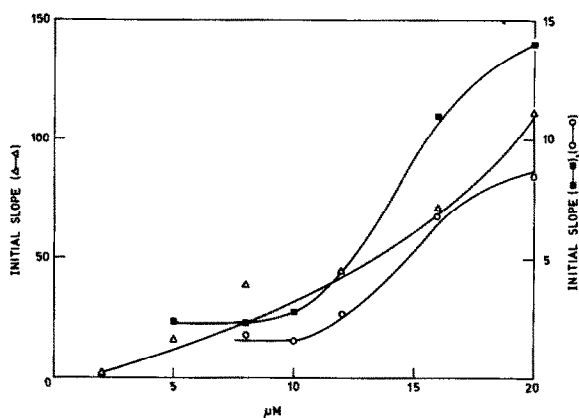


Fig.4. Plot of the initial slopes of CTC- $\text{Ca}^{2+}$  fluorescence changes against concentration of ionophore for 17 residue peptides. Note differences in scale for peptides: ( $\square$ ) Z-1-17-OH; ( $\circ$ ) Ac-1-17-OMe both using right scale; ( $\triangle$ ) Z-1-17-OMe using left scale.

a critical peptide concentration is necessary before ion translocation is possible. This may reflect the fact that the functional channel is an aggregate of peptide molecules or that preaggregation is necessary for insertion into the membrane. Evidence for the aggregation of alamethicin molecules to form pores in membranes as well as hexameric preaggregates at the membrane interface, exists [18]. Studies with fluorescent labelled emerimicin fragments have also provided evidence for aggregation in aqueous solution [19]. Conformational studies on alamethicin fragments strongly favour a  $3_{10}$  helical structure in which ion passage through the helix interior is not possible [3–5]. This indirectly emphasizes the importance of aggregation in generating transmembrane channels. The contrasting activity of Z-1-13-OMe and Z-1-13-OH suggests that in the latter charge repulsion, between the terminal COOH groups, prevents effective aggregation necessary for channel formation. It had been shown that the amino terminal nonapeptide ester of emerimicin aggregates even at  $8 \mu\text{M}$  in aqueous solution, whereas the corresponding acid does not [19]. In the case of the 17 residue fragments both esters (Z-1-17-OMe and Ac-1-17-OMe) and the acid (Z-1-17-OH) exhibit ionophore activity. Thus, it appears that with increasing chain length charge effects are no longer dominant. However, Z-1-17-OMe is effective as an ionophore even at  $2 \mu\text{M}$  in contrast to Z-1-17-OH, which is appreciably active only at  $>14 \mu\text{M}$ . The differences between Z-1-17-OMe and Ac-1-17-OMe presumably reflect the more facile aggregation of the former to yield channels. This may be facilitated by the hydrophobic benzyloxycarbonyl (Z) group. Both synthetic and natural alamethicin are more effective ion translocators, than the 17 residue fragments (fig.2), supporting the view that channel formation is favoured with increasing chain length. In charged peptides like alamethicin and suzukacillin the C-terminal negative charge, due to glutamic acid, may serve to orient the peptide in the membrane. An additional interaction may involve hydrogen bonding between the terminal hydroxyl group and lipid carbonyl or phosphate functions. Such an anchoring interaction may also be important in neutral peptides like antiemoebin, emerimicins, trichotoxin A-40 and hypelcins. Additionally the helix dipole moment in these predominantly helical peptides would also play an important part in membrane orientation [18]. These results provide some insight into the structural requirements of an alamethicin transmembrane channel. Further studies are required to establish the

detailed structure of the channel, the molecular basis for charges in channel conductance states and the gating phenomenon [20].

### Acknowledgements

This research was supported by the Department of Science and Technology and the University Grants Commission. R. N. and M. K. M. are recipients of senior fellowships from the Council of Scientific and Industrial Research and the Indian Council of Medical Research, respectively.

### References

- [1] Mueller, P. and Rudin, D. O. (1968) *Nature* 217, 713–719.
- [2] Pandey, R. C., Carter Cook, J. jr and Rinehart, K. L. jr (1977) *J. Am. Chem. Soc.* 99, 8469–8483.
- [3] Nagaraj, R., Shamala, N. and Balaram, P. (1979) *J. Am. Chem. Soc.* 101, 16–20.
- [4] Rao, Ch. P., Nagaraj, R., Rao, C. N. R. and Balaram, P. (1979) *FEBS Lett.* 100, 244–248.
- [5] Rao, Ch. P., Nagaraj, R., Rao, C. N. R. and Balaram, P. *Biochemistry* 19, 425–431.
- [6] Pandey, R. C., Carter Cook, J. jr and Rinehart, K. L. jr (1977) *J. Am. Chem. Soc.* 99, 5205–5206.
- [7] Jung, G., Konig, W. A., Liebfritz, D., Ooka, T., Janko, K. and Boheim, G. (1976) *Biochim. Biophys. Acta* 433, 164–181.
- [8] Fujita, T., Takaishi, Y. and Shimomoto, T. (1979) *J. Chem. Soc. Chem. Commun.* 413–414.
- [9] Pandey, R. C., Meng, H., Carter Cook, J. jr and Rinehart, K. L. jr (1977) *J. Am. Chem. Soc.* 99, 5203–5205.
- [10] Irmscher, G., Bovermann, G., Boheim, G. and Jung, G. (1978) *Biochim. Biophys. Acta* 507, 470–484.
- [11] Caswell, A. H. and Hutchinson, J. D. (1971) *Biochem. Biophys. Res. Commun.* 43, 625–630.
- [12] Caswell, A. H. and Hutchinson, J. D. (1971) *Biochem. Biophys. Res. Commun.* 42, 43–49.
- [13] Mathew, M. K. and Balaram, P. (1980) *J. Inorg. Biochem.* in press.
- [14] Nagaraj, R. (1980) PhD thesis, Indian Institute of Science, Bangalore.
- [15] Brunner, J., Skrabal, P. and Häuser, H. (1976) *Biochim. Biophys. Acta* 455, 322–331.
- [16] Caswell, A. H. and Pressman, B. C. (1972) *Biochem. Biophys. Res. Commun.* 49, 292–298.
- [17] Eisenberg, M., Hall, J. E. and Mead, C. A. (1973) *J. Membr. Biol.* 14, 143–176.
- [18] Boheim, G. and Kolb, H. A. (1978) *J. Membr. Biol.* 38, 99–150.
- [19] Nagaraj, R. and Balaram, P. (1979) *Biochem. Biophys. Res. Commun.* 89, 1041–1049.
- [20] Hall, J. E. (1978) in: *Membrane Transport in Biology. Concepts and Models* (Tosteson, D. C. ed) vol. 1, pp. 475–531, Springer-Verlag, Heidelberg, New York.