BBA 75996

ION TRANSFER ACROSS LIPID MEMBRANES IN THE PRESENCE OF GRAMICIDIN A

II. THE ION SELECTIVITY

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(Received March 6th, 1972)

SUMMARY

A detailed examination has been carried out of the ion selectivity of gramicidin A. Membranes of pure lipids and of various thicknesses have been used, and electrolyte concentrations have been varied over a wide range. It was first confirmed that anion permeability is low, even at very acid pH. Only in $CaCl_2$ was the transference number of the cation found to be less than unity (0.8). The measurement of biionic potentials was used to give the selectivity sequence for univalent cations. The results are compared with those already in the literature and with the conductivity data for the gramicidin single channel reported in a previous paper. The latter comparison suggests that a complex theoretical model is required to account for the kinetics of ion transfer.

Semi-quantitative considerations of the univalent cation selectivities lead to the conclusion that there is probably a continuous file of water molecules in the gramicidin pore, and that the binding of ions by the gramicidin does not wholly determine the relative permeabilities. Aspects of the pore structure are discussed briefly in the light of the new data.

INTRODUCTION

The linear polypeptide antibiotic gramicidin A has been shown to induce ion permeability in both natural and artificial lipid membranes. In the membranes of mitochondria[1–3], erythrocytes[4] and electroplax[5,6], as well as in artificial lipid bilayers formed from a variety of different lipids[7–11], the membranes become permeable to alkali metal cations, although no very detailed investigation has been carried out of the ion selectivity in any of these systems.

In a preceding paper[12] it has been shown that gramicidin A produces ion permeability by the formation of a pore through the membrane, and the properties of an isolated pore were examined at some length. Urry and coworkers[13,14] have pointed out that gramicidin A could form a variety of left-handed $\pi_{(L,D)}$ helical structures and have proposed that two such molecules linked head to head constitute the pore

Abbreviation: PTFE, polytetrafluorethylene.

through the membrane. It has been argued by Hladky and Haydon[12,15] from the results of their single channel experiments that if Urry's suggestions are correct then, of the various possible helices, the $\pi^{6}_{(L,D)}$, which has 6.3 residues per turn and a pore size of approx. 4 Å, is the most probable. Although structural evidence is still required, gramicidin seems to be unique in being the first reasonably well-characterized pore-forming substance.

It is of special interest therefore to have detailed information concerning the ion permeability of the pore, which can be used to test mathematical treatments of the ion transfer kinetics. Some data on the ion selectivity of gramicidin A have already been published[7,9–11] but for various reasons are unsuitable for present purposes. Principally, there are appreciable discrepancies between the existing data, the origin of which is not clear. One possibility lies in the diversity of poorly characterized lipids which have been used for membrane formation. Another is that earlier investigators may not have recognized and taken account of the fact that diffusion layers constitute a hazard in the measurement of biionic potentials. These considerations will be amplified below.

In the present paper the ion selectivity of gramicidin A in membranes of pure lipids, has been examined as a function of electrolyte concentration and of membrane thickness and composition. In a subsequent paper these results, together with those for the single channel[12], will be compared with the predictions of a theoretical model.

MATERIALS AND METHODS

Membrane formation and the experimental cell

Optically black lipid membranes were formed from solutions of the various lipids in hydrocarbon solvents. Gramicidin was added to the lipid solution either as the solid (when it was dispersed by ultrasonic treatment) or in solution in ethanol, the final lipid solution containing less than 1 % (v/v) ethanol. The membranes were formed in a 1 mm hole in a vessel of polytetrafluorethylene (PTFE). The complete cell is illustrated in Fig. 1. A manometer was used to introduce the lipid solution into the hole in the vertical partition which, in the vicinity of the hole, was 1 mm thick. While the cell was still dry the manometer was filled with the lipid solution and raised until the inside of the hole was wetted with the non-polar phase. When, ultimately, the cell was filled to the appropriate level with the aqueous solutions and a thick biconcave lens of lipid solution was present in the hole, gentle lowering of the manometer caused a black membrane to form. The PTFE inner vessel was a tight push fit in the outer perspex cell, an arrangement which completely excluded the possibility of streaming potentials between the two compartments.

The first stage in the measurement of the dilution or biionic potentials was to establish equal concentrations of the same electrolyte in both inner and outer compartments, and to check that the potential difference across the membrane was zero. The aqueous solution in the outer compartment was then changed by means of a U-tube device, the two arms of which were isolated from each other by a mercury seal. The spaces in each limb above the seal, and in the polyethylene tubes connecting the two sides of the U-tube to the cell, were completely filled with the original and new aqueous solution, respectively. Rotation of the U-tube then caused the solution

in the cell to be withdrawn into one tube and replaced in equal volume through the other. Although the changing of the solutions could be carried out in the presence of a black membrane it was also possible to thicken the membrane during this process and so minimise the likelihood of breakage and interdiffusion of the two solutions.

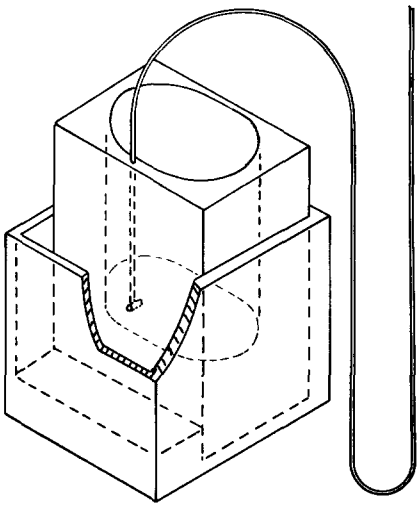

Fig. 1. The experimental cell, showing the manometer.

The manometer method is better suited to the formation of membranes from the hydrocarbon-soluble lipids such as glyceryl monooleate, than to those formed from the insoluble phospholipids. For work with the latter a brush technique was used in a cell of similar dimensions to that of Fig. 1.

The PTFE vessels were cleaned by washing in detergent solution, rinsing, immersing in chromic–sulphuric acid cleaning mixture for 5 min and rinsing very thoroughly with distilled water.

Membrane conductances were measured by means of a conventional circuit[16]. For potential measurements the electrodes were connected directly to a Vibron electrometer. Large coiled Ag–AgCl electrodes were used, either directly or separated from the cell by 3 M KCl–agar salt bridges, whichever seemed most suitable. In general the electrode asymmetry potential was < 0.1 mV. All experiments were carried out at room temperature (20 $\pm$ 1 °C).

Materials

The glyceryl monooleate was obtained from Sigma and was found to be >99 % pure 1-isomer. The egg yolk phosphatidylcholine was supplied by Mr. N. Miller of the Agricultural Research Council Institute of Animal Physiology, Babraham, and the human erythrocyte lipids by Miss F. Dagger of this Laboratory. The latter were extracted by methods described in the literature[17,18]. All the hydrocarbons were obtained from Koch-Light Ltd. and were of puriss grade. They were further purified by passage through an alumina column. The gramicidin A was also obtained from Koch-Light and the cholesterol was a puriss grade specimen from Fluka. All other reagents were of Analytical Reagent grade, the NaCl and KCl being roasted at 700 °C

to remove organic impurities. The water was twice distilled, the second time from a pyrex still. Its pH was approximately 5.6.

RESULTS

It was first established that the anion permeability of the lipid membranes in the presence of gramicidin was negligible compared to that for the alkali metal ions. This was achieved by the determination of the transference numbers of the ions in certain systems. Different concentrations of the various electrolytes were placed on either side of the membranes and the resulting E.M.F. measured. The results were interpreted according to the equation

$$E = \left(t_- \frac{\nu}{\nu_+} - 1\right) \frac{RT}{z_+ F} \ln \frac{a_2}{a_1} \tag{1}$$

where E is the E.M.F. of the high concentration side relative to the low concentration side of the cell, t_- is the transference number of the anion, ν is the total number of ions and ν_+ the number of cations produced by each electrolyte molecule, z_+ is the valence of the positive ion and a_2 and a_1 are the activities of the high and low concentrations, respectively, of the electrolyte. The specific conductances of the membranes before the addition of gramicidin were approximately $10^{-8}\ \Omega^{-1}\ cm^{-2}$, and afterwards were in the range 10^{-2} to $10^{-5}\ \Omega^{-1}\ cm^{-2}$. The results for NaCl, KCl, NH_4Cl, HCl and NaF, shown in Fig. 2, all yield within experimental error, $t_+ = 1.0$. The result for $CaCl_2$, shown in the inset of Fig. 1, gives $t_+ = 0.8$.

The selectivity sequence for the cations was found by the measurement of the biionic potentials. The latter were interpreted according to the Hodgkin–Katz–

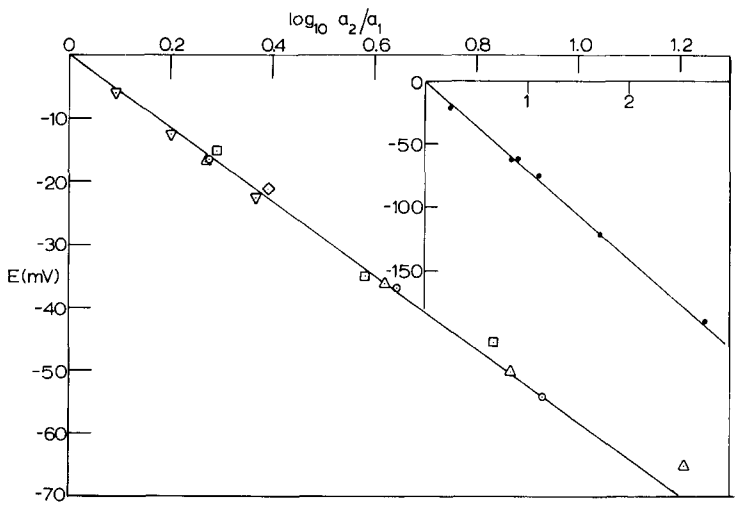

Fig. 2. Cell E.M.F. values as a function of the electrolyte activity ratio across the membrane. ▽, NaCl; △, NH_4Cl; ⊙, KCl; ⊡, HCl; ◇, NaF. Inset, ●, $CaCl_2$. Membranes were of glyceryl monooleate and *n*-decane.

Goldman equation[19,20] which, as the presence of the anion in the uni-univalent electrolytes could be ignored, may be expressed

$$E = \frac{RT}{F} \ln \frac{P_2 a_2}{P_1 a_1} \qquad (2)$$

where P_2 and P_1 are the permeabilities to the cations "2" and "1" and a_2 and a_1 are the mean activities of the two electrolytes. In these experiments, it was necessary to control carefully the specific conductance of the membrane. Thus, the potency of gramicidin is such that very small amounts are capable of producing specific conductivities in excess of $10^{-2}\ \Omega^{-1}\ cm^{-2}$. Highly conducting membranes are, however, not usually suitable for studies of the cation selectivity as the diffusion layers in the adjacent aqueous phases may influence the measured potentials. For example, the ion permeability of a membrane of specific conductance $10^{-2}\ \Omega^{-1}\ cm^{-2}$, in electrolyte of 10^{-2} mole l^{-1}, is approx. $3 \cdot 10^{-4}$ cm s^{-1}. The total diffusion layer thickness in this type of system, in the absence of mechanical stirring is approx. 0.5 cm[21,22] and hence its permeability is approx. $4 \cdot 10^{-5}$ cm s^{-1}. In stirred systems this value might increase to approx. 10^{-4} cm s^{-1}. As these estimates are both less than that expected for the membrane itself it is obvious that a very considerable part of the potential change may

TABLE I

BIIONIC POTENTIALS (E) AND PERMEABILITY RATIOS (P_{M^+}/P_{Na^+}) FOR MEMBRANES FORMED FROM GLYCERYL MONOOLEATE

Electrolyte	*Concentration (mole l^{-1})*	*Membrane: Glyceryl monooleate plus*			
		n-Decane (47 Å)		*n-Hexadecane (31 Å)*	
		E (mV)*	P_{M^+}/P_{Na^+}	E (mV)*	P_{M^+}/P_{Na^+}
LiCl:NaCl	10^{-1}	31	0.29		
KCl:NaCl	3	− 38	5.7		
	1	− 34	4.2	− 37	4.7
	10^{-1}	− 31	3.5	− 34	3.9
	10^{-2}	− 30	3.3	− 33	3.7
	10^{-3}	− 31	3.4	− 31	3.4
RbCl:NaCl	10^{-1}	− 32	3.6		
CsCl:NaCl	10^{-1}	− 38	4.6		
NH_4Cl:NaCl	3	− 58	11.7		
	1	− 56	9.8	− 55	9.4
	10^{-1}	− 46	6.3	− 45	6.0
	10^{-2}	− 44	5.7	− 45	5.9
	10^{-3}	− 38	4.5	− 40	4.9
HCl:NaCl	10^{-1}	− 96	43	− 92	38
	10^{-2}	−100	53	−103	60
	10^{-3}	−101	55	− 98	49
$(CH_3)_4NCl$:NaCl**			$<4 \cdot 10^{-4}$		

* $V(M^+) - V(Na^+)$.

** In the system NaCl (10^{-3} M) / NaCl (10^{-3} M), $(CH_3)_4NCl$ ($8 \cdot 10^{-3}$–$4 \cdot 10^{-2}$ M).

TABLE II

BIIONIC POTENTIALS (E) AND PERMEABILITY RATIOS (P_{M^+}/P_{Na^+}) FOR MEMBRANES FORMED FROM VARIOUS LIPIDS

Electrolyte	*Concentration (mole l^{-1})*	*Membrane*					
		Lecithin + n-decane		*Lecithin + cholesterol + n-decane*		*Erythrocyte lipids + n-decane*	
		E (mV)*	P_{M^+}/P_{Na^+}	E (mV)*	P_{M^+}/P_{Na^+}	E (mV)*	P_{M^+}/P_{Na^+}
LiCl:NaCl	10^{-1}			28.5	0.32	28	0.33
KCl:NaCl	10^{-1}	− 34	3.9	− 28.5	3.1	− 34	3.9
RbCl:NaCl	10^{-1}			− 36	4.3	− 42.5	5.5
CsCl:NaCl	10^{-1}			− 43	5.6	− 44	5.8
NH_4Cl:NaCl	10^{-1}	− 52	7.9	− 48	6.8	− 55	8.9
HCl:NaCl	10^{-1}	−115	94	−148	344	−127	150
	10^{-2}	−123	132				

* $V(M^+) - V(Na^+)$.

occur across the diffusion layers (*cf.* Helferrich[23]). As a consequence of these considerations the specific conductances for the membranes in the various systems were controlled to rather less than 10^{-2} Ω^{-1} cm^{-2} in concentrations of 10^{-1} M and above, and to less than 10^{-3} and 10^{-4} Ω^{-1} cm^{-2} in 10^{-2} M and 10^{-3} M solutions, respectively.

The results for the biionic potential experiments are shown in Tables I and II. Except for HCl, the measured potentials were reproducible to within 1 mV and the values quoted are the mean of at least six determinations. The two types of membrane, glyceryl monooleate *plus* *n*-decane and *n*-hexadecane, respectively, differ in thickness and also in the composition of the hydrocarbon interior, but not in the composition of the polar group layer (Andrews, Manev and Haydon[24]; Fettiplace, Andrews and Haydon[25]). Although the majority of the systems were studied at only one electrolyte concentration, two, KCl:NaCl and NH_4Cl: NaCl, were examined more thoroughly. As can be seen, the permeability ratio does not remain constant over the whole concentration range. The results for KCl: NaCl and RbCl:NaCl are very close to each other and were confirmed by the examination of RbCl : KCl. In the systems containing HCl the potentials, while steady in any one experiment, were not very reproducible from one experiment to the next. This may account for the apparent inconsistency in the data in both Tables I and II. The result for tetramethylammonium could not be obtained accurately by the usual procedure owing to the high impedance of the final system and the tendency of the membranes to rupture.

The effect of varying the polar group of the lipid is shown in Table II. The lecithin and lecithin *plus* cholesterol membranes were comparable to those whose composition was reported by Cook *et al.*[26].

DISCUSSION

Some data on the ion selectivity of gramicidin in black lipid membranes has been given by Mueller and Rudin[7], Tosteson *et al.*[9], Goodall[11] and Liberman *et al.*[10] It seems to have become generally accepted that the gramicidin channel is poorly

permeable to anions although no convincing evidence of this has so far been presented. The present data on the transference numbers therefore fills a gap and confirms the current widely held view. It is of special interest that even at pH 1 the membranes are very highly conducting and the transference number for the anion still very small. Biionic potentials for a range of electrolytes and lipid membranes have been reported[7,9–11] but they are not in good agreement between themselves or with the present data. Thus, Liberman *et al.*[10] give alkali metal ion sequences for several concentrations. Their sequence for 0.1 mole/l differs from that presented here but at 0.05 mole/l is in agreement with that given by Mueller and Rudin[7]. The quantitative discrepancies between the results of Liberman *et al.* and of Mueller and Rudin are nevertheless often larger than the discrepancies between either set of results and those of Tables I and II. Goodall[11] has given the only previous data for NH_4^+ and he reports $K^+ > NH_4^+ > Na^+$ as opposed to the present $NH_4^+ > K^+ > Na^+$ for all concentrations. Although the differences between the phospholipid and glyceride membranes examined here are minimal, it is possible that the nature of the bovine brain lipids used by both Mueller and Rudin and by Liberman *et al.* may account for the discrepancies. Thus the presence of an appreciable surface charge on the membrane could, through both specific and non-specific electrical interactions, produce differing concentrations of the two electrolytes on the two sides of the membrane. This could affect the occupancy of the pores and hence the permeability ratio. Owing to lack of experimental detail given in previous papers, however, it cannot be ruled out that the discrepancies may arise partly from the neglect of diffusion layer effects.

Before discussing the ion selectivity in terms of the structure of the conducting channel, it is of interest to compare the permeability ratios calculated from Eqn 2 with the conductance ratios for the single channel, reported in Part I[12]. (Although the conductance ratios were measured at 100 mV applied, they do not differ significantly from the limiting values for zero applied potential.) Unless carried out within the framework of a theoretical model this exercise does not yield very specific information. Nevertheless, it does show that the interpretation of the ion transfer kinetics for gramicidin is likely to be complicated. In Fig. 3 the permeability ratios and conductance ratios for K^+ and Na^+ are plotted against the electrolyte concentration. The permeability ratios are always higher than the conductance ratios and, moreover, with increasing concentration the two ratios vary in opposite directions. The explanations for these differences are rather involved, but are to some extent provided by a pore model which will be described in a later paper. The selectivity sequence for univalent cations at 0.1 M is $H^+ > NH_4^+ > Cs^+ > Rb^+ > K^+ > Na^+ > Li^+ > (CH_3)_4N^+$. Ion permeabilities are determined by both the binding of ions by the membrane sites, and by the mobility of the ions in the membrane. Gramicidin A has no ionic groups, but is well-endowed with dipolar acyl groups. Eisenman[27] has examined simple electrostatic models for the binding of ions to such groups. He has shown that according to the dehydration free energies of the ions and the free energy of the ion–dipole interactions in the membrane any of eleven selectivity sequences may be expected for the alkali metal ions. The result for gramicidin is, in fact, the first of the eleven sequences. The significance of this observation is doubtful, however, as there are reasons to suppose that the ion mobilities may be of some importance in the determination of the sequence. Thus, according to the simple binding model H^+ should be to the right of Li^+, whereas it is actually on the extreme left. This is the

position that H^+ would occupy if its permeability paralleled its mobility in water. The possibility comes to mind, therefore, that there may be a continuous row of water molecules in the pore, along which the proton could jump. Other considerations reinforce the ideas that the binding of ions may be weak and that water may be present in the pore.

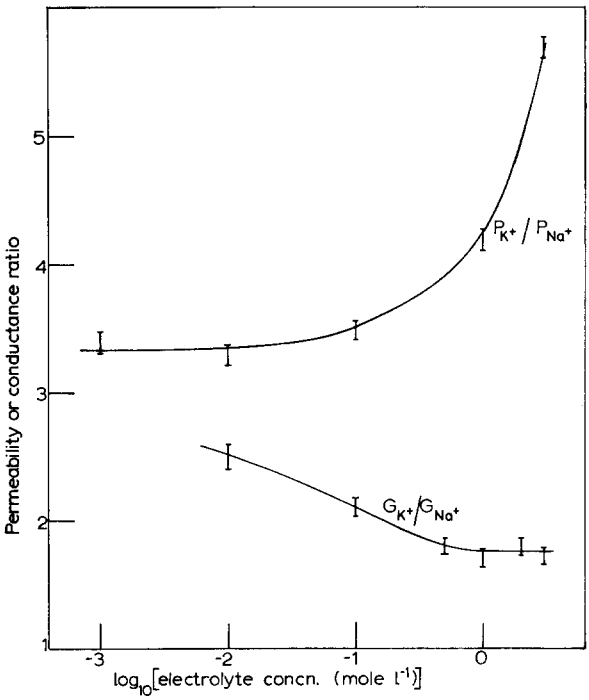

Fig. 3. A comparison of the permeability ratios calculated from Eqn 2 with the single channel conductance ratios of Part I (ref. 12).

The binding free energies of the ions in the pore will be discussed in a quantitative manner in a later paper, but some indications of these parameters may be gained from the conductance-activity curves for the single channel given previously[12]. An estimate of the free energies of transfer of ions from the aqueous phase to their sites in the pore is obtained by assuming that for a given ion, the conductance is proportional to the number of ions in the pore. If it is further assumed that the uptake of the ions follows a simple localized adsorption equation, the required free energy change is readily calculated from the activity for the half-maximal conductance. Using the standard states of unit mole fraction in both solution and pore it is found that the free energy change for K^+ is approx. —1.8 kcal/mole, and that for Na^+ approx. —1.7 kcal/mole. If both the absolute magnitude and the differences between these values are compared with the results of calculations of the free energy of transfer, assuming the ions in the pore to be dehydrated, it is found that the experimental data are smaller by at least an order of magnitude. As Eisenman[27] has pointed out, such a result would be expected if the dehydration of the ions were incomplete. This, therefore, constitutes further evidence for the presence of water in the gramicidin pore.

The apparently complete impermeability to $(CH_3)_4N^+$ is not readily explicable in terms of binding energies. Thus, rough calculations on the lines used for the alkali metal ions suggest not only that the binding energy relative to the other ions would be unable to account for the complete exclusion of $(CH_3)_4N^+$, but also that this ion might even be preferred to Cs^+. Most probably $(CH_3)_4N^+$ is excluded purely because it is too large (approx. 7 Å in diameter) to enter the pore.

The precise manner in which the helical structures proposed by Urry and coworkers[13,14] might account for the observed ion selectivity is not very clear. Thus, the preference for cations rather than anions arises presumably either from complexation of the cations with the negative ends of the acyl group dipoles, or simply from the existence of a region of negative potential within the pore.

No convincing arguments have so far been produced in support of either possibility. The differences between the activation energies for the diffusion of cations through water and through the channel are very small (0.5–1.5 kcal/mole)[12] and do not obviously allow the breaking of H bonds of the helix and the freeing of the acyl oxygens. On the other hand, it seems possible, even in the completely unperturbed helix, that by a combination of steric and coulombic interactions the hydrogen-bonded acyl groups could favour cation rather than anion adsorption. From another point of view, the relative permeability of cations to anions would be accounted for if the average potential within the pore were approx. −60 mV. Such a potential could arise simply from weak dipole orientations at the sides of the pore.

ACKNOWLEDGEMENT

Miss V. B. Myers acknowledges the award of a Medical Research Council Scholarship for Training in Research Methods.

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