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The role of acyl chain character and other determinants on the bilayer activity of A21978C an acidic lipopeptide antibiotic

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An acidic lipopeptide A21978C has previously been shown to have a powerful antibiotic activity against Gram-positive organisms. Due to its ability to increase the K^+ permeability of bacterial cells and its specific calcium requirement, which is similar to a previously described ionophore CDA, its effect on planar bilayer membranes has been studied. Although it produces significant increases in the conductivity of lipid bilayers it is shown that this alone cannot account for its in vivo activity. Similarly, unlike the in vivo results, the Ca^{2+}-induced increases in bilayer conductivity can be mimicked by Mg^{2+} and charged lipids. Results from a series of homologues differing in the length of the acyl moiety show a close similarity between bilayer conductance and LD_{50} trends from in vivo studies. A complex activity is proposed which depends upon incorporation in, rather than disruption of, the bilayer membrane.

Introduction

The lipopeptide A21978C is produced by *Streptomyces roseosporus* and is novel in that it is an acidic cyclic peptide antibiotic (Fig. 1). Similarly unusual are its highly bacteriocidal effects against certain otherwise resistant enterococcal infections and strong requirement for Ca^{2+} ions [1]. Its site of action appears to be the membrane/cell wall region as it has been shown to cause potassium leakage from cells of *Staphylococcus aureus* L form as well as an inhibition of an early stage in cell wall biosynthesis [2]. This behaviour is reminiscent of CDA, a calcium-dependent antibiotic of unknown structure from *Streptomyces coelicolor*, which has been shown to produce ion-conducting channels in planar bilayer membranes [3]. Similarly, other well characterized lipopeptide antibiotics [4,5] have specific effects on bilayer permeability. Moreover, cyclic and other peptides, not possessing a lipid side chain also exhibit ionophore activity [6,7]. It is thus a well established general mechanism of bacteriocidal action. The fatty acyl chain length of A21978C has been shown to be significant both in the antibacterial and in the toxicity tests. In fact the best version from both viewpoints is LY146032 an artificially produced 10 carbon straight chain version [2]. In summary, during the in vivo studies calcium ions, acyl chain length and membrane permeability changes have all been shown to be involved in the antibiotic activity. The following biophysical studies were made to clarify the mode of action of A21978C and they indicate a more complex mechanism than simple ionophore activity.

Materials and methods

Materials. Samples of A21978C and LY146032, an A21978C analogue (prepared by Eli Lilly Co.)

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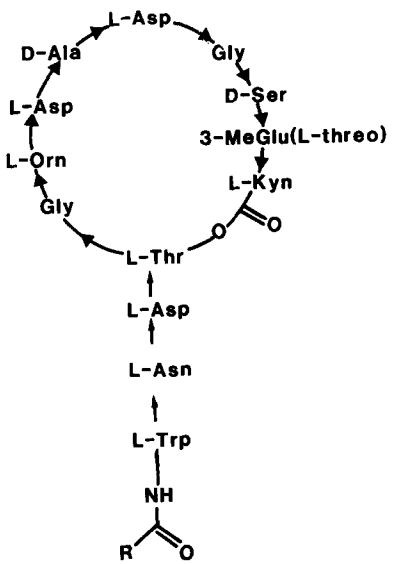

Fig. 1. The structure of A21978C antibiotics. The fatty acid chains of the three forms A21978C_1, A21978C_2 and A21978C_3 are, respectively, 11 carbon atoms and *anteiso*, 12 carbon atoms and *iso*, 13 carbon atoms and *anteiso*. LY146032 has the same general form as the others with straight chain 10 carbon atom fatty acid.

were a gift from Professor D.A. Hopwood. All salts were of 'Analar' grade, $CaCl_2$ and $MgCl_2$ were prepared from 'AVS' (B.D.H.) stock solutions. Egg lecithin was prepared by the method of Singleton et al. [8], cholesterol (Fluka, puriss) was recrystallized from N_2-saturated ethanol [9], stearylamine (octadecylamine) (Koch Light Labs) was recrystallised twice from ethanol-chloroform and phophatidyl inositol was prepared by a modification of the method of Coulon-Morelec and Faure [10]. All the solvents were of laboratory grade or better, *n*-decane was redistilled and, like the hexadecane, dried through an alumina column. Double-glass distilled water was used throughout. ^{86}Rb was supplied as RbCl by Research Products, Amersham.

Bilayer conductivity. Membranes were formed either on the ends of a set of five polythene tubes [11] or in an aperture drilled in a Teflon cell [12]. In the former, conductance per unit area was measured as described previously [11]. When using the Teflon cell, however, such measurements were made by an Apple II microcomputer fitted with an AI13 A/D converter and an AO03 D/A converter (Interactive Structures Inc, Bala, Cynwyd, PA, U.S.A.). Voltage pulses from the AO03 were fed via a high resistance to the membrane. The resulting current transients were recorded and integrated by the microcomputer thus providing a measure of the capacitance and conductance of the bilayer. By assuming a value of 3.8 $mF \cdot m^{-2}$ for the capacitance of lecithin-cholesterol/*n*-decane [11], 3.9 $mF \cdot m^{-2}$ for monoolein/*n*-decane and 5.8 $mF \cdot m^{-2}$ for monoolein/hexadecane bilayers [13] the capacitance per unit area was calculated on-line.

All the measurements of ion selectivity were carried out using the polythene tubes. The membranes were formed in a low concentration bathing solution joined by a KCl-agar bridge to another cell of identical solution containing the reference electrode. In this way both the recording and reference electrodes were in identical solutions after a concentration gradient was formed across the bilayer. This was achieved by adding a concentrated salt solution to that present outside of the membrane tubes. Transmembrane potential differences indicating an ion-selectivity [14] could then be measured directly.

Light scattering. Fresh human heparinised erythrocytes were prepared by repeated centrifugation and washing in 0.9% NaCl, 10 mM Hepes, 2 mM $CaCl_2$ (pH 7.2). Packed cells were used at a dilution of 1/800 in all light scattering experiments following the method of Selwyn et al. [15].

Liposome permeability. Single-shelled liposomes were formed by injecting an ethanolic lipid solution into a stirred 50 mM NaCl, 50 mM RbCl, 20 mM $CaCl_2$ aqueous phase [16]. The liposomes were then concentrated in a concentrator (Amicon model 52) using a 10000 mol. wt. filter under N_2 at 10^5 Pa. The resulting suspension was mixed with A21978C or valinomycin and the experiment started by adding an aliquot of stock solution containing $^{86}Rb^+$. At intervals, 100 μl samples were removed and the liposomes separated from the external isotope by running them through mini columns of Sephadex G-50 in Pasteur pipettes. The resulting 1 ml of solution was then mixed with 4 ml of scintillator (Pico-fluor 15) and the amount of $^{86}Rb^+$ measured by a Phillips PW-4700 liquid scintillation counter.

Results

Conductance as a function of antibiotic concentration

Lipid bilayer conductance was measured over a concentration range for each of the A21978C analogues. After adding aliquots from concentrated stock solutions, the bathing medium (100 mM NaCl, 25 mM $CaCl_2$) was mixed to ensure symmetry about the membrane. In each case the bilayer conductivity rose with increasing antibiotic concentration. The greatest effect was observed on egg lecithin/cholesterol membranes (2 : 1, w/w) using *n*-decane (Fig. 2) whilst monoolein/*n*-decane membranes were almost an order of magnitude less sensitive (Fig. 3). However, both types of membrane were subject to breakage at high lipopeptide concentrations illustrated by the maximum readings shown in Figs. 2 and 3. Egg lecithin/*n*-decane membranes were so prone to breakage that no reliable conductivity measurements were obtained. Similarly, monoolein/hexadecane membranes were more sensitive to the destructive effects of the lipopeptide without showing a concomitant increase in induced conductivity at sub-breakage concentrations (results not shown). On the other hand lecithin/cholesterol membranes containing a high proportion of cholesterol were virtually immune from the deleterious effects of A21978C and thus larger conductivity/concentration ranges were achieved with these bilayers (Fig. 4). It is, though, important to note that the induced conductivity at any one lipopeptide concentration is no higher in a high cholesterol membrane than in one containing only a small proportion of cholesterol.

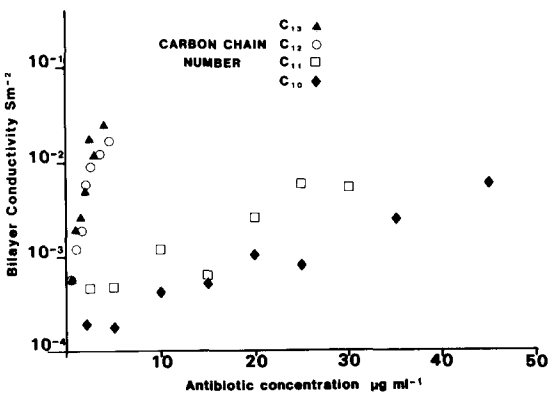

Fig. 2. Bilayer conductivity as function of lipopeptide concentration. Bilayers were formed from egg lecithin-cholesterol (2:1, w/w)/*n*-decane in 100 mM NaCl, 25 mM $CaCl_2$ at 25°C. Each point is the average of at least four membranes measured 5 min after formation. ▲, A21978C_3; ○, A21978C_2; □, A21978C_1; ◆, LY146032.

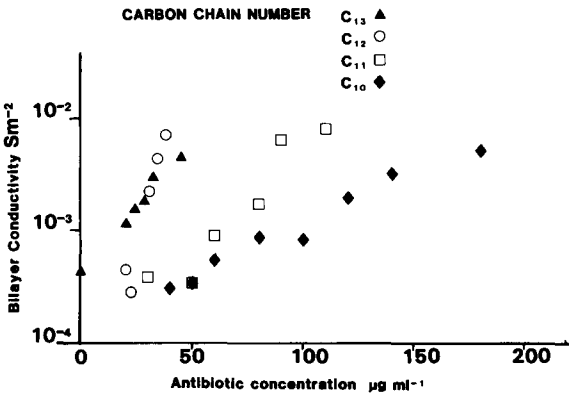

Fig. 3. Bilayer conductivity as function of lipopeptide concentration. Bilayers were formed from monoolein/*n*-decane in 100 mM NaCl, 25 mM $CaCl_2$ at 25°C. Each point is the average of at least four membranes measured 5 min after formation. ▲, A21978C_3; ○, A21978C_2; □, A21978C_1; ◆, LY146032.

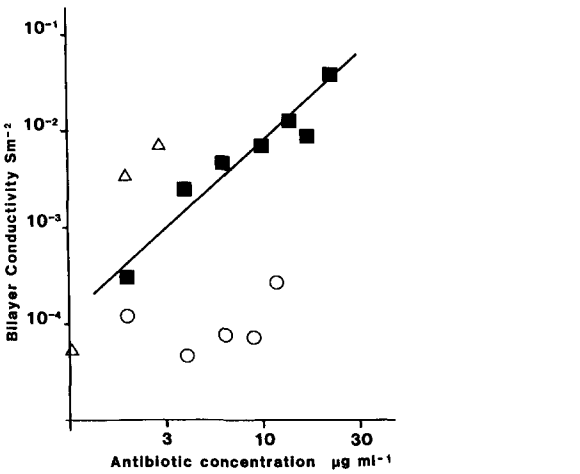

Fig. 4. Bilayer conductivity as function of lipopeptide C_2 concentration and surface charge. Bilayers were formed from: (■) egg lecithin-cholesterol (1 : 10, w/w)/*n*-decane, (△) stearylamine-lecithin-cholesterol (1 : 1 : 10, w/w)/*n*-decane, (○) phosphatidylinositol-lecithin-cholesterol (2:1:10, w/w)/*n*-decane, in 100 mM NaCl, 25 mM $CaCl_2$ at 25°C. Each point is the average of at least five membranes measured 5 min after formation. Line drawn by method of least squares gives a slope of 1.82 ± 0.28 for neutral bilayer.

Conductance as a function of fatty acid chain length

The effect of the variable fatty acid moiety of the lipopeptide can easily be seen from the results illustrated in Figs. 2 and 3. The ability to cause conductance increases (or membrane breakage) mostly follows the relation $C_3 > C_2 > C_1 >$ LY146032, although in some cases it is difficult to differentiate the effects of C_3 and C_2.

Conductance as a function of calcium concentration

Using a C_2 concentration of 4 μg/ml and lecithin-cholesterol (2 : 1, w/w)/decane bilayers, 1 M $CaCl_2$ was added to the 100 mM NaCl bathing solution and the conductance measured after each addition. The results are shown in Fig. 5 and it can be seen that as the $CaCl_2$ concentration rises from 2 to 20 mM the bilayer conductivity similarly increases by an order of magnitude.

Conductance as a function of bilayer surface charge

Using the stable high cholesterol bilayer as a base, net negative charge was introduced by adding phosphatidylinositol, whilst stearylamine was employed to induce net positive charge. In each case the amount added was calculated to provide a 10% mole fraction of the charged species, although the final concentration in the bilayer was not known. Using 100 mM NaCl, 25 mM $CaCl_2$ as the aqueous phase, the results indicate that negative charge significantly reduces the conductivity induced by the lipopeptide. The presence of stearylamine increases the effect of A21978C under the same conditions (Fig. 4) and in 100 mM NaCl markedly lessens the calcium dependence (results not shown).

Ion selectivity

For each salt, membranes were formed from lecithin/cholesterol (1 : 20, w/w) in 25 mM salt, 25 mM $CaCl_2$, 4 μg/ml A21978C_3 (25 mM $CaCl_2$ in the case of measurements of calcium selectivity). 5 min after bilayer formation, concentrated salt solution was added to increase the concentration outside up to a maximum of 500 mM. In each case (NaCl, KCl, $CaCl_2$) no distinct membrane potential difference was measured with values varying between $\pm$7mV in an apparently random fashion. Similar results were achieved with a proton gradient of 2 pH units instead of a salt gradient in an otherwise similar system.

Haemolysis

Increasing quantities of A21978C_3 were added

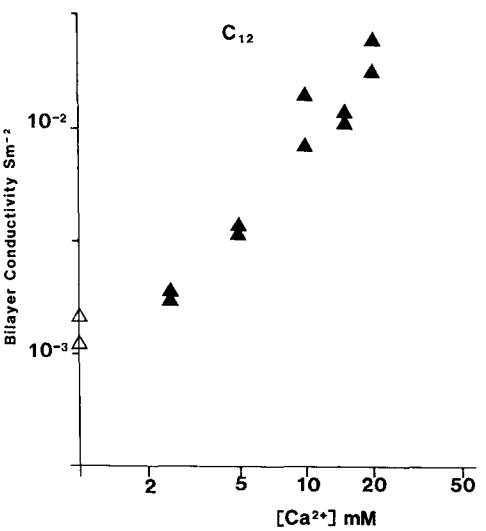

Fig. 5. Bilayer conductivity as a function of calcium concentration. Bilayers were formed from egg lecithin-cholesterol (2 : 1, w/w)/*n*-decane in 100 mM NaCl, 25 mM $CaCl_2$, 4 $\mu g \cdot ml^{-1}$ A21978C_2 at 25 °C. Each point is the average of five membranes measured 5 min after formation.

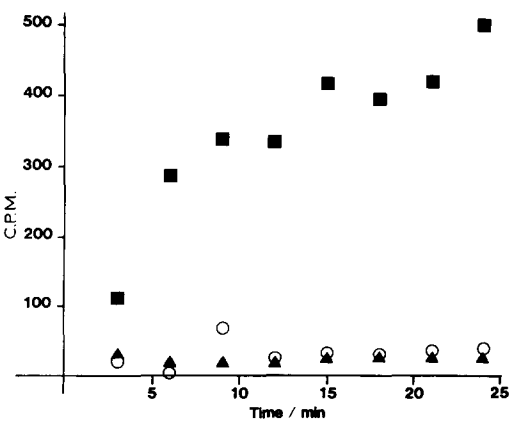

Fig. 6. Carrier-mediated influx of ^{86}Rb. Each vessel contained 2 ml of liposomes in 50 mM NaCl, 50 mM RbCl, 20 mM $CaCl_2$ and ^{86}Rb. In addition: (■) contained 5×10^{-5}M valinomycin, (▲) contained $2 \cdot 10^{-4}$M A21978C_3 and (○) was a control. 200 μl samples were taken every three minutes and passed through a Sephadex G-50 column. The activity of the eluted fraction was measured by scintillation counting and the background, due to ^{87}Rb, etc., subtracted.

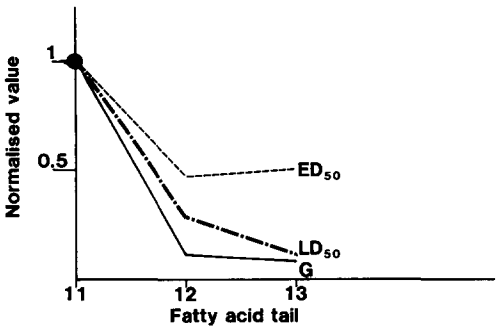

Fig. 7. A comparison of the effect of carbon chain length on the ED_{50} (mice; *Streptococcus pyogenes* infection), LD_{50} (mice), and concentration required to cause $10^{-2}S \cdot m^{-2}$ bilayer conductivity (G). All values normalised to A21978C_1 (11 carbon fatty acid tail). ED_{50} and LD_{50} values from Ref. 17.

to well stirred erythrocytes in a light scattering apparatus. The maximum concentration tested was 200 μg/ml A21978C_3 and after 1 h incubation at 25 °C no haemolysis had occurred.

Ion permeability studies with ^{86}Rb

In the presence of $5 \cdot 10^{-5}$M valinomycin, lecithin/cholesterol (1 : 1, w/w) liposomes showed an uptake of radioactive ^{86}Rb. When the valinomycin was replaced by $2 \cdot 10^{-4}$M (250 μg/ml) A21978C_3 the results shown in Fig. 6 are no different to that of the control. In a further experiment, positively charged liposomes containing 10% mole fraction of stearylamine were used. In this case neither valinomycin nor A21978C_3 treated liposomes showed a significant influx of ^{86}Rb.

Discussion

All four of the A21978C analogues increase the conductivity of planar lipid bilayers but this effect decreases as the length of the lipid tail is reduced. The lack of non-polar amino acids in the peptide headgroup and the complete loss of biological activity when the lipid moiety is removed helps to confirm the initial conclusion that the membrane interaction should decrease as the hydrophobic part of the molecule declines in size. The series C_1 to C_3 is not a truly homologous series as the C_2 has an *iso*-branched fatty acid whereas the other two contain *anteiso* groups. Were the series homologous, one might expect the effect of the 12 carbon fatty acid containing peptide C_2 to be intermediate between the other two. In the event, the effects of the C_2 and C_1 peptides are indistinguishable, the most probable explanation being that from steric considerations an *iso*-branched fatty acid would be expected to interact better with the membrane than its *anteiso* form. Further to this point it should be noted that LY146032 has an acyl group the same length as A21978C_1 and that the observed difference is solely due to the removal of the methyl branch. This change from branched to straight chain lipid has even more interesting in vivo effects: LY146032 having an LD_{50} identical to that of A21978C_1 but an ED_{50} similar to A21978C_3 [17].

Previous studies on the role of the fatty acid moiety of lipopeptides have concentrated on the way that varying lipid length affects the antimicrobial properties of polymixins. Chihara et al. [18,19] showed that with prepared colistin nonapeptide derivatives, the activity against Gram-negative bacteria increased with the length of the *n*-fatty acyl portion. In several cases, however, the corresponding *iso*-fatty acyl derivatives were considerably less effective than the straight chain version. Storm et al. [5], using unbranched derivatives of octapeptin, found an optimum chain length for the minimal inhibitory concentrations of 8 carbon atoms against *E. coli* and 12–14 carbon atoms against *B. subtilis*. Such an optimum, implying that some fatty acid chains can be too long, has not been reached in the LD_{50} [17] and conductivity measurements of the A21978C series (Fig. 7) but occurs at 13 carbon atoms for MIC measurements and 10 carbon atoms for ED_{50} in the case of the straight chain versions synthesised by Debono et al. [17]. Thus in spite of the complexity of the role of the fatty acid in lipopeptide action, the conductivity results do show that membrane interactions are a fundamental part of the action in vivo.

The clear difference between the results gained from glycerol monooleate-decane and lecithin-cholesterol-decane bilayers would appear to be a function of the differing headgroups involved as the membrane lipid carbon chains are very similar. Although A21978C is negatively charged in solution, this is unlikely to be a major factor as it

has been shown that SDS-solubilised (and therefore negatively charged) porins incorporate more freely into glycerol monooleate bilayers than into lecithin/cholesterol films [14]. Glycerol monooelate-decane bilayers have a much higher bifacial tension than those formed from lecithin/cholesterol (Lakey, J.H. and Lea, E.J.A., unpublished observations) and a role for this parameter in lipopeptide incorporation cannot be discounted without further study.

The peptide contains few non-polar amino acids and one of these, a tryptophan residue sits at the top of the lipid chain. The rest of this acidic peptide must carry a clear amount of negative charge although the exact state of ionisation of the groups is not known. As with CDA [14] the activity of the molecule against bacteria [1] and bilayers is increased in the presence of calcium ions. This raises the question as to whether the calcium is required to neutralise the charges on the molecule in order to aid its penetration of the membrane. The results presented in this paper show that the calcium dependence of the bilayer activity is rather non-specific with positively charged lipids allowing significant conductance changes to be induced in the absence of calcium. Magnesium can effectively replace calcium in bilayer conductivity measurements but not in MIC determinations where Eliopoulos et al. [1] found no increase in A21978C activity in media supplemented with Mg^{2+}, Zn^{2+}, Ba^{2+} or spermine^{4+}. The role of calcium in the bilayer experiments can be simply illustrated by the use of thin-layer chromatography. The R_f value (on silica gel plates) for A21978C_2 preincubated in 500 mM $CaCl_2$ is > 0.9 with distilled water as the solvent, 0.4 using 100 mM NaCl and < 0.05 in 100 mM NaCl + 50 mM $CaCl_2$ solvent. Hence, calcium decreased the aqueous solubility of the lipopeptide but the distilled water result suggests that calcium is not tightly bound.

The inhibition caused by phosphatidylinositol has a parallel with the effect of divalent cations on the activity of polymyxins and octapeptins which have positively charged headgroups. It is thought that the latter group of antibiotics compete with these divalent cations for sites on the outer membranes of bacteria [4]. Hence the obvious mutual repulsion between two sets of negative charges could also involve competition for the available calcium in the double layer. The effectiveness of simple electrostatic repulsion is, however, amply demonstrated by the complete blocking of valinomycin-mediated ^{86}Rb flux in liposomes containing stearylamine.

The toxicity involved with these and other lipopeptides is thought to be due to their interaction with mammalian cell membranes and haemolysis has been shown to occur in trials using crystalomycin [20]. The erythrocyte haemolysis and liposome experiments described here may help to elucidate this problem. A21978C_3 does not cause any observable damage to red blood cells at levels almost three times higher than its LD_{50}. Clearly, haemolysis is an extreme effect that might be expected to occur at much higher levels than the more insidious toxicity effects, but the absence of any observable effects may require another explanation. It may be that the large degree of negative charge present on the surface of the erythrocyte prevents these lipopeptides from penetrating the cell membrane, and the results of the liposome experiments lend some support to this view.

The mechanism by which these particular molecules increases the conductivity of lipid bilayers is not clear from the results presented here. Transmembrane channels have been extensively studied in this laboratory and it is clear that they are not a component of the A21978C-induced conductance. This is due because, firstly, no channels have been observed under extremely varied conditions of lipid, applied potential difference or salt solution and secondly because the zero-current experiments indicated that no ion selectivity was induced with the conductance. Given the absence of significant ion selectivity it seems unlikely that either a specifically structured membrane channel or an ion carrier is involved. However, a carrier which transports ions as a neutral complex and returns as a simple charged molecule is not subject to the predictions of the Goldman-Hodgkin-Katz equation in the same way as a carrier that forms a charged complex with the permeant ion (e.g., valinomycin). This is because it is the transmembrane concentration gradient of the charged species that causes the zero-current potential to arise when selectivity exists between the different components of the charged species. Hence if

A21978C transported K^+ as a $A21978C^-K^+$ complex and returned as $A21978C^-$ alone it would be the transmembrane concentration difference of the lipopeptide that would determine the size and sign of the potential. Attempts were made to carry out such an experiment but although up to 10^{-5}M of $A21978C_3$ was applied to one side of the film no potentials were observed. However, the low concentrations involved did not inspire confidence in the results.

The liposome influx experiments show that A21978C does not transport rubidium across the lipid bilayer. It is also unlikely that other cations are transported or that such a negatively charged molecule should carry anions. (Yet it is not impossible that in the presence of calcium ions, the acidic groups will be neutralised and that the positively charged ornithine residue will carry anions.)

The most adequate explanation of the conductivity mechanism of A21978C is that it transports protons as an uncharged complex. Hence in the membrane there will be an equilibrium in which the uncharged form will predominate:

$$AH \rightleftharpoons A^- + H^+ \tag{1}$$

Any potential difference will cause current to be carried by the charged form (A^-), with hydrogen ions as the means by which uncharged AH molecules may return in the direction of the cathode, thus maintaining a source of A^- on that side. Calcium ions would allow a greater partition of A21978C into the bilayer, whilst stearylamine would increase and phophatidylinositol decrease the amount of A^- compared with AH in the membrane. To produce a given level of conductivity [14], the amount of charge carrier in the bilayer may be expressed by Eqn. 2

$$C = \frac{l}{D} \cdot \frac{RT}{(zF)^2} \cdot G \tag{2}$$

where z is the number of charges on the carrier, R the gas constant, T the absolute temperature, D the diffusion coefficient, l the membrane width, C the concentration of charge carriers in membrane. For $G = 10^{-2}$ S · m^{-2} and assuming $l = 5$ nm, $D = 10^{-10}$m^2 · s^{-1} the value of C for an A^{4-} carrier would be $8.4 \cdot 10^{-9}$M; i.e. approx. 10^{-3} that of the aqueous solution (see Fig. 4). The ability of a peptide to enter the hydrophobic portion of a membrane is, limited by its charge density. This can be most easily shown by using the Born relation [21] in which $U(r)$ the energy required to move a charge from a region of dielectric constant 80 to a region of dielectric constant 2 is given by

$$U(r) = \frac{q^2}{8\pi r \varepsilon_0}\left(\tfrac{1}{2} - \tfrac{1}{80}\right) \tag{3}$$

(assuming that A^{4-} has radius r)

The concentration of the ion in the organic phase C_o is related to its aqueous concentration C_w by a partition coefficient which can be derived from the Boltzmann distribution

$$C_o = C_w \, e^{-U(r)/kT} \tag{4}$$

where k is Bolzmann's constant.

Using this relation and assuming A21978C to have a radius of 1.5 nm it indicates that an A^{4-} ion would have an organic/aqueous partition coefficient of $< 10^{-60}$. On the other hand if it carries only a single charge the concentration of charge carrier required by Eqn. 1 rises to $1.3 \cdot 10^{-7}$M and the ion concentration predicted by the Born relation (same radius) is $1.5 \cdot 10^{-6}$. Hence its possible role as a simple transmembrane carrier molecule is certainly physically unfavourable and the assumptions in this simple treatment may not be applicable. In conclusion, these results indicate that the strong calcium dependence and bacterial activity are not reproduced in a simple lipid bilayer. Both these effects appear to demand the presence of a component that is not present in the model system and the observed ionophore activity cannot account for the K^+ leakage in vivo. However, strong correlation was observed between the bilayer activity of the different versions and their ED_{50}, LD_{50} results. Hence, membrane interactions are a basic step in the action of this molecule, and seem especially relevant to the toxicity data. It would appear that the novel action of A21978C strongly depends upon membrane incorporation but that this is the first stage in reaching a more specific site.

Acknowledgements

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