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MINI-REVIEW

Gramicidin A–Phospholipid Model Systems

Bruce Cornell[1]

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Abstract

Gramicidin A forms ion-conducting channels which can traverse the hydrocarbon core of lipid bilayer membranes. The structures formed by gramicidin A are among the best characterized of all membrane-bound polypeptides or proteins. In this review a brief summary is given of the occurrence, conformation, and synthesis of gramicidin A, and of its use as a model for ion transport and the interaction of proteins and lipids in biological membranes.

Key Words: Gramacidin A; phospholipid bilayers; ion channels; lipid–protein interactions; ionophores; model membranes; protein conformation; protein folding patterns; facilitated transport; membrane protein.

Introduction

Gramicidin A is a channel-forming ionophore, capable of selectively facilitating the transport of ions across lipid bilayer membranes. It is currently the best studied example of such a molecule.

The published literature on gramicidin A may be grouped into three major research themes. The first of these is the conformation of gramicidin A when in the conducting state; the second is the kinetics of facilitated ion conduction across black lipid films; and the third, and the area principal interest to this review, is the interaction of gramicidin A with its supporting lipid membrane. In the present report each of these areas is discussed and incorporated into an overview of the interaction of gramicidin A with its supporting membrane.

[1]Commonwealth Scientific and Industrial Research Organization, P.O. Box 52, North Ryde, N.S.W., 2113, Australia.

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The results of these studies on gramicidin A provide some insights into, and raise many questions on, the interactions of lipids and proteins in biological membranes. This class of information is invaluable as a guide to studying more complex systems in which the protein component is less clearly defined than gramicidin A.

Gramicidin A

Occurrence

Gramicidin A is one of a large number of polypeptide antibiotics which are produced by the genus *Bacillus*. The role of the antibiotics within *Bacilli* is not known, although two possible functions include the inhibition of competing organisms or as part of a trigger mechanism for the germination of *B. brevis* itself (Katz and Demain, 1977).

The synthesis of these linear gramicidins within the *Bacilli* is unusual. It does not occur directly via ribosomes but, rather, through a mixture of enzymes and an essential family of thioesters which code for the amino acids and catalyze the formation of the peptide bonds.

Primary Structure

The linear gramicidins include a family of structurally related compounds denoted gramicidins A, B, C, and D. These polypeptides are predominantly composed of amino acids, although they include a mixture of L and D configurations which is not found in proteins (Sarges and Witkop, 1964). The internal hydrogen bonding allowed by this alternating sequence results in the bacterial polypeptides being considerably more stable than mammalian polypeptides in which the amino acids are exclusively L-isomers.

Although many of these bacterial antibiotics are cyclic, the gramicidins A, B, C, and D are linear polypeptides, each with a very similar sequence of 15 hydrophobic amino acids. These compounds differ in the amino acid group at position 11 (tryptophan, gramicidin A; phenylalanine, gramicidin B; or tyrosine, gramicidin C). Other variations include a replacement of the valine at position 1 in gramicidin A with isoleucine, yielding isoleucine gramicidin A.

The compound description gramicidin D needs to be interpreted with caution. It is most commonly used as a generic term for the linear gramicidins, as first isolated by Dubois (*Merck Index*, 1983). It has also been used for the isoleucine analogue of gramicidin A (Ramachandran, 1963) and yet again to describe a minor ($< 0.3\%$) component of the linear gramicidins found in *B. brevis*. This latter compound is similar to gramicidins

$$\text{CHO–L-Val}_1\text{–Gly}_2\text{–L-Ala}_3\text{–D-Leu}_4\text{–L-Ala}_5\text{–D-Val}_6\text{–L-Val}_7\text{–D-Val}_8\text{–}$$
$$\text{L-Trp}_9\text{–D-Leu}_{10}\text{–L-Trp}_{11}\text{–D-Leu}_{12}\text{–L-Trp}_{13}\text{–D-Leu}_{14}\text{–L-Trp}_{15}\text{–NHCH}_2\text{CH}_2\text{OH}$$

Fig. 1. The primary sequence of gramicidin A. The amino acid isomers alternate, permitting extensive intramolecular hydrogen bonding in the secondary folding. The N terminus is blocked by a formyl group and the C terminus by an ethanolamine group.

A, B, and C but possesses an altered amino acid sequence (Sarges and Wiktop, 1965d).

The primary structure of gramicidin A is shown in Fig. 1.

A Dubois extract of the linear gramicidins from *B. brevis* contains a mixture of gramicidins A, B, C, and D in which the major fraction (> 85%) is gramicidin A. The mixture is sometimes denoted gramicidin A′ (Urry, 1972).

Synthesis and Characterization

Bauer *et al.* (1972) have synthesized a polypeptide with the amino acid sequence of gramicidin A using an enzyme mixture extracted from *B. brevis*. The object of the study was primarily to demonstrate the biosynthetic pathway for gramicidin A, and many reports are now available of procedures for its chemical synthesis.

Sarges and Wiktop (1965b, c) sequenced gramicidin B and C, and using conventional methods synthesized gramicidin A and isoleucine gramicidin A (Sarges and Witkop, 1965a). Noda and Gross (1972) used solid-phase techniques (Merrifield, 1963) to synthesize gramicidins B and C. Yonezawa *et al.* (1976) used fragment condensation to improve the yield of gramicidin A obtained from the solid-phase technique.

Modifications to the linear gramicidins have included a covalently linked malonyl gramicidin dimer (Urry *et al.*, 1980a, b) and F^{19} and C^{13} labelled analogues (Weinstein *et al.*, 1979, 1980). Charged modifications to the N and C termini have been prepared to aid in identifying the dimer structures formed by gramicidin A in its ion-conducting state (Bamberg and Janko, 1977). Wholly synthetic preparations of gramicidin A were first reported by Prasad *et al.* (1982) and Urry *et al.* (1982). An [L-Ala^7] analogue of gramicidin A has been reported recently by Prasad *et al.* (1986).

The chromatographic purification and isolation of gramicidin A has been found by Veatch *et al.* (1974) to be complicated by an interconversion of the molecule between four conformational species. Each is revealed as a single spot by TLC. The population of each species depends on the polarity and period of storage within a particular solvent system. Some aspects of the kinetics and aggregation properties of this phenomenon were reported by Veatch and Blout (1974).

Nuclear magnetic resonance (NMR) is frequently used to measure the purity of gramicidin A. Many studies have been reported employing different solvent systems and one- on two-dimensional high-resolution C^{13} and H^1 NMR spectroscopy (see, for example, Glickson *et al.*, 1972; Fossel *et al.*, 1974; Heitz *et al.*, 1979; Urry *et al.*, 1982; Arseniev *et al.*, 1984, 1985a, b, 1986).

Circular dichroism and infrared spectroscopy have frequently been used to "fingerprint" the conformations of gramicidin A in both solutions and lipid bilayers (see, for example, Veatch and Blout, 1974; Wallace, 1983).

Assays based on a colorimetric determination of the tryptophan content have been devised to measure the concentration of the polypeptide in dispersions with lipid (Urbaneja *et al.*, 1985).

Conformation of Gramicidin A

Diffraction Studies of Gramicidin A Crystals

A commonplace approach to determining the conformation of a molecule in solution, or as is relevant in this case, in a membrane, is to develop a model from the structure revealed by X-ray or neutron diffraction patterns obtained from single crystals. Many reports have been published of diffraction studies on crystals of gramicidin A (Cowan and Hodgkin, 1953; Koeppe *et al.*, 1978, 1979; Koeppe and Schoenborn, 1984; Wallace, 1983, 1984; Wallace and Hendrickson, 1984; Wallace, 1986; Hedman *et al.*, 1985). A summary of the developments in this field has been reported by Wallace (1986).

It was not until recently, however, (Wallace and Hendrikson, 1984) that the phase problem was solved and an unequivocal structure obtained for crystalline gramicidin A. The problem in solving the structure of gramicidin A is suggested by Wallace to have been its intermediate size, falling as it does between the small molecules for which direct phase determination is possible, and the large macromolecules for which multiple isomorphous replacement may be used to determine phase. In the study by Wallace (1984), phasing was based on anomalous scattering from cesium which was cocrystallized with the gramicidin A. Isotopic replacement of deuterons for hydrogen has been used to seek a solution to the structure of gramicidin A using neutron diffraction. A neutron diffraction study to 0.5 nm of an ion-free crystal of gramicidin A, reported by Koeppe and Schoenborn (1984), yielded a helical dimer, but failed to resolve the structure as either a single or double helix.

The structure determined by Wallace (1986) for crystals of gramicidin A prepared from CsCl solutions was in fact a left-handed double helix, formed

from two antiparallel-β strands. By comparing the CD spectrum obtained from gramicidin A in its conducting state with that from solutions in which the gramicidin A was known to be in a double helix, it is clear that the conformations are very different. This establishes the important principle that the structure of a protein as determined by X-ray diffraction from single crystals need not bear any relationship to its structure in solution or in a membrane.

Gramicidin A in Solutions of Organic Solvent

Gramicidin A is virtually insoluble in water. Convenient solvents for gramicidin A include dimethyl sulfoxide, methanol, ethanol, ethyl acetate, acetone, chloroform, benzene, and dioxane. The aggregation kinetics and folding patterns of gramicidin A in a number of the above solvents were first discussed by Veatch *et al.* (1974) and Veatch and Blout (1974). These authors identified four species which included a variety of helical folding patterns with both parallel and antiparallel-β hydrogen-bonded structures and clockwise and anticlockwise handedness. They also concluded that all of these structures were dimers. Similar observations were reported by Urry *et al.* (1975), and the dimeric nature of the conducting channel confirmed by Veatch and Stryer (1977).

Several authors have reported NMR studies of gramicidin A in different solvents. These include H^1 NMR (Glickson *et al.*, 1972; Urry, 1972; Heitz *et al.*, 1979; Lorenzi *et al.*, 1984), C^{13} NMR (Fossel *et al.*, 1974), and N^{15} NMR (Hawkes *et al.*, 1984). The first direct observation of the conformation of gramicidin A in a solvent system was obtained using two-dimensional H^1 NMR by Arseniev *et al.* (1984). In this case gramicidin A in dioxane solution was shown to be an antiparallel double helix with 5.6 residues per turn.

In a series of publications this same group has now identified the conformation of gramicidin A in a variety of solvents, and the effects on the conformation of cesium ions (Arseniev *et al.*, 1985a, b). A summary of this and other works employing the NMR techniques is described by Arseniev *et al.* (1986). The structures that have been identified include a left-handed antiparallel double helix and a right-handed parallel double helix, both found in dioxane. A 7.2 residue per turn antiparallel helix was found for the Cs^+ complex of gramicidin A in CD_3OH–$CDCl_3$.

Wallace *et al.* (1981) and Wallace (1986) have used CD spectra to identify different folding geometries for gramicidin A in solvents, micelles, and liposomes. Although these studies have served as a reference to establish the folding patterns in the different environments, they do not provide direct evidence of the conformation.

Other techniques that have been employed to study the conformation of gramicidin A include Raman and infrared spectroscopy. In a series of reports, Naik and Krimm (1984, 1986a, b) have measured the vibrational spectra of gramicidin A in dioxane. These data have been related to specific folding patterns through theoretical estimates of the normal modes supported by each geometry. The calculations depend on force field profiles that have been estimated from model compounds whose structure is known. Although in general agreement with the NMR data, the conclusions that may be drawn from this approach are less direct.

Gramicidin A Codispersed with Surfactant Micelles

Two micellar systems have been used to study dispersions of gramicidin A in water. The purpose of using these dispersions is to probe the structure of gramicidin A in an environment that is similar to that in a biological membrane and yet is accessible to those techniques which require the sample be presented as an isotropic solution. This is particularly important in high-resolution NMR spectroscopy.

Key publications in this area are those by Urry *et al.* (1982, 1983c) and Arseniev *et al.* (1985a, b, 1986). Urry and his co-workers have used micelles of lysolecithin whereas Arseniev *et al.* (1986) employed micelles of sodium dodecylsulfate (SDS) to support molecules of gramicidin A in aqueous solution. Ion binding and CD studies have been reported by both groups in support of their claims that the micelle-packaged state of gramicidin A is equivalent to the ion-conducting conformation in liposomes.

Urry *et al.* (1983b) have observed the carbon-13 NMR spectrum of specifically labelled L-residue-peptide carbonyl groups along the backbone of gramicidin A in lysolecithin micelles. The chemical shift of some of these resonances is altered by the presence of thallium ions. By relating the frequency shift caused by the thallium ions to the structure of a range of models for thallium-gramicidin A, all structures apart from the left-handed $\beta^{6.3}$ end-to-end helix were excluded.

Arseniev *et al.* (1985a, b, 1986) have obtained high-resolution two-dimensional H^1 NMR maps of gramicidin A dispersed in deuterated SDS micelles. From these maps they derived NOE connectivities between certain of the $N_iH \cdots C_jH$ protons. That these groups cross-relax indicates they are in close spatial proximity which severely constrains the model for the structure formed by gramicidin A. The authors propose a right-handed $\beta^{6.3}$ helix, of opposite handedness to that suggested by Urry (1971) and Urry *et al.* (1983b).

It is possible that the different micelles support different folding patterns for the gramicidin A, although no report is available of a two-dimensional H^1

NMR study of the lysolecithin micellar system which may be used for direct comparison.

Gramicidin A in Lipid Bilayers

None of the above approaches has permitted an estimate of the conformation of gramicidin A while it is in a lipid bilayer. Sychev *et al.* (1980) and Sychev and Ivanov (1982) have reported CD and infrared spectra of the conformation of gramicidin A in dipalmitoylphosphatidylcholine vesicles. They conclude a β_{DL}-hairpin conformation of gramicidin A. However, Naik and Krimm (1986a, b) point out that exchange between the amide protons and deuterons from the D_2O solvent would frequency-shift these resonances and confuse the interpretation. In lipid suspensions Naik and Krimm conclude that a $\beta^{6.3}$ helix is the more likely structure. Difficulties arising from amide exchange are thought to have occurred in other studies employing the infrared technique (Urry *et al.*, 1983a).

Fluorescence energy transfer and quenching studies have shown that the tryptophan residues of gramicidin A reside near the surface of lipid bilayers (Haigh *et al.*, 1979). This is supported by NMR studies by Feigenson *et al.* (1977) and Weinstein *et al.* (1979, 1980, 1985) who measured the accessibility to ions of the COOH and NH_2 terminal groups of gramicidin A. By observing the perturbation to their resonance frequencies it was possible to eliminate the double helix, in favor of the NH_2-terminus to NH_2-terminus helical dimer as the dominant conformation in lipid bilayers.

Wallace *et al.* (1981) and Wallace (1984, 1986) have made extensive use of CD to observe the effect of lipid concentration, ion concentration, and lipid structure on the conformation of gramicidin A. Within the concentration range appropriate to the lamellar phase, none of the variables appeared to significantly alter the folding pattern of the gramicidin A.

Solid-state NMR has been used by Pauls *et al.* (1985) and Datema *et al.* (1986) to observe the H^2 resonance of exchangeable groups on the gramicidin A formed when it is incubated in excess CH_3OD at 40°C. This modified analogue of gramicidin A was dispersed in hydrated dipalmitoylphosphatidylcholine liposomes. Although these authors were unable to assign the many resolved splittings to the different exchangeable sites in gramicidin A, they were able to demonstrate the onset of an axial rotation by the peptide in the bilayer, when the lipid passed into the fluid phase.

A C^{13} solid-state NMR study by the present author and co-workers (Smith and Cornell, 1986; Cornell *et al.*, 1987a, b) has used the carbon-13 shielding tensor orientation of specifically labelled sites within the backbone of the molecule to determine the orientation of the carbonyl C=O bonds at the gly^2, ala^3, and val^7 positions. From these directions in aligned multilayers

of dimyristoylphosphatidylcholine, it is possible to eliminate all but the $\beta^{6.3}$ models for gramicidin A. At this stage, however, it is not possible to decide between the left- and right-hand helices. Similar information has been gleaned from doubly labelling gly^2 at the C=O and alpha carbon sites and observing the C^{13}–C^{13} dipolar splitting. Cross (1986) and Moll *et al.* (1987) have demonstrated the potential of N^{15} solid-state NMR for obtaining an even more constrained view of the polypeptide structure.

A particularly interesting result of the C^{13} NMR study is the independence of the folding pattern of gramicidin A on the transition from the crystalline to fluid state of the supporting lipid membrane.

Facilitated Transport of Ions Through the Gramicidin A Channel

Conductance Studies

When added to black lipid membranes (BLM's), gramicidin A forms dimeric ion-conducting channels which at low polypeptide to lipid mole ratios of approximately 1 : 10 000 are in equilibrium with a nonconducting monomeric form of the polypeptide (Hladky and Haydon, 1972; Bamberg and Lauger, 1973; Veatch *et al.*, 1975; Apell *et al.*, 1977). The current model for the ion-conducting state is a head-to-head dimer of two $\beta^{6.3}$ helices (Urry, 1971). The monomeric form is thought to be insufficiently long to transverse the membrane. The conduction mechanism is essentially that of a polar channel linking either side of the membrane (Hladky and Haydon, 1972).

The lifetime of the conducting dimers in BLM's is of order 10 sec and has been shown to depend upon the thickness of the lipid and the interfacial tension between the aqueous and lipid phase (Pope *et al.*, 1982; Elliott *et al.*, 1983; Ring, 1986; Buchet *et al.*, 1985). The fraction of time for which the gramicidin A is in the dimeric form also depends upon the concentrations of the polypeptide in the lipid, although at high concentrations, over the range of polypeptide-to-lipid ratios of 1 : 15 and 1 : 363, Wallace (1984, 1986) has shown that the dimerized state dominates. Direct measurement of the dimerization constant is only possible in BLM's at relatively low concentrations of gramicidin A.

Once a dimeric channel is formed, its conductance is virtually independent of the properties of the surrounding lipid and depends primarily on the structure of gramicidin A itself. The single-channel conductance is of order 15 pS with an optimal unidirectional sodium flux through a channel at zero applied potential of 2×10^6 ions sec^{-1}. The flux of water molecules through the channel is higher at approximately 6×10^7 molecules sec^{-1} under the same conditions (Finkelstein and Andersen, 1981). The resistivity of sodium transport in a gramicidin A channel is 6000 ohm-cm which is

3×10^9 times that of copper, and approximately 10^{-13} times that of an insulator such as Teflon (*Reference Data for Radio Engineers*, 1982).

The channels are selective in their conductance, and, relative to the sodium permeability, the monovalent cations may be ranked H^+ (150) > NH_4^+ (8.9) > Cs^+ (5.8) > Rb^+ (5.5) > K^+ (3.9) > Na^+ (1.0) > Li^+ (0.33) (Myers and Haydon, 1972).

Multivalent ions such as Tl^{3+} or Ca^{2+} block the transport of monovalents due to their very much greater binding to the gramicidin channel (Urry, 1984).

An observation which at first sight appears inconsistent with the channel model is the dependence of the channel conduction on the amino acid sequence (Bamberg *et al.*, 1977; Morrow *et al.*, 1979; Heitz and Gavach, 1983; Mazet *et al.*, 1984). As the amino acid residues are directed away from the pore of the gramicidin A, they are not in direct contact with the permeating ions and thus would not be expected to influence conduction. However, measurements on modified gramicidin A conclusively demonstrate a strong dependence of conduction on the side chain sequence and species.

An explanation for this effect has been sought through modification of the end group amino acids (Barrett Russell *et al.*, 1986; Durkin *et al.*, 1986), with the logic that modification of the binding potential of ions at the entrance to the channel is the principal cause of the altered conductance. One of the few exceptions to this approach has been published by Urry *et al.* (1984a) who synthesized an analogue of gramicidin A in which Val^7 and Val^8 were omitted, producing a channel that was 0.3 nm shorter.

The shorter channel possessed a reduced conductance relative to native gramicidin A. These authors accounted for the result in terms of an increased electrostatic repulsion for a second ion entering an already occupied channel. This interpretation further led them to suggest that the ion-conducting state was one in which the channel was occupied by two ions. Jordan and Vayl (1985) have questioned this interpretation, and argue that a conducting state involving a singly occupied channel could equally well explain the effect. The key point of their argument is that entry to the channel is the rate-limiting step for conductance. The shorter gramicidin A analogue thus requires a deeper penetration of an ion into the bilayer before it may enter the channel and thus it must overcome a greater potential barrier. Although Jordan and Vayl propose experiments that would distinguish between the two models, these have yet to be reported.

In a more recent study, Prasad *et al.* (1986) have shown that replacing L-Val^7 with L-Ala^7 causes a substantial increase in conductance of the channel. This result demonstrates more directly the dependence of the channel conductance on the amino acid sequence and implies an interplay of the packing of the amino acid residues and the gramicidin conformation.

Once a dimerized channel is formed, its conductance is not necessarily constant. This has been claimed even for the chemically pure gramicidin A (Prasad *et al.*, 1982). Possible mechanisms for this dispersity in conductance include different channel configurations or different end-to-end associations of the amino acid side chains. Urry *et al.* (1984b) have synthesized a gramicidin A analogue in which L-Ala^5 is replaced by L-Leu^5 whose bulkier side chain, they predicted, would limit the range of configurations adopted by the neighboring Trp^9. The conductance dispersity did indeed narrow to a significant extent, supporting the view that side-chain conformation is a source of the variation in conductance. However, Busath *et al.* (1987) and Durkin *et al.* (1987) have recently presented data in which the conductance dispersity of native L-Ala^5 gramicidin A was comparable to that of the L-Leu^5 analogue reported by Urry *et al.* This caution is required before incorporating this result into a general model for the conductance properties of gramicidin A.

Ion Binding and Exchange Rates

NMR has proven to be a valuable tool in studying ion binding and ion exchange in lipid dispersions containing gramicidin A. These data have been complementary to the direct conductance measurements and yield similar values for the binding constants as those derived from fitting the transport data (Urry, 1984). All of the alkali metal cations have now been studied using NMR techniques ($^+Li^7$, $I = 3/2$, Urry *et al.*, 1983d; $^+Na^{23}$, $I = 3/2$, Urry *et al.*, 1980a, b; $^+K^{39}$, $I = 3/2$, Urry *et al.*, 1985b, 1986; $^+Rb^{87}$, $I = 3/2$, Urry *et al.*, 1986; $^+Cs^{133}$, $I = 7/2$, Urry *et al.*, 1985a). The general approach is to follow the change in relaxation rates of the NMR signal derived from these ions as a function of the concentration of gramicidin A in a particular lipid–water–ion dispersion. The analysis assumes a rapid exchange of ions between any bound species and ions freely solvated in water. The substantial quadrupolar moments possessed by all of the monovalent alkali cations ensures that should the electric field gradient become nonzero upon their binding to gramicidin A, the relaxation rates will become shorter. Additional effects arising from the large chemical shift range expressed by these nuclei will also influence the relaxation times. The only nuclear spin $I = 1/2$ ion studied to date in this context is the thallous ion, $^+Tl^{205}$. Although lacking a quadrupolar moment, the large chemical shift of Tl^{205} perturbs the relaxation rate sufficiently to permit a measure of its binding constant to gramicidin A (Turner *et al.*, 1982; Hinton *et al.*, 1982, 1985; Koeppe *et al.*, 1987).

The results of such studies are usually expressed as plots of excess relaxation rate versus ion concentration (James and Noggle, 1969). Breaks in the slope of these plots are interpreted in terms of populations of bound ions associated with gramicidin A. Typically two bound states are found.

C^{13} NMR suggests that the binding sites are at the mouth of the channel (Urry *et al.*, 1985b). The so-called tight binding state is when the channel is singly occupied, and the weak binding state when the channel is doubly occupied. The binding constants for the monovalent alkali ions are in the range 14 to 55 mol^{-1} (strong binding state) and 0.5 to 4 mol^{-1} (weak binding state) for the monovalent cations. Depending on concentration, the thallous ion has also been shown to have strong and weak binding states with binding constants of 10^3 and 70 mol^{-1} (Hinton *et al.*, 1985).

Dielectric relaxation measurements have been used to observe the translocation of thallous ions in dispersions of malonyl gramicidin A and lysolecithin (Henze *et al.*, 1982). Malonyl gramicidin A is cross-linked at the formyl terminal to produce a trans-membrane monomer. When an ion jumps from one singly occupied site to the neighboring site within the same channel, a change in dipole moment occurs equivalent to 100 debye (Urry, 1984). The jump rate is reported to be 4×10^6 sec^{-1}, which is five orders of magnitude slower than expected for free diffusion in solution over a similar distance. This arises from the rate-limiting step for conduction being the binding site at the channel mouth, and not the passage of the ion through the channel.

Models of Ion Conductance

Thermodynamic Model. The simplest model of ion conduction in gramicidin A consistent with the experimental data is a channel-forming dimer which spans the membrane lipid and possesses a binding site for ions at its mouth on both sides of the membrane. Three barriers to conduction have been proposed to exist. Two of these are in the aqueous phase and need to be overcome for ions to enter the binding sites. A third and weaker barrier has been proposed at the center of the channel opposing the flip of an ion from the binding site on one side of a membrane to the binding site at the opposite end of the channel on the other side of the membrane (Urban *et al.*, 1978).

The binding sites are located adjacent to the Trp^9–Trp^{11} carbonyl groups near the mouth of each channel (Urry *et al.*, 1985b). In the absence of a potential across the membrane, five rate constants have been used to describe the transitions connecting the four possible states in which the binding sites are occupied by zero, one, or two ions (Urry, 1984). The effect of applying a potential across the membrane has been calculated to first order by Urry (1984) using Eyring rate theory (Zwolinski *et al.*, 1949) and the binding constants obtained by Na^{23} NMR. This model is shown to be consistent with the experimentally observed sodium fluxes over a potential range of 50–200 mV.

Electrostatic Model. A more mechanistic approach to describing the conduction of ions through gramicidin A has been to consider the Coulombic interaction of the transported ion with the carbonyl groups of the gramicidin A channel (Parsegian, 1975; Levitt, 1978; Lauger, 1982; Fischer *et al.*, 1981; Monoi, 1983; Brickmann and Fischer, 1983; Jordan, 1984). In its most sophisticated form the electrostatic model includes contributions to the potential energy from five sources (Jordan, 1984). These are (a) the difference in the ion-solvating ability of the channel pore and bulk water; (b) image charges induced by the ion; (c) interactions of an ion with the dipole moment of the membrane; (d) interactions with the charges down the channel; (e) interactions with the diffuse double layer at the membrane–water interface.

Assuming an averaged charge distribution, it is found that the image potential of an ion within the nonpolar interior of the membrane is equal and opposite to the potential of the surface dipoles lining the channel. This similarity indicates that the cationic property of gramicidin A is primarily due to the local geometry of the hydrogen-bonded amide and carbonyl groups down the center of the channel. The local variations in energy caused by the individual amide and carbonyl groups have been modelled as minor variations on a background of the substantially large barrier and binding energies. The barriers at the entrance to the channel are associated with the need to partially dehydrate the ion when it enters the gramicidin A.

The energy trajectory of ion conduction has been described in terms of the energy profile across the dimer channel in a membrane (Pullman and Etchebest, 1983). Conduction is modelled as the passage of a single file of water molecules and ions. The proposed mechanism for the transport of an ion is a reduction in the potential depth of the binding site at the entrance to the channel due to the mutual electrostatic repulsion contingent upon binding a second ion. With both sites occcupied, a thermally driven fluctuation in the position of one ion down the channel results in the opposing ion retreating, drawing the first ion further down the channel. According to the model, a positive feedback exists for this process favoring the elimination of the opposing ion from the channel and the transfer of the original ion across the bilayer. With only a single site occupied, the ions remain bound in the deeper potential well at the entrance of the channel.

Molecular Dynamics Simulations. Molecular dynamics simulations of the transport properties of gramicidin A have been extensively discussed (Fischer *et al.*, 1981; Mackay *et al.*, 1984; Kim *et al.*, 1985). In a recent series of reports by Clementi and co-workers (Fornili *et al.*, 1984; Kim and Clementi, 1985; Kim *et al.*, 1985), the dynamics of the conduction of K^+ and Na^+ ions across a solvated gramicidin A transmembrane channel has been simulated by molecular dynamics techniques. In addition to the inclusion of

bulk water at the mouth and along the channel, these studies have provided a detailed insight into the short-term order and dynamics of the water and ions.

Despite the sophistication of these studies, the simulations have yet to include the motion of the carbonyl groups lining the channel. This refinement is forcast as the next step in developing the approach. These studies have questioned the earlier picture of a single-file transport of water and ions down the channel and of the validity of the energy profiles modelled using Eyring rate theory. In particular, the central energy barrier, which was an important feature of the thermodynamic models of ion conduction, is not evident in these simulations. The principal barrier to conduction is the depth of the binding sites at the mouth of the channel.

Phospholipid–Gramicidin A Dispersions as a Model for Lipid–Protein Interactions

The Effect of Gramicidin A on Lipid Order

The use of gramicidin A′ as a model for intrinsic membrane proteins was first reported by Chapman *et al.* (1977). In this study many techniques were employed to observe the effect of gramicidin A′ on the structure of phospholipid dispersions over a concentration range from 1 : 200 to 1 : 1 mole ratio of gramicidin A′ in lipid. The techniques included differential scanning calorimetry, X-ray diffraction, Raman and electron spin resonance spectroscopy, and optical and electron microscopy. The principal effect of gramicidin A′ at concentrations below 1 : 5 was a disruption of the *static* crystalline order of the hydrocarbon chain packing of the low-temperature lamellar phase of the lipid. No effect was seen on the *dynamic* order of the high-temperature lamellar phase.

Static order is the degree of spatial regularity that exists in a material. Dynamic order relates to the range of angles over which a restricted, although extremely rapid, motion occurs.

The use of the natural extract gramicidin A′ in the early studies rather than gramicidin A does not appear to be significant and the superscript has been omitted.

At concentrations above 1 : 5, the lamellar repeat distance of both the high- and low-temperature phases becomes disordered, with two phases being apparent by ESR and X-ray diffraction. It was unclear whether this disorder was dynamic or static.

Using H^2 NMR of selectively deuterated lipids, Rice and Oldfield (1979) observed an increase in the *dynamic* order of the lipid chains of dimyristoylphosphatidylcholine at molar ratios of up to 1 : 15 gramicidin A to lipid. At

higher concentrations this trend was reversed and the order decreased. Above 1:4 the lipid dynamic order underwent an even more dramatic reduction, suggesting a change in the phase structure of the dispersion. A similar pattern of dynamic ordering at low concentrations followed by disordering at high concentrations was evident in the chemical shift anisotropy of the lipid carbonyl groups studied by C^{13} NMR (Cornell and Keniry, 1983). Related effects have been observed using infrared spectroscopy (Cortijo and Chapman, 1981; Cortijo *et al.*, 1982), electron spin resonance of phospholipid spin labels (Tanaka and Freed, 1985), and by P^{31} NMR of the lipid phosphate groups (Rajan *et al.*, 1981). Unlike the effect of the lipid chains, gramicidin A induces only disorder in the polar groups.

The dependence of the lipid dynamic order on the concentration of gramicidin A has been ascribed to at least three different effects. The first is that the rough surface of gramicidin A disorders the boundary lipids which then present a smooth rigid surface and order in the remaining lipid (Rice and Oldfield, 1979). Pink *et al.* (1981) argue that it is unlikely that a disordered boundary layer of lipid should increase the order of the remainder of the lipid dispersion.

An alternative view is that gramicidin A produces highly curved surfaces in the lipid dispersion. In the presence of rapid translational diffusion over these surfaces the NMR splittings will be averaged, thus giving the appearance of disorder (Cortijo and Chapman, 1981; Pink *et al.*, 1981; Cortijo *et al.*, 1982; Killian *et al.*, 1985a). This interpretation has recently been confounded by observing the same gramicidin A-induced disorder in aligned lipid multilayers in which the sample morphology denies such a geometry (Cornell, 1986; Cornell *et al.*, 1987c). Further evidence against this curvature model is the similarity of the effects seen by C^{13} NMR (Cornell and Keniry, 1983) and H^2 NMR (Rice and Oldfield, 1979). This is despite a more than tenfold difference between the size of the carbonyl group chemical shift anisotropy and the methylene quadrupolar splittings. Likewise the time scale defined by the infrared spectroscopy results reported by Lee *et al.* (1984) is shorter than any possible diffusive process. These data are all consistent with the induced disorder being an intrinsic property of the resultant bilayer and not of changes in bilayer curvature.

A further model to account for these effects has been proposed by Pink *et al.* (1981) who suggested that if lipid is adjacent to one gramicidin A molecule, it is ordered, and if sandwiched between two or more gramicidins, it is disordered. By selecting arbitrary values for the quadrupolar splittings of the various sites, these authors generated a reasonable fit to the NMR data. It was assumed that the lipid exchanged rapidly between all such sites on the time scale of a few tens of microseconds. The principal limitation of the model is in its phenomenological approach. No attempt is

made to justify the underlying physical chemistry of the gramicidin A–lipid interaction.

The Effect of Gramidicin A on Lipid Phase

A review of work in this area has recently been published by Killian and de Kruijff (1986).

Gramicidin A Promotes the H_{II} Phase. The ability of gramicidin A to promote the formation of the hexagonal H_{II} phase in aqueous dispersions of dielaidoyl- and dioleoylphosphatidylethanolamine and dioleoylphosphatidylcholine was first reported by van Echteld *et al.* (1981). At elevated temperatures, ethanolamine dispersions form the hexagonal phase without gramicidin A, although addition of gramicidin lowered the temperature at which this transformation occurs. The phosphatidylcholines only form the hexagonal phase in the presence of gramicidin A. The transition to the H_{II} phase is broad and occurs over a range of gramicidin A concentrations, typically from 1 : 25 to 1 : 5. The presence of the hexagonal phase seems to depend on both the nature of the lipid headgroup and on the length and saturation of the hydrocarbon chain (van Echteld *et al.*, 1982). The greater the bulk of the chain, or the smaller the bulk of the headgroup, the more disposed is the dispersion to forming an hexagonal phase.

Killian and co-workers have explored many aspects of this phenomenon. Killian and de Kruijff (1985a), using differential scanning calorimetry and P^{31} and C^{13} NMR, conclude that aggregation of the gramicidin A plays a crucial role in the formation of the H_{II} phase. They have also shown that the state of hydration of the gramicidin A–lipid dispersion is an important factor in triggering the phase transformation (Killian and de Kruijff, 1985b). The effect of gramicidin A on dispersions of negatively charged lipids was similar to its effect on the neutral phosphatidylcholines (Killian *et al.*, 1986b).

The importance of the tryptophan groups in the ability of gramicidin A to induce the H_{II} phase was investigated by N-formylating the tryptophans (Killian *et al.*, 1985b). This totally blocked the formation of the H_{II} phase in dioleoylphosphatidylcholine. To explore whether this result was peculiar to the tryptophans, the same authors (Killian *et al.*, 1986b) prepared desformyl-gramicidin A, *N*-succinyl-gramicidin, and *O*-succinyl-gramicidin, and tested the influence of these charged groups on the H_{II} phase-forming ability of the polypeptides. It was found that neither modification produced a significant change.

A recent study by the same group has reported that gramicidins A and B are very similar in producing the H_{II} phase, although gramicidin C was found to be less effective (Killian *et al.*, 1987). These results have been

interpreted as arising from the variations in the self-aggregation properties of gramicidin A and its analogues.

Gramicidin A–Lysolecithin Lamellar Phase. A further aspect of the phase-modifying ability of gramicidin A was reported independently by two groups (Killian *et al.*, 1983; Spisni *et al.*, 1983). These authors observed that lysolecithin, which normally forms micellar solutions in water, can form lamellar structures in the presence of gramicidin A. The two groups differ in their estimates of the stoichiometry, Killian *et al.* (1983) obtaining a ratio of one gramicidin A per four lysolecithins, and Spisni *et al.* (1983) one gramicidin A per 8–10 lysolecithins. Spisni *et al.* observed an additional nonstoichiometric phase. These observations have been seen as further evidence of the ability of gramicidin A to self-aggregate and produce a variety of gramicidin-rich phases (Killian *et al.*, 1986a). Aslanian *et al.* (1986) have reported a Raman scattering study of gramicidin A–lysolecithin micelles in which little, if any, interaction was observed between the tryptophan groups on the gramicidins. They question the concept of tryptophan-stacking as a basis for the effect of gramicidin A on the properties of lipid bilayer phase structure. However, in cocrystals of gramicidin A and lipid some protein–protein interactions are observed (Short *et al.*, 1987).

A Model for the Gramicidin A–Lipid Interaction

Many explanations for the interaction of gramicidin A with lipid dispersions have been discussed in the preceding text, although none have attempted to address more than the specific results of the studies they accompany. The following is an outline of a crude general model of the interaction of gramicidin A and lipid bilayers (Cornell, 1986; Cornell *et al.*, 1987c).

The basis of the model is an assumed balance of forces within the process of amphiphile self-assembly, specifically, the forces on the polar groups and the forces on the chains. When the forces on the polar groups dominate, the curvature of the resulting phase will be positive or zero, i.e., micelles, hexagonal I, or lamellar phase. When the forces on the chains dominate, the curvature will be negative, i.e., reverse micelles or hexagonal II phase. How this balance changes with the addition of gramicidin A is developed from a number of observations.

First, the dimensions and location of gramicidin A within a bilayer are now sufficiently characterized to propose that its inclusion in a membrane occurs substantially within the hydrocarbon interior (Elliott *et al.*, 1983; Huang, 1986) and results in a greater area and thus freedom for the lipid polar groups (Rajan *et al.*, 1981; Killian *et al.*, 1986a; Phonphok *et al.*, 1984).

An approximate description of this greater area, A_h, is given by $A_h = A^*(1 + aR)$, where A^* is the area per lipid molecule without gramicidin A, a the ratio of the area occupied per gramicidin A to that of the lipid, and R the mole ratio of gramicidin A in the dispersion. In the present simplified model the only constraints arising from the molecular geometry are the interfacial area and the volumes of the polar and nonpolar groups.

Another important observation is that surface pressure measurements of monolayers of gramicidin A and lipid indicate that the surface tension K_2, of dispersions containing substantial quantities of gramicidin A is 2.5–3 times lower than that of similar dispersions of pure lipid. The fall in surface pressure with the addition of gramicidin A has been incorporated empirically, based on monolayer data (Cornell *et al.*, 1977; Davion-van Mau *et al.*, 1987).

The analysis proceeds by estimating the forces acting on the heads and chains that have a net effect tending to increase the area per molecule, and those forces acting at the interface and elsewhere in the dispersion tending to decrease the area per molecule.

The pressure-area curve of the bilayer is approximated by a hyperbola of the form $2K_1/(A^* - A_{\min})$, where K_1 is determined empirically from a fit to the π–A monolayer curve of a phospholipid such as dimyristoylphosphatidylcholine, and $A_{\min}$ is the fully compressed area. The factor of 2 accounts for the two monolayers per bilayer. Two such expressions are added, one to account for the polar head groups, $2K_{1h}/(A_h - A_{\min})$, and one for the nonpolar chains, $2K_{1c}/(A_c - A_{\min})$. An estimate of the relative contributions of K_{1h} and K_{1c} is taken from the free energies of transfer between polar and nonpolar solvents of alkanes and phospholipids (Tanford, 1973).

Comparing this total pressure per unit edge of the bilayer with the opposing surface pressure, $K_2(A_c - A_{\min})$, yields the sign of the membrane curvature. Since there is a concentration dependence incorporated in both the head area A_h and in the surface tension K_2, this comparison determines the concentration at which gramicidin A will cause a reversal of phase.

The model makes a reasonable prediction of the concentration of gramicidin A required to induce the hexagonal phase in dimyristoylphosphatidylcholine multilayers. By assuming that the lateral pressure on the chains is proportional to their length, the model also accounts for the lower concentration of gramicidin A required to induce the H_{II} phase in a variety of longer-chain lipids.

A consequence of the balance of lateral pressures at different polypeptide concentrations is that the chain area A_c changes. By assuming that the dynamic order measured by NMR relates to the available lateral area, the model also accounts for the initial ordering and subsequent disordering of the chains. The ordering at low concentrations arises from the lipid headgroups being spaced by the inclusion of gramicidin A. At higher concentrations the

disorder is a result of the fall in surface pressure. The first effect decreases and the latter effect increases the chain area. It also accounts for the progressive loss of order by the polar heads as A_h monotonically increases with R. The ability of gramicidin A to transform lysolecithin micelles into a lamellar phase is also a consequence of this approach.

Although broadly successful, the model has many shortcomings, and in particular it does not take account of the phase separation and possible interprotein aggregation. The rapid diffusion coefficients reported by Tank *et al.* (1982) of dansyl-labelled gramicidin C in dimyristoylphosphatidylcholine bilayers suggests that aggregation is not dominant in the distribution of gramicidin A. Limited lateral aggregation of gramicidin A associated with the lamellar-to-hexagonal phase transition may be the cause of the range of concentrations over which this transition occurs.

Summary

The conformation of gramicidin A in lipid bilayers is now generally thought to be a $\beta^{6.3}$ helix, although the handedness remains in doubt. The location and orientation of this polypeptide within the lipid bilayer is now well understood as are the general features of its ion-conduction properties.

The molecular interactions which occur between gramicidin A and the surrounding lipid are well characterized but the theoretical insight into the nature of these interactions is in its infancy.

The gramicidin A–lipid system is devoid of most of the functional properties of many biologically interesting proteins. However, the study of these manageable model systems provides the basis on which we may build an understanding of the much broader range of mechanisms which underlie the structure and function of biological membranes.

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