PROPOSED MECHANISM FOR H_{II} PHASE INDUCTION BY GRAMICIDIN IN MODEL MEMBRANES AND ITS RELATION TO CHANNEL FORMATION

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ABSTRACT A model is proposed for the molecular mechanism of H_{II} phase induction by gramicidin in model membranes. The model describes the sequence of events that occurs upon hydration of a mixed lipid/gramicidin film, relating them to gramicidin channel formation and to relevant literature on gramicidin and lipid structure.

INTRODUCTION

Gramicidin is a hydrophobic linear pentadecapeptide, which is produced by *Bacillus brevis* before sporulation (1–3). It is believed to be involved in gene regulation by inhibiting RNA polymerase (4–6) or, together with tyrocidine, by affecting the superhelical structure of DNA (7). The structure of gramicidin A (the dominant species in the natural mixture) is

1 5
HCO–L–Val–Gly–L–Ala–D–Leu–L–Ala–D–Val
10
–L–Val–D–Val–L–Trp–D–Leu–L–Trp–D–Leu–L
15
–Trp–D–Leu–L–Trp–$NHCH_2$–CH_2OH.

Besides its apparent function in *B. brevis* the peptide has two other functional properties for which it is well known. First, in biological and model membranes gramicidin can form transmembrane channels with a selectivity for small cations (8, 9). The channels are believed to consist of two single-stranded helices in a left-handed $\beta^{6.3}$ conformation with the NH_2 terminals linked together by hydrogen-bonding (10, 11). A second interesting and well-investigated property of gramicidin is its ability to modulate lipid phase behavior (for recent review see reference 12). Upon incorporation of the peptide in lysophosphatidylcholine (LPC), which in the absence of the peptide organizes into micelles, lamellar structures are formed (13, 14). In typical bilayer-forming lipids, such as the zwitterionic phosphatidylcholine (PC) (15, 16) and the negatively charged phosphatidylserine and phosphatidylglycerol (17) and even in the erythrocyte membrane (18), gramicidin can induce a transition from a bilayer organization to a hexagonal H_{II} phase. Upon incorporation in phosphatidylethanolamines (PEs), which lipid undergoes a temperature-dependent bilayer → H_{II} phase transition, the peptide lowers the onset temperature of this transition (15, 19).

The phase preference of lipids has often been related to the dynamic shape of the molecules (20, 21). According to this shape–structure concept LPC with only one acyl chain has an overall cone shape, the hydrophobic moiety being the smaller end of the cone, which fits best into micellar structures. Bilayer-forming lipids are assumed to have an overall cylindrical shape and H_{II}-type lipids an inverted cone shape, with the polar headgroup now being the smaller end of the cone. The usefulness of this concept may be illustrated by the observation that mixtures of LPC and PE, which, due to their complementary shapes, can be expected to form an overall cylindrical complex, organized in lamellar structures (22).

Interestingly, space filling models show that the gramicidin molecule in the single-stranded $\beta^{6.3}$ conformation also has a pronounced cone shape (23), due to the bulky tryptophan residues, which are all located at the COOH-terminal part of the molecule. According to the shape–structure relationship, gramicidin, when oriented with its NH_2 terminal at the lipid/water interface, like H_{II}-type lipids, can be predicted to form lamellar structures with LPC. Similarly, in bilayer-forming lipids such an orientation of gramicidin could be responsible for its H_{II} phase-inducing activity. It was calculated (23) that due to the hydrophobicity of the tryptophan residues, this orientation of the peptide, in which the tryptophans are buried in the hydrophobic core of the lipid bilayer, is energetically more favorable than the reversed orientation, even when for the

latter the energy of interaction by intermolecular hydrogen bonding of the NH_2 terminals to form an N–N dimer is taken into account. However, it should be noted that although tryptophans are considered very hydrophobic on most hydrophobicity scales, recently some alternative ideas on this matter have emerged (24).

So far the shape–structure concept explains well the lipid structure modulating effect of gramicidin. However, there appears to be one major problem: in the reported channel configuration of the peptide, the tryptophans are located near the lipid/water interface (10, 11). Such an orientation, which possibly is stabilized by interactions between the lipid carbonyl region and the carboxy terminal of gramicidin (25), was proven to be the dominant orientation of the peptide in dimyristoyl-PC model membranes (26–28). In this paper we will address the question of whether this orientation still can be compatible with the cone shape of gramicidin as a basis for its effect on lipid structure.

We will propose a mechanistic model in which the peptide adopts the NH_2 terminal to NH_2 terminal (N–N) dimer configuration (channel configuration) upon hydration of a mixed lipid/peptide film and we will show under what conditions, according to the shape–structure relationship, such a configuration can lead to H_{II} phase formation in model membranes.

FROM CHANNEL FORMATION TO H_{II} PHASE INDUCTION

A Schematic Model

Upon hydration of a mixed gramicidin/lipid film a number of structural reorganizations take place. For instance, in mixtures of gramicidin with dioleoyl-PC in a 1:10 molar ratio of peptide to lipid, it was shown that in the absence of water the lipids are in a bilayer organization, while in excess water H_{II} phase formation occurs (16). Apparently, despite the fact that a low water content in general favors H_{II} phase formation (29), gramicidin can only induce this phase in the presence of water. Since upon hydration of the mixed film it is the peptide that takes up water, even in preference to the lipid headgroup (16), it is likely that gramicidin undergoes a conformational change and needs to be in a hydrated conformation to induce H_{II} phase formation. The following model gives a stepwise and comprehensive description of the sequence of structural reorganizations that occurs upon hydration of a mixed gramicidin/PC film. The individual steps are represented schematically in Figs. 1 and 2.

Step 1. Upon hydration of a mixed gramicidin/lipid film, the conformation of the peptide converts from an antiparallel double-stranded helix to a single-stranded NH_2-terminal to NH_2-terminal hydrogen-bonded dimer.

Gramicidin can adopt a variety of conformations (30, 31). Recently, Naik and Krimm (32, 33), based on Fourier transform infrared and Raman spectroscopy data, proposed that in dried lipid systems gramicidin is present as an antiparallel double helix, whereas the peptide adopts a single-stranded $\beta^{6.3}$ conformation in hydrated lipids. A similar transition can be observed when gramicidin is added to hydrated lipid dispersions as a dry powder (11, 33), in which it is assumed to have an antiparallel double helical conformation (32–34) or from an organic solvent in which the peptide also has this conformation (25, 35). In those cases subsequent heating results in incorporation of the peptide as an N–N dimer (11, 25, 33, 35).

Apparently, in a lipid environment in the presence of water, gramicidin prefers a single-stranded conformation. But why should this be an N–N terminal dimer? We propose the following. It has been suggested that double helices can form by intertwining of two single-stranded helices via a "zipper-mechanism" (36, 37). Similarly, the double helix then, upon hydration, could unwind according to such a mechanism. If the unwinding starts at the COOH-terminals, as is depicted in Fig. 1, *A–C*, an N–N dimer can be formed without physical constraints. Whereas in the antiparallel double helix conformation the tryptophan rings are believed not to be stacked in parallel (25), a partial unwinding of the helices could already result in stabilization by stacking interactions between tryptophan-15 and tryptophan-9 (38–40). The unwinding is accompanied by a change in the geometrical dimension of the gramicidin dimer. In the antiparallel double-stranded conformation the eight tryptophans are spread evenly throughout the length of the dimer, which therefore has a cylindrical shape (Fig. 1 *A*). Upon transition to the N–N dimeric single-stranded conformation, however, the tryptophan residues become located at the mouth of the channel leading to a relatively large cross-sectional diameter at both ends of the dimer (Fig. 1 *C*). This transition is

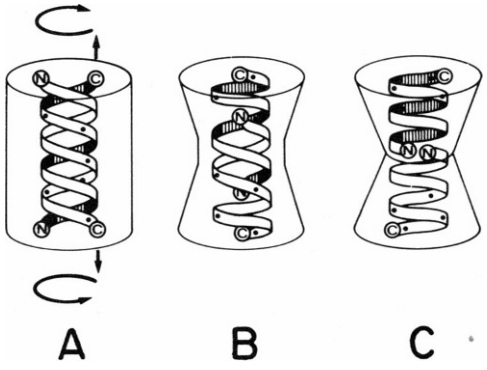

FIGURE 1 Schematic representation of the conversion of the conformation of gramicidin from an antiparallel double helix to an N-N terminal single-stranded dimer (step *1* in Fig. 2) upon hydration of a mixed gramicidin-lipid bilayer according to the "zipper-mechanism." The unwinding starts at the COOH terminus in a direction as given by the arrows. The sites of attachment of the tryptophan residues are indicated by black spots on the backbone of the helices. See text for further details.

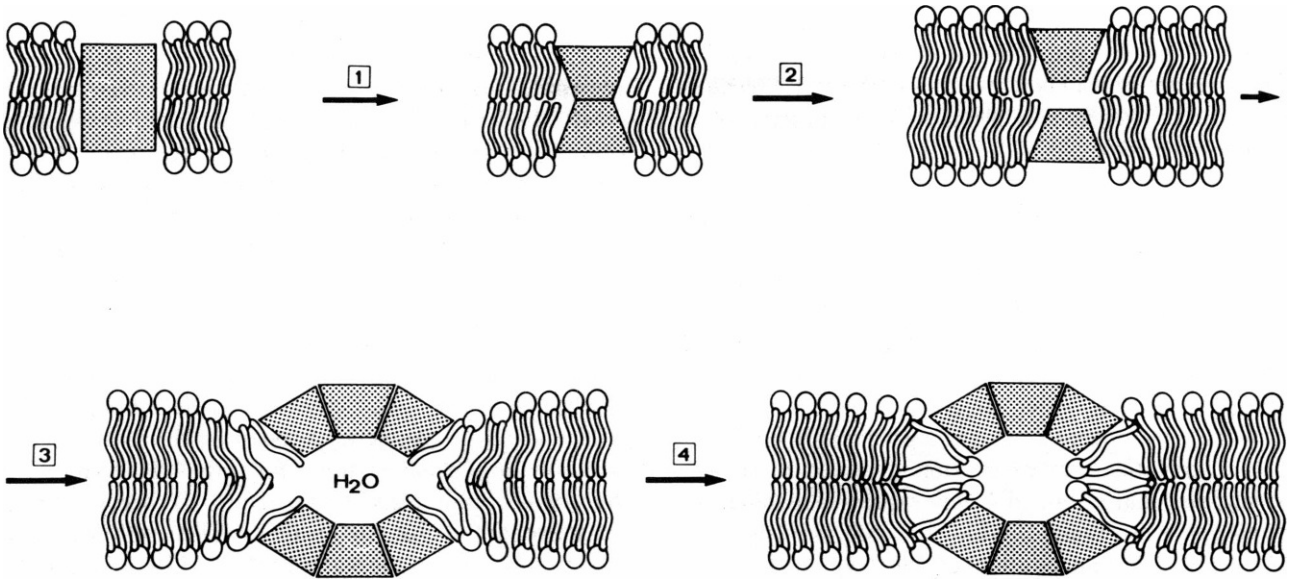

FIGURE 2. Schematic representation of steps *1–4* of the mechanism of H_{II} phase formation after hydration of a mixed gramicidin/lipid film. The gramidicin molecule in the antiparallel double helical conformation is depicted with a cylindrical shape, whereas the single-stranded $\beta^{6.3}$ conformation is represented by a cone shape, with the COOH-terminal part as the basis of the cone. All drawings are side views. For details see text. Upon hydration of the film, the conformation of the peptide converts from an antiparallel double helix to a single-stranded $\beta^{6.3}$ helix (step *1*). When the bilayer thickness exceeds the length of the gramicidin dimer, the dimer dissociates and water fills the space between both monomers (step *2*). The monomers undergo lateral self-association (step *3*), whereby the specific structure of the aggregate causes a strong decrease in the order of the adjacent lipids and the lipids flip (step *4*). Further intra- and interbilayer aggregation then leads to H_{II} phase formation.

depicted schematically in Fig. 2, step *1*. Let us now consider the situation when unwinding would start at the NH_2 terminals of the gramicidin molecule. Keeping in mind that the four bulky tryptophan residues and also the relatively large leucine residues are located at the COOH-terminal part of the peptide, it easily can be visualized that already upon partial unwinding, a large steric hindrance will be encountered when the COOH-terminals approach each other. This most likely prevents complete unwinding and subsequently inhibits the formation of C-C dimers.

We therefore propose that the single-stranded N–N configuration of gramicidin is not necessarily the energetically most favorable orientation of gramicidin in a lipid environment, but also could be a result of limitations in the unwinding process of the double-stranded dimer.

In the dry gramicidin/lipid film the peptide is very attractive to water molecules (16). Indeed, for a transition from an antiparallel double-helix conformation to a single-stranded one, it is likely that water is needed, since all intermolecular hydrogen bonds (~30 for an antiparallel $\beta^{5.6}$ helix; reference 30) must be broken and new intra- as well as intermolecular hydrogen bonds must be formed (6 and 22, respectively, for a single-stranded $\beta^{6.3}$ helix; reference 30). Since gramicidin can only induce H_{II} phase formation in the presence of water (16, 41), we propose that the peptide needs to be in a single-stranded conformation. The observation, that the ability of gramicidin to induce the formation of an H_{II} phase furthermore depends on the lipid acyl chain length (42), is the next important mechanistic clue towards an understanding of the relationship between channel and H_{II} phase formation.

Step 2. If the acyl chain length exceeds the length of the gramicidin monomer, dissociation of the dimer occurs, upon which water fills the space between both monomers.

Various experimental observations indicate that dissociation of gramicidin dimers may occur, depending on the thickness of the hydrophobic part of the lipid layer. Based on a decrease of channel lifetime (43, 44) and on circular dichroism measurements (25, 45), it was concluded that the dissociation constant for the dimer–monomer transition changes in favor of monomer formation upon increasing the bilayer thickness.

The length of the single-stranded $\beta^{6.3}$ N–N terminal dimer is ~28 Å (30), a value almost similar to that of the hydrophobic part of dipalmitoyl-PC in the liquid-crystalline state (±30 Å [46]). Since it was shown that gramicidin, when present in a 1:10 molar ratio to lipid, only can induce H_{II} phase formation in PCs when the acyl chain length exceeds 16 C-atoms (42), we propose that for H_{II} phase formation the peptide dimer must dissociate (Fig. 2, step *2*).

Upon dissociation the space between both monomers may be filled by water aggregates (47). Since the presence of water in the hydrophobic part of the membrane is unlikely to yield a stable situation, we propose as the next step in our model that lateral self-association of the

monomers plays an important role in stabilizing this dissociated state. It must be noted that the structure of these monomers is not known; therefore, we will arbitrarily assume the $\beta^{6.3}$ helical conformation in the description of our model from now on.

Step 3. The gramicidin monomers undergo lateral self-association.

It has been shown that gramicidin under some conditions has a high tendency to self-associate. Recent studies on the hydration properties of dioleoyl-PC/gramicidin mixtures showed no evidence of self-association of the peptide at very low water content (16, 41), whereas at intermediate water content and at a gramicidin/lipid ratio >1:50, aggregation was clearly found to occur (16, 41). This suggests that gramicidin only expresses its tendency to aggregate when present in the $\beta^{6.3}$ conformation, which might be due to the position of the tryptophans after unwinding of the double helix. A recent energy calculation (48) showed that indeed in the $\beta^{6.3}$ conformation, mainly due to intermolecular tryptophan–tryptophan stacking interactions involving all four tryptophan residues, aggregation is an energetically highly favorable process.

Based on hydration studies (16, 41), DSC measurements (19, 49), and sucrose-density centrifugation experiments (50), it was proposed that aggregation is an obligatory step in the gramicidin-induced H_{II} phase formation. The observation that the tryptophan residues of the peptide are essential for H_{II} phase formation (50, 51) led to the proposal that these residues play an important role in determining the macroscopic organization of the aggregates (50).

What then could be the nature of these aggregated structures? For the $\beta^{6.3}$ helix two possible modes of aggregation were calculated (48). The lowest energy of interaction (−10 kcal/mol) was obtained for a model of self-association in which the molecules form a highly curved aggregate, such that 12 gramicidin molecules aggregated in this way can form a circle with the tryptophan-containing COOH-terminal parts pointing outward. A second possible, but less favorable mode of self-association (−6 kcal/mol) was calculated to be in a linear way, perpendicular to the curved aggregate, thus in the plane of the membrane.

We now propose that upon dissociation of the gramicidin dimers, the monomers undergo lateral self-association during which both types of aggregation occur. This will result in the formation of a lipid/peptide aggregate with a semi-tubular type of structure of which a cross-section is shown in Fig. 2 (step *3*). Although in this model the dissociation and lateral self-association are depicted as subsequent steps, they are thought to be interactive processes, which also might occur in the reversed order. Thus, in the case of a very high peptide content, the tendency of the gramicidin molecules to form curved aggregates could in principle already result in dissociation of the dimers.

If self-association of gramicidin occurs, as shown in step *3* (Fig. 2), the lipids adjacent to the curved peptide aggregate will become highly disordered. ^2H- and ^{31}P-NMR studies on the hydration properties of gramicidin/dioleoyl-PC mixtures showed that the self-association of the peptide which takes place at intermediate water content is, indeed, accompanied by the formation of a population of lipids with highly disordered acyl chains and polar headgroups (16, 41).

Step 4. The lipids flip.

The orientation of the lipids next to the gramicidin aggregates is now such that the polar headgroup is adjacent to the hydrophobic tryptophan residues and the acyl chains to the aqueous environment at the NH_2-terminals of the gramicidin molecule. Therefore, it becomes more attractive for the lipids to flip and to orient with their polar headgroups towards the water at the NH_2 terminals of the peptide (Fig. 2, step *4*). Recently it has been demonstrated by Classens et al. (52) that in erythrocyte membranes, gramicidin, when incorporated at low molar ratios of peptide to lipid (gramicidin/phospholipid > 1:2,000; molar), indeed enhances lipid flip-flop, whereas at higher concentrations (gramicidin/phospholipid > 1:80) the peptide induces H_{II} phase formation (18).

Step 5. Further gramicidin aggregation results in phase separation and H_{II} phase formation.

Let us now consider the stage of hydration in which the lipid headgroups are hydrated but the bilayers are not yet maximally swollen. Then, since (*a*) gramicidin aggregates may act as nucleation sites for further peptide aggregation (50), (*b*) at intermediate water content a phase separation occurs between a gramicidin-rich phase and a gramicidin-poor bilayer (16, 41) and (*c*) upon further hydration this gramicidin-rich phase converts to a hexagonal H_{II} phase (16, 41), we propose that the gramicidin/lipid aggregate, depicted in step *4*, can increase in size by further self-association in the plane of the membrane and also, possibly, by interbilayer contacts. Upon further hydration this self-association process results in a macroscopic phase separation between a gramicidin-poor bilayer, which then allows normal swelling and a gramicidin-rich phase, which rapidly rearranges in its energetically most favorable organization, a hexagonal packing of the tubular structures and, thus, an H_{II} phase.

In the model emphasis is put on the shape of the gramicidin monomer and the mismatch between the length of the gramicidin dimer and the lipid acyl chain length. However, it should be realized that the cone shape of the gramicidin–lipid complex (42) could contribute to H_{II} phase formation and that lipids with longer acyl chains fit better into an H_{II} phase (29, 53) and, therefore, might more readily organize in this phase with gramicidin.

A schematic drawing of one tube of the gramicidin-induced H_{II} phase is shown in Fig. 3. The gramicidin

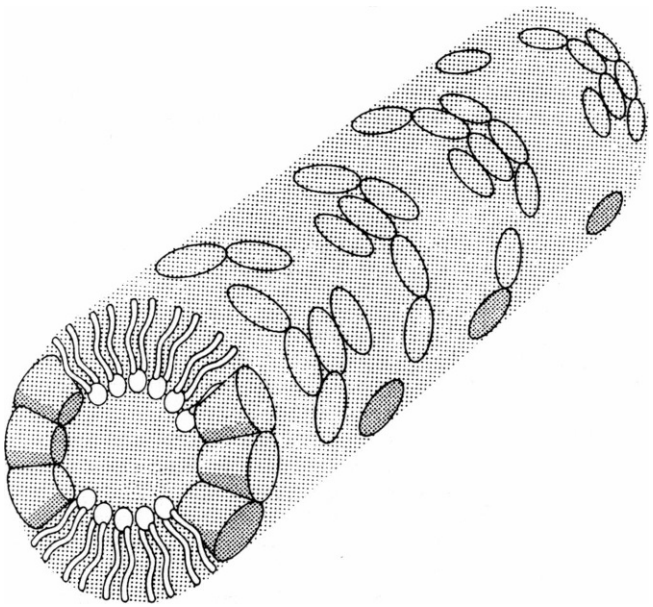

FIGURE 3 Schematic representation of one tube of a gramicidin-rich H_{II} phase.

molecules that are aggregated in clusters are oriented with their long axis perpendicular to the aqueous channels of the H_{II} phase. Since it has been demonstrated that fast exchange occurs between water molecules in the gramicidin channels and in the tubes of which the H_{II} phase is composed (16), the water molecules may flow freely from the inside of one tube to another through the gramicidin pores. The importance of the tryptophan residues for H_{II} phase formation can be illustrated by the observation that replacement of only one tryptophan by phenylalanine drastically reduces the H_{II} phase inducing activity of gramicidin (50).

Our model proposes that gramicidin, itself, is the structural backbone for the H_{II} phase. This view is supported by the following experimental data.

(*a*) The gramicidin-induced H_{II} phase is very rich in gramicidin (16, 50). A minimum of 1 gramicidin molecule per 7 lipid molecules is present, but much higher gramicidin concentrations can be accommodated in this phase.

(*b*) The tube diameter and the acyl chain order in the gramicidin-induced H_{II} phase in PC systems are independent of temperature, in contrast to the behavior of pure lipids in the H_{II} phase (40).

(*c*) The tube diameter is virtually independent of the type of lipid used (17, 41, 48).

There are two ways to get insight in the tube diameter from the geometry of the gramicidin molecule. First, it can be calculated for a gramicidin $\beta^{6.3}$ helix with a length of 14 Å (30) and diameters of 137 Å at the COOH-terminal and 47 Å at the NH_2-terminal part (23) and by assuming a perfect cone shape for the gramicidin molecule that nine gramicidin molecules can organize in a circle that then has a diameter of 68 Å. Second, extrapolation from the radius of curvature calculated from the lowest energy state of gramicidin aggregation results in a diameter of 70 Å (48). These values are similar to the tube diameter of 72 Å experimentally found for the gramicidin-induced H_{II} phase (17).

CONCLUDING REMARKS

The proposed model sheds new light on the mechanism of gramicidin-induced H_{II} phase formation in PC systems and relates it to channel formation. Although the model is proposed specifically for the interaction of gramicidin with diacylphosphatidylcholines, the basic principles could also be applied to the gramicidin-induced H_{II} phase formation in other diacyl phospholipids (17, 19). However, additional lipid headgroup specific factors, such as intermolecular electrostatic and hydrogen binding interactions between headgroups, can be expected to affect individual steps in the mechanism of gramicidin-induced H_{II} phase formation.

Our model heavily relies on the channel conformation proposed for gramicidin. It should be realized, however, that the structure of the peptide in the various lipid systems is not known and we would, therefore, like to emphasize that in our view a critical test of the model requires detailed knowledge of the conformational and aggregational behavior of the peptide in different lipid environments.

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Note added in proof: A recent study in which gramicidin was added from various organic solvents to preformed dioleoyl-PC model membranes (Tournois, H., J. A. Killian, D. W. Urry, O. R. Bokking, J. de Gier, and B. de Kruijff. 1987. *Biochim. Biophys. Acta.* 905:222–226) strongly suggests that indeed the $\beta^{6.3}$ helical configuration of gramicidin is responsible for H_{II} phase induction.

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