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Influence of cholesterol on gramicidin-induced H_{II} phase formation in phosphatidylcholine model membranes

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The influence of cholesterol incorporation on gramicidin-induced hexagonal H_{II} phase formation in different phosphatidylcholine model systems was investigated by ^{31}P- and ^{2}H-NMR, small-angle X-ray diffraction and differential scanning calorimetry. In liquid-crystalline distearoylphosphatidylcholine systems cholesterol inhibits gramicidin-induced H_{II} phase formation. In dioleoylphosphatidylcholine the opposite effect is observed. Cholesterol appears to preferentially interact with gramicidin under liquid-crystalline conditions in both systems. Two phenomena that had been reported for gramicidin-treated erythrocyte membranes and derived liposomes (Tournois, H., Leunissen-Bijvelt, J., Haest, C.W.M., De Gier, J. and De Kruijff, B. (1987) Biochemistry, 26, 6613–6621) could also be observed in more simple dioleoylphosphatidylcholine-gramicidin-cholesterol systems. These are (i) an increase in tube diameter in the gramicidin-induced H_{II} phase with increasing temperature, which is ascribed to the presence of cholesterol in this phase, and (ii) the loss of the hexagonal H_{II} phase related ^{31}P-NMR line shape at lower temperatures despite the presence of this phase as demonstrated with X-ray diffraction. This latter phenomenon appears to be due to restrictions in the rate of lateral diffusion of the phospholipids around the H_{II} tubes due to the presence of gramicidin.

Introduction

Knowledge of the molecular aspects of protein–lipid interactions is a prerequisite to understand biomembrane structure and function. The linear pentadecapeptide gramicidin A has been extensively investigated as a peptide model to understand basic principles of polypeptide–lipid interactions both with respect to its function as ion channel (for reviews, see Refs. 1–3) and with respect to its profound and specific lipid structure-modulating activity (for reviews, see Refs. 4, 5). One example of this latter effect is the strong hexagonal H_{II} phase inducing capacity of the peptide in model systems made of different phospholipids when the acyl chain length exceeds 16 carbon atoms [6,7] as well as in erythrocyte membranes and derived liposomes [8]. Inverted non-bilayer lipid structures related to the hexagonal H_{II} phase have been suggested to play different structural and functional roles in biological membranes [9]. In good agreement with this suggestion is the observation of a gramicidin-induced large increase

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in the rate of transbilayer movement of several lipids in the red cell membrane at peptide concentrations which precede H_{II} phase formation [10].

The four tryptophans at positions 9, 11, 13 and 15 play an important role in the gramicidin-induced H_{II} phase formation. Their substitution by phenylalanine [11] or their formylation [12] drastically lowers the H_{II} phase-inducing ability of the peptide. It was suggested that these residues give the peptide a cone shape which, within the shape structure concept of polymorphism [13] and in conjunction with the strong tendency of the peptide to undergo lateral self-association [11,14,15] again involving the tryptophans but now by virtue of their ability to cause intermolecular ring stacking interactions [15] leads to H_{II} phase formation. In this respect gramicidin A can be considered to be the first example of a new class of membrane polypeptides which in combination with the appropriate lipids can organize in stable intermembrane cylindrical structures such as found for instance in the tight junction [16].

In this paper we report on the influence of cholesterol on the gramicidin-induced H_{II} phase formation in phosphatidylcholine systems. The rationale behind this study is two-fold. First, although a number of studies indicated that sterols can greatly influence the polymorphism of pure lipid systems [17–20], nothing is known about the effect of cholesterol on peptide-induced changes in polymorphism. Second, in recent studies on gramicidin-induced H_{II} phase formation in erythrocyte membranes [8] several unusual features were observed such as a temperature-dependent increase in H_{II} phase tube diameter and a loss of the H_{II} 'related' ^{31}P-NMR line shape at lower temperatures despite the presence of this phase at these temperatures. Since the erythrocyte membrane is very rich in cholesterol we suggested that this lipid was responsible for these effects.

Using a combination of ^{31}P- and ^{2}H-NMR, X-ray diffraction and differential scanning calorimetry techniques, which has given a detailed molecular description of the dioleoylphosphatidylcholine–gramicidin interaction [14,21], we show and discuss that cholesterol, depending on the nature of the acyl chains, can either inhibit or promote gramicidin-induced H_{II} phase formation and that the unusual features uncovered in the erythrocyte membrane indeed can in part be accounted for by the presence of cholesterol in that membrane.

Materials and Methods

Materials

Gramicidin (from *Bacillus brevis* consisting for 80% of gramicidin A) and cholesterol were purchased from Sigma (St. Louis, MO). 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (14 : 0/14 : 0-PC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (16 : 0/16 : 0-PC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (18 : 0/18 : 0-PC), 1,2-dierucoyl-*sn*-glycero-3-phosphocholine (22 : 1_c/22 : 1_c-PC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (18 : 1_c/18 : 1_c-PC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (18 : 1_c/18 : 1_c-PE) were synthesized according to [22,23]. 1,2-[11,11-$^{2}H_2$]dioleoylphosphatidylcholine ([11,11-$^{2}H_2$]DOPC) and 1,2-[11,11-$^{2}H_2$]dioleoylphosphatidylethanolamine ([11,11-$^{2}H_2$]DOPE) were synthesized as described before [21]. All other chemicals were of analytical grade.

Sample preparation

Lipid or lipid-gramicidin dispersions were prepared for NMR as described earlier [14] by mixing 40 μmol of phospholipid and the required amounts of gramicidin and/or cholesterol in chloroform/methanol (2 : 1, v/v) followed by subsequent drying and hydration with an excess of 50% by total weight of H_2O. In experiments with [11,11-$^{2}H_2$]DOPC and [11,11-$^{2}H_2$]DOPE these lipids were 1 : 1 diluted with non-labelled phospholipid. Model membrane compositions are given as molar ratios of the individual components.

^{31}P-NMR and ^{2}H-NMR

121.5 MHz proton-noise decoupled ^{31}P-NMR spectra and 46.1 MHz ^{2}H-NMR spectra were recorded on a Bruker MSL-300 spectrometer, using a VSP probe head as described before [21]. All spectra were recorded after 15–30 min of temperature equilibration. In case of quadrupolar splittings larger than $\pm$10 kHz, ^{2}H-NMR spectra were recorded with similar results using a 10 mm high power probe head.

Small-angle X-ray diffraction

X-ray experiments were performed on a Kratky camera as described before [14]. Samples used for NMR experiments were mounted between two sheets of cellophane in a slit of a temperature-controlled sample holder. X-ray diffraction profiles were obtained from 5–10 min exposure times after 15 min of temperature equilibration.

Differential scanning calorimetry (DSC)

Samples of 10 μmol of 16 : 0/16 : 0-PC and the required amounts of gramicidin and/or cholesterol in chloroform/methanol (2 : 1, v/v) were dried under a stream of N_2 and stored overnight under high vacuum. The samples were then dispersed in 1 ml of distilled H_2O at 55 °C for 2 h. Subsequently the samples were spun for 30 min at 4 °C at 30 000 × *g*. The pellet was transferred to a sample pan of a Perkin-Elmer DSC-4 calorimeter and thermograms were recorded and data analyzed as described before [24].

Results

Saturated phosphatidylcholines

Gramicidin incorporation induces a hexagonal H_{II} phase in 18 : 0/18 : 0-PC dispersions at liquid-crystalline phase state temperatures [6]. Fig. 1 confirms this by ^{31}P-NMR using a gramicidin/PC 1 : 10 molar ratio. Below 48 °C the broadened asymmetrical ^{31}P-NMR line shape of gel state phospholipids [25] is observed. Above 48 °C the spectrum consists of two superimposed asymmetrical line shapes, one with a residual chemical shift anisotropy ($\Delta\sigma$) of approximately −42 ppm, which originates from PC in a liquid-crystalline lamellar organization and one with a reversed asymmetry and a $\Delta\sigma$ of 20 ppm which originates from the peptide-induced H_{II} phase (for a description of ^{31}P-NMR line shapes in relation to lipid phase structure, see Ref. 13). This latter component is absent when no gramicidin is present. Incorporation of equimolar amounts of cholesterol with respect to 18 : 0/18 : 0-PC converts the ^{31}P-NMR line shape at all temperatures into one which is characteristic for the liquid-crystalline bilayer (Fig. 1). This illustrates that the well-known liquefying effect of cholesterol [26] is also operative in this peptide-containing sample and, more important,

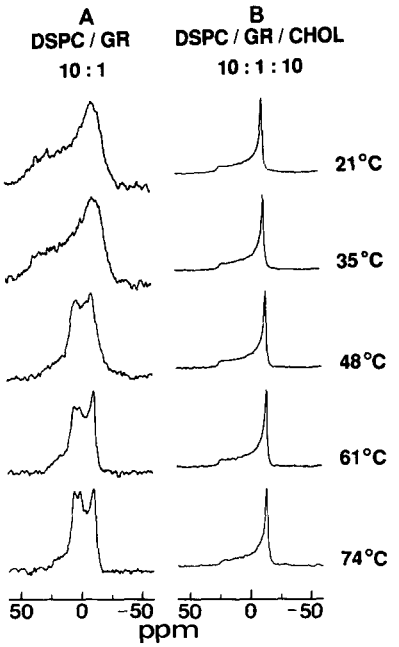

Fig. 1. 121 MHz proton decoupled ^{31}P-NMR spectra of aqueous dispersions of (A) 18 : 0/18 : 0-PC/gramicidin (10 : 1) and (B) 18 : 0/18 : 0-PC/gramicidin/cholesterol (10 : 1 : 10) recorded at the indicated temperatures.

demonstrates that the presence of cholesterol eliminates the ^{31}P-NMR line shape related to the gramicidin-induced H_{II} phase. Similar results were obtained for equimolar cholesterol-18 : 0/18 : 0-PC samples containing twice the amount of gramicidin (data not shown) excluding the possibility that this loss of H_{II} phase is the result of simple dilution of the peptide due to the increased amount of lipid. X-ray diffraction measurements showed that in these samples only sharp reflections with the periodicity of a multilamellar organization were present in contrast to the 18 : 0/18 : 0-PC-gramicidin (10 : 1) sample where at 48 °C and at higher temperatures in addition a '$1/\sqrt{3}$' reflection diagnostic of the H_{II} phase [9,14] was observed (data not shown). These data prove that cholesterol mitigates against H_{II} formation by gramicidin in this lipid system. In 14 : 0/14 : 0-PC and 16 : 0/16 : 0-PC systems gramicidin incorporation does not result in H_{II} phase formation [6]. In view of this and the foregoing it is not surprising that ^{31}P-NMR analysis carried out on these lipid systems in the absence and presence of equimolar cholesterol and 10 mol% gramicidin did

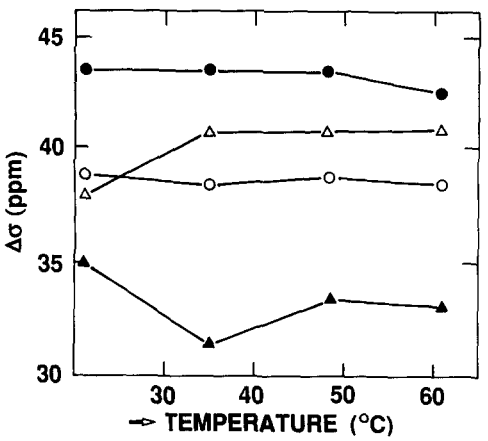

Fig. 2. ^{31}P residual chemical shift anisotropy ($\Delta\sigma$) of 16:0/16:0-PC (●———●), 16:0/16:0-PC/cholesterol (1:1; ○———○), 16:0/16:0-PC/gramicidin (10:1; ▲———▲) and 16:0/16:0-PC/gramicidin/cholesterol (10:1:10; △———△) at various temperatures.

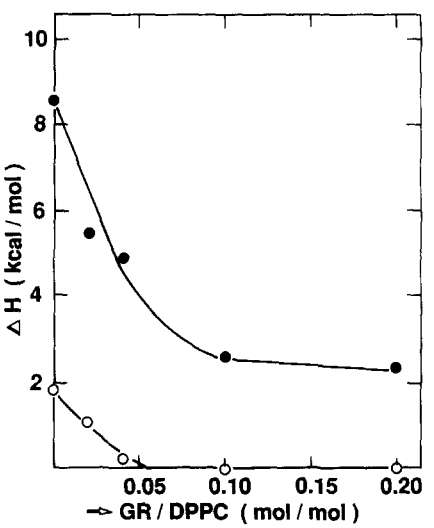

Fig. 3. Influence of gramicidin incorporation on the enthalpy of the main gel-liquid-crystalline phase transition in 16:0/16:0-PC (●———●) and 16:0/16:0-PC/cholesterol (72.5:27.5; ○———○) dispersions.

not reveal the presence of a H_{II} phase for temperatures up to 75°C. Only $\Delta\sigma$ was affected, an example of which is shown for 16:0/16:0-PC in Fig. 2. Incorporation of cholesterol or gramicidin results in a decrease in $\Delta\sigma$, the effect of the peptide being the strongest. These reductions in $\Delta\sigma$ most likely are due to a decrease in PC headgroup order due to a spacing effect of these molecules [14,25]. Surprisingly, in samples containing both cholesterol and gramicidin the decrease in $\Delta\sigma$ is much less suggesting a preferential association between cholesterol and gramicidin thereby reducing the interaction between these molecules and PC. Since similar results were obtained for 14:0/14:0-PC and 18:0/18:0-PC (data not shown), the inhibition of gramicidin-induced H_{II} phase formation in 18:0/18:0-PC by cholesterol could be the result of such a preferential peptide–cholesterol interaction.

To follow up on the possibility of selective gramicidin–cholesterol interactions DSC experiments were performed on 16:0/16:0-PC as a test lipid, using the strategy successfully employed previously to establish specific interactions between polyene antibiotics and cholesterol [27] (Fig. 3). In agreement with previous data [28] and only shown here for comparative purposes gramicidin decreases the ΔH of the gel → liquid-crystalline phase transition in a biphasic way. Incorporation of 27.5 mol% cholesterol in 16:0/16:0-PC causes a large decrease in ΔH due to the liquefying effect. Subsequent incorporation of gramicidin causes a similar decrease in ΔH as in the cholesterol-free case, suggesting that no extensive gramicidin–cholesterol interactions occur at gel state temperatures because this should result in a less marked decrease or even an increase in ΔH as observed before for polyene antibiotics under similar conditions [27].

18:1_c/18:1_c-Phosphatidylcholine

The 18:1_c/18:1_c-PC–gramicidin interaction has been studied extensively [14,21,34] and therefore this system was chosen to characterize the influence of cholesterol in more detail. Fig. 4A shows by ^{31}P-NMR that above 25°C independent of the temperature gramicidin induces the H_{II} phase, which is in agreement with previous data [34]. However, cooling down the sample to 5°C leads to a large change in the ^{31}P-NMR spectrum not described so far. The H_{II}-specific line shape decreases in intensity and converts into a broadened 'isotropic' line shape. That this is not due to a loss of the H_{II} phase at lower temperatures is unambiguously demonstrated by X-ray diffraction in Fig. 5, where the characteristic '$1/\sqrt{3}$' reflec-

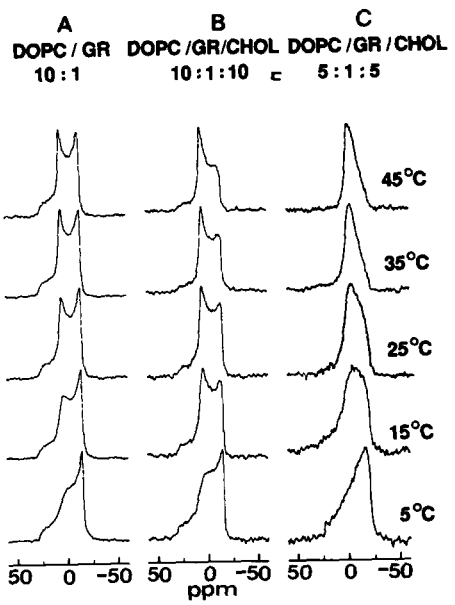

Fig. 4. 121 MHz proton decoupled ^{31}P-NMR spectra of aqueous dispersions of (A) 18 : 1_c/18 : 1_c-PC/gramicidin (10 : 1), (B) 18 : 1_c/18 : 1_c-PC/gramicidin/cholesterol (10 : 1 : 10) and (C) 18 : 1_c/18 : 1_c-PC/gramicidin/cholesterol (5 : 1 : 5) recorded at the indicated temperatures.

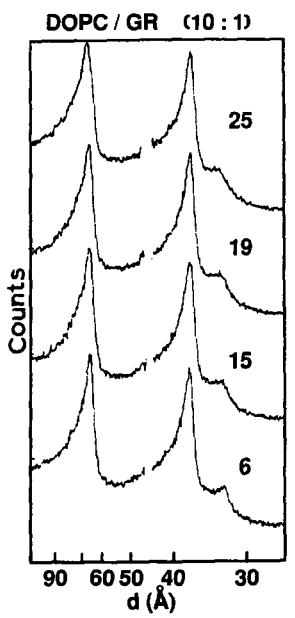

Fig. 5. Small-angle X-ray diffraction measurements of an aqueous dispersion of 18 : 1_c/18 : 1_c-PC/gramicidin (10 : 1) recorded at the indicated temperatures. The scattering intensity is given in arbitrary units.

tion with a repeat distance of 38 Å is present with similar intensity over the 6–25 °C temperature range. It thus appears that the change in line shape at lower temperatures is due to changes in the motional properties of the phospholipid, most likely a decrease in the rate of lateral diffusion of the lipids around the tubes of which this phase is formed. Incorporation of equimolar cholesterol causes in contrast to the situation described for 18 : 0/18 : 0-PC an increase in the H_{II} phase related ^{31}P-NMR line shape at all temperatures tested (Fig. 4B). Together with the spectra shown in Fig. 4C for the sample with the same gramicidin/total lipid ratio as the cholesterol-free control sample the ^{31}P-NMR data thus clearly demonstrate strong H_{II} phase promotion by cholesterol in this system above 25 ° C. Also in both cholesterol containing samples, the marked temperature-dependent changes in the ^{31}P-NMR spectra are not due to a decrease in the amount of H_{II} phase as inferred from X-ray diffraction (data not shown).

The tube diameter (e.g. the sum of the diameter of the aqueous channel and two times the length of the lipid molecule) of the H_{II} phase is twice the repeat distance of the '$1/\sqrt{3}$' reflection and is presented in Fig. 6. In contrast to the temperature-independent tube diameter in the 18 : 1_c/18 : 1_c-PC-gramicidin system, in the presence of equimolar cholesterol the tube diameter increases with increasing temperature.

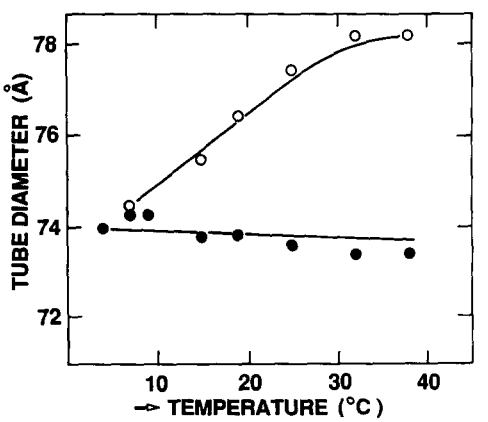

Fig. 6. Tube diameter of the H_{II} phase in 18 : 1_c/18 : 1_c-PC/gramicidin (10 : 1, ●——●), and 18 : 1_c/18 : 1_c-PC/gramicidin/cholesterol (10 : 1 : 10, ○——○) dispersions obtained from X-ray diffraction profiles measured at the indicated temperatures.

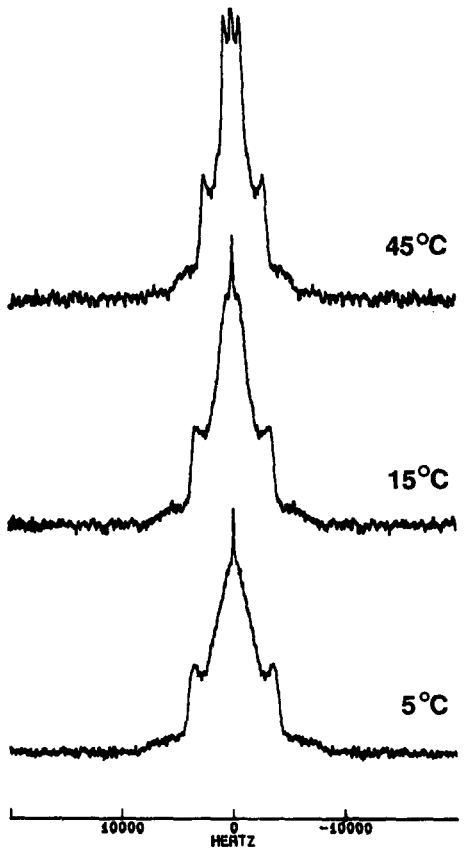

Fig. 7. 46.1 MHz ^{2}H-NMR spectra of [11,11-$^2H_2$]18:1$_c$/18:1$_c$-PC/gramicidin (10:1) dispersions recorded at the indicated temperatures. The small isotropic component arises from the natural abundant ^{2}H in H_2O.

^{31}P $\Delta\sigma$ values of these systems were recorded but are not shown or analyzed because interpretation is complicated by the complex line shapes of the spectra. More informative were ^{2}H-NMR investigations using [11,11-$^2H_2$]18:1$_c$/18:1$_c$-PC. Fig. 7 shows some selected spectra and Fig. 8 summarizes the derived values of the quadrupolar splitting ($\Delta\nu_q$). In the bilayer organization the quadrupolar splitting of the deuterium in dispersions of this lipid is $\pm$6 KHz [21,29]. At 25°C and higher, gramicidin incorporation causes the appearance of a second doublet in the ^{2}H-NMR spectrum with a much reduced quadrupolar splitting. This spectral component arises from the lipids organized in the H_{II} phase where the quadrupolar interaction is further averaged by the rapid lateral diffusion of the lipids around the H_{II} tubes together with a decreased chain order in this phase [21]. In parallel with the changes in ^{31}P-NMR line shape, the ^{2}H-NMR line shape changes from a defined doublet to a triangular shape upon cooling the samples below 15°C and 25°C for cholesterol-free and cholesterol/[11,11-$^2H_2$]18:1$_c$/18:1$_c$-PC (1:1, molar ratio) samples, respectively, suggesting changes in the motional properties of the entire molecule under these conditions. In these cases $\Delta\nu_q$ cannot be derived from the spectra. Cholesterol has a number of different effects on the ^{2}H-NMR specta. First, in line with the ^{31}P-NMR results for a fixed [11,11-$^2H_2$]18:1$_c$/18:1$_c$-PC-gramicidin molar ratio the intensity of the inner narrow line shape greatly increases with increasing cholesterol content, demonstrating H_{II} promotion by cholesterol in this system (spectra not shown). Second, the condensing effect of cholesterol on liquid-crystalline bilayers is manifested as a large increase in the quadrupolar splitting in both gramicidin-free and gramicidin-containing samples (Fig. 8).

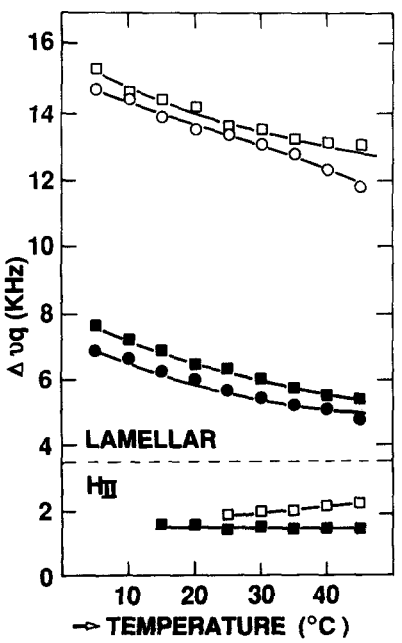

Fig. 8. Quadrupolar splitting ($\Delta\nu_q$) obtained from 46.1 MHz ^{2}H-NMR spectra of dispersions of [11,11-$^2H_2$]18:1$_c$/18:1$_c$-PC (●——●), [11,11-$^2H_2$]18:1$_c$/18:1$_c$-PC/cholesterol (1:1; ○——○), [11,11-$^2H_2$]18:1$_c$/18:1$_c$-PC/gramicidin (10:1, ■——■) and [11,11-$^2H_2$]18:1$_c$/18:1$_c$-PC/gramicidin/cholesterol (10:1:10, □——□). All values are obtained from spectra which showed clearly defined doublets.

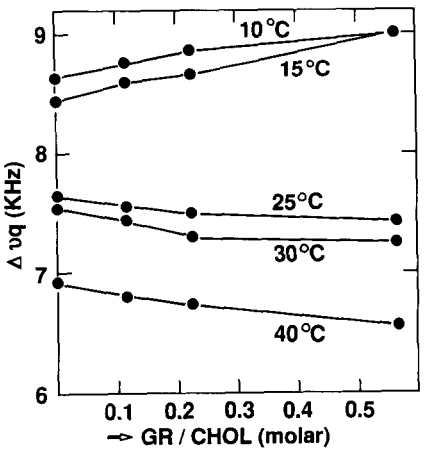

Fig. 9. Effect of gramicidin incorporation on $\Delta\nu_q$ in [11,11-$^2H_2$]18:1_c/18:1_c-PC/cholesterol (85:15) dispersions at the indicated temperatures.

In contrast to cholesterol gramicidin itself has only a small effect on the quadrupolar splitting of [11,11-$^2H_2$]DOPC in the lamellar phase (Ref. 21, and this study). If cholesterol would preferentially interact with gramicidin the cholesterol–PC interaction would be decreased resulting in a decrease in the quadrupolar splitting. Thus, in principle, analysis of $\Delta\nu_q$ in cholesterol-containing [11,11-$^2H_2$]DOPC samples as a function of gramicidin content could reveal the existence of preferential interactions among the various membrane components. Since in systems where the bilayer and H_{II} phases coexist such an analysis is complicated by the unknown composition of the phases we decided to analyze this possibility under conditions were only the lamellar phase exists. In the absence of gramicidin, cholesterol incorporation causes an almost linear increase in $\Delta\nu_q$ of 185 Hz/mol% cholesterol from 10 to 20 mol% cholesterol, which was virtually temperature independent in the 10–40 °C range (data not shown). Therefore a value of 15 mol% cholesterol was chosen for the gramicidin titration. For gramicidin/PC ratios of 0.04 or less, a single quadrupolar splitting originating from the lamellar structure was obtained. Above this value the additional quadrupolar splitting of the H_{II} phase was observed. Fig. 9 summarizes the $\Delta\nu_q$ values. At 25 °C and higher, $\Delta\nu_q$ decreases with gramicidin concentration. Below that temperature it increases. These data suggest that a preferential interaction between cholesterol and gramicidin occurs at and above 25 °C, but that at lower temperatures cholesterol is excluded from interaction with gramicidin.

18:1_c/18:1_c-Phosphatidylethanolamine

For comparative purposes the effect of cholesterol on lipid order and phase structure of hydrated [11,11-$^2H_2$]18:1_c/18:1_c-PE samples were studied. This lipid undergoes a thermotropic bilayer → H_{II} transition at around 5 °C [17]. In agreement with data on dielaidoyl-PE [18,20] incorporation of 50 mol% cholesterol has almost no effect on this transition temperature (data not shown). Incorporation of cholesterol causes a slight decrease in tube diameter (Fig. 10), without affecting its temperature dependency. At 0.5 °C, where all the lipid is in the lamellar phase, $\Delta\nu_q$ was increased from 11.7 to 16.0 kHz by incorporation of 50 mol% cholesterol (data not shown). In the hexagonal H_{II} phase at 25 °C the $\Delta\nu_q$ was proportionally similarly increased from 2.32 to 3.11 kHz.

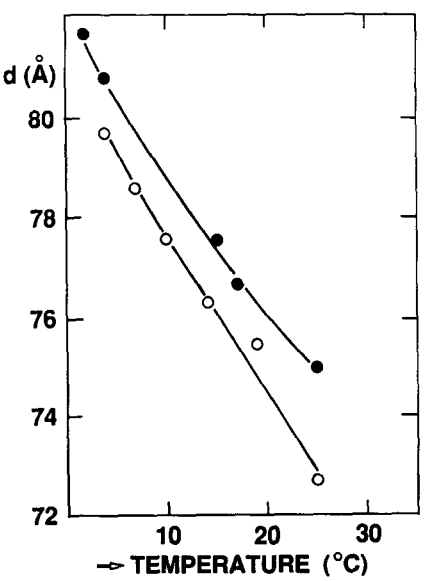

Fig. 10. Tube diameter of the H_{II} phase of hydrated 18:1_c/18:1_c-PE (●——●) and 18:1_c/18:1_c-PE/cholesterol (1:1, ○——○) obtained from the '$1/\sqrt{3}$' reflection observed in small-angle X-ray diffraction experiments performed at the indicated temperatures.

Discussion

Cholesterol appears to affect polymorphism of pure lipid systems in a lipid-dependent way. For the PE species investigated so far, cholesterol does not change overall lipid organization or causes a slight bilayer destabilization as manifested from a slight decrease in the bilayer → H_{II} phase transition temperature [17,18,20]. For saturated and unsaturated PC species with up to four *cis* double bonds in total cholesterol does not affect overall bilayer structure [36]. However, incorporation of this sterol in polyunsaturated PC species causes hexagonal H_{II} phase formation [36]. In more complex lipid systems, including PE/PC mixtures, strong bilayer destabilization and hexagonal H_{II} phase formation is observed [17,19]. The reason for the differences in extent of bilayer destabilization in these systems is unknown but could be the lipid-dependent net outcome of two counteracting effects. The small polar headgroup of cholesterol and its low hydration together with its bulky rigid hydrophobic ring system gives the molecule a cone shape which will favor H_{II} formation [13]. However, the increase in lipid order due to the condensing effect will mitigate against H_{II} phase formation. The available literature data suggest that for PC containing systems the former effect dominates. It is against this background that we would like to discuss the present results.

This study reveals an interesting fatty acyl chain-dependent modulation of the phase state in liquid-crystalline gramicidin/PC systems. Cholesterol causes either inhibition (18:0/18:0-PC) or promotion (18:1_c/18:1_c-PC) of the gramicin induced H_{II} phase formation. In addition, we observed that in the H_{II} phase of the 18:1_c/18:1_c-PC-gramicidin system cholesterol incorporation makes that the tube diameter increases with increasing temperature in contrast to the temperature-independent tube diameter of the sterol-free system. We start with an analysis of this latter observation and then return to the fatty acid chain-dependent modulation of the phase state of the lipids.

In principle, the temperature-induced increase in tube diameter of the H_{II} phase can be the result of at least four effects: (1) increased effective acyl chain length, (2) increased water content, (3) increased partitioning of cholesterol into the H_{II} phase, and (4) formation of a complex which fits better into a tube of larger diameter.

The first possibility is unlikely because usually an increase in temperature will cause a decrease in effective acyl chain length due to a decrease in lipid order (and thus a decrease in $\Delta\nu_q$). This, in fact, is the explanation for the temperature-induced decrease in tube diameter and acyl chain order for H_{II} phases formed by phosphatidylethanolamines [21]. Also it could be argued that as a result of an increase in temperature the cholesterol–18:1_c/18:1_c-PC interaction in the H_{II} phase would increase which would result in an increased effective acyl chain length due to the condensing effect. However, this we consider to be also unlikely because of the data shown in Fig. 9, which point in the opposite direction of a decreased cholesterol–18:1_c/18:1_c-PC interaction at higher temperatures. A temperature-dependent increase in the diameter of the aqueous channel in the H_{II} tubes could in principle account for the observation but most likely can be only the indirect result of changes in the molecular interactions of the components in the H_{II} phase. An increase in hydration of the individual components with increasing temperatures seems unlikely. The third possibility that cholesterol preferentially partitions in the H_{II} phase at higher temperatures can be excluded because this should result in a decrease in the cholesterol content in the coexisting lamellar phase and thus a decrease in condensing effect and a decrease in chain order, which is not observed (Fig. 8). Therefore, we favor the fourth possibility that at higher temperatures a molecular complex is formed which fits better into a tube of larger diameter. Within the terms of the shape-structure concept of polymorphism [13], this would imply a more cylindrical shape of this complex. We propose that this is a gramicidin-cholesterol complex which is formed by hydrophobic interactions between these molecules. At lower temperatures the tube diameter would be mainly determined by the gramicidin molecules themselves [15]. In favor of this proposal is the observation that the cholesterol–18:1_c/18:1_c-PC interaction decreases above 15°C (Fig. 9). Furthermore, the idea of preferential cholesterol-gramicidin interactions in liquid-crystalline systems at high temper-

atures is clearly born out by the behavior of $\Delta\sigma$ in the ^{31}P-NMR spectra of the disaturated-PC systems. With this model we can explain the bilayer stabilizing effect of cholesterol on the gramicidin/18 : 0/18 : 0-PC system. The more cylindrical gramicidin-cholesterol complex and PC species fit better into the lamellar phase of this mixed system. The strength of the cholesterol–PC interaction in ternary mixtures with gramicidin will also determine the extent of gramicidin-cholesterol complex formation. It is noteworthy that in the long chain 22 : 1_c/22 : 1_c-PC system in which gramicidin very efficiently promotes H_{II} phase formation [6] we observed a strong bilayer stabilizing effect of cholesterol (data not shown), which we propose to be the result of the reported [37], much weaker interaction between this PC species and cholesterol.

Extrapolation of these ideas to the properties of the gramicidin-induced H_{II} phase in erythrocyte membranes [8] suggests that also in this biomembrane system the cholesterol–gramicidin interaction in the H_{II} phase increases with increasing temperature.

For H_{II} phase formation a specific hydrated [14], most probably β6.3 helical conformation [30] of gramicidin is essential together with its strong tendency to undergo lateral self association into tubular structures [15]. It is thus possible that the effect of cholesterol in these gramicidin/PC systems is in addition related to an acyl chain length-dependent influence of cholesterol on gramicidin structure or organization. In this light it is intriguing to note that cholesterol greatly reduces the mean life time, without affecting the conductance of gramicidin A channels in monoolein-squalene bilayers suggesting a large increase in the dimer dissociation rate constant [31]. *N-N* dimer dissociation is considered by us [35] to be an important step in the initial stage of H_{II} phase formation and this effect thus could contribute to H_{II} phase promotion by cholesterol in the 18 : 1_c/18 : 1_c-PC system.

It is of interest to compare the effect of cholesterol on the gramicidin-induced H_{II} phase in 18 : 1_c/18 : 1_c-PC with the effect of cholesterol on the temperature-induced H_{II} phase in 18 : 1_c/18 : 1_c-PE. In the lamellar phase the PE–cholesterol interaction is largely comparable to the PC–cholesterol interaction [32]. Cholesterol causes a similar increase in $\Delta\nu_q$ of the (11,11-$^2H_2$) deuterons of 18 : 1_c/18 : 1_c-PE in the bilayer and H_{II} phase demonstrating similar increases in chain order in both phases and proving the incorporation of cholesterol in this inverted nonbilayer phase. The slight decrease in tube diameter together with the acyl chain ordering must be interpreted as a decrease in the diameter of aqueous channel within the H_{II} phase.

The loss of the hexagonal H_{II} phase specific ^{31}P-NMR line shape in the gramicidin-containing erythrocyte membrane and derived liposomes at lower temperatures despite the presence of this phase at these lower temperatures [8] could be reproduced in this study with model systems of a more simple composition. The observation that also in 18 : 1_c/18 : 1_c-PC/gramicidin this effect could be observed demonstrates that cholesterol is not essential for this effect. In line with the theoretical analysis carried out in Ref. 8 and consistent with the ^{2}H-NMR data reported in this paper, we suggest that at these low temperatures the presence of gramicidin aggregates in the H_{II} tubes reduces the rate of lateral diffusion around the tubes below the value whereby additional averaging of the chemical shift anisotropy or quadrupolar interaction occurs, leading to a loss of the characteristic line shape. That cholesterol is not essential for this effect is furthermore in line with the reported observation that this sterol has only a very small effect on lateral diffusion of 18 : 1_c/18 : 1_c-PC in model membranes [38]. At present it is not possible to simulate the ^{31}P- and ^{2}H-NMR spectral behavior of the 18 : 1_c/18 : 1_c-PC-gramicidin systems at these lower temperatures.

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