Biochimica et Biophysica Acta, 512 (1978) 480—488

BBA 78149

THE EFFECT OF GRAMICIDIN A ON THE TEMPERATURE DEPENDENCE OF WATER PERMEATION THROUGH LIPOSOMAL MEMBRANES PREPARED FROM PHOSPHATIDYLCHOLINES WITH DIFFERENT CHAIN LENGTHS

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(Received March 17th, 1978)

Summary

The permeation of water through liposomal membranes composed of various saturated phosphatidylcholines plus gramicidin A was studied as a function of temperature.

1. The presence of gramicidin in the liposomal bilayers caused an increase in water permeability. Below the phase transition temperature this effect could be measured quite clearly in all the systems we tested, but the extent of the increase was largely dependent on the length of the hydrocarbon chains.

2. Increasing amounts of gramicidin caused a gradual disappearance of the abrupt change in the rate of water permeation near the gel-liquid crystalline phase transition temperature of dipalmitoyl phosphatidylcholine liposomes. Differential scanning calorimetry analysis of the system containing these relatively small amounts of gramicidin still showed a clear transition from the liquid crystalline to the gel state with only a slight reduction in the enthalpy change.

3. In liposomes composed of dimyristoyl, dipalmitoyl and saturated egg phosphatidylcholine there was a concomitant decrease in the activation energy of water permeation in the presence of gramicidin below and above the phase transition temperature. The activation energy for water permeation through longer chained distearoyl phosphatidylcholine liposomal bilayers was the same with or without gramicidin in the bilayer.

4. It is concluded that the ability of gramicidin to form conducting channels in a gel state bilayer depends on the thickness of the paraffin core.

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Introduction

Gramicidin A is a linear pentadecapeptide isolated from *Bacillus brevis* [1,2] that has been implicated in the transport of ions across biological membranes [3,4] and model membrane systems (see ref. 5). Gramicidin A is composed of 15 hydrophobic amino acid residues with alternate D and L configuration. The molecule is electrically neutral since a formyl group blocks the amino terminus and an ethanolamine group blocks the carboxy terminus.

Recent reviews [5,6] have detailed the strong evidence that gramicidin A facilitates cation transport by forming pore-like channels, through which also water molecules can pass rapidly [30]. A model of the gramicidin channel as proposed by Urry [7—9] and Ramachandran and Chandrasekaran [10] consists of two gramicidin molecules linked in tail-to-tail (formyl end-to-formyl end) conformation as a π^6_{LD} helix with an internal diameter of about 4 Å and a length of 25—30 Å. In its dimeric form gramicidin is believed to span the lipid bilayer. The hydrophobic residues are directed towards the outer surface of the helix while the polar peptide C-O moieties line the interior of the helix. An alternative model has been proposed by Veatch and Blout [11—13]. They suggest that the gramicidin channel is composed of a double helix in which the two chains are coiled around a common axis. However, recent evidence [14—16] favours the former model.

In good agreement with the pore hypothesis for the action of gramicidin A are the findings of Krasne et al. [17] which show that the increased conductivity of a black lipid film observed when gramicidin is present is not affected by cooling the system to below the temperature at which the membrane attains the gel state. This is in contrast to the decrease in conductivity observed when the temperature of the system is lowered in the presence of valinomycin. Valinomycin is known to act by a carrier mechanism. These experiments were performed using a black lipid film composed of a mixture of glycerides and decane and it is not clear whether the gramicidin is concentrated in a small liquid portion of the membrane or whether it is actually forming a pore through the gel state membrane. Therefore it was of interest to study the action of the pore-forming ionophore in pure gel-state phospholipid bilayers as obtained in liposome systems.

Using the finding that liposomes behave as ideal osmometers, both above and below the phase transition temperature [18], we have analysed the effect of gramicidin on water permeability in liposome systems derived from lecithins with different chain lengths. Earlier experiments reported by Cohen [19,20] have already indicated that the existence of gramicidin channels in gel state bilayers can be detected using this technique.

Materials and Methods

Egg phosphatidylcholine was purified from egg yolk by acetone precipitation and subsequent chromatography over aluminium oxide and silica gel. Saturated egg phosphatidylcholine was prepared by hydrogenation of unsaturated bonds. Egg phosphatidic acid was prepared from egg phosphatidylcholine by phospholipase D degradation. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine, 1,2-dipal-

mitoyl-*sn*-glycero-3-phosphocholine and 1,2-distearoyl-*sn*-glycero-3-phosphocholine were synthesized by Mrs. A. Lancée-Hermkens as described [22]. The purity of all lecithins was checked by thin-layer chromatography. Fatty acid impurities of synthetic lipids were less than 1% as shown by gas chromatography. Gramicidin A was obtained from Sigma and used without further purification. All reagents were of analytical grade.

Multilayered liposomes, containing 4 mol% egg phosphatidic acid, were prepared at temperatures above the gel to liquid crystalline phase transition temperature as previously described [22]. To obtain gramicidin-containing liposomes an appropriate amount of gramicidin dissolved in ethanol (1 mg/ml) was added to the lipid/chloroform solution. This solution was then handled as described for liposome preparations.

Differential scanning calorimetric analyses were performed on the various liposome systems using a Perkin Elmer DSC-2 B apparatus.

For the osmotic shrinkage experiments the liposomes were prepared in 10 mM glucose and diluted in this medium to a final lipid concentration of 0.30 mM. An aliquot (9.6 ml) of this suspension was transferred into a thermostatted cuvette and vigorously stirred. After temperature equilibration an osmotic shock was given by rapidly injecting 0.40 ml of 1.0 M glucose, preincubated at the same temperature. Changes in the turbidity were determined with a spectrophotometer (Vitatron, MPS type) at 438 or 450 nm. From the recorder tracings the relative initial shrinkage velocity, $d[1/A)/dt]\%$ was calculated as previously described [22]. The activation energies presented in this paper were all calculated by the least squares method from plots in which

$$\ln \frac{\frac{d(1/A)}{dt}\%}{T}$$

was plotted against $1/T$, T being the absolute temperature [22].

Results

Fig. 1 shows the osmotic shrinkage experiments performed with dipalmitoyl phosphatidylcholine liposomes without gramicidin (Fig. 1A) and with increasing amounts of gramicidin (Fig. 1B, C, D) as a function of temperature. In the absence of gramicidin there was a dramatic decrease in water permeation upon cooling below the phase transition temperature. However, in accordance with earlier evidence [18] there was a slow but clear osmotic response when the bilayers reached the gel state. Fig. 1B, C and D shows that the presence of relatively small amounts of gramicidin facilitated the osmotic shrinkage. The effect could be noted in particular below the phase transition temperature. This increase in shrinkage rate is dependent on the amount of gramicidin added. The sharp discontinuity observed in the dipalmitoyl phosphatidylcholine liposomes at the phase transition temperature was gradually lost as the amount of gramicidin in the liposomes was increased. At a molar ratio of gramicidin to lipid of 1 : 109 the discontinuity was no longer apparent (Fig. 1D).

In order to test the effect of gramicidin on the melting behaviour of the chains, differential scanning calorimetry (Fig. 2) was performed on liposomes

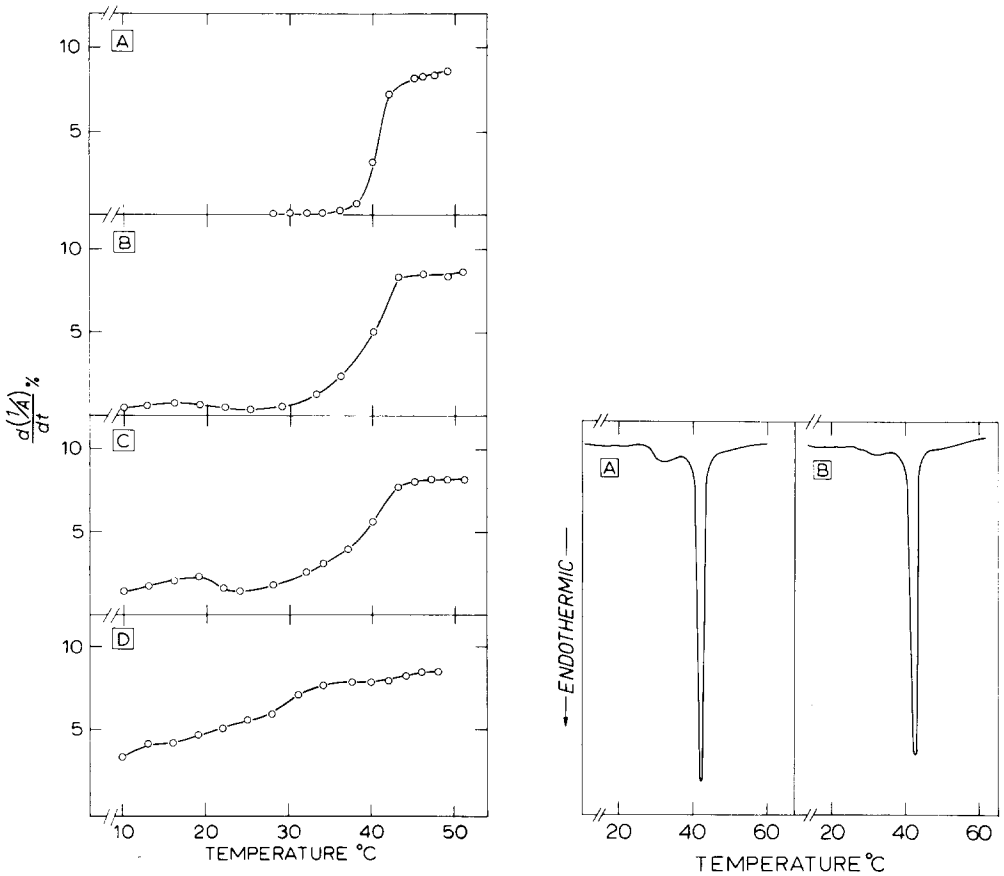

Fig. 1. Temperature dependence of the osmotic shrinkage of dipalmitoyl phosphatidylcholine liposomes following the addition of hypertonic glucose. The experiment was carried out as described in Materials and Methods with liposomes containing no gramicidin (A) and gramicidin to lipid in the molar ratio of 1 : 1054 (B); 1 : 217 (C); and 1 : 109 (D).

Fig. 2. Effect of small amounts of gramicidin on the phase transition of dipalmitoyl phosphatidylcholine liposomes. Differential scanning calorimetry was performed using liposomes prepared from dipalmitoyl phosphatidylcholine (A) and dipalmitoyl phosphatidylcholine in the presence of gramicidin (B). The ratio of gramicidin to lipid is 1 : 217. Both liposome systems were prepared in water (in the absence of glucose).

prepared from dipalmitoyl phosphatidylcholine in the presence and absence of the ionophore. A sharp transition at a temperature of 41°C was obtained with all liposome preparations tested. However, in agreement with earlier results [27], a slight reduction in transition enthalpy could be noticed. The enthalpy change was 9.2 kcal/mol for the pure lipid and 8.2 kcal/mol for a preparation to which gramicidin was added in a molar ratio of 1 : 217 lipid molecules.

The results of osmotic experiments obtained using dimyristoyl phosphatidylcholine and saturated egg phosphatidylcholine (not shown) were qualitatively the same as those shown for dipalmitoyl phosphatidylcholine liposomes. Dimyristoyl phosphatidylcholine liposomes required smaller amounts of gramicidin to obtain the same rate of water permeation shown for dipalmitoyl

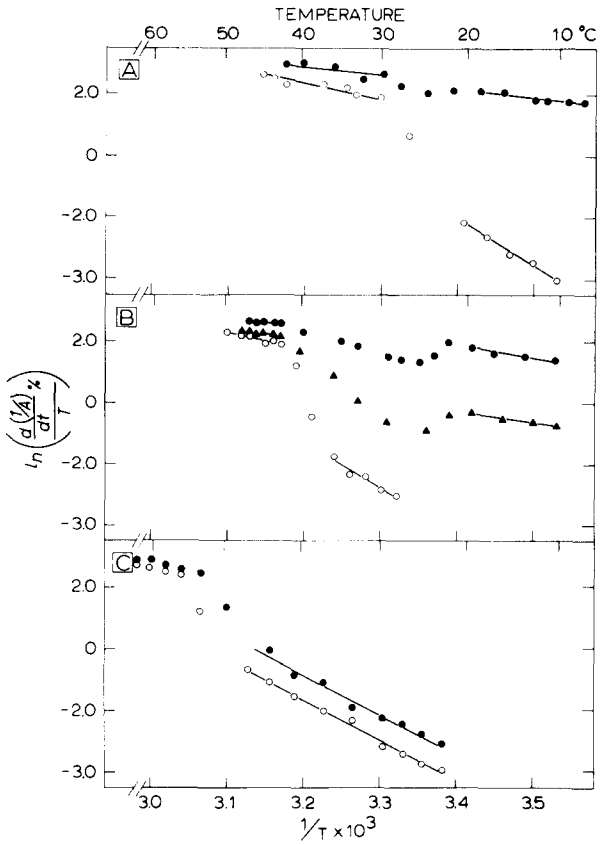

Fig. 3. Arrhenius plot of the osmotic shrinkage of various saturated liposome preparations; dimyristoyl phosphatidylcholine (A), dipalmitoyl phosphatidylcholine (B), distearoyl phosphatidylcholine (C). ○, liposomes prepared in the absence of gramicidin; ●, liposomes prepared in the presence of gramicidin in a gramicidin to lipid ratio of 1 : 217; ▲, gramicidin to lipid ratio of 1 : 1054.

phosphatidylcholine liposomes. On the other hand distearoyl phosphatidylcholine liposomes showed only a small increase in the rate of water permeation below the phase transition temperature in the presence of gramicidin. This increase was slight in comparison to the rates obtained with shorter chained phosphatidylcholine liposomes containing equal amounts of gramicidin.

Arrhenius plots of the rate of water permeation in saturated liposome systems in the presence and absence of gramicidin are shown in Fig. 3. In each case studied the plots show the sharp increase in the rate of water permeation at the phase transition in the absence of gramicidin. In liposomes prepared in the presence of gramicidin there was an increase in the rate of water transport both above and below the phase transition temperature in dimyristoyl phosphatidylcholine liposomes (Fig. 3A), dipalmitoyl phosphatidylcholine liposomes (Fig. 3B) and saturated egg phosphatidylcholine liposomes (not shown). However, the increase in rate was only slightly apparent in the distearoyl phosphatidylcholine liposomes (Fig. 3C).

Further information on the effect of gramicidin on water permeation was

TABLE I

ACTIVATION ENERGY FOR THE PERMEATION OF WATER THROUGH THE BILAYERS OF SATURATED PHOSPHATIDYLCHOLINE LIPOSOMES WITH AND WITHOUT GRAMICIDIN IN A RATIO OF 1 GRAMICIDIN MOLECULE TO 217 LIPID MOLECULES

Phosphatidylcholine species	Below the phase transition			Above the phase transition		
	Temperature range	Activation energy [a]		Temperature range	Activation energy [a]	
		without gramicidin	with gramicidin		without gramicidin	with gramicidin
Dimyristoyl phosphatidylcholine	10—20	28.3 ± 2.5 (2) [b,c]		30—45	11.6 ± 1.0 (2)	5.0 ± 1.0 (2)
	8—20		4.3 ± 0.8 (4)			
Dipalmitoyl phosphatidylcholine	28—37	28.1 ± 2.1 (2)		43—50	10.9 ± 0.8 (2)	6.1 ± 1.1 (4)
	10—20		7.0 ± 0.7 (6)			
Saturated egg phosphatidylcholine	30—43	25.8 ± 1.1 (2)				
	10—20		7.7 ± 0.6 (2)			
Distearoyl phosphatidylcholine	21—47	24.9 ± 2.0 (2)	24.5 ± 2.4 (2)			

[a] Activation energies are expressed as kcal/mol and were obtained as described in the text.

[b] The values are expressed as ±S.D.

[c] The numbers in parentheses are the number of times each experiment was performed.

obtained by calculating the activation energy for water permeation from the slopes of the lines indicated in Fig. 3. To determine the activation energies shown in Table I, lines were drawn through the linear portion of the curve obtained when the rate of osmotic shrinkage was plotted as a function of the inverse of the absolute temperature. In the case of dimyristoyl and dipalmitoyl phosphatidylcholine liposomes-containing gramicidin there was a striking decrease in the activation energy upon increasing the temperature above 20° C. Therefore, the activation energies were calculated from the slopes of lines drawn through points obtained below 20° C.

Discussion

To describe the diffusion of water and small non-electrolyt molecules through closed lipid bilayers it has been suggested [24] that mobile kinks arising from gauche-*trans*-gauche conformations in the paraffin chains act as intrinsic carriers for the movement through the hydrophobic barrier. In agreement with such a view, which implies that single permeant molecules are isolated in the non-polar membrane interior, the activation energies of the membrane diffusion processes of these small polar molecules are found to be relatively high. The actual values are clearly related to the number of hydrogen bonds the permeant molecule has in the water phase [25]. For the diffusion of water molecules the activation energy is 11 kcal/mol and this value has been found to be independent of the lipid composition in studies of a large variety of liquid crystalline liposome systems [26]. Only when the chain mobility is strongly reduced by specific lipid-cholesterol interactions or by transition from the liquid-crystalline to the gel state is the activation energy increased to even higher values (25—28 kcal/mol). This increase in activation energy may indicate that under these conditions the formation or mobility of the kinks becomes highly temperature dependent [27]. The present results show that the introduction of small amounts of gramicidin in the gel state bilayers of liposomes prepared from dimyristoyl and dipalmitoyl phosphatidylcholine causes a large increase in the water permeability while also causing a dramatic decrease in the temperature dependency of the process. The activation energies which can be calculated from the water permeability through the gramicidin containing membranes are significantly below the values obtained for the liquid crystalline bilayers (compare Table I). We take these facts as support for the existance of functional gramicidin channels through which water molecules can permeate without complete deprivation of hydrogen bonding.

On the other hand the absence of a decrease in activation energy and a much more limited increase in the rate of waterflow when gramicidin is added to distearoyl phosphatidylcholine liposomes are strong arguments that in this longer chained system no water conducting channels are formed. The slight increase in water permeability which still can be noted may be explained by a slight perturbation of the hydrocarbon chains. Such a perturbation is indicated by small changes in the enthalpy change of the transitions and also by the results of Raman studies on these systems [27].

The finding that gramicidin forms conducting channels in the gel state bilayers of dimyristoyl and dipalmitoyl phosphatidylcholine bilayers but not in

those of distearoyl phosphatidylcholine indicates that the thickness of the rigid hydrophobic core is of vital importance for the formation of such a channel. The thickness of the paraffin layer may also explain the shape of the Arrhenius curve as shown in Fig. 2B. Very remarkable is the decrease in the water permeation rate with increasing temperature. However, the temperature region where this decrease in permeation occurs corresponds to the same region where there is an increase in bilayer thickness due to a change in the tilt of the paraffin chains which has been shown by X-ray analyses on these systems [28]. A reduction in the number of conducting channels due to an increasing membrane thickness may explain the negative temperature effect, which can be noted in the temperature range of 20—30°C. Using planar liquid membranes an effect of membrane thickness on the stability of gramicidin channels has been found as well [31,32]. Although in these experiments the variations in (mean) thickness were considerably larger, the effects are less pronounced, indicating that in fluid membranes the lipid molecules have some ability to accommodate the size of the gramicidin channel.

Summarizing we conclude that the large increase in the liposomal water permeability combined with a striking decrease in the activation energy of this process supports the view that the gramicidin molecules have the ability to form channels also in the gel state bilayer. Taking into account the detailed dimensions of the phospholipid bilayer structure [28,29] it is conceivable, that dimeric channels with a length of about 30 Å can span the rigid core of the gel state bilayers of dimyristoyl phosphatidylcholine but are too short to do so in the comparable bilayers of distearoyl phosphatidylcholine. From the observations on the systems of dipalmitoyl phosphatidylcholine it appears that with this gel state bilayer we reach the critical thickness allowing the gramicidin molecules to form conducting channels.

Acknowledgement

One of the authors (B.A.B.) would like to acknowledge the support of the Cystic Fibrosis Foundation during the course of this research.

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