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Molecular aspects of the bilayer stabilization induced by poly(L-lysines) of varying size in cardiolipin liposomes

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The interaction between poly(L-lysines) of varying size with cardiolipin was investigated via binding assays, X-ray diffraction, freeze-fracture electron microscopy, and ^{31}P- and ^{13}C-NMR. Binding of polylysines to the lipid only occurred when three or more lysine residues were present per molecule. The strength of the binding was highly dependent on the polymerization degree, suggesting a cooperative interaction of the lysines within the polymer. Upon binding, a structural reorganization of the lipids takes place, resulting in a closely packed multilamellar system in which the polylysines are sandwiched in between subsequent bilayers. Acyl chain motion is reduced in these liquid-crystalline peptide-lipid complexes. From competition experiments with Ca^{2+} it could be concluded that when the affinity of the polylysine for cardiolipin was much larger than that of Ca^{2+}, a lamellar polylysine-lipid complex was formed, irrespective of whether an excess of Ca^{2+} was added prior to or after the polypeptide. When the affinity of the polylysine for cardiolipin was less or of the same order as that of Ca^{2+}, the lipid was organized in the hexagonal H_{II} phase in the presence of Ca^{2+}. These results are discussed in the light of the peptide specificity of bilayer (de)stabilization in cardiolipin model membranes.

Introduction

A considerable part of the protein component of biological membranes is only loosely associated with the membrane, mainly via electrostatic interactions with either lipids or other membrane proteins. These so-called extrinsic membrane proteins play important functional and structural roles, of which cytoskeleton-membrane associations are clear examples.

To obtain insight into the molecular aspects of the interactions between both extrinsic and water-soluble proteins with the lipid part of membranes, often well-characterized synthetic polymers of charged amino acids have been used in conjunction with model membranes. Poly(L-lysine) in particular has been popular. This highly basic (pK 9–10) linear polymer of L-lysine experiences strong electrostatic interactions with negatively charged phospholipids [1–5], resulting in an increase in temperature [1,5] and heat content [1] of the gel → liquid-crystalline phase transition. The interaction can result in vesicle fusion [6], and, in mixed lipid systems, can lead to lateral phase separations [7], resulting in the formation of ordered poly(L-lysine)-lipid domains [7,8]. Some authors claim that the polypeptide undergoes a coil → helix transition upon interaction with these lipids [9]; others assume that a coil structure is maintained [10].

Only recently, it was found by ^{31}P-NMR that poly(L-lysine) can greatly affect the polymorphism of negatively charged lipids containing model

membranes [11]. $(Polylysine)_{200}$ * acts as a bilayer stabilizer for cardiolipin in that it inhibits the ability of Ca^{2+} to induce the hexagonal H_{II} phase for this lipid. Alternatively, the polypeptide induces this phase for a fraction of the lipids in mixed phosphatidylethanolamine/cardiolipin dispersions. These structural changes are polypeptide-specific, in that other basic water-soluble polypeptides such as cytochrome *c* [12], apocytochrome *c* [13] and cardiotoxin [14] can cause a bilayer destabilization in cardiolipin dispersions. These differences in polypeptide-lipid interactions must result from differences in size, structure, amphipathicity, and/or charge of these polypeptides (see Ref. 15 for review).

To obtain a better molecular understanding of the specificity of the $(polylysine)_n$-cardiolipin interaction and its implications for the Ca^{2+}-induced polymorphism of the lipid, we characterized in this paper the peptide-cardiolipin interaction by binding experiments, small-angle X-ray diffraction, ^{31}P- and ^{13}C-NMR, and freeze-fracture electron microscopy using $(polylysines)_n$ of different size.

Materials and Methods

Materials

Poly(L-lysines) hydrobromides with polymerization degrees of 1, 2, 3 and 5, were obtained from Serva (Heidelberg, F.R.G.), and with polymerization degrees of 20, 100, 170 and 1600, from Sigma (St. Louis, U.S.A.). The molecular weights of these polylysines are 200, 400, 600, 1000, 4000, 20000, 34000 and 320000, respectively. The sodium salt of bovine heart cardiolipin was obtained from Avanti (Birmingham, U.S.A.) $^{45}Ca^{2+}$ was obtained from The Radiochemical Centre (Amersham, U.K.) as the chloride in water.

Methods

Cardiolipin liposomes were prepared by dispersing at room temperature a dry film of this lipid in 100 mM NaCl, 10 mM Tris-HCl, pH 7.0, buffer with or without polylysines by vortex mixing. Unless otherwise indicated, this buffer was used in all experiments. The amount and concentration of polylysines is always expressed on the basis of the number of constituent lysines. Thus, 1 mM $(polylysine)_n$ refers to a concentration of 1 mM of lysine. All experiments were carried out at 20°C.

Binding experiments. Three types of binding experiments were performed:

(a) Polylysine binding to cardiolipin liposomes. In a total volume of 600 μl buffer, 0.25 μmol cardiolipin (0.5 μmol cardiolipin phosphate), and 0–5 μmol polylysine (either added to preformed liposomes or present in the hydration solution) were incubated for 30 min, after which the liposomes were pelleted by centrifugation at $25000 \times g$ for 20 min. The cardiolipin and polylysine concentrations of the supernatant were determined, from which the amount of polylysine bound to the liposomes could be calculated. In no case was there any detectable amount of cardiolipin present in the supernatant.

(b) Effect of Ca^{2+} on the polylysine binding to cardiolipin liposomes. This experiment was conducted with $(polylysine)_{170}$ in a fashion similar to that described under (a), except that either 10 μl of 0.5 M $CaCl_2$ were added before the polylysine addition, or 0–25 μl 0.5 M $CaCl_2$ were added after the polylysine addition.

(c) Effect of polylysine on the $^{45}Ca^{2+}$ binding to cardiolipin liposomes. This experiment was carried out as described under (a), except that first 10 μl of a 0.5 M $^{45}Ca^{2+}$ (24000 cpm/μl) were added to the liposomes. After 10 min incubation, the hexagonally H_{II} phase organized Ca^{2+}-cardiolipin (1 : 1) complex was centrifuged for 10 min at $25000 \times g$, whereafter the supernatant was removed. Increasing amounts of a $(polylysine)_{170}$ solution were added to the pellet to a final volume of 600 μl. After 24 h incubation and subsequent centrifugation, the amount of $^{45}Ca^{2+}$ in the supernatant was determined.

Freeze-fracture electron microscopy. Pelleted stoichiometric $(polylysine)_n$-cardiolipin complexes were investigated by freeze-fracturing, using 30 vol% of glycerol as a cryoprotectant [16].

Small-angle X-ray diffraction. Small-angle X-ray diffraction of pelleted stoichiometric $(polylysine)_n$-cardiolipin complexes was performed on a Kratky camera as described before [17].

^{31}P-NMR. Broad-band proton noise-decoupled

* The poly(L-lysines) will be abbreviated as $(polylysines)_n$, in which *n* refers to the degree of polymerization.

^{31}P-NMR spectra were recorded at 36.4 MHz on a Bruker WH90 as described before [18]. Typically, 25 μmol of cardiolipin with or without polylysine or Ca^{2+} were present in 1.0 ml of the buffer, which contained in addition 20 vol% $^{2}H_2O$. In mixed 'bilayer/hexagonal' spectra, the fraction of the 'hexagonal' spectral component was determined by computer subtraction using the lineshape of the hexagonally H_{II} organized Ca^{2+}-cardiolipin (1 : 1) complex as a reference.

^{13}C-NMR. Partial proton-decoupled (broad-band 1 W input power) natural-abundance 50.32 MHz ^{13}C-NMR spectra were obtained on a Bruker WP200 spectrometer. 20 000–80 000 transients were recorded, using 18 μs 90° rf pulses, a 10 kHz sweep width and 8K data points. Chemical shifts are reported with respect to the value of the terminal $-CH_3$ group in cardiolipin.

Analytical methods. Cardiolipin was determined by a phosphorus determination [19] after perchloric acid destruction. The $(polylysines)_n$ with $n \geq 2$ were determined according to Lowry et al. [20]. Since the molar extinction coefficient depends on n, always weighted amounts of the individual polylysines were taken as standards. Free lysine ($n = 1$) was determined via a ninhydrin method [35]. $^{45}Ca^{2+}$ was determined by liquid scintillation counting.

Results

$(Polylysine)_n$-cardiolipin binding

When added to cardiolipin liposomes, the $(polylysines)_n$ cause immediate visual precipitation of the lipids when $n > 2$. The precipitation is accompanied by binding of the peptide to the lipid, as shown in Fig. 1. The binding increases sharply with n, in particular in the $n = 2$ to $n = 5$ range, suggesting that the binding is cooperative in terms of the number of lysine residues per molecule. The binding curves were analyzed in terms of the binding model given in the legend of Table I. Due to the low binding in the case of $n = 3$ no reliable binding constants could be calculated. It is obvious from the data presented in this table that the dissociation constant K_d is highly dependent on n and can reach very low values in the case of the larger polymers. Interestingly, the stoichiometry of the interaction, e.g., N the number of lipid molecules providing one $(polylysine)_n$ binding site, follows closely the value calculated assuming a stoichiometric electrostatic interaction. This sug-

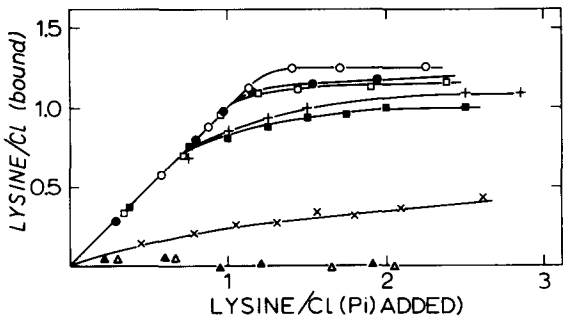

Fig. 1. Relationship between polymerization degree of $(polylysine)_n$ and the binding of the peptide to cardiolipin (CL) liposomes. $(Polylysines)_n$ used: $n = 1$ (▲), 2 (△), 3 (×), 5(+), 20 (■), 100 (□), 170 (●) and 1600 (○). The binding experiment was carried out as described in the Materials and Methods section. The amount of $(polylysine)_n$ is given as the amount of lysine monomer per cardiolipin phosphate.

TABLE I

BINDING CHARACTERISTICS OF $(POLYLYSINES)_n$ TO CARDIOLIPIN LIPOSOMES

The dissociation constant K_d and the number (N) of lipid molecules (L) that provide one binding site for the protein molecule P, were calculated from the binding curves shown in Fig. 1 and $K_d = P\,L_N/PL_N$, using an iterative non-linear regression method [21]. N calc. is the number of cardiolipin molecules bound per polylysine molecule assuming a stoichiometric electrostatic interaction.

n	K_d (μM)	Relative error in K_d (%)	N	Relative error in N (%)	N calc.
5	182	12	4.3	5	2.5
20	1.49	2	9.8	32	10
100	0.060	29	44	1	50
170	0.035	36	73	1	85
1600	0.00052	23	636	1	800

TABLE II

ACCESSIBILITY OF LIPOSOMAL CARDIOLIPIN FOR (POLYLYSINE)$_n$

The (polylysine)$_n$ was added either to preformed cardiolipin liposomes (external) or was present in the buffer used to hydrate the dry cardiolipin film (total). The lysine/cardiolipin ratios given refer to the number of lysine residues present per cardiolipin phosphate.

n	(lysine/cardiolipin) added	(lysine/cardiolipin) bound	
		external	total
170	1.5	1.2	1.0
1600	1.2	1.1	1.0

gests that all lysine residues in the oligomer interact with the cardiolipin phosphates, and in addition, that all cardiolipin molecules are accessible to the externally added (polylysine)$_n$. This latter point was proven by comparing the binding data obtained via the external addition method to those obtained from experiments in which the dry cardiolipin film was hydrated with a (polylysine)$_n$ solution (Table II). From the similarity in binding, it can be suggested that the polylysine addition to preformed cardiolipin liposomes results in a structural rearrangement of the lipids such that all cardiolipin molecules become available for interaction with the polypeptide.

Structural characteristics of (polylysine)$_n$-cardiolipin complexes

That the (polylysine)$_n$ addition to a cardiolipin dispersion results in a structural reorganization of the lipids is clearly born out by small-angle X-ray diffraction experiments. In agreement with previous data [18], a cardiolipin dispersion in 100 mM NaCl, pH 7.0, buffer only gives rise to a broad scattering profile and no sharp diffraction bands (Fig. 2A), which must be due to a lack of order and/or the large swelling of dispersions of negatively charged lipids [22]. In contrast, for $n > 3$ the stoichiometric (polylysine)$_n$-cardiolipin complex displayed sharp diffraction bands, as exemplified in Fig. 2B for the (polylysine)$_{20}$-cardiolipin system. For all (polylysine)$_n$-cardiolipin complexes a strong and a much weaker reflection were observed, which d values relate as 1 : 0.5, typical for

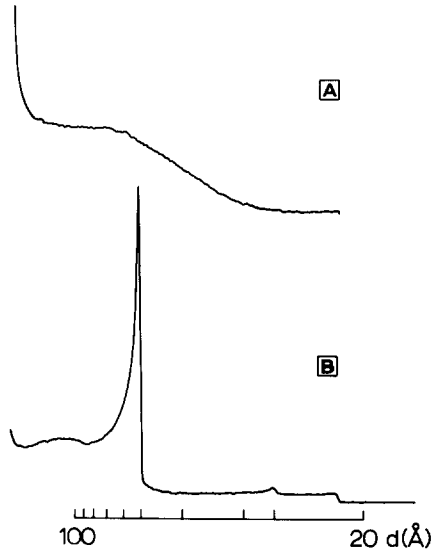

Fig. 2. Small-angle X-ray diffraction profiles of a cardiolipin dispersion (A) and the (polylysine)$_{20}$-cardiolipin complex (B). Pelleted samples were used. The polylysine was added in a 2-fold stoichiometric excess over cardiolipin.

a first- and second-order reflection of a multilameller organization (Table III). In no case did the polylysine-cardiolipin complexes display a $\frac{1}{\sqrt{3}}$ reflection typical of a hexagonal organization, as found for instance in case of the Ca^{2+} salt of cardiolipin (Table III and Refs. 18, 23).

The freeze-fracture morphology of the stoichiometric (polylysine)$_{20}$-cardiolipin complex was typical of that of a closely packed multilamellar lipid organization in that extended smooth fracture faces and closely spaced cross features were observed (data not shown). In the absence of the polypeptide, vesicle-type structures were observed with smooth fracture faces and virtually no cross features, consistent with a large separation between subsequent lipid bilayers.

The proton-decoupled ^{31}P-NMR lineshape of all stoichiometric (polylysine)$_n$-cardiolipin complexes had the asymmetrical lineshape depicted in Fig. 1B of Ref. 11 for (polylysine)$_{200}$. Such a lineshape with a high-field peak and a low-field shoulder is typical of an organization of the lipids in extended bilayers in which the chemical shift anisotropy of the ^{31}P-NMR nucleus is only partially averaged by rapid long-axis rotation [24]. This lineshape was maintained when the poly-

TABLE III

SMALL-ANGLE X-RAY DIFFRACTION DATA ON $(POLYLYSINE)_n$-CARDIOLIPIN COMPLEXES AND THE HEXAGONALLY ORGANIZED Ca^{2+}-CARDIOLIPIN COMPLEX

Pelleted samples were used in which $(polylysine)_n$ or Ca^{2+} was added in a 2-fold stoichiometric excess over cardiolipin. The relative intensities of the reflections are indicated as strong (s), medium (m) and weak (w). The values in brackets indicate the relationship between first- and higher-order reflections. n.d., not detectable.

$(Polylysine)_n$	d value (Å) of the jth order reflection		
	$j=1$	$j=2$	$j=3$
$n=$ 5	53 (s) [1]	27 (w) [2]	n.d.
20	52 (s)	25.5 (w)	n.d.
100	53 (s)	25.4 (w)	n.d.
170	52 (s)	26 (w)	n.d.
1600	52 (s)	26.3 (w)	n.d.
Ca^{2+}/cardiolipin (1 : 1)	55.8 (s) [1]	32.4 (m) $[\frac{1}{\sqrt{3}}]$	27 (m) $[\frac{1}{2}]$

lysines were added up to a 6-fold molar excess. Also the addition of 3 M NaCl which can trigger a lamellar $\rightarrow H_{II}$ transition of the pure cardiolipin [25] did not affect the ^{31}P-NMR lineshape of the stoichiometric $(polylysine)_{170}$-cardiolipin (1 : 1) complex. From the combination of the X-ray diffraction, freeze-fracture electron microscopy and ^{31}P-NMR results, we can conclude that all polylysine-cardiolipin complexes are organized in lamellar systems.

Interestingly, the interbilayer repeat distance, which corresponds to the d value of the first-order reflection, is independent of n for $5 \leqslant n \leqslant 1600$ (Table III), suggesting a very similar organization for these complexes. The $(polylysines)_n$ must be very tightly packed inbetween the subsequent bilayers, since the first-order repeat distance of 52 Å is close to the value of 50 Å reported for the lamellar cardiolipin system in the presence of high (> 3 M) salt [25]. In the case of the stoichiometric multilamellar complex between cardiolipin and the positively charged adriamycin molecule, the repeat distance is already 64 Å [26], which value further increases for other water-soluble basic polypeptides (see Discussion).

Motional characteristics of $(polylysine)_n$-cardiolipin complexes

The residual chemical shift anisotropy, $\Delta\sigma$, measured as the distance from the high-field peak and low-field shoulder in the ^{31}P-NMR spectrum, is a measure of the local order of the phosphate region of the phospholipid molecule [24]. In the case of pure cardiolipin, the $\Delta\sigma$ is 30 ppm, in agreement with previous data [18,27]. The addition of up to a 6-fold molar excess of the various $(polylysines)_n$ did not affect $\Delta\sigma$. Together with the lack of any broadening of the spectra, this demonstrates that the local order of the phosphate group in cardiolipin is not affected by the electrostatic interaction with the lysine residues and furthermore indicates that this group still experiences fast axial rotation ($\tau_c < 10^{-5}$ s), typical of a liquid-crystalline lamellar system.

Information on the motional characteristics of the fatty acyl chains was obtained by natural abundance ^{13}C-NMR. This technique, when applied to aqueous dispersions, can give useful qualitative information on the motional properties of the various carbon atoms which give rise to resolved resonances [28,29]. Fig. 3A shows the spectrum of a cardiolipin dispersion. The resonances are assigned using literature data on related lipid systems [28,30]. From low to high field resonances from carbons in the carbonyls (160 ppm), double bonds (116–118 ppm), glycerol moieties (± 50 ppm), various methylene chains (10–20 ppm) and the methyl groups (0 ppm), are respectively resolved. The good resolution in the 10–20 ppm region is due to the high linoleic acid content of bovine heart cardiolipin. All six carbons of lysine are well-resolved in a solution of $(polylysine)_{170}$ in

buffer (Fig. 3B). Addition of a 2-fold molar excess of $(polylysine)_{170}$ to the cardiolipin dispersion has two pronounced effects. Firstly, all lipid resonances broaden dramatically, demonstrating a reduction in motion of the lipid carbons. This is also apparent in the acyl chains where the linewidth of the high-field double-bond carbon resonance and the methyl resonance increase from 40 and 22 Hz to 88 and 48 Hz, respectively. Since under these experimental conditions the entire carbon spectrum of the lipids is lost when the molecules enter a gel state [31], we can conclude that in the $(polylysine)_{170}$-cardiolipin complex the acyl chains remain liquid-crystalline, but have a decreased motion as compared to the pure cardiolipin system. A quantitative interpretation of these linewidth changes in terms of changes in rate of motion and/or change in order is not possible, due to the complexity of the various factors determining the ^{13}C-NMR linewidths.

The second effect observed in the ^{13}C-NMR spectra of this $(polylysine)_{170}$-cardiolipin mixture (Fig. 3C) is that the intensities of the polylysine resonances are decreased. For instance, the intensity of the C-2 resonance relative to that of the Tris peak (which serves as an internal standard) decreases from 3.5 (Fig. 3B) to 1.7 (in Fig. 3C). This, together with the observation that at substoichiometric concentrations of polylysine no lysine signals are observed, demonstrates that due to the $(polylysine)_{170}$-cardiolipin binding approx. 50% of the polylysine signal (originating from 50 μmol polylysine) is broadened beyond detection upon interaction with the 25 μmol cardiolipin. This analysis can be made with similar results for the C_6 resonance of the lysine moiety. Apparently, in the bound state the $(polylysines)_{170}$ are highly immobilized. The linewidths of the resonances of the excess (non-bound) polylysine were found to be unaffected by the presence of the stoichiometric $(polylysine)_{170}$-cardiolipin complex, demonstrating that no rapid exchange between bound and free polylysine occurs.

Interrelationships between Ca^{2+} and polylysine in their interaction with cardiolipin

In a previous paper [11] we observed that the presence of $(polylysine)_{200}$ prevented the formation of a hexagonal H_{II} phase in the presence of a 2-fold excess of Ca^{2+}. To obtain insight into the details of this bilayer stabilization, we now investigated both the effect of Ca^{2+} on different $(polylysine)_n$-cardiolipin complexes of varying n and the effect of these polylysines on the hexagonally organized Ca^{2+}-cardiolipin complex. Using ^{31}P-NMR we quantified the fraction of bilayer and H_{II} present in $(polylysine)_n$-cardiolipin complexes in the presence of increasing amounts of Ca^{2+} (Fig. 4). In agreement with previous data [18,27], the addition of a stoichiometric amount of Ca^{2+} triggers the bilayer $\rightarrow H_{II}$ transition of pure cardiolipin, whereas the presence of $(polylysine)_{170}$

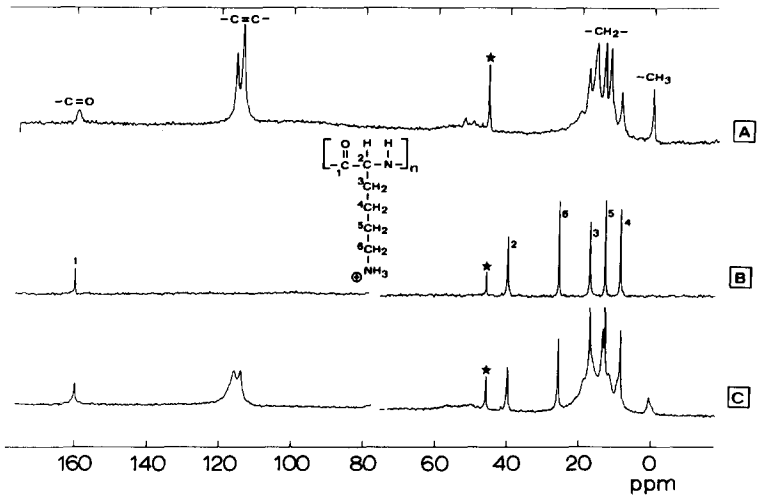

Fig. 3. Effect of $(polylysine)_{170}$ addition on the 50.32 MHz natural-abundance ^{13}C-NMR spectrum of cardiolipin liposomes. (A) 25 μmol cardiolipin in 1.0 ml buffer, (B) 100 μmol $(polylysine)_{170}$ in 1.0 ml buffer, (C) 25 μmol cardiolipin in 1.0 ml buffer incubated with 100 μmol $(polylysine)_{170}$ in 1.0 ml for 30 min. The asterisk indicates the resonance from Tris in the buffer.

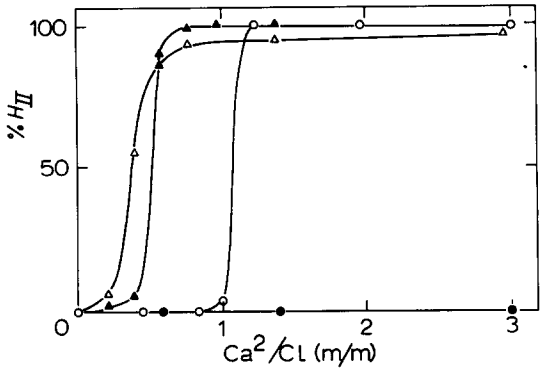

Fig. 4. Effect of Ca^{2+} on the structure of $(polylysine)_n$-cardiolipin (CL) complexes. 25 μmol cardiolipin were dispersed in 1.0 ml buffer containing 20% ^2H_2O. 100 μmol $(polylysine)_n$ were added in 100 μl buffer, followed by a 10 min incubation time after which aliquots of a 1 M $CaCl_2$ solution were added to give the indicated Ca^{2+}/cardiolipin ratios. The ^{31}P-NMR spectra were recorded 1 h after the Ca^{2+} addition, after which the amount of H_{II} signal in the spectrum was determined. Cardiolipin in the absence (○——○) and presence of $(polylysine)_n$: n = 3 (▲——▲), 5 (△——△), and 170 (●——●).

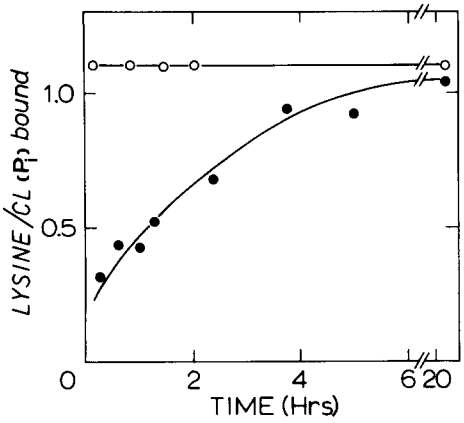

Fig. 5. $(Polylysine)_{170}$ binding to the Ca^{2+}-cardiolipin (CL) complex. 0.5 μmol cardiolipin was incubated with (●) or without (○) 10 μl 0.5 M $CaCl_2$ in a total volume of 600 μl for 10 min after which 1.5 μmol $(polylysine)_{170}$ was added and the mixture was further incubated. At particular times, samples were taken for which the $(polylysine)_{170}$ binding to the cardiolipin was determined. The 10 min incubation time with Ca^{2+} is included in the graph.

stabilizes bilayer structure up to a Ca^{2+}/cardiolipin molar ratio of 3. It can thus be concluded that the $(polylysine)_{170}$-cardiolipin interaction is stronger than the Ca^{2+}/cardiolipin interaction. However, this is highly dependent on the size of the polylysine molecule. Decreasing the size to $n = 3$ and $n = 5$, thereby decreasing the strength of the $(polylysine)_n$-cardiolipin interaction (Table I), reverses the effect in that H_{II} phase formation is already observed below stoichiometric amounts of Ca^{2+}. Most likely these small polylysines act in a cooperative manner with Ca^{2+} in terms of H_{II} phase formation, and are incorporated into the H_{II} phase.

We next studied the effect of $(polylysine)_{170}$ addition on the hexagonally organized Ca^{2+}-cardiolipin salt. Fig. 5 shows that $(polylysine)_{170}$ slowly binds to the lipids with a halftime of approx. 1.2 h, until a level is reached which is similar to that immediately obtained in the absence of Ca^{2+}. This experiment suggests that Ca^{2+} is dissociated from the Ca^{2+}-cardiolipin complex upon the interaction of the polypeptide with the lipids. That this indeed is the case is demonstrated in Fig. 6, using $^{45}Ca^{2+}$ binding experiments. Whereas in the absence of added $(polylysine)_{170}$ a stoichiometric interaction with cardiolipin occurs, increasing amounts of the peptide gradually displace $^{45}Ca^{2+}$ from its lipid binding sites until eventually virtually no $^{45}Ca^{2+}$ binding occurs anymore, suggesting that no negative charges are present in the $(polylysine)_{170}$-cardiolipin complex. This process is accompanied by a change in structure. In a typical

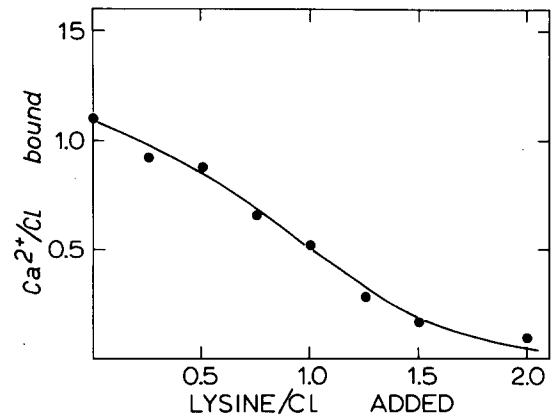

Fig. 6. $(Polylysine)_{170}$ induced release of $^{45}Ca^{2+}$ from the Ca^{2+}-cardiolipin complex. Increasing amounts of $(polylysine)_{170}$ were added to the hexagonally organized Ca^{2+}-cardiolipin complex. After 24 h of incubation, the $^{45}Ca^{2+}$ was determined in the supernatant after pelleting the lipid. Details are given in the Materials and Methods section. The release of the $^{45}Ca^{2+}$ binding is approx. 25%.

^{31}P-NMr experiment in which the time dependency of the structure change induced by the addition of 150 μmol poly(L-lysine)$_{170}$ to 1.0 ml buffer containing 25 μmol cardiolipin and 30 μl 1 M $CaCl_2$ was followed, 0, 20, 60 and 90% of the lipids were organized in bilayers, 0, 0.5, 3 and 24 h after the addition of the peptide, respectively.

Summarizing discussion

The experiments presented in this paper, together with the literature data, allow a molecular description of the events taking place when a polylysine molecule interacts with cardiolipin in an aqueous dispersion. The first step in the interaction is the electrostatic binding of the peptide to the cardiolipin phosphates present in the outermost monolayer of the liposomes. This binding is highly cooperative in terms of the number of lysine residues per (polylysine)$_n$ molecule. Whereas for the mono- and dimer of lysine virtually no binding could be detected, significant binding for the trimer and higher oligomers was observed. For n equals 5 or more, the binding results in a (transient) disruption of the outermost bilayer, allowing the polypeptide to reach additional lipid binding sites, until eventually by a process of bilayer rupture and unsealing, most likely involving fusion of the liposomes (lipid mixing) as reported by Gad for cardiolipin-containing mixed-lipid systems [6], a complex is formed between the polypeptide and the cardiolipin in which the number of positively charged lysine residues matches the number of negatively charged phosphates. The dissociation constant of these stoichiometric (polylysine)$_n$-cardiolipin complexes decreases sharply with n up to levels in the nM range for $n = 1600$, the largest oligomer tested.

The organization of the stoichiometric (polylysine)$_n$-cardiolipin complexes is schematically depicted in Fig. 7. Independent of n (for $n \geq 5$), an interbilayer surface location of the polylysines is proposed, leading to a very closely packed multilamellar system. In view of the fixed stoichiometry, very small interbilayer repeat distance (53 Å) and absence of negative charge in the polylysine-cardiolipin complex, we suggest that lysine sidechains in the oligomer interact in an alternating way with opposing cardiolipin monolayers. Analysis of space-filling models revealed that this is sterically possible.

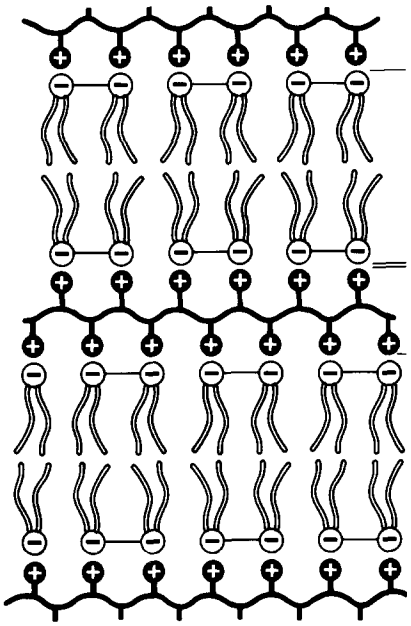

Fig. 7. Schematic representation of the stoichiometric (polylysine)$_{n \geq 5}$-cardiolipin multilamellar complex.

The ^{13}C-NMR experiments demonstrated that in the liquid-crystalline (polylysine)$_n$-cardiolipin complex the acyl chains undergo more restricted motion as compared to the peptide-free system. Electron spin resonance experiments on bovine brain phosphatidylserine model membranes, doped with 1% (w/w) of phosphatidylglycerol molecules carrying at the 2 acyl chain spin-label groups at positions 5 and 12, also revealed a reduction of acyl chain motion caused by the addition of (polylysine)$_{170}$ (A. Rietveld and D. Marsh, unpublished observations). This reduction in chain motion most likely is the result of a closer packing of the lipid molecules caused by the electrostatic interaction of the polypeptide.

It is of interest to compare the structural characteristics of the polylysine-cardiolipin complexes with those of other stoichiometric (based on net charge) water-soluble basic polypeptide-cardiolipin recombinants. Whereas (polylysine)$_n$ can be marked as a profound bilayer stabilizer, the addition of cytochrome c [12], its haem-free biosynthetic precursor apocytochrome c [13] and the snake venom cardiotoxin [14] to cardiolipin results

not only in closely packed lamellar systems, but also in lipid structures which give rise to isotropic ^{31}P-NMR signals, particulate freeze-fracture faces resembling inverted micellar structures, and hexagonal H_{II} phase (for cytochrome *c*), suggesting a bilayer destabilization. We propose that the ability of $(polylysine)_n$ to pack in a tight sheet-like manner between lipid bilayers is the determining factor causing the bilayer stabilization.

Besides the more amphipathic nature of the other polypeptides, they either are more structured in a three-dimensional manner (cytochrome *c* [32], cardiotoxin [33]), or they penetrate into the lipid bilayer (apocytochrome *c* [34], and cardiotoxin [14]). Interestingly, the repeat distance of the lamellae present in the stoichiometric apocytochrome *c*-cardiolipin complex is 97 Å (A. Rietveld, unpublished observations). In the presence of low amounts of cardiotoxin, the limiting multilamellar repeat distance was found to be 68 Å [14]. Both values are substantially larger than the 53 Å value observed in this study for the complex with polylysine, suggesting the presence of substantial amounts of apocytochrome *c* and cardiotoxin between the bilayers. Precise interpretation of the differences between the various polypeptides in their interaction with cardiolipin will await more detailed structural studies.

The interrelationship of $(polylysine)_n$ and Ca^{2+} in their interaction with cardiolipin can be understood in terms of the structure of these complexes with cardiolipin (polylysine-cardiolipin bilayer, Ca^{2+}-cardiolipin H_{II} phase) and relative binding affinity for cardiolipin. The K_d for Ca^{2+} binding to bovine heart cardiolipin was reported to be 1500 μM [18]. Thus, the addition of $(polylysine)_{170}$, which binds to cardiolipin with a K_d of 0.035 μM (Table I), to the Ca^{2+}-cardiolipin complex results in dissociation of the complex and an $H_{II} \rightarrow$ bilayer transition. Accordingly, the addition of an excess of Ca^{2+} to the $(polylysine)_{170}$-cardiolipin complex does affect the lamellar organization of the lipid, whereas the presence of the polylysines with $n = 3$ and 5, which have binding affinities of the order of that of Ca^{2+}, does not interfere with H_{II} phase formation induced by Ca^{2+}, but in fact even promotes it, most likely due to a cooperative charge-neutralizing effect.

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