Spin-Label Electron Spin Resonance Studies on the Interactions of Lysine Peptides with Phospholipid Membranes

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ABSTRACT The interactions of lysine oligopeptides with dimyristoyl phosphatidylglycerol (DMPG) bilayer membranes were studied using spin-labeled lipids and electron spin resonance spectroscopy. Tetralysine and pentalysine were chosen as models for the basic amino acid clusters found in a variety of cytoplasmic membrane-associating proteins, and polylysine was chosen as representative of highly basic peripherally bound proteins. A greater motional restriction of the lipid chains was found with increasing length of the peptide, while the saturation ratio of lipids per peptide was lower for the shorter peptides. In DMPG and dimyristoylphosphatidylserine host membranes, the perturbation of the lipid chain mobility by polylysine was greater for negatively charged spin-labeled lipids than for zwitterionic lipids, but for the shorter lysine peptides these differences were smaller. In mixed bilayers composed of DMPG and dimyristoylphosphatidylcholine, little difference was found in selectivity between spin-labeled phospholipid species on binding pentalysine. Surface binding of the basic lysine peptides strongly reduced the interfacial pK of spin-labeled fatty acid incorporated into the DMPG bilayers, to a greater extent for polylysine than for tetralysine or pentalysine at saturation. The results are consistent with a predominantly electrostatic interaction with the shorter lysine peptides, but with a closer surface association with the longer polylysine peptide.

INTRODUCTION

The binding of peripheral proteins to biomembranes plays an important role in many cellular processes. Protein kinase C, for example, may be activated by binding to acidic lipids (House and Kemp, 1987; House et al., 1989). In consequence, studies have been undertaken to investigate the effects of this type of lipid-protein interaction on the overall structure and properties of the lipid bilayer core of the membrane. Proteins bound to the membrane surface significantly alter the lipid mobility and exhibit specific preferences for certain lipid species (see, for example, Görrissen et al., 1986; Sankaram et al., 1989a,b; Marsh, 1990a). It was shown that the binding of peripheral proteins may also induce lipid phase separations in certain model membranes (Haverstick and Glaser, 1989; Yang and Glaser, 1995). Clusters of basic amino acid residues, or a generally high content of basic amino acids, are required for proteins to bind to the surface of a biological membrane with a negative electrostatic potential (Kim et al., 1991; Mosior and McLaughlin, 1992). The putative pseudo-substrate region of protein kinase C, for example, consists of 12 amino acids, of which 8 bear a positive charge (Mosior and McLaughlin, 1991), which is predicted to be required for the binding of the protein to the membrane surface, a step necessary for its subsequent activation. A further, somewhat similar example is afforded by the autoinhibitory region at the C-terminus of the plasma membrane P-type Ca^{2+}-ATPase (Filoteo et al., 1992).

For a systematic biophysical approach to characterizing the nature of the interaction of basic proteins with biomembranes, it is useful to study the interaction of oligopeptides composed of a single basic amino acid type. These constitute a simple model for specific basic amino acid clusters in proteins. The interaction of *polymers* of lysine (molecular masses ranging from 4 to 150 kDa) with negatively charged dipalmitoyl phosphatidylglycerol (DPPG) bilayers was already studied, with a focus on the secondary structure of the polypeptide (Carrier and Pézolet, 1984) and on the effect of polylysine on the cooperative gel-to-liquid crystalline phase transition of DPPG bilayers (Carrier et al., 1985; Carrier and Pézolet, 1986). More recently, the binding of short lysine peptides (Lys_n, n = 1–5) and their effects on the surface electrostatics were studied by electrophoretic mobility measurements (Kim et al., 1991; Mosior and McLaughlin, 1992).

In the present work, we focus on the interactions of oligolysine peptides with lipid bilayers of dimyristoylphosphatidylglycerol (DMPG) and mixtures with dimyristoylphosphatidylcholine. Using spin-label electron spin resonance (ESR) spectroscopy, we characterized the interactions and their lipid specificity by determining the perturbation of the lipid chain mobility on binding the peptide. Tetralysine and pentalysine were used as models for the basic amino acid clusters found in cytoplasmic proteins, and polylysine with 320 residues was chosen for comparison as representative of highly basic peripheral membrane proteins. The studies encompass conventional binding experiments and studies of lipid selectivity and of interfacial interactions via the protonation equilibria of membrane-bound fatty acids, in addition to those on lipid distribution in mixed lipid membranes with bound peptide.

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MATERIALS AND METHODS

Materials

DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine), DMPG (1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol), and DMPS (1,2-dimyristoyl-*sn*-glycero-3-phosphoserine) were obtained from Avanti Polar Lipids (Alabaster, AL) and were checked for purity by thin-layer chromatography. 3-(*N*-Morpholino)propanesulfonic acid (MOPS) was obtained from Sigma (St. Louis, MO). 5-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid (5-SASL) was synthesized as described (Hubbell and McConnell, 1971). Phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, and phosphatidic acid spin labels with the nitroxyl group on the C-5 atom of the *sn*-2 chain (5-PCSL, 5-PESL, 5-PGSL, 5-PSSL, and 5-PASL, respectively) were synthesized from the corresponding spin-labeled stearic acid (5-SASL) as described by Marsh and Watts (1982). Spin-labeled diacylglycerol (5-DGSL) was synthesized from 5-PCSL as described by Heimburg et al. (1992). All spin labels were checked by thin-layer chromatography before their use with the solvent systems $CHCl_3/CH_3OH$/ammonia (65/30/3, v/v/v) and hexane ether (1/1, v/v). Furthermore, all spin labels were checked in ethanol solution by ESR spectroscopy to determine the consistency of their concentrations. Tetralysine (Miles Labs, Elkhart, IN) and polylysine (320 residues; Sigma) were used without further purification. Pentalysine was obtained by custom synthesis from Research Plus (Bayonne, NJ) and from Multiple Peptide Systems (San Diego, CA) and used without further purification.

Sample preparation

Lipid dispersions were prepared by first codissolving the lipids (0.5 mg) with 1 mol% spin label in a chloroform/methanol solution (1:1, v/v), and then evaporating the solvent with a nitrogen gas flow and drying the sample under vacuum overnight. The dried lipid films were dispersed in various buffer solutions (10 mM buffer with 5 mM EDTA: MOPS for pH range 6.5–7.5, citric acid for pH range 3–6, sodium borate/NaOH for pH above 7.6) to a final concentration of 25 mg/ml. The samples were incubated for 1 h at 35°C. Peptide-containing samples were prepared by hydrating the dry lipid film in buffer and adding the desired amount of peptide dissolved in an equivalent volume of the same buffer. Alternatively, dried lipid films were hydrated directly with buffer containing the peptide to give the same total volume and lipid/peptide ratios. No difference in the ESR spectra was observed between the two different methods of preparation for the peptide-containing samples. Both hydrated lipid and hydrated peptide solutions were adjusted to the desired pH before they were mixed together. The pH adjustment was done so as not to exceed an ionic strength of 0.03. Peptide-containing samples were also incubated at 35°C for 1 h.

After incubation, samples were centrifuged in a tabletop centrifuge. The pH of the supernatant did not change by more than ±0.1 pH units compared with the separate solutions before mixing. The membrane pellets were then transferred to ESR capillaries. After the spectra were recorded, the samples were analyzed by protein (Lowry et al., 1951) and phosphate (Rouser et al., 1970) assays. The Lowry assay was performed with bovine serum albumin as a standard. This was calibrated with solutions of known concentrations of the lysine peptides used here.

ESR spectroscopy

ESR spectra were acquired with an E-12 Century Line 9-GHz spectrometer (Varian, Sunnyvale, CA) equipped with a TE_{102} rectangular cavity (Varian). The temperature was controlled to within ±0.1°C, by using nitrogen gas flow temperature regulation with a thin-wire thermocouple that was placed close to the sample at the top of the microwave cavity. A custom sample holder allowed positioning of the sample in the cavity with an accuracy of 0.1 mm. The spectrometer was interfaced to an IBM personal computer using a Tecmar Labmaster A/D converter for digitizing and storing the ESR spectral data. To improve the signal-to-noise ratio, 2–16 scans were accumulated, depending on the sample. The modulation frequency was 100 kHz, with a modulation amplitude of 1.25 Gauss. A time constant of 0.25 s was chosen for 4-min scans. The outer hyperfine splitting, $2A_{max}$, was used to characterize the spectra of the spin labels. Hyperfine splittings were determined by fitting the maxima and minima in the outer wings of the spectrum empirically to Gaussian curves. The outer hyperfine splittings are sensitive to both the amplitude and rate of the lipid chain motions, and can be used to characterize the strength of interaction of the various spin-labeled lipids with peptides or proteins (Lange et al., 1985; Sankaram et al., 1989a).

RESULTS

Membrane binding of different lysine peptides

The binding of tetralysine and pentalysine to anionic lipid bilayer membranes was determined by centrifugation assays with conventional chemical lipid and protein determinations. In addition, binding was studied from the dependence of the outer hyperfine splittings in the ESR spectra of 5 C-atom spin-labeled lipids on the lysine peptide/lipid ratio. The ESR spectra of the 5 C-atom spin labels in lipid dispersions correspond to axially symmetrical, partially motional-averaged, anisotropic systems. Binding of the peptide decreases the degree of motional averaging in the spectra from the spin-labeled lipid chains and thus increases the maximum outer hyperfine splitting in the ESR spectra.

Fig. 1 shows the binding behavior of the tetralysine and pentalysine peptides obtained by these two methods. In the

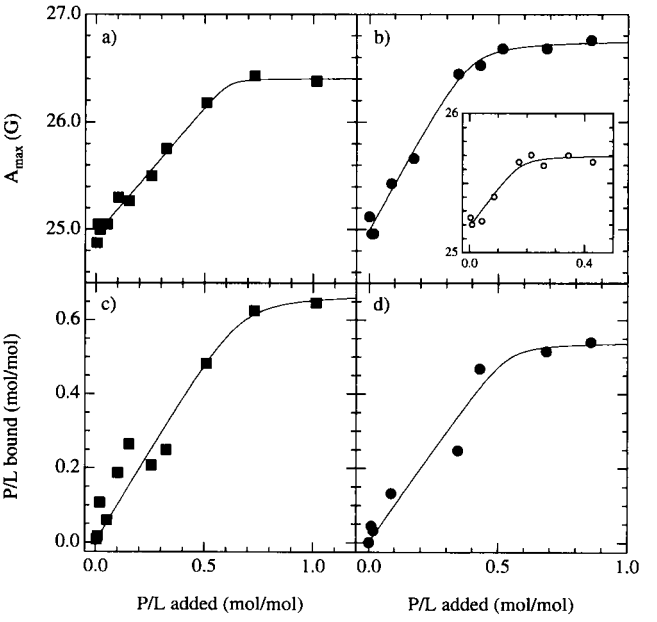

FIGURE 1 Dependence of the outer hyperfine splitting constants, A_{max}, of the 5-PGSL spin label in DMPG bilayers on the amount of peptide added to the lipid for (*a*) tetralysine and (*b*) pentalysine. The corresponding ratios of bound peptide/lipid (P/L) as a function of the added peptide/lipid ratio for (*c*) tetralysine and (*d*) pentalysine are shown below. In *b* the values of A_{max} for 5-PGSL in a mixed DMPC:DMPG (1:1 mol/mol) bilayer (○) are shown as a function of the added pentalysine/lipid ratio in the inset. Temperature: 30°C. Buffer: 10 mM MOPS, 5 mM EDTA, and 10 mM NaCl (pH 7.0).

case of tetralysine, the DMPG bilayers become saturated with peptide at a ratio of ~1.6–1.7 lipids/peptide. Chemical analysis of the membrane complexes and the point at which A_{max} achieves its limiting level yield consistent values for this binding stoichiometry. In the case of pentalysine, the lipid/peptide binding stoichiometry is increased to ~1.9–2.5 mol/mol. The range quoted corresponds to results from the chemical binding assays and the peptide/lipid dependence of A_{max}, respectively. For polylysine, a lipid/protein saturation binding ratio of 70 mol DMPG/320-residue polypeptide was estimated (data not shown).

ESR data for binding of pentalysine to a mixed membrane containing zwitterionic and anionic lipids are also given in Fig. 1 *b*. The perturbation of the overall lipid mobility is strongly reduced by admixture of the uncharged DMPC lipid component with DMPG, as seen from the lower limiting value of A_{max}. This is a result of the reduced binding of pentalysine to the mixed lipid membrane, where the saturation lipid/peptide stoichiometry is ~5 lipids/peptide.

Influence on the bilayer phase transition

The effect of pentalysine and polylysine on the thermotropic lipid phase behavior was investigated for DMPG bilayers with saturating amounts of peptide bound. Fig. 2 shows the temperature dependence of the outer hyperfine splittings in the ESR spectra of 5-PASL spin label in DMPG bilayers in the presence and absence of pentalysine or polylysine. The sharp cooperative phase transition found for the peptide-free bilayer at 23°C is significantly broadened by binding pentalysine without appreciable shift (maximally −1°) in the midpoint of the transition. The lipid chain mobility is decreased over the entire temperature range, as reflected by the increased ESR outer hyperfine splittings at any given temperature. Similar results were obtained both with other lipid spin labels and for pentalysine bound to DMPS bilayers (data not shown). These trends are further pronounced on binding polylysine rather than the smaller pentalysine oligomer. The lipid chain-melting transition is broadened even more, although any shift in the transition midpoint still remains small (maximally +1°). The outer hyperfine splittings are increased more than for pentalysine at all temperatures, with the effect being stronger in the fluid phase. The decrease in lipid chain mobility is therefore particularly pronounced on binding polylysine.

Interaction with different spin-labeled lipid species

The perturbation of the mobility of spin-labeled lipids with different polar headgroups by binding tetralysine, pentalysine, and polylysine was determined for lipid probes spin labeled at the 5 C-atom position of the chain to establish the selectivity of interaction of the different lipid species with these basic peptides (cf. Sankaram et al., 1989b). Measurements were made at 30°C on DMPG host bilayers for all three peptides and additionally on DMPS lipid matrices at 44°C for pentalysine, with a concentration of 1 mol% of the various spin-labeled lipid species. ESR spectra for the various spin labels differing in their lipid headgroup, both in the lipid matrix alone and in the lipid-peptide complexes at saturating amounts of pentalysine, are given in Fig. 3. A larger increase in the outer hyperfine splittings for a given lipid species upon addition of a peptide indicates a stronger perturbation in the mobility of that particular lipid by the peptide binding to the bilayer host. As can be seen from Fig. 3, the overall increase in spectral anisotropy when pentalysine is bound is largest in the case of protonated stearic acid and smallest in the case of diacylglycerol. However, the absolute size of the outer hyperfine splitting in DMPG bilayers alone is smaller for the neutral lipids 5-SASLH and 5-DGSL than for the spin-labeled phospholipids. For the latter, the hyperfine splittings in the peptide-free bilayers are all comparable. Table 1 summarizes the outer hyperfine splitting constants of the ESR spectra and the increase (ΔA_{max}) in these values on binding peptide for all systems investigated. Comparison of the effects of the different peptides on the outer hyperfine splittings of all spin labels shows that the longer the peptide, the stronger the perturbation of the lipid chain mobility under saturation binding conditions. For all three peptides, the protonated stearic acid is most strongly perturbed, whereas the effect is smallest on the neutral diacylglycerol. Among the different phospholipid species, the negatively charged phospholipids, 5-PGSL and 5-PSSL, exhibit a slightly stronger perturbation of the lipid acyl-chain mobility than do the zwitterionic phospholipids 5-PESL and 5-PCSL for the short peptides tetralysine and pentalysine, in both DMPG and DMPS host matrices. The lipids with small headgroups, 5-PASL$^-$, 5-PASL^{2-}, and 5-SASL$^-$, are considerably more perturbed in relation

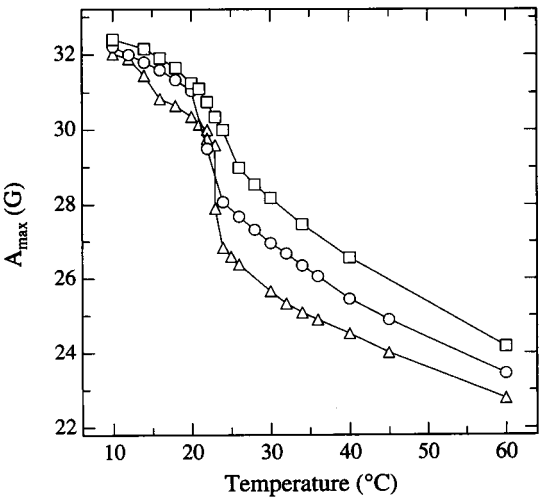

FIGURE 2 Temperature dependence of the ESR outer hyperfine splittings, A_{max}, of 5-PASL in DMPG bilayers, for the lipid bilayers without peptide (△), in the presence of a saturating amount of pentalysine (○), and in the presence of a saturating amount of polylysine (□). Buffer: 10 mM sodium borate, 10 mM NaCl, and 5 mM EDTA (pH 9.8).

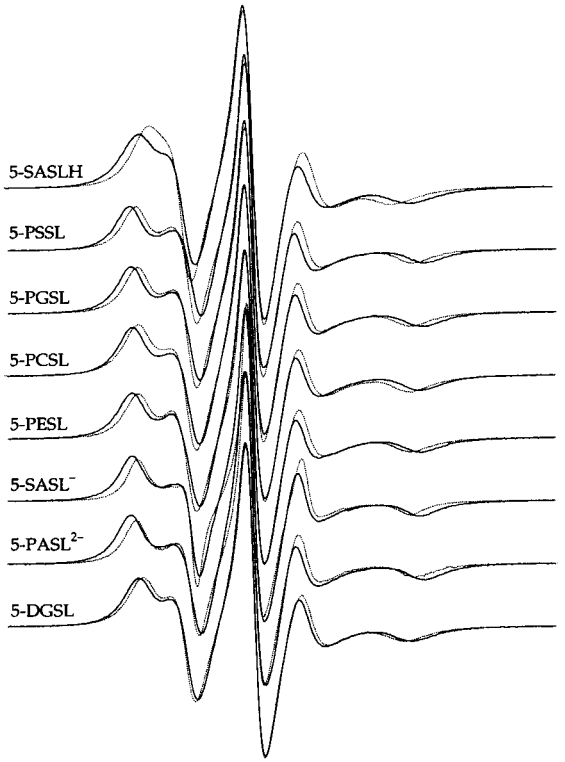

FIGURE 3 ESR spectra of different lipid species spin-labeled at the fifth position of the *sn*-2 acyl-chain, 5-XSL, in DMPG bilayers recorded at 30°C in the absence (·····) and in the presence (——) of saturating amounts of pentalysine. Total scan width: 100 G. Buffers: 10 mM MOPS, 10 mM NaCl, and 5 mM EDTA, pH 7.2, for the spin labels 5-PSSL, 5-PGSL, 5-PCSL, 5-PESL, and 5-DGSL; 10 mM citric acid, 10 mM NaCl, and 5 mM EDTA, pH 4.7, for spin label 5-SASLH; 10 mM sodium borate, 10 mM NaCl, and 5 mM EDTA, pH 9.8, for spin label 5-$PASL^{2-}$ and at pH 10.2 for spin label 5-$SASL^{-}$.

to the lipids with larger headgroups when the long polylysine is bound instead of pentalysine.

Selectivity in mixed lipid systems

The perturbation of the lipid chain mobility by binding pentalysine has also been determined for different spin-labeled lipid species in mixtures of DMPG with the zwitterionic phospholipid DMPC. The dependence of the outer hyperfine splitting constant, A_{max}, on DMPG content in DMPG/DMPC mixed bilayers in the presence and absence of peptide is given in Fig. 4 for various phospholipids spin-labeled at the 5 C-atom position. Decreasing content of the anionic phospholipid component results in a decreasing perturbation of the overall lipid mobility at saturation binding of the peptide, for all of the spin labels. As seen from Table 2, however, the overall relative perturbations of the different spin-labeled phospholipids, zwitterionic and anionic, by peptide binding are very similar, independent of the lipid composition of the mixed bilayers. (The outer hyperfine splittings of the 5-PSSL spin label are somewhat greater, in both the presence and absence of pentalysine, than are those of the other spin labels, but the perturbation induced by the peptide is very similar.) Although the background host lipid is changed, the relative selectivity of interaction of the different spin-labeled lipids with the peptide appears to be largely unchanged.

TABLE 1 Outer hyperfine splitting constants, A_{max}, of the ESR spectra from lipids spin-labeled on the 5 C-atom of the *sn*-2 acyl chain, or fatty acid spin-labeled at C-atom 5, in DMPG bilayers in the presence and absence of saturating amounts of tetralysine, pentalysine, and polylysine at 30°C

Spin label	Charge	A_{max} (gauss)		ΔA_{max} (gauss)
		DMPG	+*tetralysine*	
5-$SASL^{-}$	−*	24.9	26.2	1.3
5-PSSL	−	25.5	26.8	1.3
5-PGSL	−	25.1	26.5	1.4
5-PCSL	±	24.9	26.2	1.3
5-PESL	±	25.1	26.3	1.2
5-SASLH	0#	21.9	24.0	2.1
5-DGSL	0	24.0	25.1	1.1
		DMPG	+*pentalysine*	
5-$SASL^{-}$	−§	25.0	26.5	1.5
5-PSSL	−	25.5	27.1	1.6
5-$PASL^{2-}$	2−*	25.7	27.0	1.3
5-PGSL	−	25.1	26.7	1.6
5-PCSL	±	24.9	26.4	1.5
5-PESL	±	25.0	26.5	1.5
5-SASLH	0#	21.9	24.7	2.8
5-DGSL	0	24.0	25.0	1.0
		DMPG	+*polylysine*	
5-$SASL^{-}$	−§	24.9	27.5	2.6
5-PSSL	−	25.5	27.4	1.9
5-$PASL^{2-}$	2−*	25.7	28.3	2.6
5-$PASL^{-}$	−¶	24.9	27.4	2.5
5-PGSL	−	25.1	27.4	2.3
5-PCSL	±	24.9	27.0	2.1
5-PESL	±	25.1	27.0	1.9
5-SASLH	0#	21.9	25.6	3.7
5-DGSL	0	24.0	25.9	1.9
		DMPS	+*pentalysine*	
5-PSSL	−	24.3	25.3	1.0
5-PGSL	−	23.6	24.7	1.1
5-PCSL	±	23.5	24.4	0.9
5-PESL	±	23.6	24.3	0.7
5-DGSL	0	22.4	23.4	1.0

ΔA_{max} is the difference in A_{max} in the presence and absence of peptides. Corresponding data for the lipid spin labels in DMPS bilayers in the presence and absence of saturating amounts of pentalysine at 44°C are also given. pH was 7.2 in all samples, if not specified otherwise.
*pH 9.8.
#pH 4.7.
§pH 10.2.
¶pH 6.0.

Fatty acid pH titration

The ESR spectra of the 5-SASL stearic acid spin label in DMPG complexes with tetralysine, pentalysine, and polylysine, as well as in the lipid environment alone, were measured as a function of pH to determine the influence of the peptide association on the interfacial acid-base equilibrium of the fatty acid. Fig. 5 shows the ESR spectra ob-

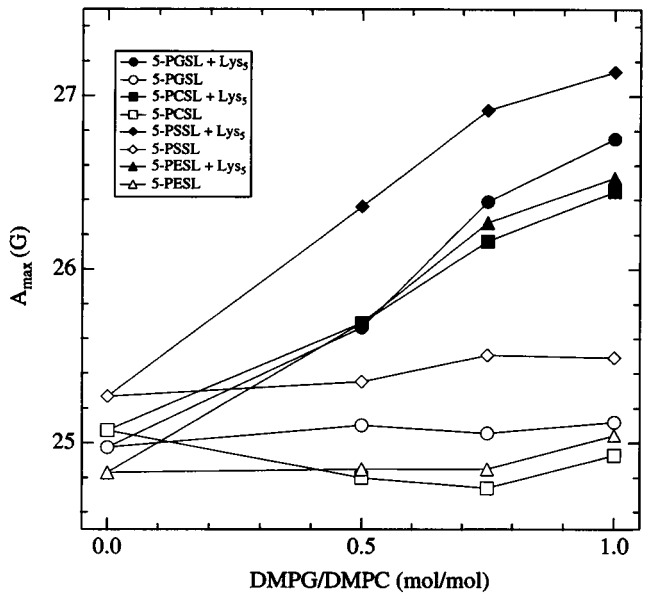

FIGURE 4 Dependence of the outer hyperfine splitting constant, A_{max}, for different spin-labeled phospholipids, 5-XSL, on the mole fraction of DMPG in DMPC/DMPG mixed bilayers, in the presence (●, ■, ◆, ▲) and absence (○, □, ◇, △) of a saturating amount of pentalysine. ○, ●, 5-PGSL; □, ■, 5-PCSL; ◇, ◆, 5-PSSL; △, ▲, 5-PESL. Buffer: 10 mM MOPS, 10 mM NaCl, 5 mM EDTA (pH 7.2). T = 30°C.

tained at different pH values for 5-SASL incorporated in DMPG bilayers alone (Fig. 5 *a*) and in DMPG bilayers saturated with pentalysine (Fig. 5 *b*). The spectral anisotropy and the maximum outer hyperfine splitting are much larger for the anionic form of the stearic acid spin label than for its protonated form. It is seen from the ESR spectra that the acid-base titration occurs between pH 7.2 and pH 8.5 in the case of DMPG alone and between pH 6.5 and 6.9 when saturating amounts of pentalysine are bound. The binding of the short pentalysine clearly shifts the pH titration downward to a lower pK.

The pH titration of 5-SASL was followed by using the outer hyperfine splittings in the ESR spectra for complexes of all four peptides with DMPG. The dependences of the outer hyperfine splittings of 5-SASL on the bulk pH are given in Fig. 6 for sample temperatures of 30°C (DMPG alone, with tetralysine, pentalysine, and polylysine) and 60°C (DMPG alone, with pentalysine and polylysine). The

TABLE 2 Increase in outer hyperfine splitting constant, ΔA_{max} (gauss), of 5-position spin-labeled phospholipids (5-XSL), on saturation binding of pentalysine to DMPG/DMPC mixed lipid membranes with different mole fractions, X_{DMPG}, of DMPG

X_{DMPG}	1.0	0.75	0.5	0.0
5-PSSL	1.6	1.4	1.0	0.0
5-PGSL	1.6	1.3	0.6	0.0
5-PCSL	1.5	1.4	0.9	0.0
5-PESL	1.5	1.4	0.8	0.0

T = 30°C, pH 7.2.

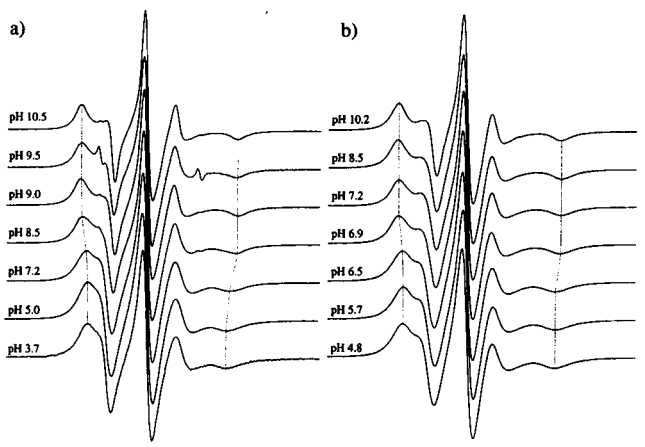

FIGURE 5 ESR spectra of stearic acid spin-labeled at the 5 C-atom position (5-SASL), in DMPG bilayers recorded at 30°C in the absence (*a*) and in the presence (*b*) of saturating amounts of pentalysine. Total scan width: 100 G. Buffers: 10 mM MOPS, 10 mM NaCl, and 5 mM EDTA, pH 6.5–7.5; 10 mM citric acid, 10 mM NaCl, and 5 mM EDTA, pH 3–6; 10 mM sodium borate, 10 mM NaCl, and 5 mM EDTA, pH 7.6–11. Dotted lines give the locus of the outer hyperfine splitting.

pH dependences of the outer hyperfine splitting, A_{max}, were fitted using the conventional expression for an acid-base titration:

$$A_{max} = \frac{A_{max}^{5\text{-SASLH}} - A_{max}^{5\text{-SASL}^-}}{1 + 10^{pH-pK}} + A_{max}^{5\text{-SASL}^-} \quad (1)$$

where $A_{max}^{5\text{-SASLH}}$ is the outer hyperfine splitting constant of the protonated form of stearic acid and $A_{max}^{5\text{-SASL}^-}$ is that of the corresponding anion, and pK is the interfacial pK_a of the fatty acid. The estimated interfacial values of pK and the shifts in pK induced by binding of the peptides are given in Table 3 for all peptides. The pK of stearic acid in DMPG is shifted down by ~1 pH unit to pK = 6.6–6.8 on saturation binding of pentalysine or tetralysine to the bilayer (measurement at 30°C). For polylysine with 320 residues, this shift is considerably larger, with an interfacial pK of 5.9,

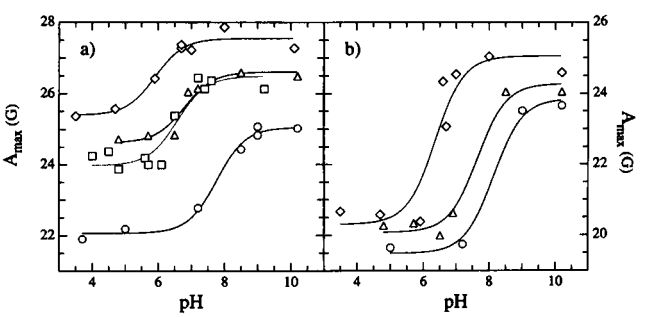

FIGURE 6 pH dependence of the outer hyperfine splitting of the stearic acid spin label, 5-SASL, at (*a*) 30°C and (*b*) 60°C in DMPG bilayers both in the absence (○) and in the presence of saturating amounts of tetralysine (□), pentalysine (△), and polylysine (◇). Buffers: 10 mM MOPS, 10 mM NaCl, and 5 mM EDTA, pH 6.5–7.5; 10 mM citric acid, 10 mM NaCl and 5 mM EDTA, pH 3–6; 10 mM sodium borate, 10 mM NaCl and 5 mM EDTA, pH 7.6–11.

TABLE 3 Interfacial pK values of 5-SASL stearic acid in DMPG bilayers in the absence and in the presence of saturating amounts of tetralysine, pentalysine, and polylysine at low ionic strength

	pK_a		ΔpK_a	
Peptide	30°C	60°C	30°C	60°C
DMPG alone	7.8	8.1	0.0	0.0
+ Tetralysine	6.6	—	−1.2	—
+ Pentalysine	6.8	7.6	−1.0	−0.5
+ Polylysine	5.9	6.4	−1.9	−1.7

ΔpK_a is the shift in pK induced by binding the oligolysine peptides.

which is −1.9 pH units lower than that of stearic acid in DMPG alone. A similar, large downward shift in pK on binding polylysine is also observed for spin-labeled phosphatidic acid, 5-PASL (data not shown). At higher temperatures (60°C, Fig. 6 *b*) the pK shifts of 5-SASL induced by the peptide are somewhat smaller: −0.5 for pentalysine (pK ≈ 7.6) and −1.7 for polylysine (pK ≈ 6.4).

DISCUSSION

Peptide binding stoichiometries

The binding stoichiometries determined for DMPG (Fig. 1) correspond to 2.4–2.5, 2.0–2.6, and 4.6 lysine residues per lipid molecule for tetralysine, pentalysine, and polylysine, respectively. Whereas the binding of the short peptides involves approximately the same number of lysine residues per DMPG molecule, significantly less lipid is bound per lysine residue in the case of polylysine. For the equimolar DMPC/DMPG mixture, the pentalysine binding stoichiometry per DMPG molecule (2 lysine residues/DMPG) is also similar to that for the short lysine peptides binding to DMPG alone. Apparently, the number of lysine residues exceeds the number of charged lipids in the saturated lipid-peptide complexes. This means that the surface potential is reversed when tetralysine, pentalysine, and polylysine bind to DMPG bilayers, in agreement with electrophoretic measurements on similar systems (Kim et al., 1991). This change should be greatest for polylysine, because of its higher lysine/DMPG ratio.

The charge stoichiometry for binding the lysine peptides can possibly be interpreted in terms of the geometric requirements for interaction of the positively charged lysine side chains with the negatively charged phospholipid headgroups. In a fluid bilayer membrane, the area per lipid is in the region of 64 Å^2 (e.g., Marsh, 1990b). Therefore the distance between phospholipid headgroups is in the region of 8 Å. Even for a fully extended peptide chain, the distance between adjacent residues is only 3.8 Å. Hence it seems likely that only every other lysine residue can interact effectively with a phospholipid headgroup. The minimum stoichiometry of lysine residues per lipid for optimum salt bridging is therefore 2, and will be somewhat higher for a more random configuration of the peptide backbone, as is observed for the saturation binding of tetralysine and pentalysine. The higher lysine/lipid stoichiometry on saturation binding of polylysine suggests that this optimum extended configuration cannot be achieved in the case of the polypeptide, probably for both steric and entropic reasons.

A minimum estimate for the membrane surface occupied by a lysine residue is ~18 Å^2 (corresponding to the area per residue in a β-sheet structure). An alternative estimate from the molar volume of 105.8 ml/mol (Cohn and Edsall, 1943) gives ~31–38 Å^2/lysine residue, on approximating the residue by a cube or a sphere. This suggests that binding one lysine residue per lipid would not give complete surface coverage, but that the stoichiometries determined for tetralysine and pentalysine, which are more than twice this, could correspond to a steric saturation of the surface. The yet larger saturation value of the lysine/lipid ratio found for polylysine implies that in this case not all residues contact the lipid surface. This could be achieved by a different surface conformation of the polylysine relative to the shorter lysine peptides, for instance any α-helical components in the conformation (cf. Carrier and Pézolet, 1986) would automatically fulfill this condition.

Bilayer phase transition temperatures

In general, it would be expected that binding of the lysine peptides might induce shifts in the chain-melting transition temperature of the anionic lipid bilayers (cf. Cevc and Marsh, 1987). The observed shifts are, however, rather small: not more than −1°C for pentalysine and +1°C for polylysine (see Fig. 2). This suggests that there is a considerable degree of compensation between the different interactions giving rise to the transition temperature shifts.

Contributions to the shift in transition temperature can come from changes in the surface electrostatics and headgroup hydration on binding the peptide (Cevc and Marsh, 1987), and possibly from the intrinsic lipid-peptide interaction itself (Heimburg and Marsh, 1996). The overall transition temperature shift would then be given by

$$\Delta T_t = \frac{\delta\Delta G_{el}}{\Delta S_t} + \frac{\delta\Delta G_{hyd}}{\Delta S_t} + \frac{\delta\Delta G_{LP}}{\Delta S_t} \qquad (2)$$

where ΔS_t is the chain-melting transition entropy, and $\delta\Delta G_i$ are differences in free energy between the bilayer fluid and gel states that arise from surface electrostatics ($i \equiv el$), headgroup hydration ($i \equiv hyd$), and the lipid-peptide interaction ($i \equiv LP$). Here it is implied that the values of $\delta\Delta G_i$ represent the differences in these quantities in the presence and absence of peptide. For DMPG bilayers alone it has been found that the electrostatic contribution to the transition temperature shift is $\Delta T_t^{el} \approx -6.5$°C, as determined from the shielding effects in the electrostatic double layer by high ionic strength (Cevc et al., 1980). The total shift in transition temperature on fully protonating the DMPG headgroups is much larger (18–19°C), however, the difference being attributed to changes in the headgroup hydration on

protonation (Watts et al., 1978; Cevc et al., 1980). Thus, even if there is no change in lipid headgroup hydration on binding the lysine peptides, it might be expected that the electrostatic shifts in transition temperature would be greater than the net shifts observed here, particularly because the electrostatic surface potential is actually reversed by the peptide binding. It therefore seems likely that the electrostatic shifts in transition temperature are almost exactly compensated by opposing ones arising from the lipid-peptide interaction. This near-compensation could explain why a small downward shift is obtained with the short lysine peptide, whereas a small upward shift is found for the larger polylysine peptide. Only a small increase in affinity for the gel phase relative to the fluid phase would be sufficient to produce compensating shifts of this magnitude (an increase of ~20% or 80% for the electrostatic or pH-induced shifts, respectively). Similar results have been found in comparing the transition shifts of dipalmitoylphosphatidylglycerol bilayers for polylysines of different chain lengths (Carrier and Pézolet, 1986).

Peptide-lipid headgroup interactions

The changes in the outer hyperfine splitting, $2A_{max}$, of the membrane-incorporated spin-labeled lipids that are induced by binding the lysine peptides can be used to obtain information on the interactions of the bound peptide with the different lipid headgroups (see Fig. 3 and Table 1). The number of lysine residues in the bound peptides considerably exceeds that of the negatively charged DMPG molecules. Therefore, it seems likely that differences in A_{max} reflect intrinsic differences in the strength of interaction with the different phospholipid headgroups in DMPG bilayers, more than a thermodynamic selectivity of association. (The latter is considered later in connection with the mixed lipid bilayers.) It is probably for this reason that the values of ΔA_{max} do not differ very greatly between the various spin-labeled phospholipids. The surface of the DMPG bilayers is saturated with lysine groups (cf. above), and therefore the perturbation in mobility of all spin-labeled phospholipids incorporated at probe amounts is dominated by the overall (saturated) interaction of the lysine peptides with the DMPG host matrix. Nonetheless, there are measurable differences in the values of ΔA_{max} that mostly indicate a slightly stronger interaction with the negatively charged phospholipids (5-PGSL, 5-PSSL, and 5-PASL) than with the zwitterionic phospholipids (5-PCSL and 5-PESL) (see Table 1). With the exception of 5-PSSL, this is particularly the case with polylysine, for which the values of ΔA_{max} are considerably greater than those found with the two shorter lysine peptides, tetralysine and pentalysine. The reason for the latter could be that polylysine is associated more closely with the lipid headgroups than are pentalysine and tetralysine. It has been suggested that the short lysine peptides may be bound ~5 Å above the negatively charged bilayer surface (Kim et al., 1991).

It is of interest to compare the size of the reduction in chain mobility induced by binding of the oligolysine peptides with that induced by binding of other peripheral proteins. The values of A_{max} for the 5-PGSL spin label in fluid DMPG bilayers at low ionic strength in the presence and absence of saturating amounts of different basic peripheral proteins are given in Table 4. The smallest perturbations of the chain mobility are for the short oligopeptides, tetralysine and pentalysine. The perturbation by polylysine is comparable to that of the classical peripheral protein, cytochrome *c* (Görrisen et al., 1986), and to that of surface-associated melittin, which is a basic peptide that is also amphipathic (Kleinschmidt et al., 1997). On the other hand, peripheral proteins that are known to penetrate the membrane partially, myelin basic protein (Sankaram et al., 1989a) and apocytochrome *c* (Görrisen et al., 1986), give larger values of A_{max}. The greatest perturbation of the lipid mobility is by α-lactalbumin in its basic form at low pH. This molten-globule state of the protein is thought, in contrast, to be fully transmembrane (Montich and Marsh, 1995). The extent of perturbation in the lipid chain mobility is therefore consistent with a closer association of the polylysine than of the shorter lysine peptides at the membrane surface. In terms of the biological implications, this corresponds to a tight binding of highly basic peripheral membrane proteins, as opposed to the reversible modulating role of association with clusters of basic residues in cytoplasmic regulatory proteins or the autoinhibitory region of the plasma membrane Ca^{2+}-ATPase.

The situation is somewhat different for the neutral spin-labeled lipids than for the charged spin-labeled lipids inter-

TABLE 4 Outer hyperfine splitting constants, A_{max}, in the ESR spectra of the 5-PGSL spin label in DMPG bilayers at 30°C, in the presence of saturating amounts of various peripheral peptides and proteins in low-ionic-strength buffers

Peptide/protein	Buffer*	A_{max} (gauss)	Ref.#
DMPG alone	10 mM HEPES, 10 mM NaCl, pH 7.2	25.1	1
+ Tetralysine	10 mM HEPES, 10 mM NaCl, pH 7.2	26.5	1
+ Pentalysine	10 mM HEPES, 10 mM NaCl, pH 7.2	26.7	1
+ Polylysine	10 mM HEPES, 10 mM NaCl, pH 7.2	27.4	1
+ Cytochrome *c*	10 mM Tris, pH 8.0	27.0	2
+ Melittin§	10 mM MOPS, pH 7.0	27.7	3
+ Lysozyme	10 mM Tris, 10 mM NaCl, pH 8.0	28.1	4
+ Myelin basic protein	10 mM Tris, 10 mM NaCl, pH 8.0	28.4	5
+ Apocytochrome *c*	10 mM Tris, pH 8.0	28.6	2
+ α-Lactalbumin	10 mM acetic acid, 10 mM NaCl, pH 4.0	30.0	6

*All buffers contained EDTA.

#References: 1. This work; 2. Görissen et al., 1986; 3. Kleinschmidt et al., 1997; 4. Sankaram et al., 1989b; 5. Sankaram et al., 1989a; 6. Montich and Marsh, 1995.

§For ditetradecylphosphatidylglycerol bilayers.

acting with the oligolysine peptides. Both the protonated form of stearic acid and diacylglycerol have lower absolute values of A_{max} in lipid bilayers without peptide (see Table 1). This is because their chains are situated deeper in the hydrophobic core of the membrane than are those of the diacyl phospholipids (Sankaram et al., 1990; Schorn and Marsh, 1996). A consequence of this is that the largest values of ΔA_{max} are found for the protonated fatty acid, 5-SASLH, presumably because the overall interaction of the lysine peptides with the DMPG host bilayers causes the single-chain fatty acid to move upward in the bilayer into a region of reduced segmental chain mobility. The two-chain diacylglycerol presumably does not have this freedom of movement and records the smallest values of ΔA_{max} because its minimal polar group is unable to interact specifically with the lysine side chains. The ionized form of the fatty acid is situated more or less in register with the diacyl phospholipid chains and senses an interaction of a strength comparable to that of the anionic phospholipids.

In DMPS bilayers, the perturbations in chain mobility of the charged phospholipid spin labels relative to the zwitterionic ones are similar to those in DMPG bilayers. The overall changes in A_{max} induced by pentalysine are smaller in DMPS than in DMPG. This may be due partly to the higher temperature of measurement (approximately equal reduced temperatures) and to the tripolar nature of the phosphatidylserine headgroup, which may modify the electrostatic interactions somewhat.

Selectivity of interaction in mixed lipid bilayers

The extent of binding of pentalysine to an equimolar DMPC/DMPG mixture is considerably reduced relative to binding to bilayers consisting wholly of DMPG (Fig. 1 *b*). This corresponds to significantly less than complete surface coverage (cf. above) and therefore would allow competition between specific spin-labeled lipids and the background host lipids for association with the peptide. This is unlike the situation with bilayers consisting only of DMPG, where the peptide binding corresponds to saturation of the lipid headgroups. Nevertheless, the selectivity between the various spin-labeled lipids for interaction with pentalysine is not greater than that found in the fully saturated system (see Fig. 4 and Table 2). This result is different from that found previously for the interaction of spin-labeled lipids with the myelin basic protein (Sankaram et al., 1989a). In the latter case, a selectivity of interaction of spin-labeled phosphatidylglycerol over that of spin-labeled phosphatidylcholine was found in protein-saturated DMPC/DMPG mixtures up to DMPC mole fractions of 0.25. A possible reason for the difference may be that pentalysine is not so closely associated with the lipid surface as is the myelin basic protein and therefore exerts its action by longer range electrostatic effects. This suggestion is in line with recent theoretical electrostatics calculations (Ben-Tal et al., 1996) and the fact that large-scale domains containing short basic peptides may be seen in bilayers of which the negatively charged lipid component represents a much smaller area fraction of the entire vesicle surface (e.g., Yang and Glaser, 1995).

Interfacial effects

The pK of spin-labeled stearic acid in zwitterionic phosphatidylcholine bilayers is shifted upward to approximately neutral pH because of the lower polarity at the membrane surface (Esmann and Marsh, 1985; Horváth et al., 1988). In anionic lipids, such as phosphatidylglycerol, this upward shift is further increased to pK ~8 (Fig. 6) because the negative surface potential enhances the interfacial proton concentration (e.g., Sankaram et al., 1990). The net shift in interfacial pK of the fatty acid on peptide binding can be expressed as

$$\Delta pK^{int} = \Delta pK^{pol} + \Delta pK^{el} + \Delta pK^{LP} \quad (3)$$

where ΔpK^{pol} is the shift due to the change in interfacial polarity, ΔpK^{el} is that due to the change in the surface electrostatics, and ΔpK^{LP} is the contribution arising from the different strengths of interaction of the two titrating stearic acid forms with the peptide. Binding the small lysine peptides to DMPG shifts the acid-base equilibrium of the fatty acid downward by ~1 pH unit to pK ~6.6 for tetralysine and pK ~6.8 for pentalysine at 30°C (Fig. 6 and Table 3). Saturation of DMPG bilayers with polylysine results in an even stronger downward shift to pK 5.9 at 30°C.

A strong downward shift in pK on binding the basic peptide is expected for electrostatic reasons. This contribution ΔpK^{el} should reduce the interfacial pK considerably below that for zwitterionic bilayers (pK ~7.0) because the surface potential, as seen from the bulk solution, is not simply reduced but reversed by the peptide binding. Any interfacial dehydration induced by peptide binding would reduce the surface polarity and give rise to an upward shift ΔpK^{pol} in interfacial pK. However, as discussed above, surface dehydration is not expected for the short lysine peptides (see Kim et al., 1991; Ben-Tal et al., 1996). The interaction with a basic peptide will tend to stabilize the anionic form of the fatty acid and therefore will give rise to a downward shift in ΔpK^{LP}. However, the contribution from direct lipid-peptide interaction most probably is relatively minor because there is little selectivity in the perturbation of spin-labeled lipids with different polar headgroups, and salt-bridge formation is not expected at a separation of 5 Å from the lipid surface (Kim et al., 1991) for tetra- and pentalysine. The contribution made by ΔpK^{LP} is therefore smaller than the downward shift due to electrostatics, ΔpK^{el}, but augments the latter, resulting in a net downward shift that is slightly greater than that arising from the reversal of the surface electrostatics. The shift for polylysine is greater than that for the short lysine peptides because the excess positive surface charge is greater (cf. above).

The reduction in the observed pK shifts at higher temperature (i.e., 60°C; see Table 3) is interesting because the temperature coefficients of the polarity-induced pK shifts ΔpK^{pol} are known to be positive for fatty acids (Bonnet et al., 1990). It is likely, however, that part of the observed shift may also be contributed by weaker binding of the peptide at higher temperature.

CONCLUSIONS

The results of this spin-label study indicate consistently that the interactions of the short oligopeptides tetralysine and pentalysine with anionic lipid membranes are principally electrostatic in nature. This conclusion is in agreement with previous electrophoretic and monolayer surface potential measurements (Kim et al., 1991) and with recent theoretical studies of the peptide-lipid association (Ben-Tal et al., 1996). The lipid redistribution that takes place on domain formation by such short basic peptides in mixed lipid membranes (Glaser et al., 1996) is presumably of a long-range electrostatic nature, because the selective perturbation of the lipid mobility that has a more localized origin and is found by spin-label ESR with highly basic peripheral membrane proteins (Sankaram et al., 1989a,b) is not observed to an appreciable extent with the short lysine peptides. It is possible that this different form of lipid-peptide association may typify the interaction with the plasma membrane of the basic residue clusters that occur in certain cytoplasmic proteins, such as the pseudo-substrate region of protein kinase C.

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