Binding of peptides with basic residues to membranes containing acidic phospholipids

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ABSTRACT There are clusters of basic amino acids on many cytoplasmic proteins that bind transiently to membranes (e.g., protein kinase C) as well as on the cytoplasmic domain of many intrinsic membrane proteins (e.g., glycophorin). To explore the possibility that these basic residues bind electrostatically to monovalent acidic lipids, we studied the binding of the peptides Lys_n and Arg_n (n = 1–5) to bilayer membranes containing phosphatidylserine (PS) or phosphatidylglycerol (PG). We made electrophoretic mobility measurements using multilamellar vesicles, fluorescence and equilibrium binding measurements using large unilamellar vesicles, and surface potential measurements using monolayers. None of the peptides bound to vesicles formed from the zwitterionic lipid phosphatidylcholine (PC) but all bound to vesicles formed from PC/PS or PC/PG mixtures. None of the peptides exhibited specificity between PS and PG. Each lysine residue that was added to Lys_2 decreased by one order of magnitude the concentration of peptide required to reverse the charge on the vesicle; equivalently it increased by one order of magnitude the binding affinity of the peptides for the PS vesicles. The simplest explanation is that each added lysine binds independently to a separate PS with a microscopic association constant of 10 M^{-1} or a free energy of ~ 1.4 kcal/mol. Similar, but not identical, results were obtained with the Arg_n peptides. A simple theoretical model combines the Gouy-Chapman theory (which accounts for the nonspecific electrostatic accumulation of the peptides in the aqueous diffuse double layer adjacent to the membrane) with mass action equations (which account for the binding of the peptides to > 1 PS). This model can account qualitatively for the dependence of binding on both the number of basic residues in the peptides and the mole fraction of PS in the membrane.

INTRODUCTION

We are interested in the binding of small basic peptides to phospholipid bilayer membranes for two reasons. First, many intrinsic membrane proteins have clusters of basic residues on their cytoplasmic surfaces (71). Glycophorin provides a well-studied example; four of the first six cytoplasmic residues after the single transmembrane region are positively charged (63). A synaptic vesicle protein homologous to the regulatory region of protein kinase C provides a more striking example; 8 of the first 12 amino acids after the putative transmembrane region are positively charged (58). The positive inside "rule" is quite general, and experiments from several different laboratories suggest that these clusters of positive charges are important in determining the orientation of the protein (28, 51). The mechanism by which these clusters of positive residues help determine the orientation of transmembrane proteins is not known. However, Hartmann et al. (28) suggest that the positive residues might sense the local electric potential produced by negatively charged phospholipids, which are preferentially located on the cytoplasmic surface of the membrane (9, 54). We would like to know how regions of proteins containing clusters of positive charges distribute themselves in the electrostatic diffuse double layer adjacent to the membrane (45) and how tightly these positive charges bind to the negative lipids.

Second, we are interested in the binding of basic peptides to membranes because a number of cytoplasmic proteins bind to the negative lipid phosphatidylserine (PS) in both biological membranes and phospholipid bilayers (2, 17, 27, 37, 52). Protein kinase C, PKC, is an important example (52, 56). When PKC is inactive in the cytoplasm, the cluster of five basic residues in the putative pseudosubstrate region of PKC (32) may bind to acidic residues in the putative substrate binding site (33). When PKC translocates to the plasma membrane and becomes active, the pseudosubstrate region presumably moves away from the substrate binding site. In the following report we explore the possibility that the basic residues on the pseudosubstrate region of PKC can bind to PS in membranes (50).

In view of the importance of the interaction of small clusters of positively charged amino acids on proteins with negatively charged lipids in membranes, there is surprisingly little information available about the energies involved in these interactions. Many studies have demonstrated that polylysines of high molecular weight (> 20 residues) bind very tightly to membranes formed from acidic lipids (14, 21, 29, 30, 36, 40, 47, 48, 65, 72).

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Furthermore, Lys_3 and Lys_5 bind significantly to membranes containing negative lipids such as cardiolipin (21) and phosphatidylserine (64). However, we know of no attempt to deduce the energy of interaction between a lysine or an arginine residue on a peptide and a PS or PG lipid in a membrane.

We wanted to deduce these energies, to investigate the specificity of the interaction, to test the suggestion (64) that the basic peptide Lys_5 did not penetrate the polar head group region of the lipid bilayer, and to determine how well the adsorption of small basic peptides to membranes could be described by a simple theory. These questions were addressed by measuring the binding of Lys_n and Arg_n (n = 1–5) to phospholipid bilayers and monolayers. To investigate how the binding energies depend on the proximity of the lysine residues, we measured the binding of peptides with five lysine residues, but with one or two alanine residues between each of the lysines. We studied the binding by making microelectrophoresis measurements on multilamellar vesicles, fluorescence measurements with the probe 2-(*p*-toluidinyl)naphthalene-6-sulfonate (TNS) on unilamellar vesicles, surface potential measurements with an ionizing electrode positioned above a monolayer, and equilibrium dialysis measurements with large unilamellar vesicles. A preliminary report of this work has appeared (49).

MATERIALS AND METHODS

We obtained similar results with egg and palmitoyl-oleoyl phosphatidylcholine (PC), with bovine brain and palmitoyl-oleoyl PS, with egg and palmitoyl-oleoyl phosphatidylglycerol (PG). These lipids, as well as dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), egg *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE), and egg *N*-(1-pyrenesulfonyl) phosphatidylethanolamine (pyrene-PE) were obtained from Avanti Biochemicals (Birmingham, AL).

Lys_n, n = 1–4, were obtained from Bachem Inc. (Torrence, CA) and Research Plus (Bayone, NJ). Arg_n, n = 1–3, were obtained from Research Plus. Lys_5 and Arg_5 were synthesized by Research Plus. Commercially available samples of Lys_5 proved unsatisfactory. Samples from three different chemical companies were beige rather than white; all contained trace concentrations of postively charged contaminants that strongly adsorbed to membranes and significantly affected the experimental results. All three samples had the same unusual counterion composition, which suggests these companies obtained the peptide from the same wholesaler. Lys_n and Arg_n, n =1–5, had free carboxyl and amino termini. We also made measurements with Lys_5, $(Lys\text{-}Ala)_4$-Lys and $(Lys\text{-}Ala\text{-}Ala)_4$-Lys with blocked (acetyl and amide) termini synthesized by Multiple Peptide Systems (San Diego, CA). Acetyl-Lys_2-amide was purchased from Research Plus (Bayone, NJ).

Purity of the peptides was checked by amino acid analysis as well as by analytical HPLC using both reverse phase and ion exchange chromatography with detection at A_{220}. The HPLC chromatograms of Lys_n, n = 3–5, showed single major peaks with <5% of other A_{220} adsorbing species. However, the chromatogram of the Arg_5 peptide revealed ~20% contamination; the polyarginine results presented in Fig. 8 should thus be regarded as preliminary.

4-Morpholinepropanesulfonic acid (MOPS) was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). 2-(*p*-Toluidinyl)naphthalene-6-sulfonate (TNS) was obtained from Sigma Chemical Co. (St. Louis, MO). Aqueous solutions were prepared with 18 MΩ water (Super-Q; Millipore Corp., Bedford, MA) that was subsequently bidistilled in an all-quartz still. They were buffered to pH 7.0 with 0.1–1 mM MOPS unless noted otherwise.

Electrophoretic mobility measurements

We followed Bangham et al. (5) in preparing multilamellar vesicles for the microelectrophoresis experiments. We measured the mobilities of the vesicles in a Rank Brothers Mark I instrument (Bottisham, Cambridge, UK) as described previously (10, 46). All reported data represent the average of at least two independent sets of measurements on 10 vesicles. The presence of 10 μM EDTA in the solution did not affect the results unless noted. Microelectrophoresis measurements are made on single vesicles and are unaffected by aggregation of the majority of vesicles in a solution. We took care that no visible peptide-induced aggregation occurred in the fluorescence, dialysis, and filtration experiments.

Fluorescence measurements

We have described previously how TNS can be used to estimate the surface potential of phospholipid vesicles (10, 25, 70, 74, 75). The 0.1 μm diam large unilamellar vesicles (LUVs) were produced by taking multilamellar vesicles through five cycles of freezing (liquid N_2) and thawing (40°C water bath) followed by extrusion (10 cycles) through two stacked 0.1 μm polycarbonate filters in an Extruder (Lipex Biomembranes, Inc., Vancouver, BC). Steady-state fluorescence measurements were made with a Spex Flurocomp (Edison, NJ).

Monolayer measurements

The methods are described in detail elsewhere (26). In brief, we used a 10-cm diam teflon petri dish (Savillex Corp., Minnetonka, MN) as a monolayer trough. Monolayers were formed by depositing lipids in a chloroform solution on a 100-ml aqueous subphase. The potentials were measured relative to a silver–silver chloride electrode in the subphase with a 20-μCi gold-coated americium-241 ionizing electrode (NRD Inc., Grand Island, NY) positioned 2 mm above the surface with a micromanipulator. The apparatus was enclosed with plexiglass to reduce contamination of the monolayer, nitrogen was introduced into the box throughout the experiment to reduce oxidation of the lipids, and the relative humidity was monitored.

Equilibrium dialysis

Two 1-ml compartments in a teflon dialysis chamber were separated by a polycarbonate membrane with 0.015 μm pores (Nuclepore Corp., Pleasanton, CA). The polycarbonate membranes adsorbed a negligible (<3%) fraction of peptides whereas cellulose membranes proved unsatisfactory because they adsorbed basic peptides. The dialysis was conducted in both directions for 24 h at 22–24°C: the LUVs (0.6–6 mM) and peptide (30 μM) were suspended in 100 mM KCl, 1 mM MOPS, pH 7.0. Phosphate analysis and light scattering measurements were used to determine that vesicles did not leak through the membrane during dialysis. The peptide concentration in both compartments was determined by a fluorescamine assay (73). The concentra-

tion of peptide in both compartments was corrected for the small positive reading that resulted from amines that contaminated the lipids (≈ 1 μM amine per 1 mM lipid); when necessary we dialyzed the LUVs against a large volume (1 l) of buffer solution for 12 h to remove these traces of amines.

Ultrafiltration

We also determined the effective binding constant of Lys_5 to 1:1, 2:1, and 5:1 vesicles in a series of ultrafiltration experiments. The mixture of LUVs and peptide was filtered through a microconcentrator Centricon 10 membrane (Amicon, Danvers, MA). Lipid leakage was negligible and peptide absorption was ~4%.

Mobility assay

The fluorescamine assay was only reliable for $[Lys_5] > 1$ μM. We measured the binding of lower concentrations of Lys_5 to 2:1 PC/PS LUVs in 0.01 M KCl, 0.001 M MOPS solutions by using mobility measurements to estimate the free concentration of the peptide. (We extended the measurements illustrated in Fig. 4 to peptide concentrations as low as 10^{-7} M to obtain a calibration curve relating free concentration of peptide to the mobility of a multilamellar vesicle.) Addition of LUVs reduced the free concentration of Lys_5 in the solution, the mobility of the few multilamellar vesicles changed, and we calculated the free concentration of Lys_5 using the calibration curve.

THEORY

We calculated the zeta potential, ζ, from the measured value of the electrophoretic mobility, u, by using the Helmholtz-Smoluchowski equation (34):

$$\zeta = u\eta/\epsilon_r\epsilon_0, \tag{1}$$

where η is the viscosity of the aqueous solution, ϵ_r the dielectric constant of the aqueous phase, and ϵ_0 the permittivity of free space. For a large smooth particle with charges at the interface (e.g., a phospholipid vesicle) ζ is the electrostatic potential at the hydrodynamic plane of shear (55, 53), which is located ~0.2 nm from the surface (3, 25, 62).

The Gouy-Chapman theory predicts and experiments confirm (45) that the electrostatic potential, $\psi(x)$, varies with distance from the surface, x, in a monovalent salt solution according to:

$$\psi(x) = \frac{2kT}{e} \ln \frac{[1 + \alpha \exp(-\kappa x)]}{[1 - \alpha \exp(-\kappa x)]}, \tag{2}$$

where

$$\alpha = \frac{\exp[e\psi(0)/2kT] - 1}{\exp[e\psi(0)/2kT] + 1}.$$

T is the absolute temperature, k the Boltzmann constant, e the magnitude of electronic charge, and $1/\kappa$ the Debye length, which is ~1 nm in a 0.1-M solution and 3 nm in a 0.01-M solution. When the potential is small, $|\psi(0)| < kT/e \simeq 25$ mV, Eq. 2 may be approximated by:

$$\psi(x) = \psi(0) \exp(-\kappa x). \tag{3}$$

The Gouy-Chapman theory predicts how $\psi(0)$ depends on the surface charge density, σ, and monovalent salt concentration, c:

$$\sinh[e\psi(0)/2kT] = A\sigma/(c)^{1/2}, \tag{4}$$

where $A = (8N\epsilon_r\epsilon_0 kT)^{-1/2}$ and N is Avogadro's number. If $|\psi(0)| < 25$ mV, Eq. 4 reduces to

$$\psi(0) = \sigma/(\epsilon_r\epsilon_0\kappa), \tag{5}$$

which also describes the potential difference between the two plates of a capacitor separated by a distance $1/\kappa$ (44).

In our analysis of the binding of positively charged peptides to negatively charged membranes, we ignore the small "screening" effect they exert on the surface potential because their concentration is always much less than the concentration of monovalent salt.[1] Furthermore, we assume for simplicity that $\zeta = \psi(0)$. Eqs. 1 and 5 demonstrate that when $|\psi(0)| < kT/e$, the mobility (zeta potential) is proportional to the surface charge density, and is thus a simple measure of the number of bound cations.

We calculate the surface charge density by making the following assumptions. The maximum surface concentration of each lipid is one lipid per 0.7 nm.[2] At $6 <$ pH < 8 the net charge on PC, PS, and PG is 0, −1, and −1, respectively. Cations bind only to the negative lipids and anions do not bind to the surface.

The bulk aqueous phase contains potassium ions at a concentration [K], and peptides of valence z at a concentration [P]. The concentrations of these cations in

[1]To illustrate that the effects of Lys_n, $n > 2$, on the zeta potential are due mainly to binding rather than screening we consider only the data obtained when the zeta potential is less negative than −25 mV. We can then linearize the Poisson-Boltzmann equation and use the concept of ionic strength from Debye-Hückel theory, $I = (\frac{1}{2})\Sigma_i c_i z_i^2$, which predicts screening effects depend on the sum of the concentration, c, times the square of the valence, z, for all ions in the solution. Fig. 1 illustrates that Lys_n, n = 3–5, do not significantly affect the ionic strength. Lys_2, at a concentration of 0.01 M, does affect the ionic strength, but a more exact treatment (46) yields a very similar curve to the one in Fig. 1.

[2]Cohen and Cohen (18) demonstrate that more sophisticated equations are required to describe mass action when either the species that adsorbs to the membrane or the membrane binding sites (lipids) are assumed to have a finite size. Their papers may be consulted for a lucid discussion of adsorption isotherms appropriate for a lattice model. S. Stankowski (personal communication) has also recently extended the Gouy-Chapman theory to account for the adsorption of large multivalent molecules. In our model, both the peptide and lipids are regarded as structureless points.

the aqueous phase immediately adjacent to the surface ($x = 0$) are related to their bulk concentrations through Boltzmann relations such as:

$$[\mathrm{P}]_0 = [\mathrm{P}] \exp[-ze\psi(0)/kT]. \quad (6)$$

The assumption that these peptides are point charges is obviously incorrect (12) and is considered in more detail below and elsewhere (8, 38, 50, 69). The surface concentrations, { }, of potassium ions and peptides bound in 1:1 complexes with the negative lipids, L, are:

$$\{K\text{-}L\} = K_{\mathrm{K\text{-}L}}\{L\}[\mathrm{K}]_0 \quad (7)$$

$$\{P\text{-}L\} = K_{\mathrm{P\text{-}L}}\{L\}[\mathrm{P}]_0, \quad (8)$$

where $K_{\mathrm{K\text{-}L}}$ and $K_{\mathrm{P\text{-}L}}$ are intrinsic association constants, expressed in conventional units (e.g., molar^{-1}).

We also assume that when a bound peptide has more than two positive moieties ($z = n = 3, 4, 5$ for Lys_n) it can interact with another negative lipid L. A simple mass action treatment[2] implies:

$$\{P\text{-}L_2\} = K_{\mathrm{P\text{-}L2}}\{P\text{-}L\}\{L\}, \quad (9)$$

where $K_{\mathrm{P\text{-}L2}}$ is a two-dimensional association constant with units meter^{2}mole^{-1}. We can express this association constant in more conventional units (e.g., molar^{-1}) by multiplying it by the thickness of the polar head group region, $d \approx 1$ nm, and dividing the three surface concentrations in Eq. 9 by d, to convert from a Gibbs-type surface or mathematical dividing plane to a Guggenheim-type surface with a finite thickness and volume (4). Similarly, for peptides with $z = 4, 5$ (e.g., Lys_4 and Lys_5), complexes can form between the peptide and three negative lipids

$$\{P\text{-}L_3\} = K_{\mathrm{P\text{-}L3}}\{P\text{-}L_2\}\{L\} \quad (10)$$

and for peptides with $z = 5$ (e.g., Lys_5)

$$\{P\text{-}L_4\} = K_{\mathrm{P\text{-}L4}}\{P\text{-}L_3\}\{L\}. \quad (11)$$

When considering the binding of a peptide of valence $z = 5$ to a membrane, the surface charge density or charge per unit area is

$$\sigma = e(-\{L\} + \{P\text{-}L_4\} + 2\{P\text{-}L_3\} + 3\{P\text{-}L_2\} + 4\{P\text{-}L\}) \quad (12)$$

and

$$\{L\} = \{L\}^{\mathrm{tot}}(1 + K_{\mathrm{K\text{-}L}}[\mathrm{K}]_0 + K_{\mathrm{P\text{-}L}}[\mathrm{P}]_0 + 2K_{\mathrm{P\text{-}L2}}K_{\mathrm{P\text{-}L}}[\mathrm{P}]_0\{L\} + 3K_{\mathrm{P\text{-}L3}}K_{\mathrm{P\text{-}L2}}K_{\mathrm{P\text{-}L}}[\mathrm{P}]_0\{L\}^2 + 4K_{\mathrm{P\text{-}L4}}K_{\mathrm{P\text{-}L3}}K_{\mathrm{P\text{-}L2}}K_{\mathrm{P\text{-}L}}[\mathrm{P}]_0\{L\}^3)^{-1}, \quad (13)$$

where $\{L\}^{\mathrm{tot}}$ is the total surface concentration of anionic lipid.

It may be helpful to consider the simplest possible model for the binding of a peptide like Lys_5 to membranes. If we assume that Lys_5 has four binding sites, that all four binding sites are identical and independent, and each binds to a negative lipid with an identical free energy or microscopic association constant k, we can write the macroscopic association constants we defined above in Eqs. 8–11 as:

$$K_{\mathrm{P\text{-}L}} = 4k \quad (8a)$$

$$K_{\mathrm{P\text{-}L2}} = (3/2)(k/d) \quad (9a)$$

$$K_{\mathrm{P\text{-}L3}} = (2/3)(k/d) \quad (10a)$$

$$K_{\mathrm{P\text{-}L4}} = (1/4)(k/d). \quad (11a)$$

The numerical factors arise because of statistical effects, which are considered in detail elsewhere (11) for the case of a macromolecule with four identical, independent binding sites.

It is convenient to define an effective or apparent association constant K that can be determined directly from equilibrium dialysis measurements. Eq. 14 relates the total surface concentration of bound Lys_5 or peptide, $\{P\}^{\mathrm{tot}}$, to its concentration in the bulk aqueous phase, [P]:

$$\{P\}^{\mathrm{tot}} = K[\mathrm{P}], \quad (14)$$

where K has the units of length. We now relate K for Lys_5 to the parameters of our binding model. By definition:

$$\{P\}^{\mathrm{tot}} = \{P\text{-}L\} + \{P\text{-}L_2\} + \{P\text{-}L_3\} + \{P\text{-}L_4\}. \quad (15)$$

By inserting Eqs. 8–11 and 6 into Eq. 15 and combining with Eq. 14 we obtain the following expression for K:

$$K = \left(\exp[-5e\psi(0)/kT]\right)\left(K_{\mathrm{P\text{-}L}}\{L\}\right)\left(1 + K_{\mathrm{P\text{-}L2}}\{L\} + K_{\mathrm{P\text{-}L2}}\{L\}K_{\mathrm{P\text{-}L3}}\{L\} + K_{\mathrm{P\text{-}L2}}\{L\}K_{\mathrm{P\text{-}L3}}\{L\}K_{\mathrm{P\text{-}L4}}\{L\}\right). \quad (16)$$

This combination of Gouy-Chapman, Boltzmann, and mass action equations is the simplest theoretical model capable of describing both the electrostatic accumulation of the basic peptides in the diffuse double layer and the binding of basic residues to acidic phospholipids in the membrane. There is now some theoretical justification for the assumptions (e.g., mean-field, smeared-charge, primitive model electrolyte) inherent in the application of the Gouy-Chapman theory to a molecularly smooth surface such as a phospholipid bilayer membrane (13, 39, 45). Several groups discuss in more detail the use of mass action equations to describe the binding of macromolecules to multiple sites on mem-

branes (15, 16, 19, 23, 61). Papers by Reynolds (61), Adam and Delbrück (1), and Berg and Purcell (7) provide excellent introductions to the differences between reactions that occur in bulk solution and those that occur on the surface of membranes.

RESULTS

Fig. 1 illustrates the effects of di-, tri-, tetra-, and pentalysine (Lys_n, n = 2, 3, 4, 5) on the zeta potential, ζ, of vesicles formed from either phosphatidylserine (PS) or phosphatidylglycerol (PG). ζ is the potential at the hydrodynamic plane of shear, which is located ~0.2 nm from the surface of a phospholipid vesicle. Eq. 3 demonstrates ζ is approximately equal to the surface potential in a 0.1-M salt solution, where the Debye length is ~1 nm. The surface potential is proportional to the surface charge density (charge/area) when the potential is smaller than $kT/e \simeq 25$ mV (Eq. 5). Thus when the potential is small there is a simple linear relationship between ζ and the number of positively charged peptides bound to a unit area of membrane.

Does the binding of these peptides to the surface depend on the chemical nature of the phospholipid head group or just on its net charge? It is apparent from Fig. 1 that the peptides bind equally well to vesicles formed from either PG (*open symbols*) or PS (*solid symbols*). None of the small basic peptides we have examined to date exhibit any significant specificity between vesicles formed from these two monovalent negatively charged lipids, and none bind significantly to vesicles formed from the zwitterionic lipid phosphatidylcholine (PC). Our mobility measurements confirm and extend the results obtained by others; spectroscopic techniques demonstrate there is little specificity in the binding of polylysine to PS and PG in membranes (66) and neither polylysine (40) nor Lys_5 (64) interact with PC.

How strong is the binding and how does it depend on the number of lysines in the peptide? To calculate a binding energy or association constant we consider the concentration of peptide required to reduce ζ to zero; the position of the plane of shear then becomes irrelevant and we can also ignore the nonspecific accumulation of peptide in the diffuse double layer. For example, the concentration of Lys_5 required to reverse the charge of the PS vesicles is of order 10^{-4} M. By extrapolation, the concentrations of Lys_4, Lys_3, and Lys_2 required to reduce ζ to zero are of order 10^{-3}, 10^{-2}, and 10^{-1} M. If we assume that one Lys_2 binds to one PS, then the surface concentration of +1 valence peptide-lipid complexes, $[P\text{-}L]$, is equal to the surface concentration of −1 valence free lipid, $[L]$, when the peptide concentration in the aqueous phase is [P] = 0.1 M. Eq. 8 illustrates the intrinsic association constant is $K_{P\text{-}L} = 1/[P] = 10\ M^{-1}$ for Lys_2. The association constants of the divalent magnesium and calcium cations with PS (46) and PG (41) are also ~10 M^{-1}. An association constant of 10 M^{-1} corresponds to a standard free energy change on binding of $\Delta G^\circ = RT \ln K_{P\text{-}L} = 5.7$ kJ/mol = 1.4 kcal/mol.

Why do Lys_3, Lys_4, and Lys_5 bind about one, two, and three orders of magnitude more strongly than Lys_2 to PS (and PG) vesicles? The simplest explanation is that these peptides bind to more than one negative lipid. If each additional lysine residue added to Lys_2 binds to a separate PS with an association constant of 10 M^{-1}, this could account for the observed increase in the binding. The theoretical curves in Fig. 1 were calculated by making this assumption.[3] (Our objective was not to choose values of the association constants that provided a best fit to the data but to illustrate that a simple model could qualitatively describe the data.)

If all the two-dimensional association constants for all the Lys_n peptides are 10^7 m^2 mol^{-1}, our model predicts that most of the peptides interact with the maximum possible number of lipids: most of the bound pentalysines interact with four negative lipids, most of the bound tetralysines interact with three negative lipids and most of the bound trilysines interact with two negative lipids. For example, Eq. 11 illustrates that the ratio of the number of pentalysines bound in 1–4 peptide-lipid complexes to the number bound in 1–3 complexes is $K_{P\text{-}L4}[L]$. The total surface concentration of the negative lipid is $(L)^{tot} = 1/(0.7\ nm^2)$, so this ratio is >10 if most of the negative lipid is free. An identical

[3]Specifically, we assumed that Lys_n (n = 2–5) forms 1–1 peptide–lipid complexes with $K_{P\text{-}L} = 10\ M^{-1}$, that Lys_n (n = 3–5) can form 1–2 peptide-lipid complexes with $K_{P\text{-}L2} = 10^7$ m^2/mol, that Lys_n (n = 4, 5) can form 1–3 peptide-lipid complexes with $K_{P\text{-}L3} = 10^7$ m^2/mol, and that Lys_5 can form 1–4 peptide-lipid complexes with $K_{P\text{-}L4} = 10^7$ m^2/mol. An association constant of 10^7 m^2/mol corresponds to an association constant in conventional units of 10 M^{-1} if we assume the thickness of the polar head group region is d = 1 nm (see Theory). To analyze the data we assumed, for simplicity, that ζ is equal to the surface potential and that potassium ions bind to the negative lipids with an intrinsic association constant of $K_{K\text{-}L} = 1\ M^{-1}$. We combined Eqs. 1, 4, 6, and 7–12 to produce the curves in Fig. 1. We assumed the valences of Lys_n (n = 2, 3, 4, 5) were 2, 3, 4, 5 in the pH 7.0 solutions, which is only approximately true (see footnotes 4 and 9), and that the peptides and charged lipids are point charges, which is not even approximately true. We obtained similar theoretical curves (deviations <3 mV) to those illustrated in Fig. 1 if we assumed that each Lys_n peptide had $n - 1$ identical independent binding sites that bind to negative lipids with a microscopic binding constant $k = 10\ M^{-1}$ or an energy of 1.4 kcal/mol. In other words, replacing Eqs. 8–11 by Eqs. 8*a*–11*a* has little effect for our conditions because most of the binding sites associate with a lipid, the last term in the third set of parentheses in Eq. 16 dominates the other terms, and the statistical factors cancel in the last term. The equations were solved using MathCAD (MathSoft Inc., Cambridge, MA).

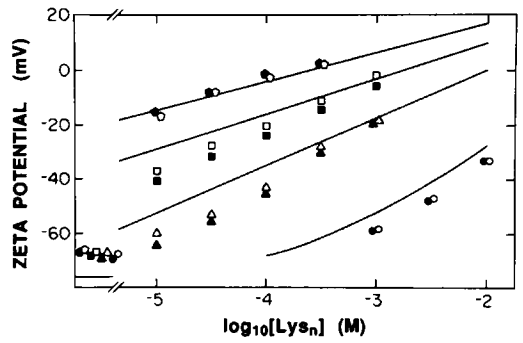

FIGURE 1 Effect of dilysine (n = 2; *circles*), trilysine (n = 3; *triangles*), tetralysine (n = 4; *squares*), and pentalysine (n = 5; *pentagons*) on the zeta potential of multilamellar vesicles formed from either palmitoyl-oleoyl phosphatidylserine, PS (*solid symbols*) or palmitoyl-oleoyl phosphatidylglycerol, PG (*open symbols*). The vesicles were formed in 0.1 M KCl buffered to pH 7.0 with 1 mM MOPS at 25°C. The data to the left of the break in the abscissa illustrate the results obtained in the absence of Lys_n molecules. Each experimental point represents the average of measurements on >20 vesicles in at least two separate experiments. The average of the standard deviations was <2 mV, smaller than the size of the symbols. The curves illustrate the predictions of a simple theory in which Lys_2 binds to negative lipids with a binding constant of 10 M^{-1} (1.4 kcal/mol) and each additional Lys residue added to the peptide binds to a separate negative lipid with this same binding constant. (See text and footnote 3 for details.)

calculation holds for the ratio of 1–3:1–2 peptide-lipid complexes for both pentalysine and tetralysine, etc. In other words, once one of the lysine residues on Lys_n (n = 3, 4, 5) binds to a negative lipid in the membrane, it is likely that the remaining n – 2 binding sites will also associate with a lipid.

The data illustrated in Fig. 1 were all obtained with peptides with unblocked termini. We measured the binding of a Lys_5 that had blocked termini to membranes, and also studied the binding of Lys_5 at pH 6 and 8. We concluded that the α-amino group of the peptides did not exert an anomalously large effect on the binding.[4]

If our model is correct, and the Lys_n (n > 2) peptides do bind to more than one negative lipid, diluting the surface concentration of negative lipid (PS or PG) with a zwitterionic lipid to which the peptides do not bind (e.g., phosphatidylcholine, PC) should decrease the binding of the peptides to the vesicles. It does.

Fig. 2 illustrates that the binding of Lys_3, Lys_4, and Lys_5 to 2:1 PC/PS (or PC/PG) vesicles is about an order of magnitude weaker than to PS (or PG) vesicles (Fig. 1). Consider Lys_5: 10^{-4} M Lys_5 reduces ζ of PS vesicles to zero (Fig. 1) but reduces ζ of the 2:1 PC/PS vesicles only to −20 mV (Fig. 2). By extrapolation of the data in Fig. 2, ~10^{-3} M Lys_5 would be required to neutralize the charge on these vesicles and reduce ζ to zero. The observation that Lys_5 binds an order of magnitude less strongly to the 2:1 PC/PS than to the PS vesicles is qualitatively consistent with the suggestion that one Lys_5 binds to more than one negative lipid. In fact, diluting the negative lipid with PC decreases the binding of these peptides to the vesicles more than expected from the simple mass action model, as we illustrate in more detail with Lys_5 in Fig. 3.

Fig. 3 *A* illustrates the effect of Lys_5 on the zeta potential of vesicles formed from PG and PC/PG mixtures. It demonstrates that the number of bound Lys_5 per PG decreases as the mole fraction of PG in the vesicles decreases. In other words, higher concentrations of Lys_5 are required to reverse the charge as the mole fraction of PG in the vesicles decreases. This observation is consistent with Lys_5 binding to more than one PG in the membrane, even when PG is diluted fivefold with PC. Fig. 3 *B* illustrates the predictions of our theoretical model, using the same values of the association constants used to describe the data in Fig. 1 (see footnote 3). There are two major discrepancies between the experimental results and the theoretical predictions: PC decreases the binding of Lys_5 to the membranes more than predicted and the slopes of the curves are greater than predicted.

We made measurements with Lys_5 in 0.01 M (Fig. 4)

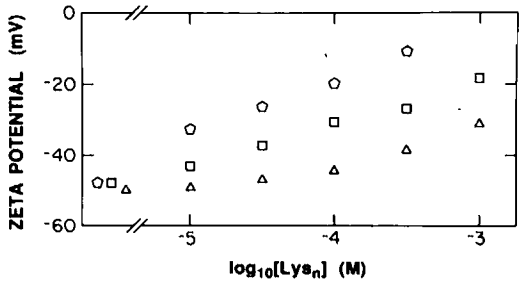

FIGURE 2 Effect of trilysine (n = 3; *triangles*), tetralysine (n = 4; *squares*), and pentalysine (n = 5; *pentagons*) on ζ of 2:1 (mol/mol) PC/PS and PC/PG vesicles. (Similar results were obtained with PC/PS and PC/PG vesicles, and the data were averaged.) These peptides had no significant effect on ζ of PC vesicles at the concentrations illustrated in the figure (data not shown). The aqueous solutions contained 0.1 M KCl buffered to pH 7.0 with 1 mM MOPS at 25°C.

[4]Compared with the results obtained at pH 7 (Fig. 1), pentalysine binds about threefold less effectively to PG vesicles at pH 8 and slightly more effectively at pH 6 (data not shown). These observations are consistent with our expectation that the pK of the α-amino group of pentalysine is ~7 (see footnote 9). Pentalysine with blocked amino and carboxyl termini binds about threefold less effectively to PG vesicles than does Lys_5 at pH 7 (data not shown). Dilysine with blocked termini binds about the same to PG vesicles as does Lys_2 at pH 7 (data not shown).

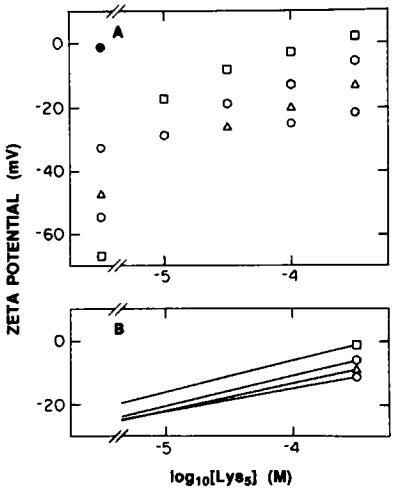

FIGURE 3 (*A*) The effect of pentalysine on the zeta potential of vesicles formed from PC/PG mixtures. The mol:mol ratio of PC/PG was 0:1, (*squares*), 1:1 (*hexagons*), 2:1 (*triangles*), 5:1 (*open circles*), and 1:0 (*solid circle*). Pentalysine has no significant effect on the zeta potential of PC vesicles (data not shown). The aqueous solutions contained 0.1 M KCl buffered to pH 7.0 with 1 mM MOPS at 25°C. (*B*) The theoretically predicted effect of pentalysine on the zeta potential using the same parameters that described the data in Fig. 1 (see footnote 5). The symbols to the right of the curves designate the PC/PG mixtures.

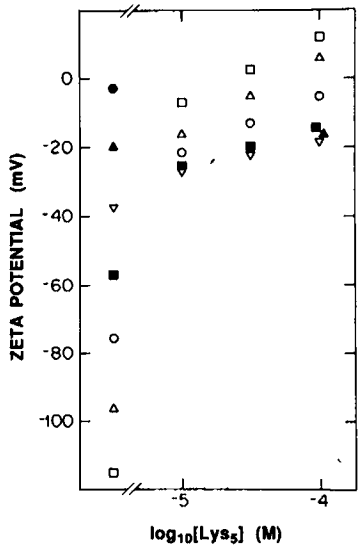

FIGURE 4 The effect of pentalysine on the zeta potential of vesicles formed from PG (*open squares*) and from mixtures of PC with PG. PC/PG 2:1 (*open triangles*), 5:1 (*open circles*), 10:1 (*solid squares*), 20:1 (*open inverted triangles*), 50:1 (*solid triangles*), PC (*solid circle*). The aqueous solutions contained 0.01 M KCl buffered to pH 7.0 with either 0.1 mM MOPS or phosphate at 25°C.

as well as 0.1 M (Fig. 3) KCl for several reasons.[5] The qualitative features of the results are similar in both 0.01 and 0.1 M KCl. Specifically, Figs. 3 and 4 illustrate that a [Lys_5] concentration of order 10^{-4} M reverses the charge on the PG vesicles in both 0.1 and 0.01 M KCl. The [Lys_5] required to reverse the charge increases as PG is diluted with PC, which implies Lys_5 binds to the PC/PG vesicles with association constants that decrease in the sequence 0:1 > 2:1 > 5:1 > 10:1 ≃ 20:1 ≃ 50:1, as predicted qualitatively from Eq. 16.

Before we compare the detailed predictions of the theoretical model with the data in Fig. 4 we note that the data cannot be described by the theoretical model if we assume the peptide forms only 1–1 complexes with the negative lipid. If we choose a 1–1 peptide-lipid intrinsic association constant high enough to account for the effect of Lys_5 on the ζ of PG vesicles, $K_{P\text{-}L} = 10^4\ M^{-1}$, the predicted effect of Lys_5 on the other vesicles is much larger than the observed effect (Fig. 5 *A*). On the other hand, if we choose a value for $K_{P\text{-}L}$ low enough to account for the small effect of Lys_5 on the ζ of the 50:1 PC/PG vesicles, $K_{P\text{-}L} = 10\ M^{-1}$, the predicted effect of Lys_5 on the other vesicles is much smaller than the observed effect (Fig. 5 *B*). If only 1–1 peptide-lipid complexes are formed, the concentration of peptide required to reverse the charge should be the same for all lipid compositions (Fig. 5 *A*), which is clearly not the case (Fig. 4). The ζ data suggest the binding is about twofold stronger in 0.01 than in 0.1 M salt; the intrinsic association constants used to produce the theoretical curves in Fig. 5 *C* were a factor of 2 larger than those used to fit the data in Figs. 1 and 3. The theoretical model (Fig. 5 *C*) can describe qualitatively the experimental data (Fig. 5).

Our data suggest that Lys_5 binds to phospholipid vesicles by interacting with several negative lipids. What is the orientation of Lys_5 when it adsorbs to the membrane? Do the lysine residues intercalate into the polar head group region? NMR experiments (64), and the

[5]First, if there is less KCl to screen the surface charges, theory predicts (Eq. 4) and experiments confirm that the zeta potential (e.g. Figs. 3, 4) of a PC/PG membrane becomes more negative. The binding of pentalysine to vesicles with low mole % PG can now be measured. Second, our assumption that anions do not bind to the peptides or membranes is more valid in 0.01 than in 0.1 M salt. Third, the finite size of the peptides affects their distribution in the diffuse double layer (12). Our assumption that the peptides are point charges is more valid in 0.01 than in 0.1 M salt because the Debye length is longer (3 vs. 1 nm). Fourth, the finite size of the adsorbed peptides can affect the electrophoretic mobility in two different ways. If the bound peptide protrudes from the surface it will exert hydrodynamic drag. If the charges on the peptide extend some distance from the surface they will have a larger effect on the mobility than if they were at the surface. These problems have been treated theoretically (22, 42, 43). Both these effects become less important as the salt concentration decreases.

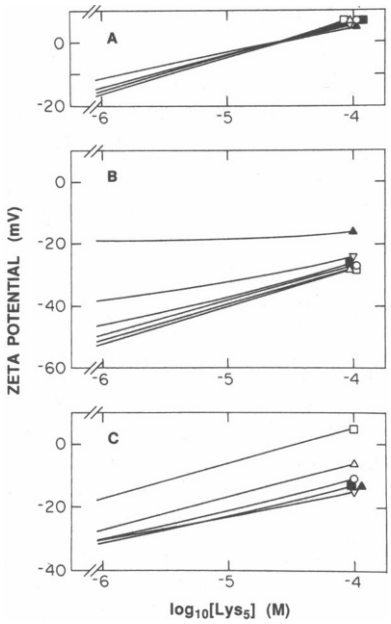

FIGURE 5 The theoretically predicted effect of pentalysine on the zeta potential of PC/PG vesicles in 0.01 M KCl. The symbols to the right of the figures designate the PC/PG mixture indicated in Fig. 4. We assume that the valence of pentalysine is +5, that the zeta potential is equal to the surface potential (plane of shear is located at the surface), and that potassium ions bind to PG with an association constant of 1 M^{-1}. (*A*) The peptide forms only 1–1 peptide-lipid complexes with an association constant (Eq. 8) $K_{P\text{-}L} = 10^4\ M^{-1}$. (*B*) The peptide forms only 1–1 peptide-lipid complexes with an association constant $K_{P\text{-}L} = 10\ M^{-1}$. (*C*) The peptide forms 1–1 peptide-lipid complexes with $K_{P\text{-}L} = 20\ M^{-1}$, but also forms 1–2, 1–3, and 1–4 complexes with two, three, and four negative lipids (Eqs. 9, 10, 11) with $K_{P\text{-}L2} = K_{P\text{-}L3} = K_{P\text{-}L4} = 2\ 10^7\ mol^{-1}\ m^2$.

results we report below, suggest that when the peptide adsorbs to membranes it remains outside the envelope of the head group.

Seelig et al. (67) developed an elegant technique to measure orientation of the head group of a phospholipid in a fluid membrane. They deuterated the head group of PC, then used NMR to determine its average orientation. The head groups of PC are normally coplanar with the surface of the membrane. When calcium or other small cations bind to the phosphate group of PC they repel the positively charged choline moiety and the average orientation shifts toward a value perpendicular to the surface. Roux et al. (64) showed that Lys_5, in contrast to calcium ions, had no effect on the quadrapole splittings of headgroup-deuterated PC, and little effect on headgroup-deuterated PS. They suggested that Lys_5 binds outside the envelope of the polar head group. If this interpretation is correct, Lys_5 should have a smaller effect on the surface potential measured by an ionizing electrode above a PS monolayer than on ζ. Although a detailed analysis of the problem is rather complicated (22, 42, 43), the theory is well developed and the essence of the phenomenon is easy to understand. We consider only a simple example. The average distance of the potassium counterions from the negatively charged surface of the membrane, the Debye length, is 1 nm in 0.1 M salt (44, 45). In other words, the surface potential may be described approximately by a capacitor equation (Eq. 5), or a model in which all the counterions in the diffuse double layer are placed a distance equal to the Debye length from the membrane. If the charges on the adsorbed Lys_5 molecules were also located 1 nm from the surface, the surface potential would not change when Lys_5 ions adsorb. Five free potassium counterions are replaced by one bound Lys_5 ion but the surface potential would still be described by the same capacitor equation. However, ζ or electrophoretic mobility would change dramatically because the adsorbed Lys_5 ions move with the vesicle when a field is applied but the free potassium ions in the diffuse double layer move in the opposite direction to the vesicle.

Fig. 6 illustrates the monolayer data obtained in 0.1 M KCl. The potential above a PC monolayer, measured relative to the clean air-solution interface, is $\Delta V = +450$ mV, a number that agrees well with the range of values in the literature (6, 26, 31, 60). This potential must be due to oriented dipoles at the membrane-solution interface, but the molecular origin of the potential is obscure. Lys_5 has no significant effect on the surface potential of a PC monolayer, a result we expected from its lack of effect on the ζ of PC vesicles. PS has a surface potential that is 110 mV more negative than PC. This result is consistent with the predictions of Gouy-Chapman-Stern

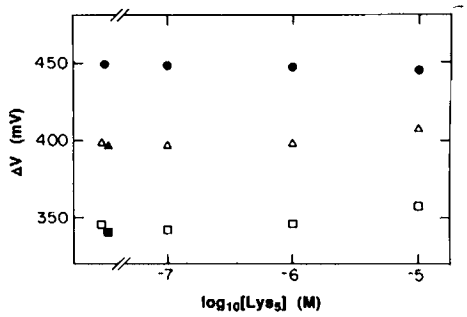

FIGURE 6 The effect of pentalysine on electrostatic potential, ΔV, above a monolayer formed from PS (*open squares*), 2:1 PC/PS (*open triangles*), or PC (*solid circles*). The potential was measured with an ionizing electrode relative to the potential of the clean air-solution interface. The aqueous subphase contained 0.1 M KCl buffered to pH 7.0 with 0.1 mM phosphate. The solid triangle and square illustrate the negligible effect of 10^{-5} M EDTA.

theory (Eqs. 4, 6, 7) if we assume the dipole potentials of PC and PS monolayers are identical and that potassium binds to PS with an intrinsic association constant $0.1 < K_{K\text{-}L} < 1\ M^{-1}$ (25). The addition of Lys_5 to a concentration of 10^{-5} M produces only small changes in the surface potential of PS (15 mV) and 2:1 PC/PS (10 mV) monolayers (Fig. 6). We observed similar small effects of Lys_5 on the surface potential of PG and 2:1 PC/PG monolayers (data not shown). In contrast, this concentration of Lys_5 produced large changes (50 mV) in the ζ of the PS and PG vesicles (Fig. 1). This difference between the zeta and surface potential results is consistent with the suggestion by Roux et al. (64) that Lys_5 adsorbs outside the envelope of the polar head group. Calcium, in contrast to Lys_5, has the same effect on the zeta and surface potentials of PS vesicles and monolayers (46), presumably because it penetrates into the polar head group region.

If the suggestion that Lys_5 adsorbs outside the envelope of the head groups is correct, the surface potential results should agree more closely with the ζ results in 0.01 M KCl (Debye length 3 nm) than in 0.1 M KCl (Debye length 1 nm). They do.

Fig. 7 illustrates the surface potential results obtained in 0.01 M KCl. The surface potential of a PC monolayer is independent of the salt concentration (Figs. 6, 7), as expected theoretically for this electrically neutral surface. The surface potentials of the PS and the 2:1 PC/PS monolayers are ~60 mV more negative than the values observed in 0.1 M KCl (compare Figs. 6 and 7), as predicted from Gouy-Chapman theory (Eq. 6). Lys_5, at a concentration of 10^{-5} M, now has a relatively large effect on the surface potential of PS monolayers (70 mV) and similar effects were observed with PG monolayers (data not shown).

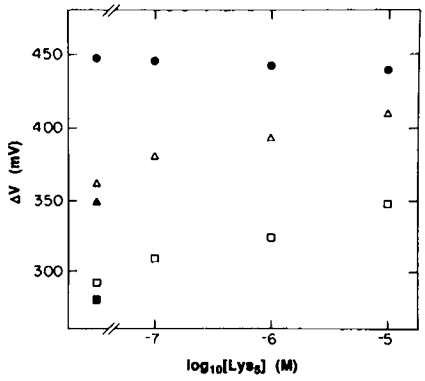

FIGURE 7 The effect of pentalysine on the potential, ΔV, above a monolayer formed from PS (*open squares*), 2:1 PC/PS (*open triangles*), or PC (*solid circles*). The aqueous subphase contained 0.01 M KCl and was buffered to pH 7.0 with either 0.1 mM phosphate or 1 mM MOPS. The filled square and triangle illustrate the effect of 10^{-5} M EDTA.

The results we obtained on large unilamellar vesicles with TNS, a fluorescent probe of the surface potential (75) were similar to those obtained with an ionizing electrode above a monolayer (Figs. 6, 7). Specifically, the potential sensed by TNS at the surface of PC vesicles did not change when 10^{-5} M Lys_5 was added. The potential sensed by TNS at the surface of PS vesicles changed much less (from −90 to −75 mV) than did ζ (from ~−70 to −20 mV, see Fig. 1) in 0.1 M KCl. However, the TNS results obtained in 0.01 M KCl were comparable with the zeta results. The potential sensed by TNS at the surface of a 5:1 PC/PS vesicle in 0.01 M KCl changed from ~−60 to −20 mV upon addition of 10^{-5} M Lys_5, a result comparable to the ζ measurement in Fig. 4 (−75 to −20 mV). In summary, a comparison of the surface potential results (obtained with an ionizing electrode above a monolayer or a TNS probe adsorbing to a vesicle) with the ζ measurements supports the suggestion (64) that Lys_5 adsorbs outside the polar head group region of the bilayer.

We wanted to know if peptides with arginine residues bind as strongly as peptides with lysine residues to vesicles formed from the negative lipids PS and PG. They do. Fig. 8 illustrates the effects of penta-, tri-, and diarginine (Arg_5, Arg_3, and Arg_2) on the ζ of PS vesicles in 0.1 M KCl. Similar results were obtained with PG vesicles (data not shown). These peptides have about the same effect on ζ as do Lys_5, Lys_3, and Lys_2 (Fig. 1). For example, the measured or extrapolated concentrations of Arg_5, Arg_3, and Arg_2 required to reverse the charge of a PS vesicle are $2\ 10^{-4}$, $3\ 10^{-3}$, and $3\ 10^{-2}$ M (Fig. 8). These numbers agree within a factor of three with the corresponding values for Lys_5, Lys_3, and Lys_2 (Fig. 1). Arg_2, Arg_3, and Arg_5 do not bind to PC at the concentrations indicated in Fig. 8, in agreement with the results obtained with lysine peptides (data not shown).

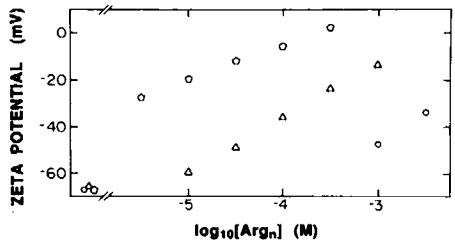

FIGURE 8 The effect of Arg_2 (*circles*), Arg_3 (*triangles*), and Arg_5 (*pentagons*) on the zeta potential of PS vesicles. The aqueous solution contained 0.1 M KCl buffered to pH 7.0 with 1 mM MOPS.

However, arginine and lysine peptides do not bind identically to negatively charged lipids.[6]

The cytoplasmic surface of a typical membrane contains ~30% negative phospholipids and the surface potential should be ~ −30 mV. How strongly does a pentavalent basic peptide bind to such a surface, how accurately does our simple theoretical model describe this binding, and how well do the electrophoretic mobility measurements presented agree with more direct measurements? To address these questions we measured the binding of pentalysine to phospholipid vesicles using both an equilibrium dialysis and a filtration technique. We made measurements at three different mole fractions of negative lipid and two different lipid concentrations. We consider one representative result. For a 2:1 PC/PG membrane, we placed a total lipid concentration ≃ 1 mM, and a total [Lys_5] = 30 μM in one half of a dialysis chamber (0.1 M KCl, pH 7.0). From the equilibrium measurement of the free [Lys_5] (≃ 10 μM, which corresponds to a zeta potential ≃ −30 mV from Fig. 2), the value of the effective binding constant K (Eq. 14) was 4 ± 1 μm (n = 3). We obtained identical results, within experimental error, from the filtration measurements. (Increasing the magnitude of the zeta potential by decreasing the [KCl] produced a large increase in the measured value of K, as we expected.) The value of K predicted from our simple model (Eq. 16) was always larger than the experimentally measured value: ~10 rather than 4 μm for the 2:1 PC/PG membrane in 0.1 M KCl.

The discrepancy between the measured and theoretically predicted values of K is not surprising because our model is very simple. We can describe the zeta potential data for the 2:1 PC/PG membrane more accurately if we choose an effective valence, z_{eff}, in the Boltzmann relation (Eq. 6), which affects the slope, and a different microscopic association constant, which affects the intercept of the essentially linear curve in Fig. 2 or 3. A value of z_{eff} = 3.6 (rather than 5) and a microscopic association constant of k = 5 M^{-1} (rather than 10) provide a good fit to the data. K calculated from the model (Eq. 16) for these parameters was equal to 4 μm, which agrees with the experimental results obtained from equilibrium dialysis and filtration experiments. Thus our simple theoretical model (Eqs. 4, 6, 7–11) overestimates the binding of peptides to PC/PS bilayer membranes because $z_{eff} < z$ (8, 38, 50, 69), but the zeta potential measurements reported here provide a good estimate of the number of bound peptides. The effective association constant of 4 μm (the distance one must move away from a planar membrane to find the same number of peptides in the aqueous phase and bound to the membrane) is comparable to the dimensions of a typical cell.

[6]For example, arginine binds about tenfold more strongly than lysine to PS vesicles (Appendix) and Arg_5 with blocked termini binds about tenfold more strongly to 4:1 PC/PG membranes than Lys_5 with blocked termini (data not shown).

DISCUSSION

Our most important experimental observation is that each lysine (Figs. 1, 2) or arginine (Fig. 8) residue added to a peptide decreases the concentration of peptide required to reverse the charge on a PS (or PG) vesicle tenfold. Nonspecific accumulation of the peptide in the aqueous diffuse double layer (Eq. 6) is absent when the net charge on the vesicle is zero; thus our observation implies that each basic residue added to a peptide increases the binding affinity of the peptide for the membrane tenfold. Our results agree qualitatively with the results obtained by de Kruijff et al. (21) for the binding of di-, tri-, and pentalysine to cardiolipin liposomes.

We considered the simplest theoretical model capable of describing this experimental observation, mass action. The Gibbs and Guggenheim formulations are mathematically equivalent. If we follow Guggenheim and consider the head group region to be an ideal solution of finite thickness (d = 1 nm), we can describe the data in Fig. 1 by assuming that dilysine and each lysine residue added to dilysine (n − 1 residues in Lys_n) are capable of binding to a PS molecule with a macroscopic association constant of 10 M^{-1}. We obtained an equally good fit to the data if we assumed there were n − 1 equivalent, independent (noninteracting) binding sites on Lys_n that bound to PS with a microscopic association constant of 10 M^{-1} or an energy of 1.4 kcal/mol (see footnote 3).

In this mass action model a peptide, P (e.g., Lys_5), from the aqueous phase binds initially to the head group of one negative lipid, L, to form a P-L complex (Eq. 8). A free lysine residue in the P-L complex then combines with another negative lipid to form a P-L_2 complex (Eq. 9) and so on. These higher order complexes between one peptide and several negative lipids only form to a significant degree because the product of the association constant and the concentration of negative lipids in the surface phase is >1. For an association constant of 10 M^{-1}, this product is ~5 for the mole fraction of negative lipid found on the cytoplasmic surface of many plasma membranes; a surface with 25% negative lipid corresponds to a concentration of 0.5 M in the surface phase (25% negative lipid ≈ 1 negative lipid/3 nm^2 ≈ 5 10^{-11} mol negative lipid cm^{-2} ≈ 0.5 M if d = 1 nm). Thus the binding energy or association constant is sufficient to produce a synergism or apparent cooperativity in the interaction of basic residues on peptides or proteins with

negative lipids (50). By this we mean that when one basic residue on a peptide binds to the membrane it places all the other binding sites on the molecule in a different phase (Guggenheim model), one where the concentration of anionic lipids is much greater than in the bulk aqueous phase; all the remaining binding sites combine with lipids via reactions that appear to be ideally cooperative or have a maximum Hill coefficient. The results in Figs. 3 and 4 are consistent with the prediction that Lys_5 interacts with several negative lipids in PC/PS membranes but we were not able to detect this slight clustering of negative lipids by making fluorescence measurements.[7] The binding does not depend strongly on either the temperature or the diffusion coefficients of the lipids in the plane of the membrane.[8]

The mass action formalism is clearly an oversimplification. It ignores the finite size of both the lipids and the peptides, and it considers the highly concentrated polar head group region to be an ideal, infinitely dilute solution. Of course the energy we deduced for the binding of the negative lipids PS and PG to lysine and arginine residues is dependent on our model. Within the framework of the mass action model, the microscopic association constant k varies from a value of 20 for 0.01 M salt to a value of 5 M^{-1} for PC/PS membranes in 0.1 M salt. Thus we conclude that a reasonable estimate for the energy would be 1–2 kcal mol^{-1}. This net free energy change, calculated from $RT \ln(k)$, is the sum of all the free energy terms involved in the binding, including the terms describing the loss of entropy that must occur (8, 23, 50). The weak energy ($\approx$1.5 kcal mol^{-1}) we deduce is consistent with the observation that the lifetime of the lysine-phosphatidylserine complex is short on the NMR timescale (64, 68).

If we consider only the concentrations of peptides required to reverse the charge on the vesicles, we can ignore nonspecific electrostatic effects. To describe the binding of peptides when the surface potential is not zero, we must account for the nonspecific accumulation of the charged peptides in the aqueous diffuse double layer (45). We did this by combining the mass action model (Eqs. 7–12) with the Boltzmann relation (Eq. 6) and the Gouy-Chapman theory (Eq. 4). Our assumption that a peptide is a point charge is obviously incorrect. (The finite size of the peptide will limit the distance of closest approach to the membrane and affect its distribution in the diffuse double layer [12]. Other factors, such as the discrete nature of the adsorbed peptides [39, 69], may also affect the adsorption.) A better fit to the zeta potential data and a better agreement between these data and the equilibrium dialysis measurements was obtained (see Results) by using an effective valence ($z_{eff} < z$) in the Boltzmann equation, an approach used to describe the binding of other basic peptides to membranes (8, 38, 50, 69).

Where are the peptides located when they bind to the membrane? It is highly unlikely, based on Born energy calculations (57), that these peptides penetrate the low dielectric hydrocarbon interior of the bilayer. Our observation that Lys_5 has a smaller effect on the surface potential than on ζ supports the suggestion (64) that the peptide does not penetrate the polar head group region. A simple calculation based on equations described elsewhere (42, 43) can reconcile the surface potential (Fig. 6) and ζ (Fig. 3) data if the charges on Lys_5 are 0.5 nm from the surface (calculations not shown).

How do neutral amino acids inserted between the positively charged lysine and arginine residues affect the binding of the peptides to membranes formed from acidic lipids? We compare the binding of a number of different peptides with five basic residues and a net charge of +5 to PS (or PG) membranes. Lys_5 (Fig. 1), Arg_5 (Fig. 8), Lys_5 with blocked termini (unpublished), and peptides that mimic cationic regions of protein kinase C (50) and phospholipase C (59) all reverse the charge on PS and PG membranes at concentrations of order 10^{-4} M. However, if two alanine residues are inserted between each Lys residue in Lys_5, the apparent association constant of these peptides with membranes decreases about an order of magnitude (data not shown).

What is the biological significance of our results? Many proteins contain regions with clusters of basic amino acids. Although the energies ($\approx$1.5 kcal/mol) and association constants ($\approx$10 M^{-1}) involved are weak, they are strong enough to ensure that when one basic residue in the cluster binds to an anionic lipid, most of the adjacent positively charged amino acids will bind as well. Thus, the four basic residues on the cytoplasmic portion of the membrane-spanning region of glycophorin could bind with a significant energy ($\approx$5 kcal/mol) to the negatively charged lipids in the membrane, as illustrated by a perceptive sketch in a cell biology text (20, see their Fig. 13–16). Furthermore, clusters of basic

[7]The effects of Lys_5 on excimer formation with pyrene-labeled negative lipids or on fluorescence energy transfer between two negative lipids with different fluorescence probes (NBD-PE and pyrene-PE) were both very small (5:1 PC/PS membranes, data not shown). A simple analysis indicates the clustering induced by peptides like Lys_5 should be difficult to detect, in agreement with our measurements with peptides and those of Jones and Lentz (35) with proteins. Spin label ESR studies may prove more sensitive (65).

[8]We did experiments with Lys_5 on 5:1 DMPC/DMPG membranes in both a gel (15°C) and liquid-crystalline (30°C) state. We obtained identical results in both cases, results very similar to those observed with unsaturated 5:1 PC/PG membranes in Fig. 4 *A* (*open circles*).

residues exist on cytoplasmic proteins, such as protein kinase C (50) and phospholipase C (59), and these residues may interact with acidic lipids in the plasma membrane.

APPENDIX

We measured the effect of lysine and arginine on the ζ of PG (and PS) vesicles to estimate the binding of these monovalent cations to negative lipids. In a 0.1-M lysine chloride, 0.001 M MOPS, pH 6.0 solution, the ζ of a PG vesicle is -60 mV (± 2 mV, $n = 20$); similar results were obtained with PS. This is identical to the ζ of a PG vesicle in 0.1 M NaCl, which suggests the intrinsic association constant of lysine with PG (and PS) is 0.6 M^{-1} (25), or of order 1 M^{-1}. Why does Lys_2 bind an order of magnitude more strongly to PG vesicles than lysine (see Fig. 1)? Although we cannot rule out the obvious possibility that Lys_2 interacts with >1 negative lipid, we suspect that induction (24) effects are also important because the α-amino group on Lys_2 binds protons an order of magnitude less strongly than the α-amino group on lysine.[9]

Arginine binds about tenfold more strongly than lysine to negative lipids. The zeta potential of a PG vesicle in a 0.1 M arginine, 0.001 M MOPS, pH 6.0 solution is -40 mV (± 2 mV, $n = 20$), which suggests the intrinsic association constant is of order 10 M^{-1}. Similar results were obtained with PS vesicles.

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