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Binding of Basic Peptides to Acidic Lipids in Membranes: Effects of Inserting Alanine(s) between the Basic Residues†

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ABSTRACT: We studied the binding of peptides containing five basic residues to membranes containing acidic lipids. The peptides have five arginine or lysine residues and zero, one, or two alanines between the basic groups. The vesicles were formed from mixtures of a zwitterionic lipid, phosphatidylcholine, and an acidic lipid, either phosphatidylserine or phosphatidylglycerol. Measuring the binding using equilibrium dialysis, ultrafiltration, and electrophoretic mobility techniques, we found that all peptides bind to the membranes with a sigmoidal dependence on the mole fraction of acidic lipid. The sigmoidal dependence (Hill coefficient >1 or apparent cooperativity) is due to both electrostatics and reduction of dimensionality and can be described by a simple model that combines Gouy–Chapman–Stern theory with mass action formalism. The adjustable parameter in this model is the microscopic association constant k between a basic residue and an acidic lipid ($1 < k < 10$ M^{-1}). The addition of alanine residues decreases the affinity of the peptides for the membranes; two alanines inserted between the basic residues reduces k 2-fold. Equivalently, the affinity of the peptide for the membrane decreases 10-fold, probably due to a combination of local electrostatic effects and the increased loss of entropy that may occur when the more massive alanine-containing peptides bind to the membrane. The arginine peptides bind more strongly than the lysine peptides: k for an arginine residue is 2-fold higher than for a lysine residue. Our results imply that a cluster of arginine and lysine residues with interspersed electrically neutral amino acids can bind a significant fraction of a cytoplasmic protein to the plasma membrane if the cluster contains more than five basic residues.

Several classes of proteins contain clusters of positively charged amino acids that may bind to negatively charged phospholipids, which are located preferentially on the cytoplasmic surface of a plasma membrane (Op den Kamp, 1979; Bishop & Bell, 1988). The intrinsic or membrane-spanning proteins comprise one class. The cytoplasmic domain of these proteins often contains clusters of basic residues, which may help determine the orientation of these proteins in the mem-

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brane (Hartman et al., 1989; Nilsson & von Heijne, 1990), possibly by binding to acidic phospholipids (Hartman et al., 1989).

The protein kinase C (PKC)[1] substrates neuromodulin (aka GAP-43, P-57, B-50, F-1), neurogranin, and the MARCKS (myristoylated alanine-rich C kinase substrate) protein comprise another class. Genetic arguments (Houbre et al., 1991) and the binding measurements we discuss below suggest that the clusters of basic residues on these proteins may interact transiently with acidic lipids; this would allow membrane-bound PKC to phosphorylate the Ser residues contained within these clusters (Graff et al., 1989a; Apel et al., 1990; Baudier et al., 1991). Calmodulin binds strongly to the unphosphorylated form of these proteins, probably by interacting with these basic clusters (Andreasen et al., 1983; Cimler et al., 1985; Alexander et al., 1987; Graff et al., 1989b; Baudier et al., 1991; McIlroy et al., 1991) but binds only weakly to the phosphorylated form of the proteins (Alexander et al., 1987; Graff et al., 1989b). Thus, as suggested by Storm and co-workers (Alexander et al., 1987) and discussed by other groups (Graff et al., 1989b; McIlroy et al., 1991; Houbre et al., 1991), these proteins may act as "molecular switches" that release calmodulin upon activation of the calcium–phospholipid second messenger system. Most clusters of basic residues in proteins, including the clusters in these three PKC substrates, have interspersed electrically neutral amino acids. This study examines how inserting the electrically neutral amino acid alanine into simple basic peptides modifies their adsorption to membranes.

Both polylysines of high molecular weight (Kimelberg & Papahadjopoulos, 1971; Montal, 1972; Hartmann et al., 1977; Hartmann & Galla, 1978; de Kruijff et al., 1985; Walter et al., 1986; Carrier & Pezolet, 1986; Fukushima et al., 1988; Laroche et al., 1988; Mittler-Neher & Knoll, 1989; Sankaram et al., 1989; Carrier et al., 1990) and shorter peptides containing basic residues (de Kruijff et al., 1985; Dufourcq et al., 1981; Roux et al., 1988; de Kroon et al., 1990, 1991; Kim et al., 1991) bind to membranes containing acidic lipids. Studies on Lys_n (n = 2–5) demonstrated that each basic residue in these peptides binds independently to an acidic lipid in a membrane with a free energy of 1–2 kcal/mol (Kim et al., 1991). A simple theoretical model that combines the Gouy–Chapman–Stern theory of the electrostatic potential adjacent to a charged membrane, the Boltzmann relation, and sequential mass action equations account qualitatively for the binding data (Kim et al., 1991). This model is highly oversimplified: it ignores both the local electrostatic effects of adjacent basic residues (Edsall & Wyman, 1958; Cantor & Schimmel, 1980) and any mass-dependent entropy loss that might occur when the peptide binds to a membrane and loses translational and rotational degrees of freedom (Janin & Chothia, 1978; Dwyer & Bloomfield, 1981; Finkelstein & Janin, 1989).

To explore the limitations of the model, we investigated a series of peptides with five arginine or lysine residues separated by zero, one, or two alanines. Alanine was chosen because the work of Jacobs and White (1986, 1987, 1989; White, personal communication) suggests that little free energy is required to move alanines on small peptides from the aqueous phase to the surface of a phospholipid bilayer. We also measured the binding of peptides with five adjacent lysine residues and either two or four alanine residues at each end. We studied the binding of these peptides to large unilamellar vesicles by making ultrafiltration and equilibrium dialysis measurements and to multilamellar liposomes by making electrophoretic mobility measurements. We used two types of association constants to describe the binding data. The effective binding constant K (eq 1) relates the surface concentration of the peptide, the number of adsorbed peptides per unit area of membrane $\{P\}$, to its concentration in bulk solution $[P]$. K, expressed in units of length, corresponds to the distance one must move from a unit area of membrane to obtain a volume of solution that contains the same number of peptides that are bound to the membrane. Although K can be determined directly from the experimental measurement of $\{P\}$, it is not a true constant. Its value depends on the free surface concentration of acidic lipid and the surface potential of the membrane. The microscopic association constant k characterizes the binding of a single basic residue on a peptide to an acidic lipid in a membrane. In contrast to K, k should depend on neither the surface concentration of acidic lipid nor the surface potential. As with pentalysine, we assumed that each peptide contains four identical binding sites; eq 2 gives an example from Kim et al. (1991; see their eqs 8 and 8a). $\{P–L\}$ and $\{L\}$ are the surface concentrations of the peptide–single acidic lipid complex and free acidic lipid, respectively, whereas $[P]_0$ is the free peptide concentration in the aqueous phase immediately adjacent to the membrane. This concentration is related to the concentration in the bulk aqueous phase by the Boltzmann relation. The factor 4 arises because of statistical reasons (Cantor & Schimmel, 1980).

$$\{P\} = K[P] \quad (1)$$

$$\{P–L\} = 4k\{L\}[P]_0 \quad (2)$$

Materials and Methods

Large unilamellar vesicles (LUVs) and multilamellar liposomes were formed from mixtures of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC) with either 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (PS) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (PG). The lipids were obtained from Avanti Polar Lipids (Birmingham, AL). The 0.1-μm diameter LUVs (Hope et al., 1985) were produced by taking multilamellar vesicles through five cycles of freezing (liquid nitrogen) 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). The lipid concentrations in the final sample were determined by phosphate analysis (Kim et al., 1991).

The peptides acetyl-Arg_5-amide, acetyl-$(Arg\text{-}Ala)_4$-Arg-amide, acetyl-$(Arg\text{-}Ala_2)_4$-Arg-amide, acetyl-Lys_5-amide, acetyl-$(Lys\text{-}Ala)_4$-Lys-amide, acetyl-$(Lys\text{-}Ala_2)_4$-Lys-amide, acetyl-$Ala_2\text{-}Lys_5\text{-}Ala_2$-amide, and acetyl-$Ala_4\text{-}Lys_5\text{-}Ala_4$-amide were synthesized by Multiple Peptide Systems (San Diego, CA). Purity of the first six peptides was checked by amino acid analysis and analytical HPLC. If required, the peptides were additionally purified by reverse-phase HPLC to 95%. The remaining two peptides were 95% pure from analytical HPLC and mass spectroscopic measurements and were used without further purification.

4-Morpholinepropanesulfonic acid (MOPS) was purchased from Pharmacia (Piscataway, NJ). Aqueous solutions were prepared with 18 MΩ water (Super-Q, Millipore Corp.,

[1] Abbreviations: L, acidic lipid; LUVs, large unilamellar vesicles; Lys_5, pentalysine; MARCKS, myristoylated alanine-rich C kinase substrate; MOPS, 4-morpholinepropanesulfonic acid; P, peptide; PC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; PKC, protein kinase C; PS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; k, microscopic association constant as defined in eq 2; K, effective binding constant as defined in eq 1; z_{eff}, effective valence; ζ, zeta potential.

Table I: Effective Binding and Microscopic Association Constants for Arginine and Lysine Peptides[a]

peptide	K (μM)		k (M^{-1})		
	2:1 PC/PG	4:1 PC/PG	Figure 1	Figure 2	Figure 3
acetyl-Arg_5-amide	ND	2 ± 0.4	6	10	8
acetyl-(Arg-Ala)$_4$-Arg-amide	9 ± 3	0.6 ± 0.2	4	7	6
acetyl-(Arg-Ala_2)$_4$-Arg-amide	5 ± 2	0.2 ± 0.07	3	5.5	6
acetyl-Lys_5-amide	3 ± 0.2	0.3 ± 0.04	3	6.5	4
acetyl-(Lys-Ala)$_4$-Lys-amide	0.8 ± 0.04	0.1 ± 0.02	2	4	2.5
acetyl-(Lys-Ala_2)$_4$-Lys-amide	0.6 ± 0.03	0.1 ± 0.02	1.7	3	1.4

[a] The effective binding constant K (eq 1) for the peptides was calculated from the binding data presented in Figure 1 for the two indicated lipid mixtures. Each number represents an average ± SD (n = 4). ND, not determined; the fraction of bound peptide was about 100%. The microscopic association constant of a basic residue with an acidic lipid, k, (eq 2) was obtained by fitting the experimental data shown in Figures 1–3 with the model described in the Appendix.

Bedford, MA) that was subsequently bidistilled in an all-quartz still. They were buffered to pH 7.0 with 1 mM MOPS or bicarbonate.

Multilamellar vesicles for the microelectrophoresis measurements were prepared as described by Bangham et al. (1974). We measured the mobilities of these vesicles in a Rank Brothers Mark I instrument (Bottisham, Cambridge, U.K.) as described previously (Cafiso et al., 1990; Kim et al., 1991). Equilibrium dialysis and ultrafiltration experiments were performed as described previously; peptide concentrations were determined from a fluorscamine assay (Kim et al., 1991). For experiments with the arginine peptides, Centricon-10 ultrafiltration units (Amicon, Beverly, MA) were first centrifuged with 0.1 M KCl buffered with 1 mM bicarbonate, pH 7.0, to wash out traces of substances that, after hydrolysis, reacted with fluorescamine. (The bicarbonate buffer solution was used in all ultrafiltration and equilibrium dialysis experiments with peptides containing arginine residues because MOPS yielded a significant quantity of primary amines after hydrolysis.) The peptides containing arginine residues were hydrolyzed in the vapors of 6 M HCl at 105 °C under vacuum overnight (Atherton & Sheppard, 1989) to allow measurement with the fluorescamine assay. The results obtained with the peptides were corrected for the 2–9% free peptide that binds to the ultrafiltration membrane.

Results

Figure 1 illustrates how the percent of bound peptide depends on the mol % acidic lipid, PG, in a phospholipid bilayer membrane. Three features of the results are immediately apparent. First, the percent of bound peptide depends in a sigmoidal manner on the mol % acidic lipid. Second, the arginine peptides (Figure 1A) bind more strongly than the lysine peptides (Figure 1B). Third, insertion of alanine(s) between the basic residues decreases the binding.

The sigmoidal dependence arises for two reasons. The electrostatic potential produced by the acidic lipids (McLaughlin, 1989) increases the concentration of the peptides in the aqueous diffuse double layer. The reduction of dimensionality or translocation of the peptide to the surface region that occurs when the first basic residue binds to an acidic lipid increases the effective concentration of acidic lipid sensed by the other residues and promotes their association (Mosior & McLaughlin, 1991, 1992). The curves in Figure 1 illustrate the prediction of a simple theoretical model that takes both these phenomena into account (Appendix). Although this Gouy–Chapman/mass action model is highly oversimplified, it provides a qualitative description of this sigmoidal dependence.

The peptides containing arginine residues (Figure 1A) bind 10-fold more strongly than their lysine analogues (Figure 1B) to the negatively charged membranes. If four basic residues

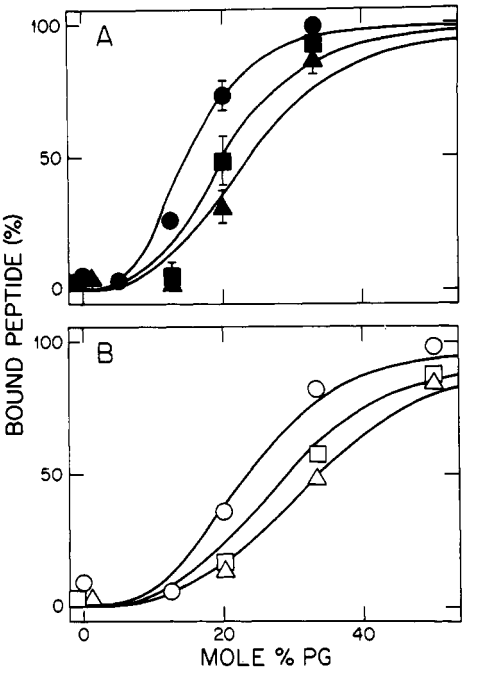

FIGURE 1: Binding of peptides with either five arginine (A) or five lysine (B) residues to large unilamellar vesicles formed from mixtures of the zwitterionic lipid PC and the negatively charged lipid PG. Peptides with adjacent basic residues are denoted by circles, those with one alanine spacer by squares, and those with two alanine spacers by triangles. The results were obtained using an ultrafiltration technique (Kim et al., 1991). The theoretical binding curves were calculated assuming the microscopic association constant between a basic residue and an acidic lipid k = 6, 4, 3 (A) or 3, 2, 1.7 M^{-1} (B) and the effective charge of the peptide z_{eff} = 2.5. The theoretical model is discussed in the Appendix. Aqueous solutions containing 0.1 M KCl, 8 mM total lipid, and 30 μM peptide were buffered to pH 7.0 at 25 °C with 1 mM bicarbonate (A) or MOPS (B). Each point represents an average of four measurements; the bars indicate SD if larger than the symbol size.

on the peptides provide independent binding sites for acidic lipids (Kim et al., 1991), this 10-fold difference in K (eq 1) is due to a 2-fold difference in the microscopic association constant k (eq 2). Thus each individual arginine residue in these peptides has a 2-fold greater affinity than a lysine residue for a monovalent acidic lipid such as PG (see Table I). The reason for this small difference in affinity is not understood.

The affinity of peptides for the membranes decreases when basic residues are spaced by alanine (Figure 1). The insertion of alanine residues has a similar effect on both arginine and lysine peptides. The binding data illustrated in Figure 1 allow us to calculate the effective binding constant (K in eq 1) of the peptides to membranes with different mol % PG (Table I). Placing an Ala–Ala pair between the basic residues decreases K about 5-fold (Table I). Conversely, if we interpret the results in terms of microscopic association constants (curves

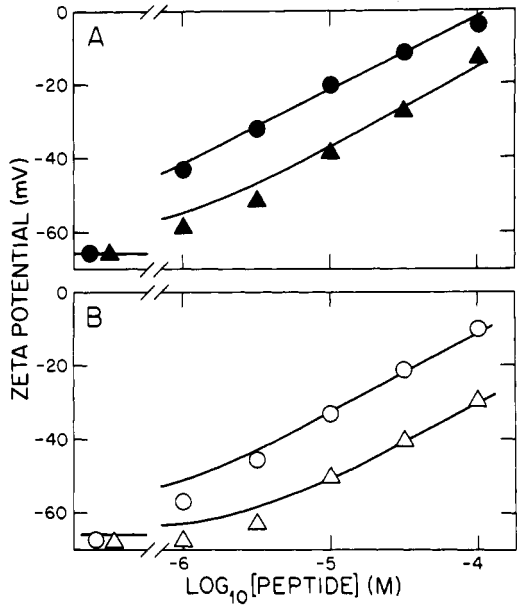

FIGURE 2: Effect of peptides with either five arginine (A) or five lysine (B) residues on the zeta potential of PS vesicles. Curves illustrate the theoretical predictions assuming microscopic association constants k = 10, 5.5 (A) and 6.5, 3 M^{-1} (B) and z_{eff} = 2.5. Symbols are explained in the legend to Figure 1. The aqueous solution contained 0.1 M KCl buffered with 1 mM MOPS to pH 7.0 at 25 °C. The symbols to the far left indicate the zeta potential of vesicles in the absence of the peptide.

in Figure 1), the Ala–Ala pair decreases k about 2-fold (Table I).

The results shown in Figure 1 were obtained by ultrafiltration. Equilibrium dialysis measurements were also made for all peptides with 2:1 PC/PG vesicles, and the results were identical within experimental error.

We made electrophoretic mobility measurements to confirm the results illustrated in Figure 1 with an independent technique and to obtain information about the effect of the peptides on the electrostatic potential. The electrophoretic mobility, the velocity of a vesicle in a unit electric field, is proportional to the zeta potential, ζ, the potential in the aqueous phase adjacent to the membrane as predicted by the Helmholtz–Smoluchowski equation (Aveyard & Haydon, 1973; McLaughlin, 1977, 1989; Hunter, 1981; Cafiso et al., 1989). The zeta potential is proportional to the surface charge density of lipid vesicles (ζ <25 mV) and is thus linearly related to number of bound peptides per unit area (Kim et al., 1991). Figure 2 shows that arginine peptides (Figure 2A) bind more strongly than lysine peptides (Figure 2B) to PS membranes and that peptides with two alanine residues separating the charges (triangles) bind 10-fold less strongly than peptides with adjacent basic residues (circles). Results obtained with peptides having one alanine spacer fall between the two curves in Figure 2 (data not shown). We obtained essentially the same results with PS (Figure 2) and PG vesicles (data not shown).[2]

We used the same theoretical model to describe the data in Figure 2 and Figure 1 (Appendix). The curves in Figure 2 were drawn assuming that the effective valence of the peptides is half the real valence. Theoretical curves with a valence of 5 instead of 2.5 have slopes half those shown in

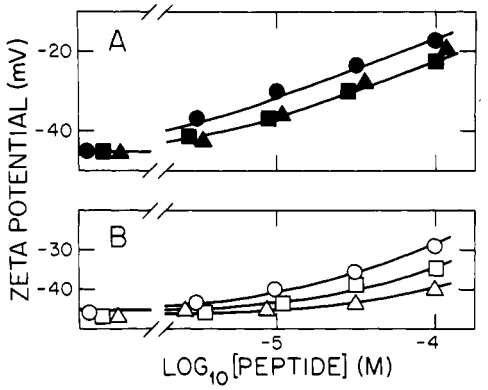

FIGURE 3: Effect of peptides with five arginine (A) or five lysine (B) residues on the zeta potential of 2:1 PC/PG vesicles. The curves represent a fit of the model with k = 8, 6, 6 (A) and 4, 2.5, 1.4 M^{-1} (B) and z_{eff} = 2.5. Other experimental conditions are as given in the legend to Figure 2; the symbols are explained in the legend to Figure 1.

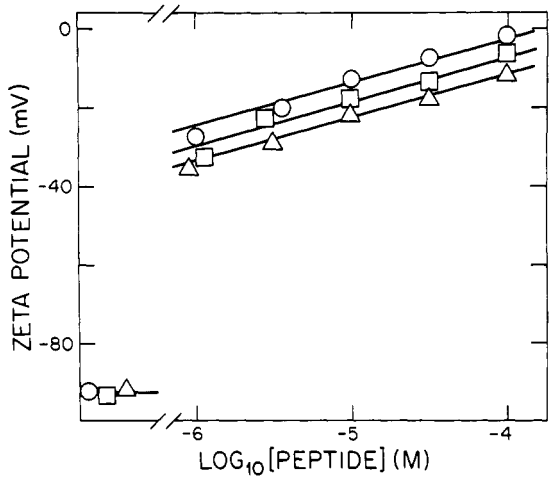

FIGURE 4: Effect of peptides with five lysine residues on the zeta potential of 2:1 PC/PS vesicles. The solutions contained 0.01 M KCl buffered with 1 mM MOPS to pH 7.0 at 25 °C. The curves represent predictions of the model for z_{eff} = 5 and k = 17, 11, 8 M^{-1}; the symbols are the same as given in the legend to Figure 1.

Figure 2 and do not describe the experimental data.[3] (An effective valence of 2.5 was also used for the theoretical curves shown in Figure 1.) Inserting two alanines between basic residues decreases the microscopic association constant k 2-fold (Table I). The arginine residues bind 2-fold more strongly to the acidic lipids than do the lysine residues (Table I). Thus, the results obtained with electrophoretic mobility measurements agree qualitatively with the results obtained from filtration measurements.[4]

[2] The concentrations of a given peptide required to neutralize the surface charge of PS (Figure 2) or PG vesicles (not shown) are identical, indicating the peptides bind these two acidic lipids equally well. The slope of zeta potential vs peptide concentration curves, however, was 20% larger for PS than for PG vesicles, for reasons that we do not understand.

[3] We and others discuss elsewhere how the finite size of the peptide, discreteness of charge effects, and the binding of anions to the peptides could all reduce the effective valence of a charged peptide that adsorbs to a membrane (Carnie & McLaughlin, 1983; Kuchinka & Seelig, 1989; Schwarz & Beschiaschvili, 1989; Langner et al., 1990; Stankowski & Schwarz, 1990; Mosior & McLaughlin, 1991; Stankowski, 1991).

[4] The k values presented in Table I from Figure 2 are about a factor of 2 larger than those deduced from Figure 1. The results from Figure 2 were obtained with PS (and PG) vesicles, whereas the results from Figure 1 were obtained from vesicles formed from PG/PC mixtures. One of the limitations of our simple theoretical model is that it does not describe the increase in k that occurs as the mole fraction of acidic lipid in the membrane increases, a phenomenon discussed in more detail in Mosior and McLaughlin (1991). The dependence of k on mol % acidic lipid also manifests itself in Figure 1 as a disagreement between the experimental points and the theoretical curve. The dependence of k on mol % acidic lipid is possibly due to the use of the mean-field Gouy–Chapman theory, which ignores all local electrostatic interactions between lipids and peptides.

We also made zeta potential measurements using 2:1 PC/PG (and PC/PS) vesicles. The results shown in Figure 3 agree qualitatively with those in Figure 1. Specifically, the arginine peptides bind more strongly than the lysine peptides, and placing alanines between the basic residues decreases the affinity of the peptide for the vesicle. The microscopic association constants deduced from the zeta potential measurements of Figure 3 agree within a factor of 2 with those obtained from the ultrafiltration measurements of Figure 1, as shown in Table I.

All the data suggest that placing a pair of alanines between the basic residues decreases k about 2-fold (Table I). We repeated the measurements in 0.01 M KCl, where the Debye length is 3 rather than 1 nm, for both theoretical and experimental reasons.[5] (The slope of the zeta potential curves in 0.01 M KCl is consistent with an effective valence equal to the real valence. This observation suggests that the deviation seen in 0.1 M salt is related to the size of the peptide relative to the Debye length.) The results shown in Figure 4 are qualitatively consistent with those illustrated above: placing two alanines between the lysine residues decreased the microscopic association constant about 2-fold, from k = 17 to 8 M^{-1}. [For the arginine peptides, k decreased from 25 to 19 M^{-1} (data not shown).]

Our filtration and zeta potential measurements (not shown) demonstrate that adding alanine residues to the ends of a peptide with five adjacent lysine residues decreases the binding to vesicles containing negatively charged lipids, but not as much as interspersing the same number of alanine residues between the lysines. For example, the zeta potential results we obtained with acetyl-Ala_4-Lys_5-Ala_4-amide lie approximately midway between the results illustrated in Figure 2B for acetyl-Lys_5-amide and acetyl-$(Lys\text{-}Ala_2)_4$-Lys-amide. The addition of Ala residues to the ends of the peptide should not change the local electrostatic interactions between the basic residues, and these interactions cannot, therefore, fully account for the effect of adding alanine residues to the basic peptides we studied.

In summary, when eight Ala residues are either interspersed between the basic residues or added to the ends of the peptide, the binding of the peptide to negatively charged membranes decreases $\leq$1 order of magnitude. A similar order of magnitude decrease in the binding can be produced by deleting one basic residue (Kim et al., 1991).

Discussion

Our experimental results lead us to four conclusions: separating charged residues with two alanines decreases 2-fold the affinity of basic residues in peptides for acidic lipids in membranes; arginine residues bind 2-fold more strongly than lysine residues to the monovalent acidic lipids PS and PG; basic residues bind PS and PG equally well; and peptides with basic residues exhibit an apparent cooperativity with respect to acidic lipids when binding to membranes.

The 2-fold decrease in the microscopic association constant k of a basic residue for an acidic lipid produced by the insertion of two alanine residues corresponds to a decrease in the standard free energy change upon association ($\Delta G° = -RT \ln k$) of 0.4 kcal/mol. Electrostatic interactions between charged groups on the peptide are one likely candidate for producing this change. Three experimental results suggest that the binding of a basic residue to an acidic lipid is due mainly to an electrostatic attraction of opposite charges: basic residues do not bind significantly to zwitterionic lipids (Figure 1), there is little specificity between acidic lipids (footnote 2), and, at least for the case of pentalysine, the bound peptides appear to be located outside the envelope of the polar head group (Roux et al., 1988; Kim et al., 1991). If the charged groups on the peptide are separated by electrically neutral alanine residues, the electrostatic potential at a basic residue due to the *other* charged residues will decrease, reducing the affinity of the basic group for an acidic lipid. A change of 20 mV in the local electrostatic potential at a basic residue would produce the 2-fold change in k. Such effects are well documented for peptides: for example, Edsall and Wyman (1958) devote an entire section of their text to electrostatic effects on the pK_a values of peptides. Recent measurements demonstrate that interactions between charged groups in proteins generally produce electrostatic energy changes of 0.3–0.5 kcal/mol (Sharp & Honig, 1990; Warshel & Åqvist, 1991), which is the correct range to account for the effects of alanine spacers between basic residues.

The insertion of alanines also increases the mass of the peptide, thus increasing the loss of free energy that occurs when the peptide binds to the membrane; in principle the peptide loses one translational and two rotational degrees of freedom. If the peptide is regarded as a rigid molecule, the theoretically calculated (Janin & Chothia, 1978; Dwyer & Bloomfield, 1981) extra free energy loss or "entropy price" that is paid when the more massive peptides bind is also sufficient to account for the experimental results illustrated in Figure 1. The experimental observation that adding alanine residues to the ends of the basic peptide also decreases the binding appears to support this interpretation. However, Finkelstein and Janin (1989) acknowledge that "translation–rotation degrees of freedom lost in a bimolecular complex do not really vanish" and that their previous calculations led to "overestimates of the entropy change". Thus we suspect that alanine residues decrease the binding of basic peptides to membranes by means of a combination of local electrostatic and other effects (e.g., steric hindrance) but admit the matter remains unresolved.

We are confident we understand why peptides with basic residues exhibit an apparent cooperativity with respect to acidic lipids when they bind to membranes. Both the electrostatic potential produced by the acidic lipids and the decrease in dimensionality that occurs when the first basic residue on the peptide binds to the membrane give rise to apparent cooperativity (Mosior & McLaughlin, 1991, 1992). We have studied several peptides with five basic residues, including ones that mimic the pseudosubstrate region of protein kinase C (Mosior & McLaughlin, 1991) or a conserved region of phospholipase C (Peterson et al., 1991) and contain neutral amino acids other than alanine. All these peptides display apparent cooperativity and binding affinity similar to those of the peptides illustrated in Figure 1.

What is the biological significance of our results? Although interspersing the electrically neutral amino acid alanine between the charged residues reduces the affinity of basic peptides for membranes and there are slight differences between

[5] The assumption in our theoretical model that the peptides are point charges is more valid in 0.01 M KCl (where the electrostatic potential decays less rapidly with distance from the membrane) than in 0.1 M KCl (the Debye lengths are 3 and 1 nm). Using electrophoretic mobility measurements and the Helmholtz–Smoluchowski equation to deduce the zeta potential (Kim et al., 1991) is more valid in 0.01 than in 0.1 M salt solutions because the finite size of the adsorbed peptides affects the electrophoretic mobility of vesicles in two ways. First, the peptide may protrude from the vesicle surface, exert hydrodynamic drag, and decrease the mobility. Second, if the charges on the peptide extend some distance from the surface they will change the mobility more than predicted from the Helmholtz–Smoluchowski equation for the reason illustrated in Figure 1 in Pasquale et al. (1986).

the affinities of arginine and lysine residues for acidic lipids, the biologically important result illustrated in Figure 1 is that all of these peptides bind significantly to membranes containing 20–30 mol % acidic lipids. This corresponds to the mole fraction of acidic phospholipids on the cytoplasmic surface of a typical plasma membrane (Op den Kamp, 1979; Bishop & Bell, 1988). The total concentration of lipid used in the experiments illustrated in Figure 1 was 8 mM, which corresponds to the membrane-surface/solution-volume ratio encountered by a protein in a spherical cell with a radius of 2 μm. Thus an exposed cluster of more than five basic amino acids on a cytoplasmic protein could bind a significant fraction of protein to the plasma membrane.

This statement requires several caveats. First, negatively charged amino acids on the bound protein located less than a Debye length from the membrane surface will decrease the interaction. Second, the structure of the protein may preclude several of the basic residues in the cluster from interacting with acidic lipids. For example, an amphipathic helix will adsorb strongly to bilayer membranes by means of hydrophobic interactions (Epand et al., 1989). Melittin binds to membranes in this manner. The basic residues on melittin may be constrained by the relatively rigid helix to point away from the surface, and the available evidence suggests they do not bind significantly to acidic lipids (Beschiaschvili & Seelig, 1990). Third, the "entropy price" paid upon binding to the membrane could be as much as 6 kcal/mol greater for a 100 kDa protein than for a 1 kDa peptide (Janin & Chothia, 1978; Dwyer & Bloomfield, 1981), although it is probably less than half this value (Finkelstein & Janin, 1989). An extra entropy price of 3 kcal/mol could be overcome by two additional basic residues in the cluster because each basic residue should increase the affinity of the protein for the membrane 10-fold. Additional basic residues added to small peptides do increase the affinity, as expected theoretically (Kim et al., 1991; Montich and McLaughlin, unpublished results). Fourth, these electrostatic interactions can act in concert with other binding interactions, such as the partitioning of hydrophobic groups from the protein into the bilayer. Phospholipase A_2, for example, appears to interact with bilayers through a combination of hydrophobic and basic residues (Scott et al., 1990). The acyl groups on neuromodulin (cysteines at positions 3 and 4 can be palmitylated) and the MARCKS protein (terminal glycine can be myristoylated) also appear to be important for membrane association (Houbre et al., 1991; Graff et al., 1989c).

Houbre et al. (1991) suggested that a conserved basic region of neurogranin and neuromodulin that includes 9 or 10 basic and 1 or 2 acidic residues binds to acidic lipids in membranes. Furthermore, they showed that neuromodulin and neurogranin exist as membrane-bound and cytosolic forms in rat brain and that the phosphorylated form of neuromodulin had a much lower affinity for vesicles containing negative lipids than the dephosphorylated protein. The MARCKS protein contains a region of 25 residues that has 13 basic and no acidic residues, plus 4 serines that are phosphorylated by protein kinase C (Graff et al., 1989a, 1991). A peptide corresponding to this region binds more strongly to membranes with acidic lipids than the peptides illustrated in Figure 1, as predicted from the Gouy–Chapman/mass action model discussed here; when we mimic the effect of phosphorylation by replacing the serines with aspartic acid residues, the affinity of the peptide for the membrane decreases (McLaughlin and Blackshear, unpublished results). Possibly both the hydrophobic energy from insertion of the acyl chain into the bilayer interior and the electrostatic energy from the association of a cluster of basic residues with acidic phospholipids are required for the MARCKS protein to associate significantly with a membrane. If so, addition of negative charges to the serine residues located within this cluster provides a simple electrostatic mechanism for the observation that phosphorylation causes the MARCKS protein to move off the plasma membrane of neutrophils (Thelen et al., 1991).

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Appendix

The details of the model used to fit both the binding (Figure 1) and the zeta potential data (Figures 2–4) are given in Kim et al. (1991), and the equation numbers below refer to this paper. The electrostatic potential produced by acidic lipids (McLaughlin, 1989) is described by the Gouy–Chapman theory (eq 4). We assumed the peptides are point charges that accumulate in the electrostatic diffuse double layer produced by the acidic lipids and described this accumulation with the Boltzmann relation (eq 6). Of course, the peptides are not point charges; they are comparable in size to the thickness of the aqueous diffuse double layer ($1/\kappa$ = 1 nm in 0.1 M salt). Thus not all charges experience the potential at the surface of the membrane [e.g., Carnie and McLaughlin (1983)]. We compensated empirically for this limitation of the model by using an effective valence of the peptides in the Boltzmann relation.[3] The effective valence was obtained from the slope of the zeta potential vs peptide concentration curves. Sequential mass action equations were used to describe the binding of basic residues to acidic lipids (eqs 8–11, 8a–11a). We assume all the pentavalent peptides have four independent and identical binding sites, as we did previously with pentalysine (Kim et al., 1991). The zeta potential was calculated from the measured value of the electrophoretic mobility using the Helmholtz–Smoluchowski equation (eq 1) and assumed to be equal to the electrostatic potential at the surface of the membrane. To describe the zeta potential of phospholipid vesicles in the absence of peptides, we assume that potassium ions bind to acidic lipids (Eisenberg et al., 1979). The calculations for the PC/PG vesicles in Figure 3 require a value of the intrinsic association constant of 1.7 M^{-1} (eq 7), which was also used to draw the theoretical curves in Figure 1. The values for the PS vesicles were 2 (0.1 M KCl) and 3.5 M^{-1} (0.01 M KCl). The choice of the intrinsic association constant for potassium ions with acidic lipids does not affect to any significant degree the values we calculated for the microscopic association constants k of basic residues with acidic lipids.

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