Peptides that mimic the pseudosubstrate region of protein kinase C bind to acidic lipids in membranes

Marian Mosior and Stuart McLaughlin
Department of Physiology and Biophysics, Health Sciences Center, State University of New York, Stony Brook, New York 11794-8661 USA

ABSTRACT The cytoplasmic form of protein kinase C (PKC) is inactive, probably because the pseudosubstrate region in its regulatory domain blocks the substrate-binding site in its kinase domain. Calcium ions cause a translocation to the membrane: maximum activation requires a negative lipid such as phosphatidylserine (PS) and the neutral lipid diacylglycerol (DAG) but the mechanism by which PS and DAG activate PKC is unknown. Pseudosubstrate region 19–36 of PKC-β has six basic and one acidic amino acids and region 19–29 has five basic and no acidic amino acids. Since any binding of basic residues in the pseudosubstrate region to acidic lipids in the membrane should stabilize the active form of PKC, we studied how peptides with amino acids equivalent to residues 19–36 and 19–29 of PKC-β bound to phospholipid vesicles. We made equilibrium dialysis, filtration, and electrophoretic mobility measurements. The fraction of bound peptide is a steep sigmoidal function of the mol fraction of negative lipid in the membrane, as predicted from a simple theoretical model that assumes the basic residues provide identical independent binding sites. The proportionality constant between the number of bound peptides/area and the concentration of peptide in the bulk aqueous phase is 1 μm for a membrane with 25% negative lipid formed in 0.1 M KCl. Equivalently, the association constant of the peptide with the membrane is 10^4 M^{-1}, or the net binding energy is 6 kcal/mol. Thus the interaction of basic residues in the pseudosubstrate region with acidic lipids in the membrane could provide 6 kcal/mol free energy towards stabilizing the active form of PKC.

INTRODUCTION

Protein kinase C (PKC), an enzyme that phosphorylates serine and threonine residues (1, 33, 37, 54–56, 58), is an important component of the calcium/phospholipid second messenger system (7–9). The binding of a variety of hormones, neurotransmitters, or growth factors to receptors activates a phospholipase C that catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into the two second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). DAG is also formed by the breakdown of phosphatidylcholine (24). IP_3 diffuses through the cytoplasm and releases calcium ions from vesicular organelles. When the cell is quiescent (concentration of free calcium $< 10^{-7}$ M), a significant fraction of PKC molecules reside in the cytoplasm and are inactive. When the intracellular concentration of free calcium rises, PKC "translocates" to or binds to the plasma membrane and actively phosphorylates its substrates (54, 65, 68). In cell-free systems, the neutral lipid DAG and the negatively charged phospholipid phosphatidylserine (PS) are required in the membrane for maximal activation (27, 28, 56, 58).

House and Kemp (30) hypothesized that the regulatory domain of PKC contains a pseudosubstrate region that interacts with a substrate binding site in the kinase domain, rendering it inactive. They noted that the amino acid sequence between residues 19 and 36 of PKC resembled a phosphorylation site, then showed that the corresponding synthetic peptide antagonizes substrate phosphorylation by PKC. Makowske and Rosen (44) provided strong support for the hypothesis by showing that antiserum raised against this peptide activates PKC in the absence of calcium and lipids. Hannun and Bell (27) also concluded that their kinetic data support this model.

The most highly developed model for PKC activation is based on studies with mixed micelles (27). In this model: "The enzyme first associates with the surface of mixed micelles in the presence of PS and Ca^{2+} but remains inactive. In the second step, the enzyme becomes activated upon DAG or phorbol ester binding." However, it is not clear how PKC binds to the membrane (5, 43), why binding is usually a prerequisite for activation, and whether negatively charged lipids such as PS are required in the membrane for both binding and activation. We hypothesize that basic residues located in the pseudosubstrate region of PKC bind to acidic lipids located on the cytoplasmic surface of the plasma membrane, and that this interaction stabilizes the active form of the enzyme.

To investigate this hypothesis, we measured how strongly peptides identical to regions 19–29 and 19–36 of the PKC-β isotype bind to membranes containing negative lipids. These peptides have five and six basic

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residues, respectively, and both have a net charge of +5. The first dozen residues of the pseudosubstrate region in the other five well-characterized isotypes also have four to six basic residues (58). Another objective was to test how well the binding of these peptides to membranes could be described by a simple theoretical expression. Our theoretical model predicts that the binding of these peptides to membranes should depend sigmoidally (i.e., with a high Hill coefficient, or high apparent cooperativity) on the mol fraction of negative lipid in the membrane, as does the activity of PKC (12, 27, 28, 52). Finally, we wanted to compare the binding observed with these peptides to the binding observed with a shorter, simpler peptide that has five basic residues, pentalysine (38).

THEORY

We use the simplest theoretical model capable of describing the binding of charged peptides to membranes (16, 38). In brief, charged lipids such as PS or PG produce an electrostatic potential at the surface of a membrane, $\psi(0)$, which we describe by the Gouy–Chapman–Stern theory of the aqueous diffuse double layer (46). This negative potential increases the concentration of the positvely charged peptides in the aqueous phase immediately adjacent to the membrane. We assume the peptide is a point charge and describe the accumulation by the Boltzmann relation. Of course the peptide is not a point charge; it is larger than the thickness of the diffuse double layer, the Debye length, which is ~1 nm in a 0.1 M salt solution. Thus not all the charges on the peptide will experience the full value of the surface potential. For this and other reasons, we use an effective valence, z_{eff}, that is less than the real valence in the Boltzmann relation.[1] To calculate the net charge/area on the membrane, we assume each adsorbed peptide has its real valence, $z = 5$.

We assume that the two peptides we studied both have four binding sites for acidic lipids (in the Appendix we discuss the evidence that only four basic residues combine with acidic lipids), that all binding sites are identical and independent, that each site binds to a negatively charged lipid with an identical net free energy or intrinsic, microscopic[2] association constant, k, and that zwitterionic lipids do not bind to the peptides. We describe the binding with mass action equations. We follow Guggenheim and assume the interactions occur in a surface phase of thickness $d \approx 1$ nm, which we regard as an ideal solution. If we follow Gibbs and assume the reactions occur on a two-dimensional surface that lacks finite thickness, we obtain mathematically identical expressions.

We define the effective binding constant of a peptide to the membrane, K, as the proportionality constant that relates the concentration of peptide in the bulk aqueous phase, $[P]$, to the number of peptide molecules bound to a unit area of surface, $\{P\}$:

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

K has the units of length. We determine K experimentally with either equilibrium dialysis or filtration experiments. In terms of our theoretical model, K is given by

$$K = \left(\exp\left(-z_{eff}e\psi(0)/kT\right)\right)\left(4k\{L\}\right)\left(1 + (3/2)(k/d)\{L\} + (k/d)^2\{L\}^2 + (1/4)(k/d)^3\{L\}^3\right). \quad (2)$$

$\{L\}$ is the number of negatively charged lipids per unit area of membrane and $\{L\}/d$ is the concentration of the lipid in the surface phase of thickness d. The term in the first pair of large parentheses describes the Boltzmann accumulation of the peptide in the aqueous phase adjacent to the membrane. The term in the second pair of large parentheses describes the binding of a lipid to one of the four identical sites on the peptide. The term in the third pair of large parentheses describes the binding of lipids to the three remaining binding sites on the peptide. The numerical factors in the second and third terms arise because of statistical effects (see reference 14).

MATERIALS AND METHODS

We obtained similar results with egg and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC), with bovine brain and 1-palmitoyl-2-

[1]There are at least four reasons why the effective valence, z_{eff}, in the Boltzmann term of Eq. 2 is less than the real valence of the peptide. First, the finite size of the peptide limits the distance of closest approach. Thus the charges on the peptide do not experience the full surface potential but the potential at some distance from the membrane. Second, the finite size of the peptide affects its distribution in the diffuse double layer (15). Third, discreteness-of-charge effects may be important for these multivalent ions (41; Stankowski, S., personal communication). Fourth, anions such as chloride may bind to the peptide. In the Appendix we consider the experimental evidence that the formal value of the effective valence in Eq. 2 is about half the true valence of the three pentavalent peptides pentalysine, PKC(19-29) and PKC(19-36).

[2]The adjectives microscopic and macroscopic refer to association constants defined with respect to individual sites on the peptide or to the entire peptide (14, see pages 850–852 and 862; 38). The adjective intrinsic refers to the assumption that all electrostatic effects are accounted for by the mean field Gouy–Chapman theory and Boltzmann relation, the term in the first set of large parentheses in Eq. 2. The 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 that must describe the loss of entropy that occurs (10, 19, 35).

oleoyl-*sn*-glycero-3-phosphoserine (PS), and with egg and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (PG). The lipids were obtained from Avanti Polar Lipids (Birmingham, AL).

The peptides PKC(19-29) and PKC(19-36) were obtained from Multiple Peptide Systems (San Diego, CA). The peptide PKC (19-36) has the sequence acetyl-Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val-His-Glu-Val-Lys-Asn-amide. 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 purity of PKC(19-36) was >98%. PKC(19-29) required additional purification using reverse phase HPLC to increase purity to 95%.

4-Morpholinepropanesulfonic acid (MOPS) was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). 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.

Multilamellar vesicles were used for the microelectrophoresis measurements (3). We measured the mobilities of these vesicles in a Rank Brothers Mark I instrument (Bottisham, Cambridge, UK) as described previously (13, 47). Equilibrium dialysis and ultrafiltration experiments were performed as described previously (38). The results obtained with PKC(19-36) were corrected for the 11% free peptide that appeared to bind to the ultrafiltration membrane.

RESULTS

We first consider a peptide identical to residues 19–36 of PKC-β, i.e., the pseudosubstrate region (30, 36, 44, 58). This peptide, PKC(19-36), has the sequence acetyl-Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val-His-Glu-Val-Lys-Asn-amide. It has three arginine, three lysine, and one glutamic acid residues and should thus have a charge of ~+5 in a pH 7 solution. The open circles in Fig. 1 illustrate how the binding of this peptide to large unilamellar vesicles (LUVs) formed in 0.1 M KCl depends on the mol % negative lipid in the membrane. (The results in Fig. 1 were obtained from filtration measurements; identical results were obtained from control equilibrium dialysis measurements on 2:1 PC/PG vesicles.) There is no significant binding to membranes formed with either 0 or 10% negative lipid. The binding increases steeply with the PG content of the membrane: >95% of the peptide is bound to vesicles formed with 50% negatively charged lipid.

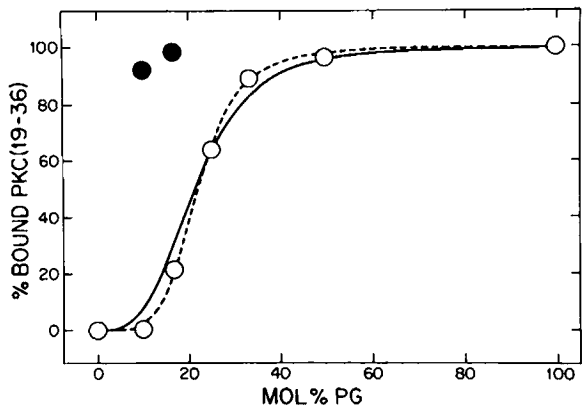

FIGURE 1 Binding of a peptide identical to residues 19–36 of PKC-β to large unilamellar vesicles (LUVs) formed from mixtures of the zwitterionic lipid PC and the negatively charged lipid PG. The aqueous solutions contained 0.1 M KCl (*open circles*) or 0.01 M KCl (*filled circles*) buffered to pH 7.0 with 1 mM MOPS, T = 25°C. They also contained 10 mM lipid (PG plus PC) and 30 μM PKC(19-36) peptide. The solid curve illustrates the prediction of Eq. 2, which ascribes the sigmoidal nature of the binding to a combination of electrostatics and reduction of dimensionality. The dashed curve illustrates the predictions of a Hill equation with a Hill coefficient of 5, which provides a satisfactory fit but a physically incorrect interpretation of the data.[3]

The sigmoidal[3] binding curve arises for two reasons. First, the negatively charged lipids produce an electrostatic potential in the aqueous phase immediately adjacent to the membrane. This potential accumulates postively charged peptides in the aqueous diffuse double layer, which increases the fraction of bound peptide. Second, an increase in the mol fraction of negative lipid increases the binding of the peptide to the membrane because of mass action; the peptide does not bind to the zwitterionic lipid PC but can bind more than one negatively charged lipid, such as PG.

The solid curve in Fig. 1 illustrates the prediction of Eq. 2. The curve is drawn with the value of k, the intrinsic microscopic[2] association constant of a basic residue on the peptide with an acidic lipid in the membrane equal to 3.5 M^{-1}, a value that gave a better least square (and visual) fit to the data than either 3.0 or 4.0. Thus the net free energy[2] of interaction of a basic

[3]The sigmoidal dependence of the percent bound peptide on the concentration of negative lipid in the membrane (Fig. 1) resembles the sigmoidal dependence of the binding of oxygen to hemoglobin. When cooperative reactions occur in three dimensions they can be described by a Hill equation; the Hill coefficient is a measure of the steepness of the sigmoidal relation and reflects the degree of cooperativity of the reaction. A Hill coefficient >1 for a reaction in three dimensions implies the binding of the first ligand produces an allosteric change that increases significantly the association constant of the second ligand. The data in Fig. 1 can also be described by an expression that is formally identical to the Hill equation. We assume an infinitely cooperative reaction occurs in the bulk aqueous phase between n soluble acidic lipids, L, and a peptide, P. It follows that the fraction of bound peptide is equal to $(K^*[L])^n/(1 + (K^*[L])^n)$, where K^* is a constant. This equation, with a Hill coefficient of n = 5 (*dashed line* in Fig. 1) can describe the binding data obtained with PKC(19–36), as well as the essentially identical data obtained with pentalysine (51). However, this is not the correct interpretation of our measurements. It is highly unlikely that the binding of an acidic lipid to one basic residue on these simple peptides increases the association constants of the other residues for acidic lipids by orders of magnitude. Furthermore, our model predicts the marked increase in binding observed when the salt concentration decreases (Fig. 1).

residue and an acidic lipid is weak, $RT\ln(k) \approx 1$ kcal/mol. (As discussed in the Appendix, the effective valence of the peptide that appears in Eq. 2 was assumed to be $z_{eff} = 2.5$, half the actual valence of the peptide.)

Our interpretation of Eq. 2 and the data in Fig. 1 follows Guggenheim and considers the polar head group region to be a surface phase of thickness d, where d is of order 1 nm. Recall that $\{L\}$ = number of acidic lipids/area. When the product of the binding constant, k, and the concentration of acidic lipid in the surface phase, $\{L\}/d$, is <1, the collection of terms in the third set of large parentheses in Eq. 2 is of order unity. For $k \simeq 4\ M^{-1}$, this occurs for $\{L\}/d < 0.25$ M (equivalent to 10 mol % acidic lipid or $\{L\} = 1$ lipid/7nm^2). Thus, for a membrane with $<10\%$ PG, the peptide combines mainly with one negative lipid, as described by the term in the second set of large parentheses in Eq. 2, and the binding is very weak, as is observed experimentally in Fig. 1. Conversely, when $k\{L\}/d > 1$, the last term in the third set of large parentheses in Eq. 2 dominates the other terms. Thus for membranes with $>10\%$ PG, the effective binding constant depends on a high power of the percent PG in the membrane because the peptide combines with more than one negative lipid. This contributes to the steep sigmoidal nature of the binding illustrated in Fig. 1.

In terms of the Guggenheim model of a surface phase of finite thickness, the binding of acidic lipids appears to be a cooperative process because the binding of the first acidic lipid to a basic residue on the peptide places the other residues in a phase where the concentration of PG is sufficiently high (for membranes with $>10\%$ PG) that most of the remaining binding sites combine with PG. In the mathematically equivalent Gibbs model of a surface, the interface is an infinitely thin dividing plane and acidic lipid concentrations are expressed in terms of number per unit area (e.g., $\{L\}$) rather than volume (e.g., $\{L\}/d$). In terms of this model, the binding of one residue reduces the dimensionality of the further reactions and allows other binding sites to interact with acidic lipids. We discuss this apparent cooperativity[3] in the binding of acidic lipids to peptides in more detail elsewhere (38, 51).

Although the sigmoidal nature of the solid curve in Fig. 1 arises from both electrostatics and a transfer to a separate phase that contains a high concentration of ligand (or, equivalently, reduction of dimensionality), it is easy to demonstrate that electrostatics alone strongly affects the binding of this pentavalent peptide. The filled circles illustrate binding measurements made in 0.01 rather than 0.1 M KCl: decreasing [KCl] dramatically increases the binding of the peptide. For a vesicle containing 10% PG, the percent bound peptide increases from ~ 0 to 90%. For a vesicle containing 17% PG the percent bound peptide increases from ~ 20 to 99%. This dramatic increase in binding occurs because decreasing the salt concentration increases the magnitude of the negative electrostatic potential at the surface of the membrane ($\psi(0)$ in Eq. 2). The results illustrated in Fig. 1 for the PKC(19-36) peptide in both 0.1 and 0.01 M KCl are essentially identical to those obtained with the shorter, simpler pentavalent peptide Lys_5 (Mosior, M., and S. McLaughlin, unpublished data).

To illustrate that the binding of the peptide to the membrane depends on the mol fraction of negatively charged lipid even when the surface potential is maintained at a constant value, we consider the effect of the peptide on the zeta potential. Fig. 2 illustrates the effect of PKC(19-36) on the zeta potential of phospholipid vesicles. The zeta potential is the electrostatic potential ~ 0.2 nm from the surface of the membrane (2, 20, 23, 60) and is approximately the surface potential, $\psi(0)$ in Eq. 2. This potential is (approximately) proportional to the net charge density at the surface of the membrane, and is thus linearly related to the number of peptides bound to a unit area of membrane. PKC(19-36) does not bind significantly to PC vesicles: 100 μM peptide does not change the zeta potential of a PC vesicle from its value of ~ 0 mV in 0.1 M KCl (data not shown). PKC(19-36) binds strongly to the PG and PS vesicles: 1 μM peptide reduces the magnitude of the zeta potential from ~ -70 to -30 mV (*open squares*). This peptide, like all the basic peptides we have examined, binds equally well to PS and PG vesicles.

The effect of mass action is most easily demonstrated by comparing the concentration of peptide required to reduce the zeta potential to the same value for PC/PS vesicles of different composition. When the zeta potential is the same, the nonspecific electrostatic accumulation of the peptide in the aqueous diffuse double layer is also the same. Consider the concentrations of peptides required to reduce the zeta potential to -30 mV: PS and 2:1 PC/PS vesicles require peptide concentrations of ~ 1 and 30 μM, respectively (Fig. 2). Thus reducing the negative lipid content of the membrane reduces the binding affinity of the vesicle for the peptide by a mass action (third set of large parentheses in Eq. 2) as well as an electrostatic (first set of large parentheses in Eq. 2) mechanism.

If we lower the concentration of KCl from 0.1 M (Fig. 2) to 0.01 M (Fig. 3), the zeta potential (approximately surface potential) of a charged vesicle becomes more negative, as predicted by the Gouy–Chapman theory. (Compare the points at the left of the graphs in Figs. 2 and 3, which were obtained in the absence of peptide). The effective binding constant of the peptide with the membrane (K in Eqs. 1 and 2) increases because of the Boltzmann term in Eq. 2, as illustrated by the results in Fig. 1. This dependence of K on salt concentration is also

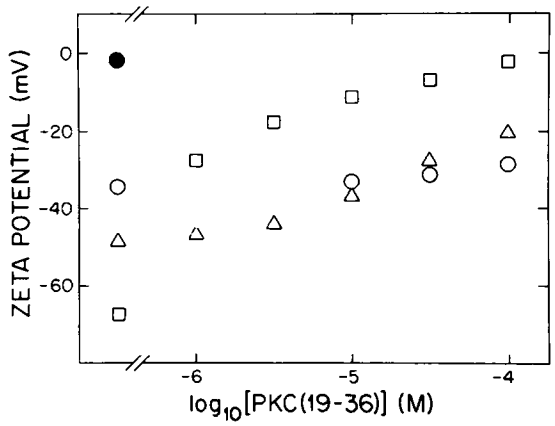

FIGURE 2 The effect of the PKC(19-36) peptide on the zeta potential of PS and PG (*open squares*), 2:1 PC/PS (*open triangles*), 5:1 PC/PS (*open circles*), and PC (*filled circle*) vesicles. The aqueous solution contained 0.1 M KCl buffered to pH 7.0 with 1 mM MOPS. Identical results were obtained with PG and PS vesicles and the measurements were averaged to produce the open squares.

apparent from a comparison of Figs. 2 and 3. For example, 10 μM peptide has no effect on the zeta potential of a 5:1 PC/PS vesicle in 0.1 M KCl (*open circles* in Fig. 2), whereas 1 μM peptide reduces the zeta potential of a 5:1 vesicle in 0.01 M KCl from −70 to −30 mV (*open circles* in Fig. 3). The effects of PKC(19-36) on the zeta potentials of PC/PS vesicles in 0.01 M KCl (Fig. 3) are very similar to those of Lys_5, another pentavalent

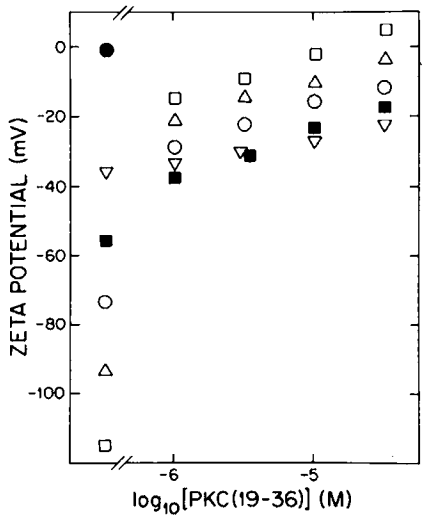

FIGURE 3 The effect of the PKC(19-36) peptide on the zeta potential of PS (*open squares*), 2:1 PC/PS (*open triangles*), 5:1 PC/PS (*open circles*), 10:1 PC/PS (*filled squares*), and 20:1 PC/PS (*open inverted triangles*), and PC (*filled circle*) vesicles. The aqueous solution contained 0.01 M KCl buffered to pH 7.0 with 1 mM MOPS.

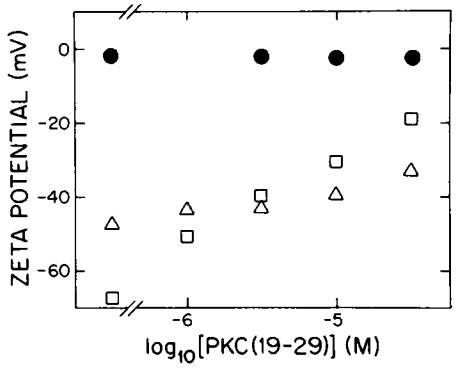

FIGURE 4 The effect of the PKC(19-29) peptide on thet zeta potential PS (*open squares*), 2:1 PC/PS (*open triangles*), and PC (*filled circles*) vesicles. The aqueous solution contained 0.1 M KCl buffered to pH 7.0 with 1 mM MOPS.

peptide (see Fig. 4 in reference 38). The results illustrated in Fig. 3 can be described theoretically[4] by the same combination of Gouy-Chapman, Boltzmann, and mass action equations we used to describe the Lys_5 results (see Fig. 5 of reference 38). We discuss the limitations of our simple model in the Appendix.

We studied PKC(19-29), which lacks the Asn-Val-Glu-Val-Lys-Asn sequence of PKC(19-36), to compare the peptides' binding to membranes. Both peptides have a net charge of +5, but PKC(19-29) has five basic and zero acidic residues. A comparison of Figs. 4 and 2 illustrates that PKC(19-29) binds with about the same affinity as PKC(19-36) to 2:1 PC/PS membranes (but with less affinity to PS membranes, for reasons that are not clear). There is no specificity in the binding of the PKC(19-29) peptide to PS vs PG; the data (not shown) we obtained with PG vesicles are identical to the PS data illustrated in Fig. 4. Similar data were also obtained with phosphatidic acid vesicles (data not shown). PKC(19-29), like PKC(19-36) and Lys_5, binds less strongly to the 2:1 PC/PS vesicles than to PS vesicles, which suggests it binds with a stoichiometry >1 to the negative lipids in the vesicle. Finally, it is similar to all the other basic peptides we have studied in that it does not bind significantly to PC vesicles (*filled circles,* Fig. 4). The fit of the theoretical curves illustrated in Fig. 3 *B* of reference 38 to the data for PKC(19-29) is about as good as for Lys_5.

We also measured how a known concentration of

[4]The data in Fig. 3 can be described qualitatively by the theoretical curves in Fig. 5 *C* of reference 38. These curves were drawn with the effective valence, $z_{eff} = z = 5$, and a macroscopic intrinsic association constant of 20 M^{-1}. We repeated the calculations using a microscopic intrinsic constant of 20: the curves are shifted about a factor of two to the left (stronger binding) than the curves in Fig. 5 *C*. We can obtain a better fit to the data if we assume that $z_{eff} = 4$, and that $k = 21\ M^{-1}$.

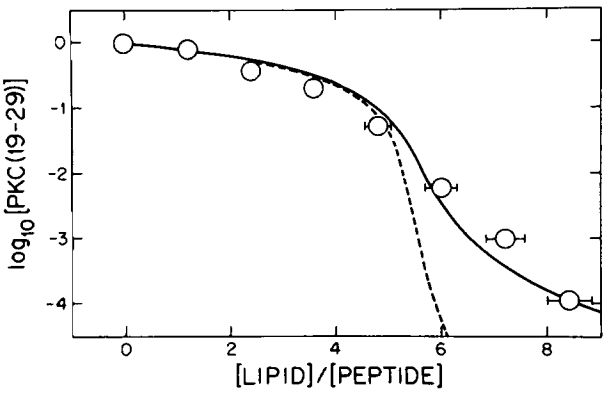

FIGURE 5 Effect of PS large unilamellar vesicles (LUVs) on the free concentration of the peptide PKC(19-29) in a 0.01 M KCl, 1 mM MOPS, pH 7 solution. The ordinate represents the fraction of peptide free in solution (logarithmic scale), measured relative to the total peptide concentration of 3 μM. The abscissa represents the concentration of PS accessible to the peptide (one-half total lipid for these LUVs), measured relative to the total concentration of peptide. The open circles represent the experimental measurement (n = 20). The solid curve is the prediction of Eq. 2, using an effective valence of z_{eff} = 3.0 and a binding constant k = 21 M^{-1}. The dashed curve illustrates the prediction of Eq. 2 with $z_{eff} = z = 5$ and k = 21 M^{-1}. In both curves we assumed the binding constant of potassium ions with PS was 3 M^{-1} to describe accurately the zeta potential data obtained in the absence of peptide.

large unilamellar vesicles decreases the free concentration of the peptide PKC(19-29). The results we obtained in 0.01 M KCl with PKC(19-29) and PS vesicles are illustrated in Fig. 5: note the precipitous decrease in the free concentration of peptide when the lipid concentration doubles.[5] When the lipid concentration (measured relative to the total peptide concentration of 3 μM) increases from four to eight, the free concentration of peptide decreases by three orders of magnitude. This behavior contrasts markedly to the binding one observes with neutral solutes. In that case, the free concentration of a neutral peptide that binds to lipid is given by $[P] = [P^{tot}]/(1 + K[L])$, where $[P^{tot}]$ is the total concentration of peptide, K is an association constant, and $[L]$ is the concentration of lipid. Doubling the lipid concentration reduces the free concentration of the neutral peptide twofold at most. We observe the large effects seen in Fig. 5 because the magnitude of the electrostatic potential adjacent to the lipid vesicles increases as less peptide is available to bind to the membranes. This markedly increases the effective binding constant (K in Eqs. 1 and 2), which is proportional to the Boltzmann term. The two theoretical curves in Fig. 5 illustrate the importance of using an effective rather than the actual valence in the Boltzmann expression. The dashed curve is drawn from Eq. 2 with $z_{eff} = z = 5$, whereas the solid curve is drawn with z_{eff} = 3.0, the value that gave a best fit to the zeta potential versus PKC(19-29) measurements (see Appendix). The solid curve provides a much better fit to the experimental data.

[5]We obtained similar results to those illustrated in Fig. 5—precipitous reduction in free peptide concentration as predicted by theory—for Lys_5 as well as PKC(19-29). We made measurements in 0.1 M KCl with PS vesicles and in 0.01 M KCl with PS and 2:1 PC/PS vesicles (data not shown).

DISCUSSION

Our main objective was to measure how strongly peptides that mimic the pseudosubstrate region of PKC bind to membranes under physiological conditions. Fig. 1 illustrates how the binding of PKC(19-36) depends on the mol % negative lipid in the membrane. The cytoplasmic surface of a plasma membrane typically contains between 20 and 30% negative phospholipids, usually PS for a mammalian cell (11, 49, 57, 66, 69). Fig. 6 illustrates how the effective binding constant depends on the mol % of negative lipid in the membrane, as deduced from the data in Fig. 1 using Eq. 1. The effective binding constant is of order 1 μm for a membrane with 25 mol % negative lipid. In other words, one must go a distance x = 1 μm away from a planar membrane of area A to find the same number of peptides bound to the membrane and in the volume Ax. By making certain assumptions,[6] we can express this effective binding constant in more conventional units, such as 10^4 M^{-1}, which corresponds to a net free energy change of 6 kcal/mol.

We now consider the implications of our study to the activation of PKC. As we illustrate in Fig. 7, PKC consists of a regulatory domain with a pseudosubstrate region (foot) and a kinase domain with a substrate-binding site (mouth). The work of House and Kemp (30) and of Makowske and Rosen (44) suggests that in the cytoplasm of a quiescent cell, this extremely flexible (25) enzyme resembles the title of a Bellow (6) story: "Him With His Foot in His Mouth". House and Kemp (30) showed that a peptide corresponding to amino acids

[6]We can express the effective binding constant K in more conventional units, such as $Molar^{-1}$, by following other investigators (17, 18, 61, 63) and assuming that the binding of a peptide, P, to n acidic lipids, L, can be described by the equation: $K' = n[PL_n]/[L][P]$. To convert our effective binding constant K into the association constant K' we divide both sides of Eq. 1 by the free surface concentration of PG and multiply them by the assumed number of lipid molecules that bind to the peptide, i.e., four. Each lipid occupies ~0.7 nm^2, so the effective binding constant of K = 1 μm for the binding of PKC(19-36) to a membrane with 25 mol% negative lipid corresponds to an association constant $K' = 6.7\ 10^3\ M^{-1} \simeq 10^4\ M^{-1}$, which is equivalent to a net free energy change on binding of $RT \ln (K')$, or ~6 kcal/mol.

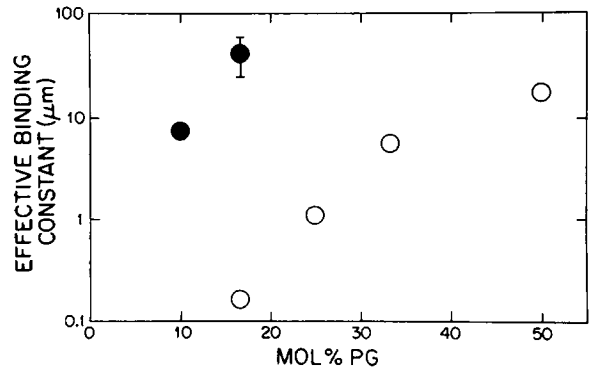

FIGURE 6 The effective binding constant, K, for the peptide PKC(19-36), plotted as a function of the mol percent negatively charged lipid in the membrane. The value of K was deduced from the data in Fig. 1 using Eq. 1 (*Open circles*) 0.1 M KCl. (*Filled circles*) 0.01 M KCl.

19–31 of PKC is as effective as peptide 19–36 in inhibiting substrate phosphorylation; this 19–31 region contains 5 basic (and no acidic) amino acids. These five basic amino acids are represented in the cartoon (Fig. 7) as the five positively charged toes of the pseudosubstrate foot. The positive charges are important for function (30, 31, 45). These five basic residues possibly interact with acidic residues in the putative substrate binding site (32).

Our working hypothesis is that the interaction of the pseudosubstrate region with acidic lipids facilitates the activation of PKC. Our measurements allow us to rule out the simple possibility that in the presence of calcium the enzyme removes its foot from its mouth, and the binding of the positively charged toes to the negatively charged lipids causes concomitant translocation and activation of the enzyme. The effective binding constant of the PKC peptides we measured[6] to membranes with 25% negative lipid (K = 1 μm, or $K' = 10^4$ M^{-1}, or net free energy change = 6 kcal/mol) is too weak to account for the observed binding of PKC to vesicles upon exposure to calcium. According to Bazzi and Nelsesteun (4), the effective binding constant of the protein is $> 10^8$ M^{-1} (or the net free energy change is > 12 kcal/mol) for vesicles containing 30% PS. Thus the protein binds with at least 6 kcal/mol more free energy to the membranes even though the protein loses more translational and rotational free energy than the peptide when it binds (10, 19, 35).

Evidence from several laboratories suggests that calcium ions can cause PKC to bind to the membrane without removing the foot from the mouth: in the absence of DAG binding to PC/PS vesicles can occur without activation (4, 22, 59). Once the enzyme has bound to the membrane, DAG and PS might act together to help pull the foot out from the mouth and thus activate PKC. Our measurements with PKC(19-29) and PKC(19-36) peptides demonstrate that ~6 kcal/mol free energy will be gained if the basic toes bind to acidic lipids when the pseudosubstrate region is removed from the substrate binding site. How much free energy is required to remove the foot from the mouth? We can crudely estimate it is ~10 kcal/mol from the observation that about 100 nM pseudosubstrate peptide is required to inhibit PKC (30). Thus the free energy recovered by interaction with negative phospholipids (6 kcal/mol for a 25 mol% PS membrane) can stabilize the active state of the enzyme but is not sufficient to fully activate the enzyme in the absence of DAG. Our hypothesis is consistent with the observation that less DAG is required as the mol % PS in the membrane increases (27).

In summary, acidic lipids may be involved in both the calcium-induced binding of PKC to the membrane, a process that is not understood (5, 43), and the activation of the enzyme. Observations in the literature provide three lines of support for the hypothesis that acidic lipids stabilize the active form of PKC by binding to basic residues in the pseudosubstrate region, as illustrated in Fig. 7. First, the activation of PKC and the binding of pseudosubstrate peptides to a membrane both depend on the mol fraction of negative lipid in the membrane in a sigmoidal manner.[7] Several groups have demonstrated that the rate at which PKC phosphorylates either substrates or itself is a sigmoidal function of the mol% PS in a micelle (12, 26, 28, 29, 34, 52, 58) or membrane (53). The steepness of the sigmoidal curve (Hill coefficient) depends on the nature of the surface, the substrate, and the PKC isotype. Hannun and Bell (26) showed their sigmoidal dependence of PKC activity on mol% PS was consistent with a Hill coefficient =5, as was the binding of the PKC(19-36) peptide in Fig. 1; the dashed line in Fig. 1 is drawn with a Hill coefficient of 5. Larger Hill coefficients are obtained with the purified α and β isotypes of PKC (12, 52). Second, the observation that PKC activation occurs with 100% PS vesicles in the absence of DAG (62) is consistent with our simple hypothesis. Fig. 6 (see also Figs. 1 and 2) illustrates that the effective binding constant of the pseudosubstrate peptide to membranes is a steep function of the mol% acidic lipid—the net free energy change upon binding increases from ~6 to >10 kcal/mol when the mol% negative lipid increases from 25 to 100%. Thus the free energy change on binding to a 100% PS membrane is

[7]The binding of the pseudosubstrate region of PKC to anionic lipids may differ from that of the PKC(19-36) peptide because their structures may be different. Even if their structures are similar, the degree of apparent cooperativity or Hill coefficients for the two processes may be different because the interaction of the pseudosubstrate region of PKC with acidic lipids may involve several binding steps, as illustrated in Fig. 7.

sufficient to compensate for the energy required to remove the pseudosubstrate region from the substrate binding site. Third, Eq. 2 demonstrates that adsorption of basic amphiphiles to membranes should reduce binding of basic peptides to membranes by reducing the magnitude of the surface potential. Many observations (21) suggest that chemically diverse basic amphiphiles such as tetracaine (50) and trifluoperazine (39, 67) inhibit PKC. Tetracaine and trifluoperazine adsorb strongly to bilayer membranes at the concentrations used in these experiments (~1 and 0.1 mM) and do change the surface potential (48).

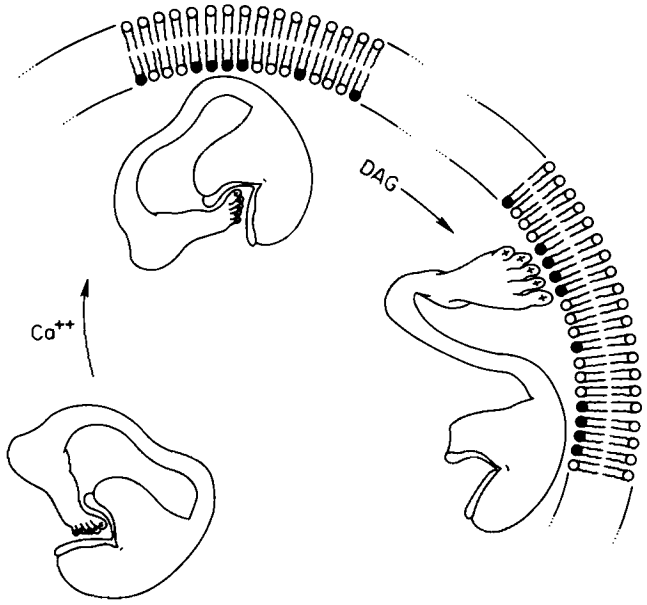

FIGURE 7 Cartoon illustrating our working hypothesis about the activation of PKC. The filled circles represent the polar head groups of negatively charged phospholipids, such as PS, which are located preferentially on the cytoplasmic monolayer of the plasma membrane. The open circles represent the head groups of zwitterionic lipids, such as PC, to which positively charged amino acids do not bind. Protein kinase C, PKC, has a kinase domain with a substrate binding site (mouth) and a regulatory domain with a pseudosubstrate region (foot) that contains five positively charged amino acids (toes). The experiments of House and Kemp (30) and Makowske and Rosen (44) suggest that PKC has its foot in its mouth when it is in the cytoplasm. Experiments from several different laboratories suggest calcium ions can cause PKC to bind to the membrane but do not cause activation; this translocation requires acidic lipids and the mechanism is not understood. We hypothesize that binding of basic residues in the foot region to acidic lipids in the membrane (and concomitant binding of the adjacent DAG binding site on PKC to DAG in the membrane) then activates the enzyme by compensating for the free energy required to remove the foot from the mouth. Our measurements with peptides indicate the binding of the pseudosubstrate region to negative lipids should decrease the free energy of the activated form of the enzyme by ~6 kcal/mol.

The observation that PS activates PKC more effectively than other anionic lipids such as PG and PA (12, 26, 34, 42,52) would appear to argue against the hypothesis illustrated in Fig. 7 because we observe little selectivity in the binding of the PKC peptides to the acidic lipids PS, PG, and PA. Complete activation of PKC, however, requires phorbol esters or DAG as well as calcium and acidic phospholipids. Phorbol esters bind more effectively to enzyme-phospholipid complexes containing PS than to complexes containing either PA or PG and this could account for the selectivity observed (26, 34).

APPENDIX

There are three major limitations to our simple model. First, we must assume the effective valence of the peptides that appears in the Boltzmann relation, z_{eff} in Eq. 2, is about half the actual valence. Second, the intrinsic microscopic association constant appears to decrease when the mole fraction of anionic lipid in the membrane decreases. Third, we have no direct information about the number of binding sites for anionic lipids on the peptides.

We initially assumed that $z_{eff} = z = 5$ and attempted to fit the experimental data presented in Figs. 1–5 with our model (Eqs. 4, 6–13, 8a–11a in reference 38), which has only one free parameter, the intrinsic microscopic association constant of a basic residue with an acidic lipid, k. The slopes of the curves illustrated in Figs. 2–4, however, were larger than the theoretically predicted values. The limiting theoretical slope is 58 mV/z_{eff} = 11 mV/decade peptide concentration if $z_{eff} = 5$. To account for the slopes of the experimental data we allowed z_{eff} to be a free parameter and obtained the following values of z_{eff}: 4 (Fig. 2, *PS membrane*), 2.5 (Fig. 2, *2:1 PC/PS*), 4 (Fig. 3, all membranes), and 2 (Fig. 4, *PS*). The necessity of choosing $z_{eff} < z$ was also apparent from the experimental results illustrated in Fig. 5: the dashed curve represents predictions of the model with $z_{eff} = z = 5$ and $k = 21\ M^{-1}$. We obtained better agreement between theory and experiment by assuming $z_{eff} = 3$ (*solid curve*), a value that also yields the best fit for the effect of PKC(19-29) on the zeta potential of a PS membrane in 10 mM KCl (not shown). The filtration binding data illustrated in Fig. 1 also indicate that $z_{eff} < 5$. The data can be described if $z = z_{eff} = 5$ and $k = 0.3\ M^{-1}$. This value of k, however, is $\ll$ the value deduced from other measurements on vesicles that had more bound peptide and a less negative surface potential. To reconcile the two sets of measurements, we assumed $z_{eff} = 2.5$, which gave the best fit to the slope of the zeta potential curve for PKC(19–36) and 2:1 PC/PS(PG) membranes in 100 mM KCl (Fig. 2). A value of $k = 3.5\ M^{-1}$ then provided an adequate fit to the data in Fig. 1 (see *solid curve*) as well as agreeing better with values for k obtained from other measurements made under different conditions. Other large basic peptides, such as melittin (10, 40, 64) and pentalysine (38), also appear to have lower effective valences. We list some reasons that z_{eff} is about half the value of z for these large peptides in footnote 1.

The value of k obtained from a least squares fit to the zeta potential data obtained with different PC/PS membranes is not a constant, but decreases as the mole fraction of acidic lipid decreases. For PKC(19–36) in 100 mM KCl, k = 10, 6, and 3 M^{-1} for 0:1, 2:1, and 5:1 PC/PS or PC/PG membranes, respectively. The direct binding measurements in Fig. 1 also illustrate the dependence of the microscopic association constant on the mol fraction of acidic lipid: the theoretical binding

curve calculated for $k = 3.5\ M^{-1}$ (*solid line*) overestimates the fraction of bound peptide to the membranes with a low mol% PG and underestimates the fraction bound to membrane with a high mol% PG. We do not understand the reasons for this deviation from the simple mass action formulation.

The peptides PKC(19–29) and PKC (19–36) have five and six basic residues, respectively, and each basic residue is a potential binding site for acidic lipids. More extensive studies on another pentavalent peptide, Lys_5, suggest it has four binding sites for acidic lipids (38), and in the absence of direct information we assume the PKC(19–29) and PKC(19–36) also have four binding sites.

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