Phosphorylation Reverses the Membrane Association of Peptides that Correspond to the Basic Domains of MARCKS and Neuromodulin

Jiyun Kim,* Perry J. Blackshear,‡ J. David Johnson,§ and Stuart McLaughlin*

*Department of Physiology and Biophysics, HSC, SUNY, Stony Brook, New York 11794; ‡Howard Hughes Medical Institute Laboratories and the Section of Diabetes and Metabolism, Division of Endocrinology, Metabolism, and Genetics, Departments of Medicine and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710; and §Department of Medical Biochemistry, College of Medicine, Ohio State University Medical Center, Columbus, Ohio 43210 USA

ABSTRACT Several groups have observed that phosphorylation causes the MARCKS (Myristoylated Alanine-Rich C Kinase Substrate) protein to move off cell membranes and phospholipid vesicles. Our working hypothesis is that significant membrane binding of MARCKS requires both hydrophobic insertion of the N-terminal myristate into the bilayer and electrostatic association of the single cluster of basic residues in the protein with acidic lipids and that phosphorylation reverses this electrostatic association. Membrane binding measurements with myristoylated peptides and phospholipid vesicles show this hydrophobic moiety could, at best, barely attach proteins to plasma membranes. We report here membrane binding measurements with basic peptides that correspond to the phosphorylation domains of MARCKS and neuromodulin. Binding of these peptides increases sigmoidally with the percent acidic lipid in the phospholipid vesicle and can be described by a Gouy-Chapman/mass action theory that explains how electrostatics and reduction of dimensionality produce apparent cooperativity. The electrostatic affinity of the MARCKS peptide for membranes containing 10% acidic phospholipids (10^4 M^{-1} = χ/[P], where χ is the mole ratio of peptide bound to the outer monolayer of the vesicles and [P] is the concentration of peptide in the aqueous phase) is the same as the hydrophobic affinity of the myristate moiety for bilayer membranes. Phosphorylation decreases the affinity of the MARCKS peptide for membranes containing 15% acidic lipid about 1000-fold and produces a rapid ($t_{1/2}$ < 30 s) dissociation of the peptide from phospholipid vesicles.

INTRODUCTION

The myristoylated alanine-rich C kinase substrate (MARCKS) protein (Aderem, 1992; Blackshear, 1993) and neuromodulin (Liu et al., 1990; Estep et al., 1990; LaBate and Skene, 1989; Coggins et al., 1991) have attracted attention because they might play important roles in the calcium/phospholipid second messenger signaling system. Both MARCKS (Albert et al., 1987) and neuromodulin (Cimler et al., 1985; Liu and Storm, 1989; Baudier et al., 1989) are present at concentrations of about 10 μM in whole brain. Calmodulin (CaM), which is present at comparable concentrations in brain (Klee and Vanaman, 1982), binds to neuromodulin in a calcium-independent manner with an association constant of 10^6 M^{-1} (Cimler et al., 1985; Alexander et al., 1987; Alexander et al., 1988) and to MARCKS in a calcium-dependent manner (half maximal effect at [Ca^{2+}] = 300 nM) with a maximum association constant of 4×10^8 M^{-1} (Graff et al., 1989c; McIlroy et al., 1991a). Thus, a considerable fraction of CaM in quiescent cells could be bound to neuromodulin and, when the [Ca^{2+}] rises, MARCKS could compete with other proteins for the calcium-calmodulin (Ca-CaM) complex. When MARCKS (Graff et al., 1989c; McIlroy et al., 1991a) and neuromodulin (Alexander et al., 1987) are phosphorylated by protein kinase C (PKC), their affinities for CaM decrease significantly. These results led to the proposal that neuromodulin might "bind and concentrate CaM at specific sites and release CaM locally in response to protein kinase C phosphorylation" (Liu et al., 1990; Skene, 1990). The observations that activation of PKC increases the level of CaM in the cytoplasm of intact cells (MacNichol and Schulman, 1992) and produces translocation of CaM from membrane to cytoplasm (Mangels and Gnegy, 1992) support this proposal.

The features of the existing models common to both proteins are illustrated in Fig. 1. On the far left, MARCKS is shown tethered reversibly to the membrane by the insertion of its myristoyl group (14 carbon acyl chain) into the lipid bilayer (Graff et al., 1989a; George and Blackshear, 1992). Neuromodulin is presumably tethered in a similar, but essentially irreversible, manner by the two palmitoyl (16 carbon acyl chain) groups covalently attached to cysteine residues near its amino terminus (Skene and Virág, 1989; Houbre et al., 1991). The CaM-binding domain of MARCKS (or neuromodulin) binds to CaM with an association constant

Received for publication 4 January 1994 and in final form 6 April 1994.

Address reprint requests to Stuart G. McLaughlin, Department of Physiology & Biophysics, Health Sciences Center, SUNY, Stony Brook, NY 11794-8661. Tel.: 516-444-3615 (3039); Fax: 516-444-3432; E-mail: smcl@epo.som.sunysb.edu.

Abbreviations used: MARCKS, myristoylated alanine rich C kinase substrate; CaM, calmodulin; PKC, protein kinase C; K_1, association constant defined in Eq. A1; K_2, partition coefficient defined in Eq. A4; K_3, partition coefficient defined in Eq. A5; K_4, association constant defined in Eq. A2; LUVs, large unilamellar vesicles; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; MARCKS(151–175), peptide corresponding to the CaM-binding domain of MARCKS; neuromodulin(37–53), peptide corresponding to the CaM-binding domain of neuromodulin; tetra-Asp MARCKS, MARCKS(151–175) with aspartic acids replacing the four serines; SUVs, small unilamellar vesicles; DPE, dansyl-phosphatidylethanolamine; K, partition coefficient defined in Eq. 1; K_p, molar partition coefficient defined in Eq. 2; k, microscopic binding constant of a basic residue, and an acidic lipid as described in Kim et al. (1991).

© 1994 by the Biophysical Society

0006-3495/94/07/227/11 $2.00

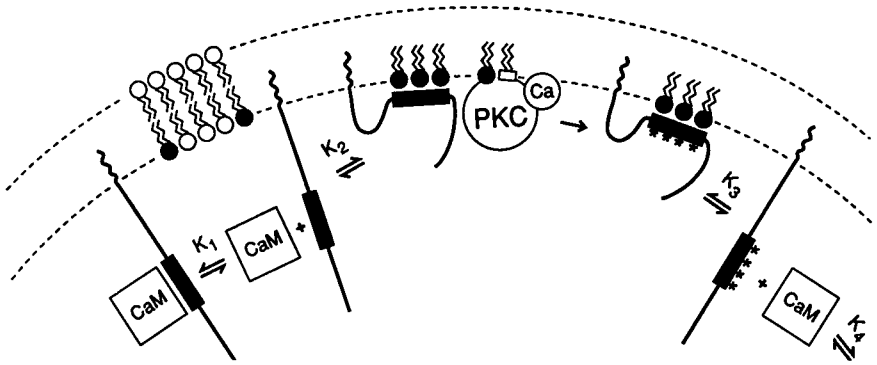

FIGURE 1 Sketch of the model. Calmodulin, CaM, (*open square*) binds to the CaM-binding domain of MARCKS, residues 151–175 (*filled rectangle*), with an association constant K_1. MARCKS is shown tethered to the plasma membrane by means of its myristoyl chain (*squiggly line*). We hypothesize that the CaM-binding domain also associates with the head groups of acidic lipids (*filled circles*) in the membrane with a partition coefficient K_2. When the concentrations of diacylglycerol in the membrane and calcium ions in the cytoplasm increase, protein kinase C, PKC (*large open circle*) translocates to the membrane and phosphorylates the CaM-binding domain of MARCKS. The phosphorylated (*) CaM-binding domain binds to acidic lipids in the membrane with a partition coefficient $K_3 < K_2$. CaM combines with the phosphorylated form of MARCKS with an association constant $K_4 < K_1$. The constants K_1 – K_4 are defined in the Appendix.

K_1. (In Fig. 1 we do not distinguish between CaM and Ca-CaM.) This domain (residues 151–175 of the bovine MARCKS protein) contains the four serines that are phosphorylated by PKC and is absolutely conserved among the bovine, avian, and human proteins (Graff et al., 1989b, c; Stumpo et al., 1989; Graff et al., 1991). The CaM-binding domain of neuromodulin (residues 39–55) contains the single serine that is phosphorylated by PKC (Houbre et al., 1991; Wakim et al., 1987; Apel et al., 1990; Nielander et al., 1990; Chapman et al., 1991) and is strictly conserved among the vertebrates (LaBate et al., 1989; Skene, 1989). On the far right of Fig. 1, phosphorylated protein is shown binding to CaM with an association constant K_4; $K_4 \ll K_1$ for both MARCKS (McIlroy et al., 1991a) and neuromodulin (Alexander et al., 1987).

Our working hypothesis is that the CaM-binding domains of MARCKS and neuromodulin,[1] which contain clusters of basic residues, also bind to acidic lipids in the plasma membrane (K_2 in Fig. 1) and that phosphorylation decreases this binding ($K_3 < K_2$): this hypothesis is reasonable because small peptides with clusters of basic residues do bind to membranes containing acidic lipids (de Kruijff et al., 1985; Kim et al., 1991; Mosior and McLaughlin, 1991; Mosior and McLaughlin, 1992a, b; Montich et al., 1993; Taniguchi and Manenti, 1993; Gawrisch et al., 1993). As reviewed elsewhere, stimulation of the calcium/phospholipid second messenger system produces diacylglycerol and inositol trisphosphate, which releases calcium ions (Berridge, 1993). PKC then translocates to the plasma membrane, binds to acidic phospholipids and diacylglycerol, becomes activated, and phosphorylates its substrates (Bell and Burns, 1991; Newton, 1993). Phosphorylation by PKC shifts the equilibrium in Fig. 1 from left to right; the net effect is that phosphorylation of MARCKS (or neuromodulin) increases the concentration of free Ca-CaM (or CaM) in the cytoplasm.

Fig. 1 does not show that phosphorylation causes a translocation of the MARCKS protein from membrane to cytoplasm in many cells (Thelen et al., 1991; Wang et al., 1989; Sawai et al., 1993). This reversible translocation is not due to removal of the myristate, which is necessary but not sufficient for membrane binding. Furthermore, this phosphorylation-induced translocation of MARCKS from membrane to solution also occurs with simple phospholipid vesicles containing acidic lipids, which demonstrates that physical factors can induce the translocation (Taniguchi and Manenti, 1993). We hypothesize that a significant fraction of MARCKS is bound to the plasma membrane only when **both** the amino terminal myristate inserts hydrophobically into the interior of the membrane **and** the cluster of basic residues interacts electrostatically with acidic phospholipids in the inner monolayer of the plasma membrane, which typically contains about 10–20% negatively charged phospholipids (Op den Kamp, 1979). Previous measurements with myristoylated peptides show that the hydrophobic insertion of myristate into a bilayer can provide at most a partition coefficient (apparent association constant with lipids) of 10^4 M^{-1} (Peitzsch and McLaughlin, 1993), which is barely enough to partition a myristoylated protein into the plasma membrane of a typical cell.

We report here both direct and indirect measurements of the membrane binding of peptides that correspond to the basic CaM-binding/phosphorylation domains of MARCKS and neuromodulin.

MATERIALS AND METHODS

Materials

The zwitterionic lipids egg and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC) as well as the acidic lipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (PG) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (PS) were obtained from Avanti Polar Lipids (Birmingham,

[1] Our model for MARCKS and neuromodulin differs slightly from the one proposed by Houbre et al. (1991) for neuromodulin. They propose that calcium ions cause dissociation of CaM from neuromodulin; calcium ions, however, appear to decrease the affinity of neuromodulin for CaM only when the ionic strength is low (Liu and Storm, 1989).

AL). 1,2-dipalmitoyl-L-3-phosphatidyl[*N*-methyl-^{3}H]choline ([^{3}H]-PC) and 1,2-di[1-^{14}C]oleoyl-L-3-phosphatidylcholine ([^{14}C]-PC) were from Amersham (Arlington Heights, IL). Dansyl-phosphatidylethanolamine (DPE) was from Molecular Probes (Eugene, OR).

The MARCKS peptide (KKKKKRFSFKKSFKLSGFSFKKNKK), which corresponds to residues 151–175 of the bovine MARCKS protein, and the tetra-Asp MARCKS peptide (KKKKKRFDFKKDFKLDGFDFKKNKK), in which aspartic acid residues replace each of the four serines, were synthesized and purified as previously described (Graff et al., 1991). MARCKS(151–175) with blocked ends (acetyl on the amino and amide on the carboxy termini) was obtained from Multiple Peptide Systems (San Diego, CA). Control electrophoretic mobility and binding measurements on peptides with blocked or unblocked termini yielded the same results. The neuromodulin peptide (acetyl-KIQASFRGHITRKKLKG-amide), which corresponds to residues 37–53 of bovine neuromodulin (Skene, 1989), also was obtained from Multiple Peptide Systems. The purity of the peptides was >94% as determined by analytical HPLC, mass spectrographic, and amino acid analysis. Rat brain protein kinase C (PKC), a generous gift from Dr. Richard Epand (McMaster University, Hamilton, Canada), contained a mixture of isotypes; 95% of the kinase activity was dependent on Ca^{2+} and phosphatidylserine (Mosior and Epand, 1993). [γ^{32}P]-ATP was from New England Nuclear (Wilmington, DE). Aqueous solutions were prepared with 18 MΩ water (Super-Q, Millipore Corp., Bedford, MA) that was subsequently bidistilled in an all-quartz still.

Electrophoretic mobility measurements

The electrophoretic mobility, u, of multilamellar vesicles (Bangham et al., 1974) was measured in a Rank Brothers Mark I instrument (Bottisham, Cambridge, UK) as described previously (Cafiso et al., 1989). The Helmholtz-Smoluchowski equation relates u to the zeta potential, ζ, the potential about 0.2 nm from the surface of a phospholipid vesicle (McLaughlin, 1989). Control experiments and theoretical calculations show that the concentration of vesicles (0.01–0.1 mg/ml) was sufficiently low that they bound a negligible fraction of the peptide. The free peptide concentration in these experiments was thus equal to the total peptide concentration. Each data point in Fig. 4 represents an average of at least two independent sets of measurements on 10 vesicles. The SDs are not shown because these are smaller than the size of the symbols (i.e., <2 mV).

Large unilamellar vesicle (LUV) preparation

Multilamellar vesicles were made by mixing PC and PG in chloroform, vacuum-drying this mixture, and resuspending in a 170 mM sucrose solution buffered to pH 7 by 1 mM MOPS. Trace amounts of [^{14}C]-PC or [^{3}H]-PC were incorporated into the lipid mixture to monitor the total lipid concentration in later steps. LUVs were produced (Hope et al., 1985) by taking multilamellar vesicles through five cycles of freezing (liquid N_2) and thawing (40°C water bath) followed by 10 cycles of extrusion through a stack of two polycarbonate filters (with 0.1 μm diameter pore size) in an Extruder (Lipex Biomembranes, Inc., Vancouver, BC). The sucrose was removed from the solution outside the vesicles by diluting the vesicle solution fivefold in a 100 mM KCl, 1 mM MOPS, pH 7 solution and centrifuging for 1 h at $10^5 \times g$ at 25°C. The supernatant was discarded, and the resuspended pellet was used for the binding assay.

Binding measurements with nonphosphorylated peptides

The binding measurements were carried out by mixing peptide (600 nM to 5 μM) with LUVs (1 mM), then separating the vesicles from the unbound peptide by centrifugation (Rebecchi et al., 1992; Mosior and Epand, 1993) for 1 h at $10^5 \times g$ at 25°C. 90% of the supernatant volume was retrieved immediately, and the concentrations of peptide and lipid (2–8%) that remained in the supernatant were determined. The pellet was resuspended, and the amount of bound peptide was determined after correcting for the small fraction of lipid that did not spin down. By measuring the peptide in both the supernatant and the pellet, we confirmed that there was no significant loss onto the polyallomer tube at these peptide concentrations. The concentration of the peptide in the supernatant and the pellet was determined by a fluorescamine assay (Weigele et al., 1972). Loss of peptide onto the polyallomer tubes and the sensitivity of the fluorescamine assay limited direct binding measurements to peptide concentrations >500 nM.

Binding measurements with phosphorylated peptides

We mixed 100–300 nM ^{32}P-labeled peptide with 1 mM sucrose-loaded LUVs, then separated the vesicles from the unbound peptide by centrifugation. We determined the peptide concentration in the supernatant by using P81 filters (Newton and Koshland, 1989; Chakravarthy et al., 1991): 100 μl of sample was spotted on a P81 filter (a cation exchanger that binds basic peptides with high affinity) and rinsed with 0.4% phosphoric acid. The filter was dried, and the peptide concentration was determined using a Beckman Model LS 3801 counter (Beckman, Palo Alto, CA).

In contrast to nonphosphorylated peptide, our preliminary experiments revealed that about 90% of the phosphorylated peptide was lost onto the polyallomer microcentrifuge tubes, presumably because of its low concentration (≈100 nM). We used three methods to decrease this loss: salinizing the tube (50% loss), adding a carrier (3 mg/ml BSA (10% loss) or 1 mg/ml gelatin (20% loss)), and increasing the concentration of the phosphorylated peptide to 3 μM, which allowed us to check a few measurements with the fluorescamine assay. Despite differences in the loss of peptide, the ratios of the bound to free peptide concentrations were comparable with all three techniques (loss should not affect these binding measurements because we determine the [peptide] in both the supernatant and precipitate); the results were averaged and are reported in Figs. 2 and 3 as single values.

Phosphorylation of peptides by Protein Kinase C (PKC)

The reaction mixture contained 20 mM MOPS, pH 7, 200 μM $CaCl_2$, 5 μM 10:1 PS/diolein sonicated small unilamellar vesicles (SUVs bath-sonicated in a 20 mM MOPS, pH 7 solution), 5 mM $MgCl_2$, 10 μM peptide, 1 mM dithiotreitol (DTT), and 1–10 nM PKC; 200 μM [γ^{32}P]-ATP was added to start the reaction (Kaibuchi et al., 1981; Epand et al., 1989). The mixture was incubated for 1 h at 30°C, and the reaction was terminated by adding 10 mM EDTA, 10 mM sodium phosphate, and 10 mM NaF. This solution was used as a source of the phosphorylated peptide without further purification. The stoichiometry of phosphorylation was 3.5 phosphates per MARCKS(151–175) and 1 per neuromodulin(37–53).

Fluorescence measurements

Fluorescent SUVs were prepared by tip sonication of a mixture of DPE (10%), PS (20%), and PC (70%). Fluorescent SUVs were separated from free DPE on a Sephadex G-50 column equilibrated with 90 mM KCl, 10 mM MOPS, pH 7.0. Fluorescence of DPE was measured at 22°C with a Perkin-Elmer LS-5 fluorescence spectrophotometer: excitation was at 340 nm, and emission was monitored at 520 nm in Fig. 6. Excitation of acrylodan-MARCKS peptide (Fig. 5) was at 370 nm, and emission was monitored at 470 nm: we performed control titrations of SUVs in the absence of peptide, which allowed us to correct for the small contribution of liposome turbidity to the fluorescence measurements.

RESULTS

The peptide neuromodulin(37–53) has two arginine, four lysine, and no acidic residues; it should have a charge of about +6 in a pH 7 solution. Fig. 2 illustrates how the binding of this peptide to phospholipid vesicles ([lipid] = 1 mM) depends on the percent acidic lipid in the membrane. Fig. 2 has

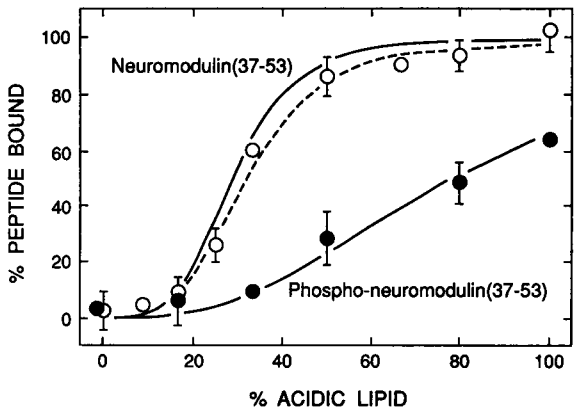

FIGURE 2 Effect of phosphorylation on the binding of neuromodulin-(37–53) to large unilamellar vesicles (LUVs) formed from mixtures of the zwitterionic lipid PC and the acidic lipid PG. The (○) represent the binding data obtained with neuromodulin(37–53); the (●) represent the data obtained with the phosphorylated form of this peptide. The aqueous solutions contained 0.1 M KCl buffered to pH 7 with 1 mM MOPS at $T = 25°C$ and 1 mM total lipid in the form of sucrose-loaded LUVs. They also contained 5 μM neuromodulin(37–53) or 100 nM to 5 μM phosphorylated peptide (see Materials and Methods). The symbols represent averages of $n \geq 3$ measurements for neuromodulin(37–53), $n \geq 6$ measurements for phospho-neuromodulin(37–53). We show the SDs only when they are greater than the size of the symbols. The theoretical curves, which are discussed in more detail in the text, represent the predictions of a simple Gouy-Chapman/mass action theoretical model. The two adjustable parameters are the microscopic intrinsic association constant of a basic residue with an acidic lipid ($k = 2$ M^{-1} or $\Delta G^\circ = RT \ln k \simeq 1$ kcal/mol for both solid curves) and the number of binding sites for acidic lipids on the peptides, which is assumed to decrease from 6 to 4 upon phosphorylation.

three salient features: neither the phosphorylated nor the non-phosphorylated form of neuromodulin(37–53) binds to electrically neutral PC vesicles; both forms bind with a sigmoidal dependence on the percent acidic lipid in the membrane, and the phosphorylated form binds with a lower affinity.

The observation that neuromodulin(37–53) does not bind significantly to PC vesicles demonstrates that hydrophobic interactions are not of paramount importance for the association of this peptide with phospholipid vesicles.[2] The binding mechanism thus differs from that of another CaM-binding peptide, melittin (Maulet and Cox, 1983), which forms an amphipathic helix and adsorbs strongly to PC vesicles (Dempsey, 1990; Kuchinka and Seelig, 1989).

The sigmoidal dependence of binding on the percent of acidic lipid in the membrane (apparent cooperativity) arises for two reasons (Mosior and McLaughlin, 1992a). First, the negatively charged lipids produce an electrostatic potential at the surface of the membrane that attracts the positively charged peptide, concentrating it in the aqueous phase immediately adjacent to the surface. Second, when the first basic residue on the peptide binds to an acidic lipid in the membrane, the remaining basic residues experience a much higher local concentration of acidic lipids. The upper solid curve in Fig. 2 illustrates the prediction of a simple Gouy-Chapman/mass action theory that accounts for these two phenomena (Mosior and McLaughlin, 1992a, c). The number of binding sites is assumed to be equal to the number of basic residues, and the intrinsic microscopic association constant between a basic residue in the peptide and an acidic lipid in the membrane is assumed to be $k = 2$ M^{-1} (Mosior and McLaughlin, 1992a, c). The limitations of this model are discussed in Mosior and McLaughlin (1992c). Although the fit of the theoretical curve[3] to the data is satisfactory, it is not unique: the dashed curve in Fig. 2 shows that the data also can be described by assuming that there are fewer binding sites (3 rather than 6) with higher association constants (4.5 rather than 2 M^{-1}).

The filled circles in Fig. 2 show that the phosphorylated peptide binds less strongly than neuromodulin(37–53) to the vesicles. For example, vesicles with 33% acidic lipid bind 60% of neuromodulin(37–53), whereas they bind only 10% of the phosphorylated peptide. Although the interpretation is not unique, we can describe the data in Fig. 2 with the Gouy-Chapman/mass action theory by assuming that the number of binding sites decreases from 6 to 4 upon phosphorylation and that the microscopic association constant k does not change.

We define a partition coefficient, K, that relates the surface concentration of the peptide (number of peptides adsorbed per unit area of membrane), $\{P\}$, to its concentration in the bulk aqueous solution, $[P]$ as

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

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. Alternatively, we can define a molar partition coefficient, K_P (M^{-1}), that relates the mole ratio of peptide in the membrane phase, χ, to the concen-

[2] Circular dichroism measurements show that both the MARCKS(151–175) and neuromodulin(37–53) peptides are random coils in solution but change conformation upon binding to vesicles containing acidic lipids (Coburn et al., 1993). Hydrophobic interactions probably contribute weakly to the binding of MARCKS(151–175). We studied the binding of a peptide similar to MARCKS(151–175) but with the phenylalanines at positions 157 and 170 replaced by alanines. Removing these Phe residues decreased the binding of the peptide to 10:1 PC:PG vesicles twofold, which suggests that hydrophobic interactions play a measurable but small role in the binding.

[3] Our Gouy-Chapman/mass action model assumes that the peptides are point charges, although they are actually larger than the thickness of the diffuse double layer, $1/\kappa \simeq 1$ nm for 0.1 M salt. We compensate for this by using an effective valence, z_{eff}, in the Boltzmann relation that describes the peptides' electrostatic accumulation in the double layer. The slopes of the curves in Fig. 4 depend on z_{eff}. We assumed z_{eff} was 6 to fit the data in Fig. 4 for MARCKS(151–175). If we use the real value of 13 in the Boltzmann relation, the slopes are one-half the value illustrated in Fig. 4. The effective valences used for the curves in Fig. 3 were $z_{eff} = 6, 4$, and 3, or half the actual valences of MARCKS(151–175), tetra-Asp MARCKS, and phosphorylated MARCKS(151–175), respectively. The effective valence of neuromodulin-(37–53), as determined from zeta potential measurements (not shown) is 3, or half the actual valence. We use this value in describing the data in Fig. 2 and assume z_{eff} is 2 for the phosphorylated peptide.

TABLE 1 Partition coefficients of peptides onto membranes

Peptide	% Acidic lipid				
	10	15	17	20	33
Neuromodulin(37–53)	0.07 30	0.3 100	0.5 200	1 400	7 3,000
Phosphoneuromodulin(37–53)	0.01 4	0.04 20	0.06 25	0.1 40	0.5 200
MARCKS(151–175)	15 7,000	500 200,000	1,500 600,000	9,000 4,000,000	
tetra-Asp MARCKS	0.5 200	2 1,000	3.5 1,500	7 3,000	141 6,000
Phos-MARCKS(151–175)	0.07 30	0.3 100	0.4 200	0.9 400	7 3,000

The partition coefficients, K (in μm; upper number) and K_p (in M^{-1}; lower number) are defined in Eqs. 1 and 2. K and K_p are calculated from Gouy-Chapman/mass action theoretical curves (solid lines) shown in Figs. 2 and 3.

tration of free peptide, [P] as

$$\chi = K_P[P], \tag{2}$$

where $\chi = 2[P]_m/[L]$. $[P]_m$ is the concentration (M) of peptide bound to the membrane, and [L] is the total concentration (M) of lipid; the factor of 2 arises because only the lipids on the outer surface of the vesicles are exposed to peptides. Each phospholipid in a membrane occupies an area of 0.7 nm^2, so $K_P = 420K$ when K_P is in M^{-1} and K is in μm. We stress that the partition coefficients K_P and K are not true constants: they depend on the percent acidic lipid and the surface potential. When the percent bound peptide in Fig. 2 is between 10 and 90%, K_P (or K) can be calculated directly from Eqs. 1 or 2 with reasonable accuracy. For example, for a membrane with 33% acidic lipid, about 60% of neuromodulin(37–53) and 10% of the phosphorylated peptide bind to vesicles ([L] = 10^{-3} M in Fig. 2, about the concentration of lipids in a spherical cell of radius 10 μm). It follows from Eq. 2 that $K_P = 2[P]_m/[L][P] = (2)(60\%)/(10^{-3}\ M)\ (40\%) = 3 \times 10^3\ M^{-1}$ for neuromodulin(37–53) and $K_P = 2 \times 10^2\ M^{-1}$ for the phosphorylated peptide. Thus, phosphorylation reduces the affinity of neuromodulin(37–53) for these membranes about 10-fold. We use the Gouy-Chapman/mass action theory as a curve fitting procedure to estimate K_P when <10% or >90% of the peptide is bound. These numbers are given in Table 1. For example, for 17 mol% acidic lipid, K_P is again about 10-fold greater for neuromodulin(37–53) than for the phosphorylated peptide.[4]

Fig. 3 illustrates the results we obtained for the binding of MARCKS(151–175) to PC/PG vesicles (*open circles*). This peptide contains 12 lysine, 1 arginine, and no acidic residues and should thus have a charge of about +13 at neutral pH. Fig. 3 shows the same three prominent features as Fig. 2: MARCKS(151–175) does not bind to PC vesicles, binding increases sigmoidally with the percent acidic lipid in the membrane, and phosphorylation (*filled circles*) reduces the affinity of the peptide for the membrane.

A comparison of Figs. 3 and 2 illustrates two quantitative differences between the MARCKS and neuromodulin peptides. First, MARCKS(151–175) binds with higher apparent cooperativity and affinity than neuromodulin(37–53): the curve describing the MARCKS(151–175) binding (Fig. 3, *open circles*) is more sigmoidal, and the midpoint is shifted to the left when compared to the curve describing the neuromodulin(37–53) binding (Fig. 2, *open circles*). For example, vesicles containing 17% acidic lipid bind >90% of the MARCKS peptide but only 10% of the neuromodulin peptide. The theoretical curves in Figs. 2 and 3 demonstrate that the Gouy-Chapman/mass action theory can account for these differences. Because MARCKS(151–175) has a higher valence than neuromodulin(37–53), the electrostatic surface potential adjacent to the negatively charged lipid membrane concentrates the MARCKS peptide to a greater extent (McLaughlin, 1977, 1989; Honig et al., 1986; Cevc and Marsh, 1987; Cafiso et al., 1989; Shin and Hubbell, 1992). Moreover, the MARCKS peptide has more binding sites (basic residues). As we discuss in detail elsewhere (Mosior and McLaughlin, 1992a, b) these two factors increase both the apparent cooperativity and the affinity of the MARCKS peptide for membranes.

The second difference between Figs. 3 and 2 is that phosphorylation (*filled circles*) decreases the membrane affinity of MARCKS(151–175) more than it decreases that of neuromodulin(37–53). Table 1 illustrates that for vesicles containing 17% acidic lipid, phosphorylation decreases the partition coefficient (K or K_P) of MARCKS(151–175) about 1000-fold, whereas it decreases the partition coefficient of neuromodulin(37–53) only 10-fold. This difference is probably due to electrostatic effects: the phosphorylated

[4] We checked experimentally that the value of $K_P = 200\ M^{-1}$ deduced for the binding of neuromodulin(37–53) to 5:1 PC:PG vesicles (Table 1) by this approach was correct by increasing the lipid concentration [L] from 1 mM (Fig. 2) to 2, 5, and 10 mM. The percent bound peptide was determined at each [L], and the average value of K_P, as determined directly from these measurements and Eq. 2, was $175 \pm 30\ M^{-1}$, which agrees with the value of $200\ M^{-1}$ predicted from the Gouy-Chapman/mass action curve-fitting procedure.

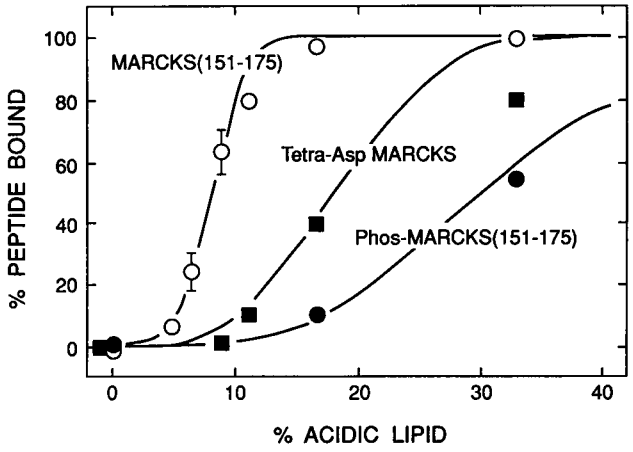

FIGURE 3 Effect of phosphorylation on the binding of MARCKS(151–175) to LUVs formed from mixtures of PC and PG. The (○) represent the binding of MARCKS(151–175) to sucrose-loaded LUVs. The (■) represent the binding data obtained with tetra-Asp MARCKS, a peptide analogous to MARCKS(151–175) with the four serines replaced by aspartic acid residues. The (●) represent the binding of phosphorylated MARCKS(151–175), i.e., peptides that had been phosphorylated by PKC to an average stoichiometry of 3.5 phosphates/peptide. Experimental conditions are as in Fig. 2. Peptide concentrations are 1 μM for MARCKS(151–175), 5 μM for tetra-Asp MARCKS and 100 nM to 3 μM for phosphorylated MARCKS(151–175). The symbols represent averages of $n \geq 4$ measurements for MARCKS(151–175), $n \geq 4$ for Tetra-Asp MARCKS, and $n \geq 11$ for phosphorylated MARCKS(151–175). The SDs for these results are shown only when they are greater than the size of the symbols. The curves represent the predictions of the Gouy-Chapman/mass action model with the microscopic intrinsic association constant of a basic residue with an acidic lipid assumed to be $k = 3\ M^{-1}$ and the number of binding sites on the peptide assumed to decrease from 13 to 7 for tetra-Asp MARCKS-(151–175) and from 13 to 4 for phosphorylated MARCKS(151–175).

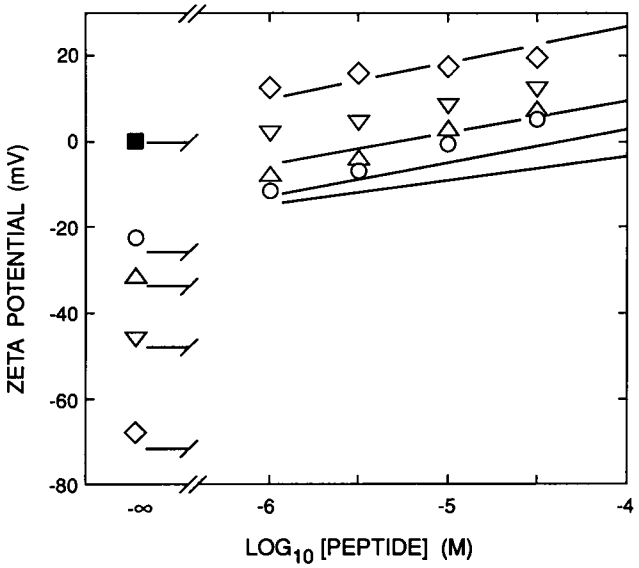

FIGURE 4 Effect of MARCKS(151–175) on the zeta potential of PS (◇), 2:1 PC/PS (▽), 5:1 PC/PS (△), 10:1 PC/PS (○), and PC (■, no effect) multilamellar vesicles. The aqueous solutions contained 0.1 M KCl buffered to pH 7 with 1 mM MOPS at 25°C. The theoretical curves represent the prediction of the Gouy-Chapman/mass action theory assuming the peptide has 13 binding sites, each with an association constant of 3 M^{-1}, the same parameters used to construct the curve in Fig. 3. The theoretical curves on the right-hand side of the figure correspond, from top to bottom, to the diamond, inverted triangle, triangle, and circle symbols, respectively.

MARCKS peptide contains 3.5 phosphates/peptide, whereas the phosphorylated neuromodulin peptide contains only 1 phosphate/peptide. An additional experiment suggests that the effect of phosphorylation is due mainly to the electrical charge (≈−2) of the phosphate group. The tetra-Asp MARCKS peptide (Fig. 3, *filled squares*), in which the four electrically neutral serines in MARCKS(151–175) are replaced by monovalent negatively charged aspartic acid residues, binds with lower affinity than MARCKS(151–175) but with greater affinity than phosphorylated MARCKS(151–175).

We made electrophoretic mobility measurements to confirm many of the results in Figs. 2 and 3. For these measurements, we used a sufficiently low concentration of multilamellar vesicles that a negligible fraction of the total [peptide] was adsorbed to the vesicles. Under our conditions, the zeta potential, to a first approximation, is related linearly to the number of adsorbed basic peptides.

Fig. 4 illustrates that MARCKS(151–175) binds strongly to PC/PS vesicles: 1 μM peptide reduces the zeta potential of the 5:1 PC/PS vesicles from about −30 to −10 mV and of the PS vesicles from about −70 to +10 mV. These results are qualitatively consistent with the predictions of the Gouy-Chapman/mass action theory used to describe the data in Fig. 3; the theoretical curves[3] in Fig. 4 were calculated with the same parameters as for MARCKS(151–175) in Fig. 3. MARCKS(151–175) has a larger effect on the zeta potential of PC/PS vesicles than does the analogous tetra-Asp peptide with four serines replaced by aspartate residues (data not shown), which agrees with the measurements illustrated in Fig. 3. The effect of neuromodulin(37–53) on the zeta potential of phospholipid vesicles are also consistent with the results illustrated in Fig. 2 (data not shown). Finally, the zeta potential results demonstrate that neuromodulin(37–53) and MARCKS(151–175) exhibit little selectivity between the monovalent acidic lipids PS and PG.[5]

Fig. 5 illustrates another independent technique we used to study the binding of MARCKS(151–175) to membranes. The peptide was synthesized with a Cys residue replacing Phe 159, then covalently labeled with the environmentally sensitive acrylodan fluorescent probe (McIlroy et al., 1991b). A large (≈10-fold) increase in acrylodan-MARCKS peptide fluorescence occurs when it binds to PC/PS vesicles; 13 μM lipid produces a half-maximal effect with 100 nM peptide (Fig. 5), and 11 μM lipid produces a half-maximal effect with 30 nM peptide (not shown). Similar effects were seen with

[5] We obtained identical results using MARCKS(151–175) with PS and PG vesicles (as well as with 5:1 PC/PG and 5:1 PC/PS vesicles), and the results were averaged in Fig. 4. Neuromodulin(37–53) has the same effect on the zeta potential of PG vesicles and PS vesicles (data not shown). The other small basic peptides that we have examined to date also exhibit little specificity between PS and PG (Kim et al., 1991; Mosior and McLaughlin, 1991, 1992a, b).

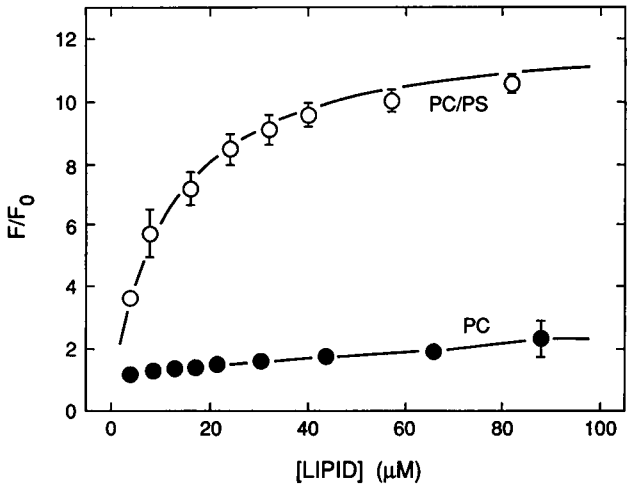

FIGURE 5 Titration of acrylodan-labeled MARCKS(151–175) with SUVs. Solutions containing 100 nM acrylodan-labeled MARCKS(151–175) were titrated with PC/PS (16% PS, ○) or PC (●) SUVs; all solutions contained 90 mM KCl, 10 mM MOPS, pH = 7. F/F_0 = Fluorescence intensity in the presence of SUVs/fluorescence intensity in the absence of SUVs. Each data point is the average of 3 titrations ± SE. Half-maximal effect for PC/PS vesicles is seen with [lipid] = 13 μM.

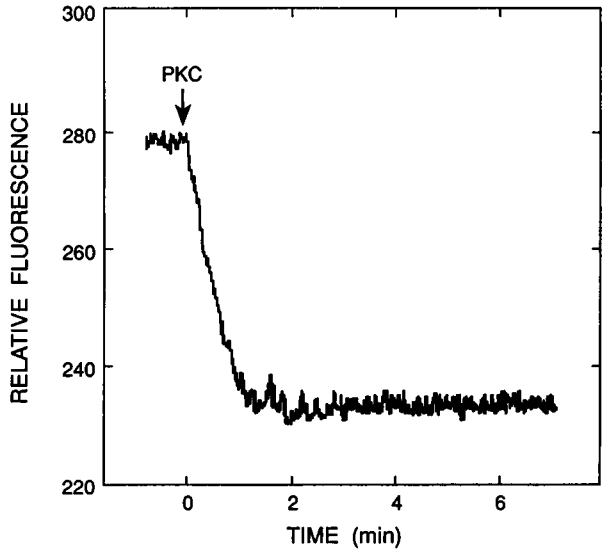

FIGURE 6 Phosphorylation of MARCKS(151–175) by PKC produces rapid desorption of the peptide from membranes. The 1-ml solution contained DPE/PS/PC SUVs (1 μM PS), 90 mM KCl, 10 mM MOPS, 0.1 mM EGTA, 0.2 mM $CaCl_2$, 5 mM $MgCl_2$, 2.5 μg diacylglycerol, and 1 mM ATP at pH = 7. Addition of 100 nM MARCKS(151–175) produced a 20% increase in the fluorescence. At the indicated time, 10 μg of PKC was added to initiate phosphorylation of MARCKS(151–175).

SUVs (Fig. 5) and LUVs (not shown). Little binding is observed to PC vesicles. These results suggest that $K_P \approx 10^5$ M^{-1} for the binding of the peptide to PC/PS vesicles containing 16% acidic lipid, a number that agrees qualitatively with the value estimated from the data in Fig. 3 using the Gouy-Chapman/mass action theory (Table 1).

Fig. 6 illustrates that phosphorylation of MARCKS(151–175) by PKC produces a rapid desorption of the peptide from lipid vesicles. The vesicles, formed from a mixture of dansylphosphatidylethanolamine, DPE (10%), PS (20%), and PC (70%), contained 30% monovalent acidic lipid. (Control zeta potential measurements showed that the peptide binds equally well to PC/DPE and PC/PS vesicles.) When MARCKS(151–175) binds to these vesicles, it produces a 20% increase in the DPE fluorescence (not shown). We then added PKC to the vesicle-peptide preparation and monitored the decrease in DPE fluorescence as a function of time. PKC induces a rapid (half-maximal effect in 30 s) decrease in the fluorescence. In the absence of ATP, PKC has no effect on the fluorescence. The simplest interpretation of Fig. 6 is that PKC binds to the vesicles and phosphorylates membrane-bound peptides, decreasing their affinity for the vesicles; the phosphorylated peptides desorb from the membranes, reducing the fluorescence. This interpretation is consistent with the results illustrated in Fig. 3 and Table 1, which show that phosphorylation decreases the membrane affinity of MARCKS(151–175).

Finally, we examined the competition between acidic lipids and CaM for MARCKS(151–175). We first mixed 0.6 μM acrylodan-labeled MARCKS peptide and sucrose-loaded vesicles (1 mM total lipid) together in 100 mM KCl, 1 mM MOPS, pH 7, centrifuged the vesicles, and measured the fluorescence signals from ethanol suspensions of the supernatant and precipitate to calculate the fraction of peptide bound to the vesicles. Vesicles containing either 10 or 33% acidic lipid bound most of the fluorescent peptide, as expected from the data illustrated in Fig. 3. We then added CaM (2 μM) and Ca^{2+} (50 μM) to the solutions and repeated the experiment. A simple analysis[6] predicts that most of the peptide should move off vesicles containing 10% acidic lipid and become bound to Ca-CaM, whereas most should remain bound to vesicles containing 33% acidic lipid. Our experimental results agree with these predictions. Most of the peptide is bound to the vesicles containing 10% PG in the absence of CaM (80 ± 3%, n = 3), or when CaM (2 μM) is present with 1 mM EDTA (75 ± 3%, n = 2). Addition of CaM (2 μM) + Ca^{2+} (50 μM) decreases markedly the percent of peptide bound to these vesicles (to 11 ± 3%, n = 3). Addition of CaM + Ca^{2+} to the solution with vesicles containing 33% PG produced no significant decrease in the fraction of peptide bound to the vesicles.

[6] We describe the binding of CaM to peptide, P, with a conventional mass action expression: [P-CaM] = K_1[CaM][P], where [P-CaM] is the concentration of the peptide-CaM complex. The concentration of peptide bound to lipid can be calculated from Eq. 2: [P-L] = K_P[L][P]/2. From these two equations we obtain: [P-L]/[P-CaM] = K_P[L]/(2K_1[CaM]). Our [L] = 10^{-3} M corresponds to a spherical cell of radius 10 μm. For vesicles with 10% acidic lipid $K_P = 7 \times 10^3$ M^{-1} (Table 1). $K_1 = 4 \times 10^8$ M^{-1} in the presence of Ca^{2+} (McIlroy et al., 1991a) and [CaM] ≃ 2×10^{-6} M, so the ratio of lipid-bound peptide to CaM-bound peptide should be ≃ 1/100. Thus, most of the peptide should be bound to CaM. Conversely, when the vesicles contain 33% acidic lipid, $K_P > 4 \times 10^6$ (Table 1) and most of the peptide should be bound to the vesicles rather than CaM.

DISCUSSION

Fig. 1 illustrates the hypothesis that the clusters of basic residues in neuromodulin[1] and the MARCKS protein bind not only to CaM, but also to acidic lipids in the plasma membrane, as suggested previously by Houbre et al. (1991) for neuromodulin and by Taniguchi and Manenti (1993) for MARCKS. We studied the membrane binding of peptides corresponding to these CaM-binding domains to test this hypothesis. Our results demonstrate that the peptides neuromodulin(37–53) and MARCKS(151–175) bind strongly to membranes containing acidic lipids and that phosphorylation of these peptides reduces this binding. For example, phosphorylation produces about a 1000-fold reduction in the affinity of MARCKS(151–175) for membranes containing 15% acidic lipids (Fig. 3 and Table 1).

Could the interaction between peptides and phospholipid vesicles documented in Figs. 3–6 for MARCKS(151–175) occur to an appreciable extent between this region of the native protein and the plasma membrane in cells, where CaM and other proteins such as actin (Aderem, 1992; Hartwig et al., 1992) can compete with lipids for the CaM-binding domains? Although the question cannot be answered with certainty from the available data, a simple theoretical model developed in the Appendix allows us to make a prediction. In this model, we consider both the tethering of MARCKS to the membrane via the myristoyl chain and the competition between CaM and acidic lipids for the cluster of basic residues on MARCKS. The model and our data predict that acidic lipids in the membrane could compete successfully with CaM (and actin) for this cluster of basic residues in MARCKS.

Although MARCKS is the major substrate for PKC in many cells, its function remains unclear: it could act as a buffer for Ca-CaM (Blackshear, 1993) or be involved in the reversible interaction of cytoskeletal elements with the plasma membrane (Aderem, 1992). Although answers to these questions obviously require experiments with the intact protein, we argue that a reductionist approach to the interaction of myristoylated proteins with membranes is worth exploring. If we know how strongly the myristate moiety and the cluster of basic residues interact with membranes, can we predict the membrane-binding behavior of the intact protein?

As noted in the Introduction, MARCKS moves reversibly off the plasma membrane in many (Thelen et al., 1992; Wang et al., 1989; Sawai et al., 1993), but not all, cells (James and Olsen, 1989) when phosphorylated. Our working hypothesis is that both the hydrophobic energy obtained from the insertion of the myristoyl chain into the membrane and the electrostatic energy obtained from the binding of the basic residues to acidic lipids are required for the stable binding of MARCKS to the plasma membrane. The maximum hydrophobic energy obtained from myristate penetration corresponds to a partition coefficient (defined in analogy with Eq. 2 above) of 10^4 M^{-1} (Peitzsch and McLaughlin, 1993). Let K_H be the hydrophobic partition coefficient of the myristate chain on MARCKS into a bilayer. The intact MARCKS protein binds to PC vesicles with a $K_H = 3 \times 10^3$ M^{-1} (J. Kim, T. Shishido, A. Aderem, S. McLaughlin, unpublished data). The work reported here (Table 1) shows that the electrostatic energy corresponds to a partition coefficient of $K_P = 10^4$ M^{-1} ($K = 15$ μm) if the membrane contains 10% acidic lipids, a minimal estimate for a plasma membrane. According to a simple model developed in the Appendix (see Eq. A7), neither hydrophobicity nor electrostatics alone can provide a strong anchoring of MARCKS to the membrane, but together they can accomplish the task. Phosphorylation reduces the partition coefficient of the MARCKS(151–175) peptide onto a membrane with 10% acidic lipid more than 100-fold (Fig. 3, Table 1): this result can explain the observation that phosphorylation of the MARCKS protein produces a translocation from membrane to solution with phospholipid vesicles containing acidic lipids (Taniguchi and Manenti, 1993).

Myristoylated proteins other than MARCKS also appear to utilize clusters of basic residues to anchor themselves to plasma membranes. For example, the first 17 residues of $pp60^{src}$ contain 1 acidic and 6 basic residues. Experiments with both the native $pp60^{src}$ protein and a myristoylated peptide that mimics the amino terminal region of this protein demonstrate that both protein and peptide bind about 1000-fold more strongly to membranes containing 30% acidic lipid than they do to membranes containing only the zwitterionic lipid phosphatidylcholine (Buser et al., 1994). This result, when combined with measurements of the binding of the nonmyristoylated peptide, agrees satisfactorily with the prediction of Eq. A7 (Buser et al., 1994).

In summary, biophysical experiments with the component parts of complicated myristoylated proteins such as MARCKS and $pp60^{src}$ provide information that can be used to test theoretical models that predict how strongly these proteins bind to phospholipid vesicles and biological membranes.

APPENDIX

Equations that describe the model in Fig. 1.

In a cell with a free concentration of CaM = $[CaM]_{free}$ and a free concentration of basic residues on the MARCKS protein (the cluster of basic residues is bound neither to lipids nor to CaM) = $[M]_{free}$, we can describe the binding of CaM to MARCKS with a conventional mass action expression

$$K_1[CaM]_{free}[M]_{free} = [M\text{-}CaM], \quad (A1)$$

where [M-CaM] is the concentration of the MARCKS-CaM complex in the cell. The reaction between CaM and the phosphorylated MARCKS protein, M*, can be described in a similar manner as

$$K_4[CaM]_{free}[M^*]_{free} = [M^*\text{-}CaM]. \quad (A2)$$

Although CaM is free to move within the entire volume of the cell, the CaM-binding domain, residues 151–175 of MARCKS, is tethered to the plasma membrane (Fig. 1). We represent the region of the protein between residues 1 and 150 as a dimensionless, flexible, electrically neutral string of length r attached at one end to myristoyl chain inserted into the membrane and at the other end to the the CaM-binding region, M. We ignore the dimensions of M. As $r \ll R$, the radius of the cell, the CaM-binding region M (and the phosphorylated region M*) is confined within a hemisphere of volume $v = (2/3)\pi r^3$ rather than the cell volume. We assume the concentration of M is constant within this hemisphere. The complexes between

CaM and M (or M*) also are confined within these hemispheres, and these volumes cancel from Eq. A1 (or Eq. A2), where all concentrations are expressed in terms of the cell volume.

When we calculate an expression for the binding of M (or M*) to the surface of the membrane, however, we must consider that the membrane is exposed to a higher concentration of M than if M were free in the cell. Equation 1 is written as a partition coefficient onto the surface for a peptide; we can write an analogous equation for the CaM-binding region of MARCKS, M, where M-L denotes a complex between M and the acidic lipids in the plasma membrane:

$$K_2[M]^t_{free} = \{M\text{-}L\}^t. \quad (A3)$$

The superscript t indicates that M is tethered in a volume $v = (2/3)\pi r^3$ and can bind to an area of membrane $a = \pi r^2$. Multiplying both sides of Eq. A3 by the volume of the hemisphere, v, and the area of membrane enclosed by this hemisphere, a, and dividing by the cell volume, V, we obtain

$$K_2[M]_{free} = [M\text{-}L](v/a), \quad (A4)$$

where $v/a = 2r/3$ and the concentrations in Eq. A4 are expressed in terms of the cell volume. (Alternatively, we could consider the basic clusters, M, confined to, and uniformly distributed within, a spherical shell of volume $4\pi R^2 r$. In this case $v/a = r$.) Crothers and Metzger (1972) give a more extensive discussion of this problem.

We obtain a similar relation between the concentration of the phosphorylated CaM-binding domain that is bound neither to CaM nor to lipids, $[M^*]_{free}$, and the concentration of M* bound to acidic lipids in the membrane, [M*-L]:

$$K_3[M^*]_{free} = [M^*\text{-}L](v/a). \quad (A5)$$

Both acidic lipids in the membrane and calmodulin can bind to the calmodulin binding domain M. Which wins in this simple model? In other words, what is the ratio of [M-L], and [M-CaM], where both concentrations are expressed in terms of the cell volume. By combining Eqs. A1 and A4 and taking $v/a = 2r/3$ we obtain

$$[M\text{-}L]/[M\text{-}CaM] = 3K_2/(2K_1 r[CaM]_{free}). \quad (A6)$$

To estimate this ratio, we must assume a value for the length of the 150 residue "string" attaching M to the membrane: $r = 10$–50 nm is a reasonable guess. The value of K_1 is 4×10^8 M^{-1} when the $[Ca^{2+}]$ is high, but is much lower at the concentrations of calcium found in a quiescent cell (McIlroy et al., 1991a). The value of $[CaM]_{free}$ is probably of order 1 μM. The value of K_2 for the native MARCKS protein is probably significantly lower than the value listed in Table 1 for the MARCKS(151–175) peptide because there are many negatively charged acidic residues adjacent to the basic region that spans residues 151–175. Furthermore, there is an uncertainty of two orders of magnitude about the value of K_2 because the percent free acidic lipid in a plasma membrane could be as low as 10% or as high as 20% (Op den Kamp, 1979), and the value of K_2 depends steeply on this parameter (Fig. 3). Thus, we can only state that Eq. A6 predicts the CaM-binding domain of MARCKS should be bound to lipids rather than CaM when the $[Ca^{2+}]$ in the cell is low. The main advantage of this analysis is that Eq. A6 can be used to estimate the value of K_2 from competition experiments with CaM using the intact MARCKS protein and phospholipid vesicles of defined composition.

The flexible string model also allows us to calculate an expression for the overall partition coefficient of a myristoylated peptide or protein onto a phospholipid vesicle, K' (M^{-1}). Let K_H be the hydrophobic partition coefficient of the myristate onto the membrane. $K_H = 10^4$ M^{-1} for small myristoylated peptides (Peitzsch and McLaughlin, 1993), and $K_H = 3 \times 10^3$ M^{-1} for the intact MARCKS protein (J. Kim, T. Shishido, A. Aderem, S. McLaughlin, unpublished data). If r is the length of the electrically neutral, flexible string between the myristate and the cluster of basic residues, and K_2 (defined in units of length as in Eq. 1) is the partition coefficient of the cluster of basic residues onto the membrane, then it is easy to show that

$$K' \simeq K_H(1 + 3K_2/2r). \quad (A7)$$

By measuring how the value of K', the partition coefficient of the MARCKS protein onto phospholipid vesicles, depends on the fraction of acidic lipids in the vesicles, one can deduce values of K_2 for the intact protein and compare these values with the values of K shown in Table 1 for the MARCKS-(151–175) peptide.

We thank Dr. Richard Epand for a generous gift of Protein kinase C and Drs. Alan Aderem and Marian Mosior for many helpful discussions.
This work was supported by National Institutes of Health Grant GM24971 and National Science Foundation Grant DMB-9117526 (to S. McLaughlin) and by National Institutes of Health Grant DK33727 (to J. D. Johnson). P. J. Blackshear was an Investigator of the Howard Hughes Medical Institute.

REFERENCES

Aderem, A. 1992. The MARCKS brothers: a family of protein kinase C substrates. *Cell.* 71:713–716.

Albert, K. A., A. C. Nairn, and P. Greengard. 1987. The 87-kDa protein, a major specific substrate for protein kinase C: purification from bovine brain, and characterization. *Proc. Natl. Acad. Sci. USA.* 84:7046–7050.

Alexander, K. A., B. M. Cimler, K. E. Meier, and D. A. Storm. 1987. Regulation of calmodulin binding to P-57. *J. Biol. Chem.* 262:6108–6113.

Alexander, K. A., B. T. Wakim, G. S. Doyle, K. A. Walsh, and D. A. Storm. 1988. Identification, and characterization of the calmodulin-binding domain of neuromodulin, a neurospecific calmodulin-binding protein. *J. Biol. Chem.* 263:7544–7549.

Apel, E. D., M. F. Byford, D. Au, K. A. Walsh, and D. R. Storm. 1990. Identification of the protein kinase C phosphorylation site in neuromodulin. *Biochemistry.* 29:2330–2335.

Bangham, A. D., M. W. Hill, and N. G. A. Miller. 1974. Preparation and use of liposomes as models of biological membranes. *Methods Membr. Biol.* 1:1–68.

Baudier, J., C. Bronner, D. Kligman, and R. D. Cole. 1989. Protein kinase C substrates from bovine brain. *J. Biol. Chem.* 264:1824–1828.

Bell, R. M., and D. J. Burns. 1991. Lipid activation of protein kinase C. *J. Biol. Chem.* 266:4661–4664.

Berridge, M. 1993. Inositol trisphosphate and calcium signalling. *Nature.* 361:315–325.

Blackshear, P. J. 1993. The MARCKS family of cellular protein kinase C substrates. *J. Biol. Chem.* 268:1501–1504.

Buser, C., C. Sigal, M. Strzelczyk, L. Mooney, M. D. Resh, and S. McLaughlin. 1994. Membrane binding of myristoylated peptides corresponding to the NH_2-terminal region of $pp60^{v\text{-}src}$. *Biophys. J.* 66:60*a*. (Abstr.)

Cafiso, D., A. McLaughlin, S. McLaughlin, and A. Winiski. 1989. Measuring electrostatic potentials adjacent to membranes. *Methods Enzymol.* 171:342–364.

Chakravarthy, B. R., A. Bussey, J.-F. Whitfield, M. Sikorska, R. E. Williams, and J. P. Durkin. 1991. The direct measurement of protein kinase C (PKC) activity in isolated membranes using a selective peptide substrate. *Anal. Biochem.* 196:144–150.

Chapman, E. R., D. Au, K. A. Alexander, T. A. Nicolson, and D. R. Storm. 1991. Characterization of the calmodulin binding domain of neuromodulin. *J. Biol. Chem.* 266:207–213.

Cevc, G., and D. Marsh. 1987. Phospholipid Bilayers. Wiley & Sons, New York. 442 pp.

Cimler, B. M., T. J. Andreasen, K. I. Andreasen, and D. R. Storm. 1985. P-57 is a neural specific calmodulin-binding protein. *J. Biol. Chem.* 260: 10784–10788.

Coburn, C., J. Eisenberg, M. Eisenberg, S. Mclaughlin, and L. Runnels. 1993. Calculations of electrostatic potentials adjacent to membranes and peptides that mimic the calmodulin-binding domains of neuromodulin and the MARCKS protein. *Biophys. J.* 64:60*a*. (Abstr.)

Coggins, P. J., and H. Zwiers. 1991. B-50(GAP-43): biochemistry and functional neurochemistry of a neuron-specific phosphoprotein. *J. Neurochem.* 56:1095–1106.

Crothers, D. M., and H. Metzger. 1972. The influence of polyvalency on the binding properties of antibodies. *Immunochemistry.* 9:341–357.

de Kruijff, B., A. Rietveld, N. Telders, and B. Vaandrager. 1985. Molecular aspects of the bilayer stabilization induced by poly(L-lysines) of varying size in cardiolipid liposomes. *Biochem. Biophys. Acta.* 820:295–304.

Dempsey, C. E. 1990. The actions of melittin on membranes. *Biochim. Biophys. Acta.* 1031:143–161.

Epand, R. M., A. R. Stafford, R. Bottega, and E. H. Ball. 1989. Studies on the mechanism of action of a bilayer stabilizing inhibitor of protein kinase C: cholesterylphosphoryldimethylethanolamine. *Biosci. Rep.* 9:315–328.

Estep, R. P., K. A. Alexander, and D. R. Storm. 1990. Regulation of free calmodulin levels in neurons by neuromodulin: relationship to neuronal growth, and regeneration. *Curr. Top. Cell. Reg.* 30:161–180.

Gawrisch, K., K.-H. Han, J.-S. Yang, L. D. Bergelson, and A. Ferretti. 1993. Interaction of peptide fragment 828–848 of the envelope glycoprotein of human immunodeficiency virus type I with lipid bilayers. *Biochemistry.* 32:3112–3118.

George, D. J., and P. J. Blackshear. 1992. Membrane association of the myristoylated alanine-rich C kinase substrate (MARCKS) protein appears to involve myristate-dependent binding in the absence of a myristoyl protein receptor. *J. Biol. Chem.* 267:24879–24885.

Graff, J. M., J. I. Gordon, and P. J. Blackshear. 1989a. Myristoylated and nonmyristoylated forms of a protein are phosphorylated by protein kinase C. *Science.* 246:503–506.

Graff, J. M., R. R. Rajan, R. R. Randall, A. C. Nairn, and P. J. Blackshear. 1991. Protein kinase C substrate, and inhibitor characteristics of peptides derived from the myristoylated alanine-rich C kinase substrate (MARCKS) protein phosphorylation site domain. *J. Biol. Chem.* 266: 14390–14398.

Graff, J. M., D. J. Stumpo, and P. Blackshear. 1989b. Characterization of the phosphorylation sites in the chicken, and bovine myristoylated alanine-rich C kinase substrate protein, a prominent cellular substrate for protein kinase C. *J. Biol. Chem.* 264:11912–11919.

Graff, J. M., T. N. Young, J. D. Johnson, and P. Blackshear. 1989c. Phosphorylation-regulated calmodulin binding to a prominent cellular substrate for protein kinase C. *J. Biol. Chem.* 264:21818–21823.

Hartwig, J. H., M. Thelen, A. Rosen, P. A. Janmey, A. C. Nairn, and A. Aderem. 1992. MARCKS is an actin filament crosslinking protein regulated by protein kinase C, and calcium-calmodulin. *Nature.* 356: 618–622.

Honig, B. H., W. L. Hubbell, and R. F. Flewelling. 1986. Electrostastic interactions in membranes, and proteins. *Annu. Rev. Biophys. Biophys. Chem.* 15:163–193.

Hope, M. J., M. B. Bally, G. Webb, and P. R. Cullis. 1985. Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume, and ability to maintain a membrane potential. *Biochim. Biophys. Acta.* 812:55–65.

Houbre, D., G. Duportail, J.-C. Deloume, and J. Baudier. 1991. The interactions of the brain-specific calmodulin-binding protein kinase C substrate, neuromodulin (GAP-43), with membrane phospholipids. *J. Biol. Chem.* 266:7121–7131.

James, G., and E. N. Olson. 1989. Myristoylation, phosphorylation, and subcellular distribution of the 80-kDa protein kinase C substrate in BC_3H1 myocytes. *J. Biol. Chem.* 264:20928–20933.

Kaibuchi, K., Y. Takai, and Y. Nishizuka. 1981. Cooperative roles of various membrane phospholipids in the activation of calcium-activated, phospholipid-dependent protein kinase. *J. Biol. Chem.* 256: 7146–7149.

Kim, J., M. Mosior, L. A. Chung, H. Wu, and S. McLaughlin. 1991. Binding of peptides with basic residues to membranes containing acidic phospholipids. *Biophys. J.* 60:135–148.

Klee, C. B., and T. C. Vanaman. 1982. Calmodulin. *Adv. Prot. Chem.* 35: 213–321.

Kuchinka, E., and J. Seelig. 1989. Interaction of melittin with phosphatidylcholine membranes. Binding isotherm, and lipid head-group conformation. *Biochemistry.* 28:4216–4221.

LaBate, M. E., and J. H. P. Skene. 1989. Selective conservation of GAP-43 structure in vertebrate evolution. *Neuron.* 3:299–310.

Liu, Y. C., and D. R. Storm. 1989. Dephosphorylation of neuromodulin by calcineurin. *J. Biol. Chem.* 264:12800–12804.

Liu, Y. C., and D. R. Storm. 1990. Regulation of free calmodulin levels by neuromodulin: neuron growth, and regeneration. *Trends Pharmacol. Sci.* 11:107–111.

MacNichol, M., and H. Schulman. 1992. Cross-talk between protein kinase C and multifunctional Ca^{2+}/calmodulin-dependent protein kinase. *J. Biol. Chem.* 267:12197–12201.

Mangels, L. A., and M. E. Gnegy. 1992. Carbachol stimulates binding of a photoreactive calmodulin derivative to calmodulin-binding proteins in intact SK-N-SH human neuroblastoma cells. *J. Biol. Chem.* 267: 5847–5854.

Maulet, Y., and J. A. Cox. 1983. Structural changes in melittin and calmodulin upon complex formation, and their modulation by calcium. *Biochemistry.* 22:5680–5686.

McIlroy, B. K., J. D. Walters, P. Blackshear, and J. D. Johnson. 1991a. Phosphorylation-dependent binding of a synthetic MARCKS peptide to calmodulin. *J. Biol. Chem.* 266:4959–4964.

McIlroy, B. K., J. D. Walters, and J. D. Johnson. 1991b. A continuous fluorescence assay for protein kinase C. *Anal. Biochem.* 195:148–152.

McLaughlin, S. 1977. Electrostatic potentials at membrane-solution interface. *Curr. Top. Membr. Transp.* 9:71–144.

McLaughlin, S. 1989. The electrostatic properties of membranes. *Annu. Rev. Biophys. Biophys. Chem.* 18:113–136.

Montich, G., S. Scarlata, S. McLaughlin, R. Lehrmann, and J. Seelig. 1993. Thermodynamic characterization of the association of small basic peptides with membranes containing acidic lipids. *Biochem. Biophys. Acta.* 1146:17–24.

Mosior, M., and R. Epand. 1993. Mechanism of activation of protein kinase C: Roles of diolein, and phosphatidylserine. *Biochemistry.* 32:66–75.

Mosior, M., and S. McLaughlin. 1991. Peptides that mimic the pseudosubstrate region of protein kinase C bind to acidic lipids in membranes. *Biophys. J.* 60:149–159.

Mosior, M., and S. McLaughlin. 1992a. Electrostatics and reduction of dimensionality produce apparent cooperativity when basic peptides bind to acidic lipids in membranes. *Biochim. Biophys. Acta.* 1105:185–187.

Mosior, M., and S. McLaughlin. 1992b. Binding of basic peptides to acidic lipids in membranes: effects of inserting alanine(s) between the basic residues. *Biochemistry.* 31:1767–1773.

Mosior, M., and S. McLaughlin. 1992c. Electrostatics and dimensionality can produce apparent cooperativity when protein kinase C and its substrates bind to acidic lipids in membranes. *In* Protein Kinase C, Current Concepts and Future Perspectives. D. S. Lester and R. M. Epand, editors. Ellis Horwood Limited, Chichester, England. 157–180.

Newton, A. C. 1993. Interaction of proteins with lipid headgroups: lessons from protein kinase C. *Annu. Rev. Biophys. Biomol. Struct.* 22:1–25.

Newton, A. C., and D. E. Koshland. 1989. High cooperativity, specificity, and multiplicity in the protein kinase C-lipid interaction. *J. Biol. Chem.* 264:14909–14915.

Nielander, H. B., L. H. Schrama, A. J. van Rozen, M. Kasperaitis, A. B. Oestreicher, W. H. Gispen, and P. Schotman. 1990. Mutation of serine 41 in the neuron-specific protein B-50 (GAP-43) prohibits phosphorylation by protein kinase C. *J. Neurochem.* 55:1442–1445.

Op den Kamp, J. A. F. 1979. Lipid asymmetry in membranes. *Annu. Rev. Biochem.* 48:47–71.

Peitzsch, R. M., and S. McLaughlin. 1993. Binding of acylated peptides and fatty acids to phospholipid vesicles: pertinence to myristoylated proteins. *Biochemistry.* 32:10436–10443.

Rebecchi, M., A. Peterson, and S. McLaughlin. 1992. Phosphoinositide-specific phospholipase C-δ_1 binds with high affinity to phospholipid vesicles containing phosphatidylinositol 4,5-bisphosphate. *Biochemistry.* 31:12742–12747.

Sawai, T., M. Negishi, N. Nishigaki, T. Ohno, and A. Ichikawa. 1993. Enhancement by protein kinase C of prostacyclin receptor-mediated activation of adenylate cyclase through a calmodulin/myristoylated alanine-rich C kinase substrate (MARCKS) system in IC2 mast cells. *J. Biol. Chem.* 268:1995–2000.

Shin, Y.-K., and W. L. Hubbell. 1992. Determination of electrostatic potentials at biological interfaces using electron-electron double resonance. *Biophys. J.* 61:1443–1453.

Skene, J. H. P. 1989. Axonal growth-associated proteins. *Annu. Rev. Neurosci.* 12:127–156.

Skene, J. H. P. 1990. GAP-43 as a "calmodulin sponge" and some implications for calcium signalling in axon terminals. *Neurosci. Res. Suppl.* 13:S112–S125.

Skene, J. H. P., and I. Virág. 1989. Posttranslational membrane attachment and dynamic fatty acylation of a neuronal growth cone protein, GAP-43. *J. Cell Biol.* 108:613–624.

Stumpo, D. J., J. M. Graff, K. A. Albert, P. Greengard, and P. J. Blackshear. 1989. Molecular cloning, characterization, and expression of a cDNA encoding the "80- to 87-kDa" myristoylated alanine-rich C kinase substrate: a major cellular substrate for protein kinase C. *Proc. Natl. Acad. Sci. USA.* 86:4012–4016.

Taniguchi, H., and S. Manenti. 1993. Interaction of myristoylated alanine-rich protein kinase C substrate (MARCKS) with membrane phospholipids. *J. Biol. Chem.* 268:9960–9963.

Thelen, M., A. Rosen, A. C. Nairn, and A. Aderem. 1991. Regulation by phosphorylation of reversible association of a myristoylated protein kinase C substrate with the plasma membrane. *Nature.* 351:320–322.

Wakim, B. T., K. A. Alexander, H. R. Masure, B. M. Cimler, D. R. Storm, and K. A. Walsh. 1987. Amino acid sequence of P-57, a neurospecific calmodulin-binding protein. *Biochemistry.* 26:7466–7430.

Wang, J. K. T., S. I. Walaas, T. A. Sihra, A. Aderem, and P. Greengard. 1989. Phosphorylation, and associated translocation of the 87-kDa protein, a major protein kinase C substrate, in isolated nerve terminals. *Proc. Natl. Acad. Sci. USA.* 86:2253–2256.

Weigele, M., S. D. DeBernarde, J. P. Tengi, and W. Leimgruber. 1972. A novel reagent for the fluorometric assay of primary amines. *J. Am. Chem. Soc.* 94:5927–5928.