Membrane Domains Containing Phosphatidylserine and Substrate Can Be Important for the Activation of Protein Kinase C†

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ABSTRACT: The relationship between lipid domains and enzyme activity was studied *via* the direct visualization and quantitation of domains by fluorescence digital imaging microscopy. The substrate used in these experiments was a basic peptide derived from a prominent cellular substrate (MARCKS) of protein kinase C. The MARCKS peptide and phosphatidylserine, which were labeled by two different fluorophores, colocalized into domains in large vesicles (5−10 μm). Increasing the ionic strength disrupted the domains of the MARCKS peptide and phosphatidylserine, and this was accompanied by a decrease in protein kinase C activity. Dansylpolylysine, which inhibits protein kinase C, was similar to the MARCKS peptide in forming domains enriched in phosphatidylserine. The degree of enrichment of the MARCKS peptide in the phosphatidylserine domains decreased proportionally with protein kinase C activity when polylysine was added. Polylysine caused the MARCKS peptide to be displaced from the domains into the nondomain areas of the vesicles. This suggested that binding of the substrate to the vesicles was not the critical factor for protein kinase C activity, but rather it was the organization of the substrate into domains that was related to the activation of the enzyme. Gramicidin, which was chosen to represent a neutral membrane protein, was excluded from the domains with phosphatidylserine, and it had no effect on the enrichment of the domains or the enzyme activity. The results of this study show that the formation of membrane domains can be important for the activation of protein kinase C and the activity can be inhibited by disrupting the domains.

Most biological membranes are not uniform and have a heterogeneous distribution of lipids and proteins (Glaser, 1992, 1993). Lipid domains can exist in a variety of different forms and be caused by different mechanisms. A variety of basic proteins or peptides bind to acidic phospholipids, and some cause the formation of domains or effect phase separations [*e.g.*, Birrell and Griffith (1976), Boggs et al. (1977), Hartmann et al. (1977), Dufourcq and Faucon (1978), Mustonen et al. (1985), Laroche et al. (1988), Haverstick and Glaser (1989), Desormeaux et al. (1992), Gawrisch et al. (1993), and Wang et al. (1993)]. The concentration of certain components into domains or the partitioning of components either into or out of specific domains can have numerous functional consequences (Vaz, 1992). Clustering of phospholipids appears to be important in activating protein kinase C (PKC)[1] (Bazzi & Nelsestuen, 1987a; Newton & Koshland, 1989), and this enzyme may provide a good example of how membrane domains may function in regulating enzyme activity. Fluorescence digital imaging microscopy, used in this study, provides a method for the direct visualization and quantitation of membrane components in domains (Rodgers & Glaser, 1993a,b; Luan & Glaser, 1994). However, no information has been reported about the functional role of domains visualized by this technique.

PKC has attracted considerable attention for its critical role in signal transduction pathways within cells as well as for its activation by lipids. Receptor-mediated, extracellular stimuli can cause the breakdown of phosphoinositides to inositol 1,4,5-triphosphate and DAG. The second messenger, inositol triphosphate, leads to increased levels of cytoplasmic Ca^{2+}. The increased Ca^{2+} and DAG in turn serve as activators of PKC (Bell & Burns, 1991). The full activation of Ca^{2+}-dependent isoforms of PKC requires Ca^{2+}, DAG, and PS (Kikkawa et al., 1982; Hannun et al., 1985, 1990; Boni & Rando, 1985; Bell et al., 1986; Newton & Koshland, 1989; Bazzi & Nelsestuen, 1990; Farago & Nishizuka, 1990; Newton, 1993). The details of the PKC−membrane interaction as well as of the exact mechanism of PKC activation still are not well understood.

PKC substrates have been classed into three categories according to their different requirements for phosphorylation by PKC (Bazzi & Nelsestuen, 1987b). Substrates in the first category, such as protamine, require no additional factors for phosphorylation by PKC. Substrates in the second category, such as myelin basic protein, require phospholipids for phosphorylation. And substrates in the third category, which include histone, myelin light protein, and troponin I, require the presence of both Ca^{2+} and phospholipids to be phosphorylated by PKC. The variable requirements of the substrates are thought to indicate their differing interactions with phospholipids and the enzyme. This study uses the

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[1] Abbreviations: acrylodan, 6-acryloyl-2-(dimethylamino)naphthalene; DAG, *sn*-1, 2-dioleoylglycerol; dansyl, 5-(dimethylamino)-naphthalene-1-sulfonyl; DOPS, dioleoylphosphatidylserine; MARCKS, myristoylated alanine-rich C kinase substrate; Mops, 3-(*N*-morpholino)-propanesulfonic acid; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; NBD-PC, 1-acyl-2-[6-(*N*-NBD-amino)caproyl]phosphatidylcholine; NBD-PS, 1-acyl-2-[6-(*N*-NBD-amino)caproyl]phosphatidylserine; PC, phosphatidylcholine; PKC, protein kinase C; Tris, tris(hydroxymethyl)aminomethane.

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MARCKS peptide, which is derived from a prominent cellular substrate of PKC and is frequently used in studies of the enzyme. The MARCKS peptide falls in the third category of PKC substrates, according to the report of McIlroy et al. (1991). The experimental results reported in this paper illustrate the role of membrane domains in activating and regulating the activity of PKC.

MATERIALS AND METHODS

Purification of Protein Kinase C. The purification of PKC was carried out by the procedure described by Kitano et al. (1986) except that a threonine−Sepharose column (Sigma Chemical Co., St. Louis, MO) was used instead of a threonine−agarose column. One bovine brain yielded 50−150 μg of PKC, and the enzyme gave a single band on sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

Synthesis and Labeling of the MARCKS Peptide. The 25 amino acid MARCKS peptide (CKKKKRFSFKKSFKLSGFSFKKNKK) containing the PKC phosphorylation sites of the MARCKS protein was synthesized by the Biotechnology Center Genetic Engineering Facility at the University of Illinois and was labeled with acrylodan on the single cysteine residue in the peptide (McIlroy et al., 1991). The labeling ratio was 0.97 mol of acrylodan/mol of MARCKS peptide determined by the acrylodan absorbance at 340 nm and the Coomassie protein assay (Pierce Chemical Co., Rockford, IL).

Protein Kinase C Assay. The activity of PKC was assayed by the procedure of McIlroy et al. (1991). This assay measures the change in fluorescence intensity of the acrylodan-labeled MARCKS peptide induced by PKC phosphorylation. The changes in fluorescence intensity during phosphorylation of the acrylodan-MARCKS peptide were monitored with an SLM 8000 fluorometer in the slow time mode using an emission wavelength of 480 nm and an excitation wavelength of 370 nm. The assay was carried out in 1 mL containing 10 mM Mops, pH 7.0, 3 mM $MgCl_2$, 0.3 mM $CaCl_2$, 0.1 mM EGTA, 0.1 mM ATP, 35 μM lipid vesicles (molar ratio egg PC:DOPS:DAG = 85:10:5), 0.1 μM acrylodan-MARCKS peptide, and 0.5 μg of pure PKC. Polylysine (average molecular weight either 4500 or 80 000) and gramicidin were purchased from the Sigma Chemical Co. (St. Louis, MO) and were added to the complete PKC assay mixture at the desired concentration 5 min before the reaction was initiated with PKC. When NaCl was added, it also was added to the complete assay mixture 5 min before the reaction was initiated with PKC.

Preparation of Lipid Vesicles. NBD-PC, egg PC, DOPS, and DAG were obtained from Avanti Polar Lipids (Alabaster, AL). NBD-PS was synthesized from NBD-PC by transphosphatidylation using cabbage phospholipase D (Yang et al., 1967; Comfurius et al., 1977), and the product was purified by lipid extraction and thin-layer chromatography (Haverstick & Glaser, 1987). Large unilamellar vesicles for microscopy were prepared according to the procedure of Haverstick and Glaser (1987). The ratio of all components was the same as that used in the PKC activity assay. One slide contained 10 μL of solution with 35 μM lipid vesicles (molar ratio egg PC:DAG:DOPS:NBD-PS = 85:5:9.5:0.5), 0.1 μM acrylodan-MARCKS peptide, and either 20 μM polylysine, 20 μM gramicidin, or 140 mM NaCl. In addition, the solution contained 0.1% agarose in order to immobilize the vesicles. The slide was maintained for 5 min at room temperature before it was viewed under the microscope.

Fluorescent Labeling of Gramicidin and Polylysine. Gramicidin was fluorescently labeled with dansyl chloride using the procedure described by Haverstick and Glaser (1989). Polylysine was labeled with the same probe by a method similar to that used by Gilmore and Glaser (1982). Specifically, 3.1 μmol of dansyl chloride in methanol and 18 μmol of sodium deoxycholate in acetone were evaporated under a stream of nitrogen to obtain a thin film. The detergent−dansyl chloride mixture was dispersed by addition of 1.5 mL of 0.1 M $NaHCO_3$, pH 9.0, and vortexing. Polylysine (1.25 μmol) dissolved in 1.5 mL of 0.1 M $NaHCO_3$ was added so the final detergent concentration was 6 mM. After 1 h of reaction at room temperature, the labeled polylysine was purified using chromatography on a 20-mL Sephadex G-25 column using 20 mM Tris, pH 7.0, with 10 mM β-mercaptoethanol. The dansylpolylysine and dansylgramicidin had a fluorescence excitation maximum at 340 nm and an emission maximum at 505 nm.

Instrumentation. The vesicles were viewed with the fluorescence digital imaging microscope described previously (Haverstick & Glaser, 1987) except the image was processed with a Macintosh IIci computer. Because the wavelength of excitation was 470 nm for NBD, 370 nm for acrylodan, and 340 nm for dansyl, a filter between 420 and 480 nm was used for the excitation of NBD images and another filter between 310 and 390 nm was used for the excitation of the acrylodan and dansyl images. The images were collected at an emission wavelength >515 nm for NBD and >430 nm for acrylodan and dansyl.

Image Processing. The background fluorescence was subtracted from each image, and the image was normalized to a mean radiance value of 100 ± 4. The fold enrichment of a component in a domain was expressed as the ratio of the highest radiance value in the domain to the lowest radiance value in the vesicle. The average enrichment was determined for a population of 55 vesicles, and the error in determining the mean was less than 5%. The images were displayed using a pseudo-color scheme where the lowest radiance value was dark blue and the highest radiance value was red (Figure 1 C).

RESULTS

Ability of the MARCKS Peptide, Polylysine, and Gramicidin To Form Domains in PS Vesicles. Fluorescence digital imaging microscopy allows the direct visualization of the lateral distribution of membrane components. For these studies, large unilamellar vesicles composed of 85% egg PC, 0.5% NBD-PS, 9.5% DOPS, and 5% DAG were formed in 10 mM Mops buffer, pH 7.0. NBD images of the vesicles showed that NBD-PS was distributed evenly and no domains were present in the vesicles (Figure 1A). Since 12 of the 25 residues of the MARCKS peptide are basic, it was expected that it would bind to acidic phospholipids along with polylysine. Kim et al. (1994) measured the extent of binding of the MARCKS peptide to membranes containing acidic phospholipids and also showed that phosphorylation reverses its membrane association. It was also expected that the MARCKS peptide would form membrane domains as shown with cytochrome *c* (Haverstick & Glaser, 1989). When the MARCKS peptide and polylysine were added to the vesicles, PS was no longer evenly distributed but rather it was enriched in domains (Figure 1D,E). The domains had formed in the 2 min necessary to place the vesicles on a

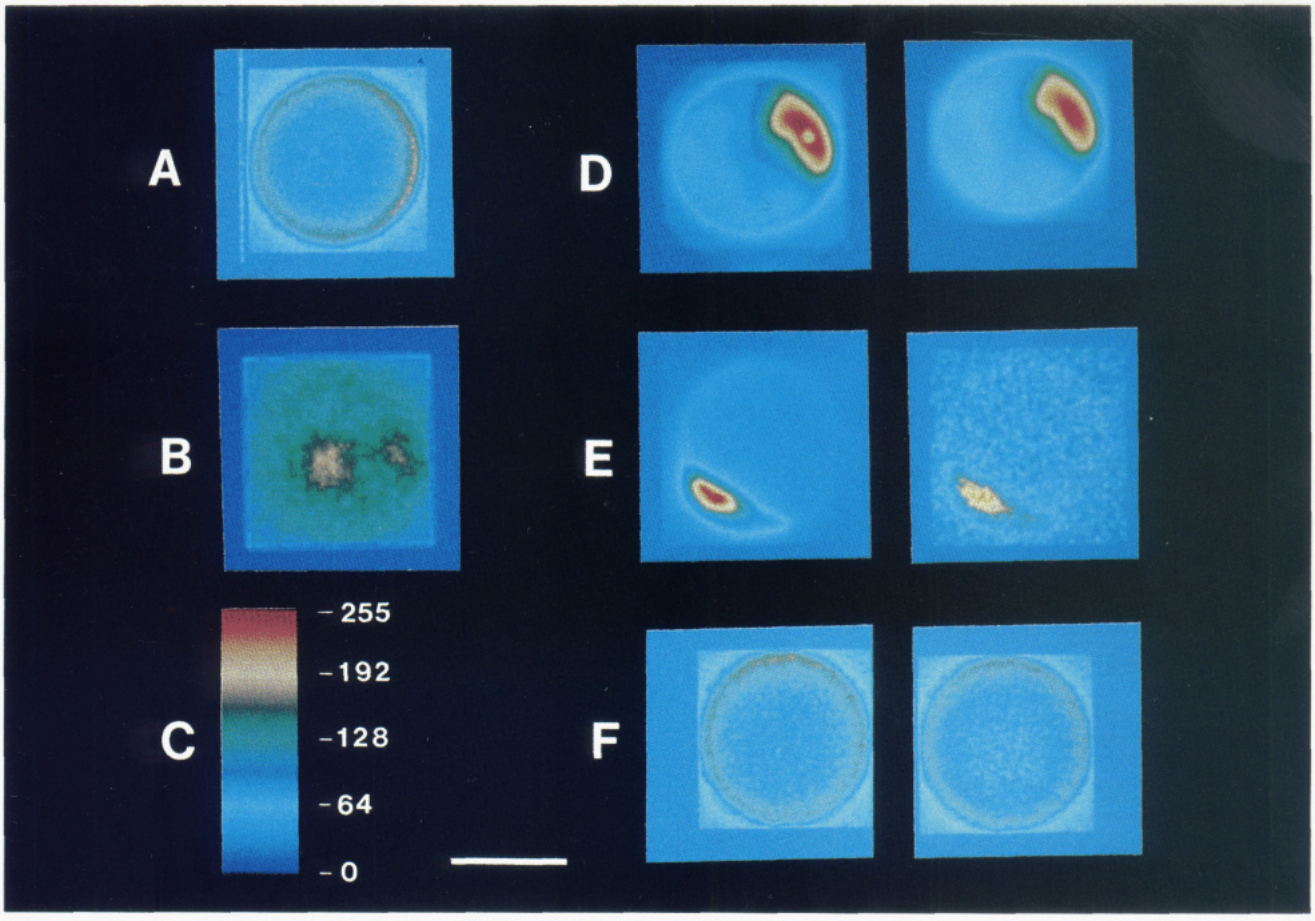

FIGURE 1: The MARCKS peptide and polylysine induced the formation of domains in phospholipid vesicles containing 0.5% NBD-PS, 9.5% DOPS, 5% DAG, and 85% egg PC. The distribution of NBD-PS in the vesicles was uniform in 10 mM Mops buffer, pH 7.0 (A). Ca^{2+} in the PKC assay buffer induced the formation of domains enriched in NBD-PS (B). The addition of either 0.1 μM MARCKS peptide (D) or 20 μM polylysine (E) to the vesicles in Mops buffer also induced the formation of domains enriched in NBD-PS (images on the left), but 20 μM gramicidin did not (F). The images on the right in each panel were taken of the same vesicle as on the left but viewed for either the acrylodan-MARCKS peptide (D), dansylpolylysine (E), or dansylgramicidin (F). All images were normalized to a mean gray value of 100 $\pm$ 4. The pseudo-color scheme applied to the images is shown in panel C with the equivalent radiance values. The bar indicates 4 μm.

slide after the addition of the substrates or polylysine and capture an image. All the vesicles studied were single unilamellar vesicles not in contact with other vesicles. The left and the right images in Figure 1D are images of the same vesicle viewed for either NBD-PS (left) or the acrylodan-MARCKS peptide (right), respectively. Figure 1E shows another vesicle viewed for either NBD-PS (left) or dansylpolylysine (right). The addition of gramicidin (1F) and NaCl (data not shown) did not induce domains in the vesicles, and the distribution of NBD-PS remained uniform. In Figure 1F, the left image is for NBD-PS and the right image is for dansylgramicidin in the same vesicle.

Domains enriched in PS were also observed when vesicles were formed in the PKC assay buffer rather than the Mops buffer even without the MARCKS peptide or polylysine. Shown in Figure 1B is a NBD-PS image of a vesicle containing the same lipids as used in the above experiments but formed in 10 mM Mops, pH 7.0, 3 mM $MgCl_2$, 0.3 mM $CaCl_2$, 0.1 mM EGTA, and 0.1 mM ATP. As reported by Haverstick and Glaser (1987), PS domains can be induced by Ca^{2+}.

Inability of the MARCKS Peptide, Polylysine, and Gramicidin To Form Domains in PC Vesicles. Vesicles were made containing 99.5% egg PC and 0.5% NBD-PC, and the same series of experiments were conducted as described for the PS-containing vesicles. In this case, the NBD-PC was always distributed uniformly, and there were no differences before and after addition of the MARCKS peptide. Some of the MARCKS peptide did bind to the vesicles, however, as judged by the total acrylodan intensity that was 14% of the intensity of the PS-containing vesicles measured over the whole vesicle. The acrylodan-MARCKS peptide was distributed uniformly in the PC vesicles as opposed to being sequestered into domains that were observed in the PS-containing vesicles.

The same experiments as before were conducted using the PC vesicles with the addition of either 20 μM dansylpolylysine, 20 μM dansylgramicidin, or 140 mM NaCl. The distribution of NBD-PC remained uniform, and no domains were formed by polylysine, gramicidin, or NaCl (data not shown). Ca^{2+} also did not cause the formation of domains in these vesicles.

Effects of Polylysine, Gramicidin, and NaCl on the Activity of Protein Kinase C. The fluorescence change during the phosphorylation of the acrylodan-MARCKS peptide by PKC was monitored using the same large unilamellar vesicles as in the microscopy experiments which were composed of DOPS, DAG, and egg PC (molar ratio 10:5:85). Sonicated vesicles were also used with similar results. The activity of PKC decreased with the addition of polylysine to the assay (Figure 2). The presence of NaCl also reduced the activity of PKC, but gramicidin had no effect. Also, the activity in

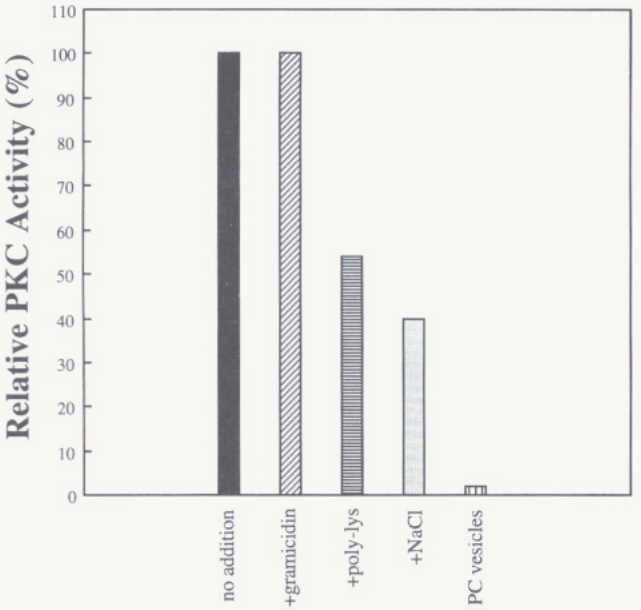

FIGURE 2: Effects of gramicidin, polylysine, and NaCl on the activity of protein kinase C in PS and PC vesicles. Protein kinase C was assayed using the acrylodan-MARCKS peptide as the substrate in egg PC vesicles containing DOPS and DAG. Either 20 μM gramicidin, 20 μM polylysine (MW 4500), or 140 mM NaCl was added to the assay mixture. The last bar is the activity of PKC in vesicles containing only PC.

PC vesicles containing no PS was only 2% of the activity found in the PS-containing vesicles. Since some of the substrate still bound to the PC vesicles but did not form domains, the binding itself was not sufficient for PKC activity.

Effects of Polylysine and NaCl on the Domains of the MARCKS Peptide and PS. In order to determine how polylysine and NaCl inhibited PKC activity, their effects on the domains were investigated in more detail. Figure 3 shows the domains of NBD-PS and the acrylodan-MARCKS peptide in vesicles formed under PKC assay conditions. The left images in Figure 3 were viewed for NBD-PS, and the right images were viewed for the acrylodan-MARCKS peptide. The domains of NBD-PS and the acrylodan-MARCKS peptide (Figure 3A) in the presence of polylysine (Figure 3B), gramicidin (Figure 3C), and NaCl (Figure 3D) were still present but appeared to be changed when polylysine or NaCl was present. When polylysine was added, the NBD-PS domains were still prominent, but the acrylodan-MARCKS peptide domains were diminished. The addition of NaCl, on the other hand, appeared to reduce both the NBD-PS and the acrylodan-MARCKS peptide domains. In order to examine the effects of polylysine and NaCl in more detail and determine the significance of the changes, a large population of vesicles was examined for each experimental condition.

In the three-dimensional graphs shown in Figure 4, the X, Y, and Z axes correspond to the highest radiance of the acrylodan-MARCKS peptide in a given domain, the highest radiance of NBD-PS in the domain, and the number of domains with those values. The intensity of each image was normalized to 100 ± 4, so a peak falling on the $X-Y$ diagonal corresponds to domains with equal enrichment of both NBD-PS and the acrylodan-MARCKS peptide.

Figure 4A shows the distributions of NBD-PS and the acrylodan-MARCKS peptide in the domains of vesicles formed under the assay conditions. The values are clustered together at the upper corner of the $X-Y$ plane, indicating

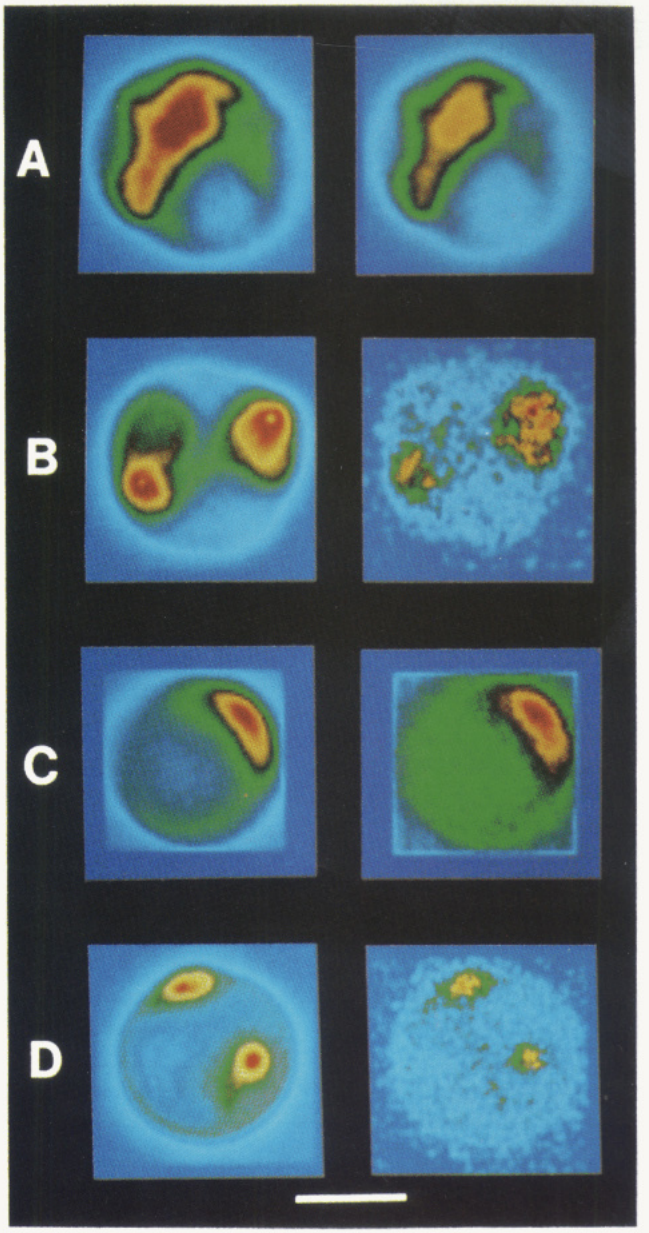

FIGURE 3: (A) Vesicles containing 0.5% NBD-PS, 9.5% DOPS, 5% DAG, and 85% egg PC were made in PKC assay buffer and incubated with the acrylodan-MARCKS peptide for 5 min. In each panel two images were taken of the same vesicle and viewed for either NBD fluorescence (left) or the acrylodan-MARCKS peptide (right). Vesicles after the addition of either 20 μM polylysine (MW 4500) (B), 20 μM gramicidin (C), or 140 mM NaCl (D) are shown. The bar represents 4 μm.

that the domains were enriched in both NBD-PS and the acrylodan-MARCKS peptide.

When polylysine was added to the vesicles, the values were spread out on the left side of the $X-Y$ plane, indicating that some domains had reduced amounts of the MARCKS peptide even though they were still enriched in PS (Figure 4B). Those domains with low amounts of the MARCKS peptide were presumably occupied by polylysine because polylysine also induced the formation of PS-enriched domains (Figure 1E). Since polylysine was not labeled in this experiment, its participation in the domains was reflected indirectly by the relatively low radiance values of the acrylodan-MARCKS peptide. Evidently polylysine had the ability to compete with the MARCKS peptide and to replace it in the PS domains rather than forming separate PS-enriched domains.

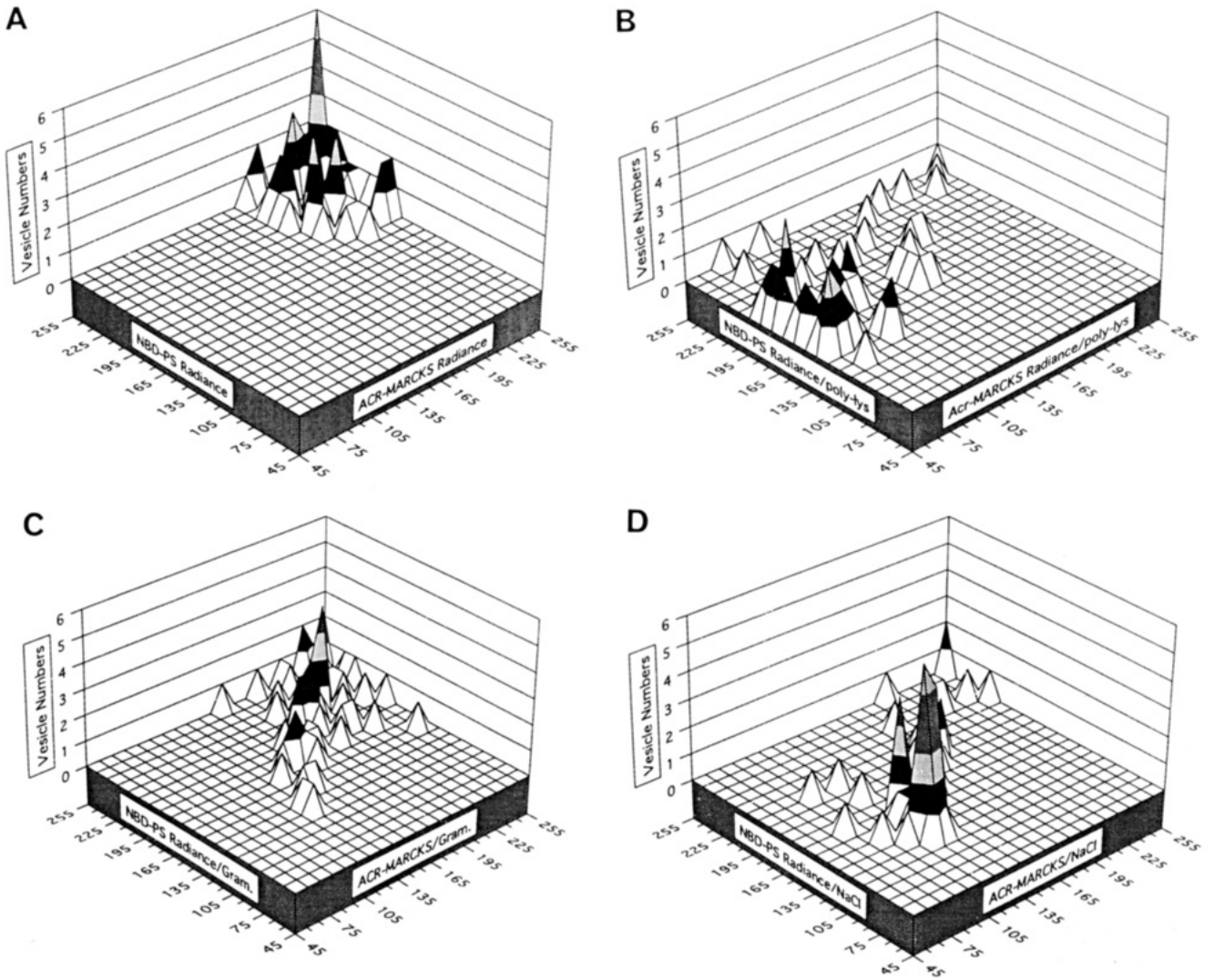

FIGURE 4: Effects of polylysine, NaCl, and gramicidin on the enrichment of the MARCKS peptide and PS in domains. Images were collected from a population of vesicles (sample size 55) under the same conditions as the vesicles shown in Figure 3. (A) The distribution of maximum radiance values of NBD-PS and the acrylodan-MARCKS peptide in domains under PKC assay conditions is shown. The same vesicles in the presence of either 20 μM polylysine (MW 4500) (B), 20 μM gramicidin (C), or 140 mM NaCl (D) are shown.

This raised the question as to where the MARCKS peptide went. That is, did the peptide come off the vesicles or was it displaced to the nondomain areas of the vesicles? The mean radiance value of acrylodan for the whole vesicle (*i.e.*, the mean radiance value per pixel in the image before normalization) was 111 for the population of vesicles without polylysine and 109 for the population of vesicles with polylysine. Thus, the acrylodan-MARCKS peptide was displaced by polylysine in the domains, and the peptide moved to the nondomain areas because the total amount of the acrylodan-MARCKS peptide on the vesicles was similar. The change in the domains caused by polylysine in this experiment resulted in a 47% loss of PKC activity.

The effect of NaCl on the domains is shown in Figure 4D. In this case, most of the radiance values were shifted to the middle of the $X-Y$ plane. The mean radiance value of acrylodan for the whole vesicles before normalization was 67 for the population of vesicles after the addition of NaCl. The decreased number indicates that NaCl displaced the MARCKS peptide from the vesicles. This result is consistent with the binding of the MARCKS peptide to the PS being primarily electrostatic in nature, which could be disrupted by high ionic strength. The decreased binding of the acrylodan-MARCKS peptide resulted in less enrichment for both PS and the substrate in the domains. The decreased enrichment in the domains caused a loss of 60% of the PKC activity in this experiment.

Gramicidin served as a control since it had no effect on the activity of PKC. No significant differences in the distribution of the MARCKS peptide−PS domains were found in vesicles containing gramicidin as shown in Figure 4C.

Since domains were found in PS vesicles formed in the PKC assay buffer containing Ca^{2+} before adding the MARCKS peptide, the ability of Ca^{2+} to induce PS domains was evaluated in order to understand the role of Ca^{2+} in the above experiments. Ca^{2+} could either act with the MARCKS peptide to cause an enrichment of PS in the domains or compete with the MARCKS peptide in forming the domains as did polylysine. The enrichment of the acrylodan-MARCKS peptide and PS with and without Ca^{2+} was measured in a population of vesicles. Also, the enrichment of PS induced by Ca^{2+} alone was measured. The enrichment was defined as the highest radiance value of the domain over the lowest radiance value in the vesicle. The results showed that the MARCKS peptide alone caused the enrichment of PS 2.8 times higher than that produced by Ca^{2+}. When both the MARCKS peptide and Ca^{2+} were added together, the result was the same as with the MARCKS peptide alone. Thus, the MARCKS peptide had a greater ability to induce

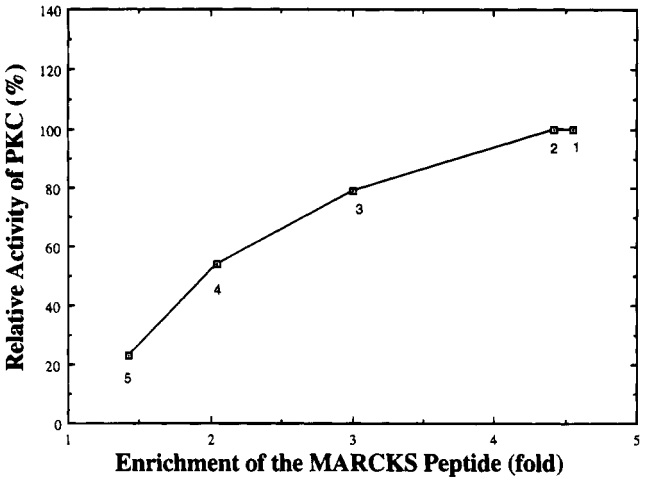

FIGURE 5: Effects of polylysine on the enrichment of the MARCKS peptide and the activity of protein kinase C. The enrichment of the acrylodan-MARCKS peptide under the PKC assay conditions was determined for a population of vesicles (sample size 55). The enrichment was defined as the highest radiance value of the acrylodan-MARCKS peptide in the domains over the lowest radiance value in the vesicle. The activity of PKC was measured using the same vesicles with no additions (1) or in the presence of either 20 μM gramicidin (2), 10 μM polylysine (MW 4500) (3), 20 μM polylysine (MW 4500) (4), or 20 μM polylysine (MW 80 000) (5).

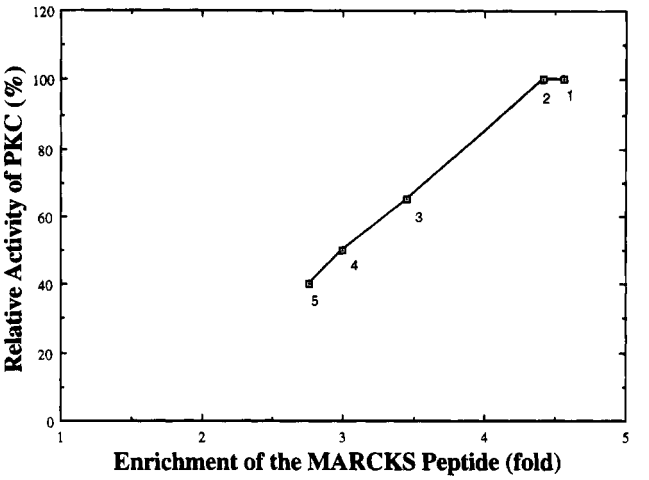

FIGURE 6: Effects of NaCl on the enrichment of the MARCKS peptide and the activity of protein kinase C. The conditions were the same as in Figure 5. The enrichment of the MARCKS peptide and the PKC activity were measured with no addition (1) and in the presence of either 20 μM gramicidin (2), 50 mM NaCl (3), 100 mM NaCl (4), or 140 mM NaCl (5).

PS domains than Ca^{2+} and the MARCKS peptide in effect outcompeted Ca^{2+} to form the PS domains.

The enrichment of PS caused by polylysine alone was also measured. The result showed that polylysine had comparable ability to enrich PS into domains as the MARCKS peptide. This would explain why polylysine could compete with the MARCKS peptide in forming PS domains.

Relationship between the Enrichment of the MARCKS Peptide and the Activity of Protein Kinase C. The activity of PKC was varied by using different concentrations of polylysine or different molecular weight polylysines. Changing the molecular weight from 4 500 to 80 000 did not appear to cause any additional effect other than the effective change in concentration of positive charges. The enrichment of the MARCKS peptide with the addition of polylysine was studied, and the relationship with PKC activity is given in Figure 5. The activity of PKC showed a strong dependence on the enrichment of the MARCKS peptide in the domains. In the absence of polylysine the enrichment of the MARCKS peptide was 4.5-fold (highest radiance value of the domain to the lowest radiance value in the vesicle). When the enrichment of the substrate was reduced to approximately 1.5-fold by the addition of polylysine, the activity of PKC dropped to 23% of the control value.

There was a similar result when the domains of the MARCKS peptide and PS were reduced by the addition of NaCl (Figure 6). With 140 mM NaCl the activity of PKC was reduced to 40% of the control value, and the enrichment of the substrate was 2.7-fold.

Several control experiments were carried out to assess the effect of the NBD group on the formation of domains. When dansyl-PS was used instead of NBD-PS, the average enrichment of dansyl-PS was 11.4-fold in the domains after the addition of the MARCKS peptide to the vesicles in 10 mM Mops buffer, pH 7.0. This compares with a value of 10.9 when NBD-PS was used. The acrylodan-MARCKS peptide was enriched 4.5-fold in this experiment. When the acrylodan-MARCKS peptide was added to unlabeled PS vesicles (all 10% DOPS), the peptide also formed domains and was enriched 4.7-fold. In addition, when unlabeled MARCKS peptide was added to vesicles with NBD-PS, the enrichment of NBD-PS was 11.5-fold. Thus, there was similar domain formation using either NBD-PS, dansyl-PS, or unlabeled PS, and in addition, there was similar domain formation using either the labeled or unlabeled MARCKS peptide.

DISCUSSION

Multiple components are necessary for the activation of PKC, and their organization within the membrane may be important for understanding the mechanism of PKC activity. Fluorescence digital imaging microscopy offers the advantage of direct visualization of the lateral distribution of membrane components and the quantitation of both the PKC substrate and PS in domains. All factors that affect the domains could presumably change the PKC activity.

The binding of basic proteins such as cytochrome *c* to membranes containing acidic phospholipids is usually inhibited at high salt concentrations (Haverstick & Glaser, 1989), and PKC activity also is inhibited by high salt concentrations (Bazzi & Nelsestuen, 1987b; Hannun & Bell, 1990). PKC activity decreased in the presence of NaCl in this study (Figure 6), and the enrichment of the MARCKS peptide decreased proportionally. The increased ionic strength reduced the binding of the MARCKS peptide to the vesicles, and consequently, there was a reduction in activity. The experiment could only be carried out by addition of 140 mM NaCl to the vesicles because higher concentrations of NaCl caused the vesicles to collapse. The MARCKS peptide was mixed with the vesicles, and the domains formed immediately. The addition of NaCl or polylysine was done after the formation of the MARCKS peptide−PS domains, and the results showed that the binding of the MARCKS peptide to PS was reversible.

Polylysine decreased the concentrations of the MARCKS peptide in the domains by a different mechanism than NaCl. The ability of polylysine and the ability of the MARCKS peptide to cause the enrichment of PS were comparable in these experiments. This property made polylysine a competitor in forming the domains of PS and the MARCKS peptide. When polylysine was added to the vesicles contain-

ing the MARCKS peptide, the domains were still enriched in PS to the same extent, but some of the MARCKS peptide in the domains was replaced by polylysine. The loss of PKC activity corresponded to the lower enrichment of the MARCKS peptide (Figure 5).

The total radiance values of the acrylodan-MARCKS peptide in vesicles with or without polylysine were similar. This showed that the MARCKS peptide did not come off the vesicles, but rather it was replaced by polylysine in the domains and it moved to the nondomain areas. This could occur because the concentration of the MARCKS peptide was not high enough to saturate all the PS in the vesicles and all the PS was not in the domains. These data were especially interesting because they showed that binding of the substrate to the vesicles was not sufficient for full PKC activity. High PKC activity required the formation of domains.

The experiment with gramicidin served as a control since it did not cause domains to be formed or affect the activity of PKC. Another control experiment with vesicles containing PC only, and no PS, showed that domains were not induced by the MARCKS peptide and almost no PKC activity was found in the vesicles. The PKC activity also was similar in both sonicated vesicles and large unilamellar vesicles used for microscopy.

Either the MARCKS peptide or Ca^{2+} could induce domain formation, but the MARCKS peptide caused PS to be enriched about 2.8 times higher than Ca^{2+}. In the PKC assay mixture, this resulted in the enrichment of PS and the MARCKS peptide being the same in either the absence or presence of Ca^{2+}. Ca^{2+} is a very important component in the activation of PKC, but its role does not appear to be in the forming of domains, at least with this substrate under the experimental conditions. In these experiments, a saturating concentration of Ca^{2+} (0.3 mM) for PKC activity was used. A much lower concentration of Ca^{2+} (approximately 0.6 μM) could induce domains, however, in the absence of the MARCKS peptide. It has been proposed that Ca^{2+} acts as a bridge between PKC and the negatively charged phospholipids (Bazzi & Nelsestuen, 1990; Burns & Bell, 1991), and it seems reasonable that Ca^{2+} might be important to link PKC to the domains of the substrate and PS.

In summary, the results reported in this paper show that domains are important for the activation of PKC. Agents that disrupted the domains of the MARCKS peptide and PS reduced the activity of the enzyme. While NaCl caused the peptide to dissociate from the vesicles, polylysine displaced the peptide from the domains into the other areas of the vesicles. Thus, binding of the substrate to the vesicles was not sufficient for full activity; rather the MARCKS peptide had to be organized into domains. This illustrates the function of the domains visualized by fluorescence digital imaging microscopy for enzyme activity and illustrates how the organizations of membrane components can regulate membrane processes.

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