Formation of Membrane Domains during the Activation of Protein Kinase C†

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ABSTRACT: The lateral membrane organization of phosphatidylserine, diacylglycerol, substrate, and Ca^{2+}-dependent protein kinase C in large unilamellar vesicles was investigated by using fluorescence digital imaging microscopy. The formation of phosphatidylserine domains could be induced by either Ca^{2+}, the MARCKS peptide, or protein kinase C. However, only Ca^{2+} could induce diacylglycerol to partition into the phosphatidylserine domains. In the complete protein kinase C assay mixture, two separate triple-labeling experiments demonstrated the colocalization of phosphatidylserine, protein kinase C, diacylglycerol, and the MARCKS peptide in domains. The amounts of all the labeled components in whole vesicles and in domains were measured at various concentrations of either phosphatidylserine, Ca^{2+}, diacylglycerol, or the MARCKS peptide or with the addition of polylysine. The role of each component in forming membrane domains and in mediating the enzyme activity was analyzed. The results indicated that the inclusion of the MARCKS peptide in the domains, not just the binding of the substrate to vesicles, was especially important for PKC activity. The formation of PKC domains required the presence of DAG and Ca^{2+} at physiological ionic strength. The PKC activity was proportional to the amounts of PKC and substrate in the domains. The results also showed that the MARCKS peptide left the domains after being phosphorylated. A model for the activation of protein kinase C involving sequestering of the reaction components into membrane domains is proposed. The efficiency of the reaction was greatly increased by concentrating the activators, the enzyme, and the substrate into domains.

The activation of protein kinase C (PKC)[1] represents one of the best examples of how extracellular signals are transduced across the cell membranes to alter cellular metabolism. This ubiquitous protein is a family with at least 11 members (Bell & Burns, 1991; Liscovitch & Cantley, 1994; Dekker & Parker, 1994). Most of them are regulated by phosphatidylserine (PS) and diacylglycerol (DAG), but only some of them require Ca^{2+} for activity (Nishizuka, 1986; Bell & Burns, 1991; Newton, 1994). The activation of PKC in cells is believed to occur through signal transduction cascades. When a variety of agonists bind to specific receptors on the cell surface, one response is increasing hydrolysis of phosphoinositides (PIP_2) to produce inositol 1,4,5-triphosphate (IP_3) and DAG. IP_3 mobilizes Ca^{2+}; then the increased Ca^{2+} and DAG activate PKC (Bell & Burns, 1991).

A number of studies on PKC have been directed at the cofactors involved in the PKC reaction. The activation of PKC by PS, DAG, and phorbol ester has been investigated in mixed micelles to obtain defined conditions (Hannun et al., 1985; Lee & Bell, 1989). The association of PKC with membranes was observed using light-scattering and fluorescence energy transfer measurement (Bazzi & Nelsestuen, 1987a,b, 1991a,b, 1992), and for example, PKC binding shows a steep dependence on the concentration of PS (Newton & Koshland, 1989; Hannun & Bell, 1990; Mosior & Epand, 1993).

Fluorescence digital imaging microscopy provides a sensitive means to study the lateral distribution of membrane components (Rodgers & Glaser, 1993a,b). Domains visualized by this technique can be important in activating PKC (Yang & Glaser, 1995). The method can also be used for measuring the compositions and enrichment of particular molecular species in membrane domains (Luan et al., 1995). In this paper the ability of labeled PKC (Ca^{2+}-dependent isoforms), PS, DAG, and a substrate (the MARCKS peptide) to form domains in vesicles was examined. The MARCKS peptide was chosen as a model PKC substrate since it is often used to assay PKC. The 25 amino acid basic peptide contains the phosphorylation sites of MARCKS (*m*yristoylated *a*lanine-*r*ich C *k*inase *s*ubstrate), which is a prominent cellular substrate of PKC. The roles of the substrate and all the components necessary to activate PKC were examined for their ability to form membrane domains and to mediate enzymatic activity.

MATERIALS AND METHODS

Materials. Poly(L-lysine) (4500 Da) was purchased from Sigma Chemical Co. (St. Louis, MO).

Purification of Protein Kinase C. Purification of Ca^{2+}-dependent isoforms of PKC from cow brain homogenate was achieved using a two-step method. Preparation of inside-out vesicles from erythrocyte ghosts was performed using

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[1] Abbreviations: acrylodan, 6-acryloyl-2-(dimethylamino)naphthalene; AMP-PNP, adenylyl imidodiphosphate; DAG, *sn*-1, 2-dioleoylglycerol; dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; C_{11}-dansyl-PC, 1-acyl-2-[11-(*N*-dansylamino)undecanoyl]phosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IP_3, inositol 1,4,5-triphosphate; MARCKS, myristoylated alanine-rich C kinase substrate; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; NBD-PS, 1-acyl-2-[6-(N-NBD-amino)-caproyl]phosphatidylserine; PC, phosphatidylcholine; PIP_2, phosphatidylinositol 4,5-diphosphate; PKC, protein kinase C; PMSF, phenylmethanesulfonyl fluoride; PPO, 2,5-diphenyloxazole; PS, phosphatidylserine; Tris, tris(hydroxymethyl)aminomethane.

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the method described by Bennett et al. (1983) and Wolf et al. (1985a,b). Binding of PKC from cow brain homogenate to the inside-out vesicles was accomplished following the procedures described by Wolf et al. (1985a,b) and Niedel et al. (1983). PKC was bound to the inside-out vesicles in the presence of Ca^{2+} but was released from the vesicles upon the addition of EDTA and EGTA. Phenyl-Sepharose chromatography was used for the last step in the purification. The method permits the complete purification of approximately 250 μg of PKC per cow brain, and it gave a single band on a sodium dodecyl sulfate−polyacrylamide electrophoresis gel.

Protein Kinase C Activity Assay. PKC activity was quantitated by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into the MARCKS peptide following the procedures of Chakravarthy et al. (1991) and Edashige et al. (1992). The reaction mixture was composed of 0.1 mM lipid vesicles (molar ratio of DOPS:DAG:egg PC = 10:5:85), 10 mM Tris, pH 7.0, 10 mM $MgCl_2$, 50 μM ATP, 0.25 μCi of [γ-^{32}P] ATP, 0.3 mM $CaCl_2$, 8 μM MARCKS peptide, and 0.5 μM PKC in a final volume of 200 μL. The reaction was carried out for 10 min at room temperature and stopped by addition of 100 μL of cold 10% acetic acid. After a 10 min incubation on ice, individual samples were filtered and washed with cold 10% acetic acid on 2 cm^2 GF/C glass filters (Whatman, Hillsbora, OR). The filters were dried and suspended in scintillation fluid (0.5% PPO in toluene), and the ^{32}P was counted in a Beckman LS7000 scintillation counter.

Preparation of the Lissamine Rhodamine Labeled PKC. The purified protein kinase C was labeled by lissamine rhodamine B sulfonyl chloride using the procedure provided by Molecular Probes (Eugene, OR). The protein (0.2 mg) was mixed with the fluorescent probe (14 μg) in a final volume of 2 mL in 20 mM Tris, pH 7.0. The pH of the solution was adjusted to 9.0 by adding one-tenth volume of 1.0 M Na_2CO_3. The solution was stirred in the dark at room temperature for 30 min and dialyzed overnight against a buffer containing 10 mM Tris, pH 7.0, and 1 mM PMSF to remove the majority of the free dye. Phenyl-Sepharose chromatography was used to ensure the complete removal of the free fluorescent probe. The molar ratio of rhodamine to protein was determined to be approximately 1:1 by rhodamine absorbance at 566 nm using an extinction coefficient of 93 000 M^{-1} cm^{-1} and protein absorbance at 280 nm assuming an extinction coefficient of 1 mg^{-1} cm^2. The labeled PKC had the same activity as the unlabeled protein as determined by the activity assay.

Preparation of the Dansyl-Labeled Diacylglycerol. Dansyl-DAG was prepared from C_{11}-dansyl-PC (Omann & Glaser, 1984) *via* a method similar to that used by Cunningham et al. (1989). C_{11}-dansyl-PC (1 μmol) was dissolved in 1.0 mL of diethyl ether, and then 1.0 mL of 0.2 M Tris-HCl, pH 7.3, containing 12 mM $CaCl_2$ and 93 units of phospholipase C was added to the solution to remove the phosphate head group of C_{11}-dansyl-PC. After the solution was stirred for 2 h at room temperature, dansyl-DAG was extracted three times with 3 mL of hexane. The hexane phase was evaporated under N_2, and the products were resuspended in chloroform. The chloroform containing dansyl-1,2-DAG and dansyl-1,3-DAG was loaded onto a silica gel G 20 × 20 cm plate (Analtech, Newark, DE) and developed in chloroform/acetone, 95:5 (v/v), at room temperature for about 25 min (Kodali et al., 1990). In this solvent system, dansyl-1,3-DAG migrates faster than dansyl-1,2-DAG, with R_f values of 0.41 and 0.28, respectively. The concentrations of the products were determined by dansyl absorbance at 335 nm with an extinction coefficient of 4300 M^{-1} cm^{-1}. The yields of dansyl-1,2-DAG and dansyl-1,3-DAG were 22% and 78%, respectively.

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 as determined by acrylodan absorbance at 360 nm and the Coomassie protein assay (Pierce Chemical Co., Rockford, IL).

Vesicle Preparation. Vesicles used for microscopy were prepared using the procedure described by Darszon et al. (1980). The phospholipids (250 nmol) in $CHCl_3$ at the desired molar ratios were placed in a small glass vial (15 × 45 mm) and dried to a thin film along the bottom of the vial *via* a gentle stream of N_2. The lipids were hydrated slowly in 500 μL of the desired buffer. After a 48 h incubation at room temperature in the dark, large unilamellar vesicles (2−10 μm) were obtained from the upper layer of the clear solution and were ready for microscopy.

Instrumentation. The vesicles were viewed using fluorescence digital imaging microscopy as described previously (Haverstick & Glaser, 1987). A filter between 420 and 480 nm (Leitz G filter system) was used for the excitation of NBD images, another filter between 310 and 390 nm (Leitz A filter system) was used for the excitation of acrylodan and dansyl images, and a third filter between 530 and 560 nm (Leitz B2 filter system) was used for the excitation of rhodamine images. The images were collected at an emission wavelength >515 nm for NBD, >430 nm for acrylodan and dansyl, and >550 nm for rhodamine. Besides using the Leitz filter systems, some Corning filters were also used in the excitation and emission paths. In the excitation path, filter 3−72 was used for NBD, 7-60 for dansyl and acrylodan, and 3−69 for rhodamine. In the emission path, 4−94 was used for NBD, dansyl, and acrylodan and 3−67 was used for rhodamine.

Image Processing. The background fluorescence was subtracted from each image. 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 as shown in Figure 1D.

Compositional Analysis of the Domains. Fluorescence digital imaging microscopy permits quantitative analysis of the concentration of a fluorescent molecule in the domains (Luan et al., 1995). Standard curves (radiance value versus molar concentration) of rhodamine and acrylodan were constructed by using uniform vesicles labeled with different amounts of these fluorescent probes. A population of vesicles was collected for each point of the standard curve and for the compositional analysis of the domains. If the initial concentration of a component in the vesicles is known, *e.g.*, a phospholipid, the molar percentage of the component

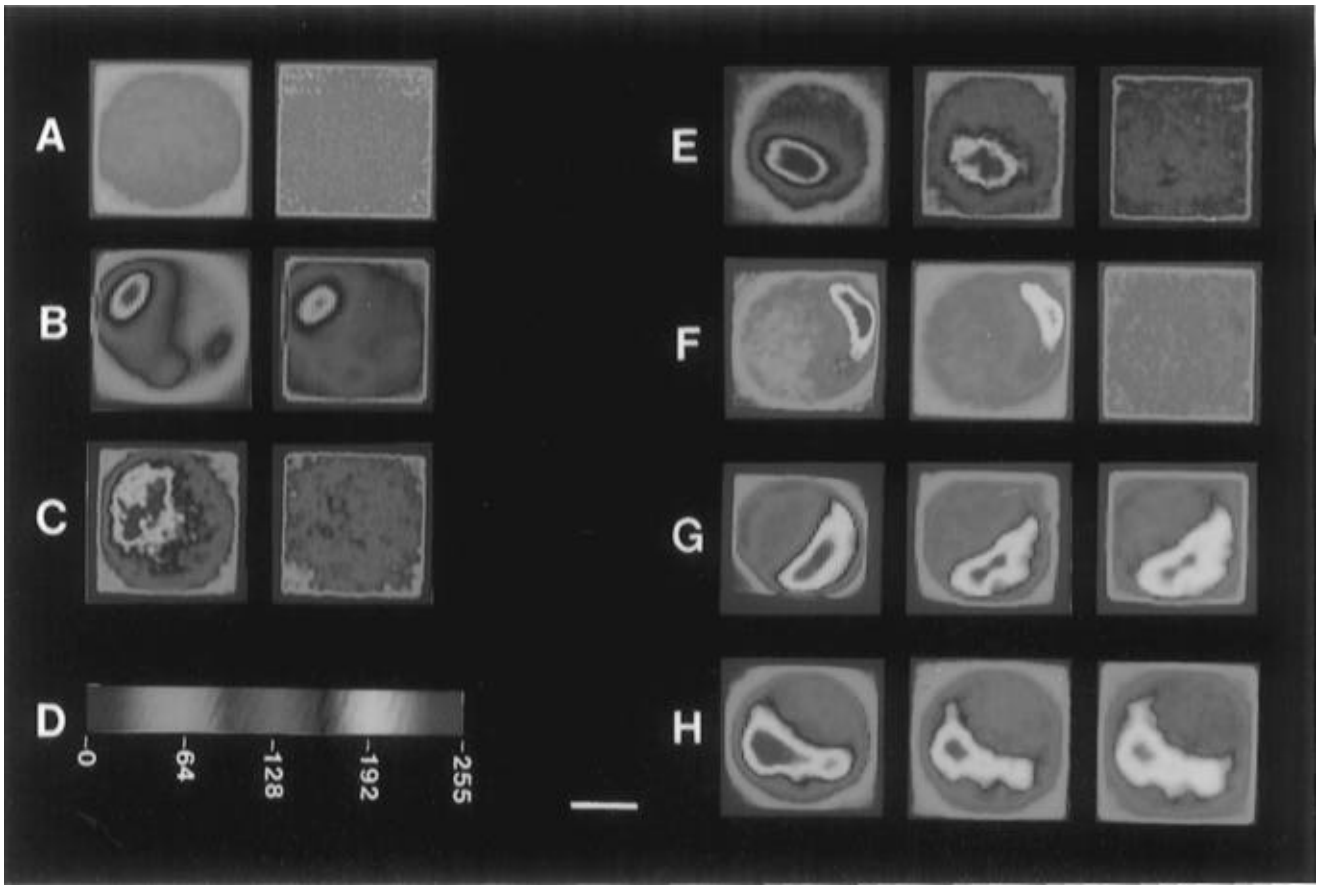

FIGURE 1: Formation of domains containing PS, DAG, Ca^{2+}, substrate, and PKC. Images in panels A−F were obtained from vesicles composed of 10% DOPS (including 0.3% NBD-PS), 5% DAG (including 1.5% dansyl-DAG), and 85% egg PC in 10 mM Tris, pH 7.0. The individual images in each panel of this figure were captured from the same vesicle, but they represent the distribution of different fluorophores as described below. (A) The images of NBD-PS (left) and dansyl-DAG (right) show the uniform distribution of both PS and DAG in the vesicle. (B) The addition of 0.3 mM Ca^{2+} to the vesicles induced the formation of a NBD-PS (left) and a dansyl-DAG (right) domain. (C) The addition of 8 μM MARCKS peptide to the vesicles (without calcium) induced a NBD-PS (left) but not a dansyl-DAG (right) domain. The addition of either 0.5 μM rhodamine−PKC (E) or 0.5 μM rhodamine−PKC plus 8 μM MARCKS peptide (F) (without calcium) did not induce dansyl-DAG domains (right in E and F) but did induce NBD-PS (left in E and F) and rhodamine−PKC domains (middle in E and F). Vesicles in panels G and H had the same overall compositions but were formed in the complete PKC assay buffer (10 mM Tris, pH 7.0, 10 mM $MgCl_2$, 50 μM AMP-PNP, 8 μM MARCKS peptide, 0.3 mM $CaCl_2$, and 0.5 μM PKC). (G) One vesicle was viewed for NBD-PS (left), rhodamine−PKC (middle), and dansyl-DAG (right). In this experiment, rhodamine-labeled PKC was added to the vesicles labeled with 0.3% NBD-PS and 1.5% dansyl-DAG. (H) Another vesicle was viewed for NBD-PS (left), rhodamine−PKC (middle), and the acrylodan−MARCKS peptide (right). In this experiment, rhodamine-labeled PKC and acrylodan-labeled MARCKS peptide (20% labeled) were added to the vesicles labeled with 0.3% NBD-PS. Panel D shows the pseudo color scheme applied to the images with the lowest radiance value represented by dark blue and the highest radiance value represented by red. The white bar indicates 4 μm.

in the domains can also be directly calculated by determining the enrichment of the component in the domains compared to the radiance of the whole vesicle (fold enrichment) from the images.

RESULTS

Formation of PS and DAG Domains. To visualize the distribution of PS and DAG, double-labeled vesicles containing 10% DOPS (including 0.3% NBD-PS), 5% DAG (including 1.5% dansyl-DAG), and 85% egg PC were formed in 10 mM Tris, pH 7.0. The images in each panel of Figure 1 were captured from the same vesicle, but viewed for different labeled components. The images of NBD-PS (left image in Figure 1A) and dansyl-DAG (right image in Figure 1A) showed a uniform distribution of both PS and DAG. The addition of 0.3 mM Ca^{2+} to the vesicles induced the formation of NBD-PS domains (Figure 1B, left) and dansyl-DAG domains (Figure 1B, right). The pattern of domain formation was different in every vesicle, but domains were induced in all the vesicles of the preparation, which is the case in all the examples of domains that have been examined. The addition of 8 μM MARCKS peptide to the vesicles without calcium induced NBD-PS domains (Figure 1C, left) but not dansyl-DAG domains (Figure 1C, right). The addition of either 0.5 μM PKC (Figure 1E) or 0.5 μM PKC plus 8 μM MARCKS peptide (Figure 1F) without calcium did not induce the formation of DAG domains either (right images in Figure 1E,F), although NBD-PS domains (left images in Figure 1E,F) were formed in these vesicles. Rhodamine−PKC colocalized in the PS domains (middle images in Figure 1E,F). These results indicated that the formation of PS domains could be induced by either Ca^{2+}, the MARCKS peptide, or PKC individually, but the formation of DAG domains only occurred upon the addition of Ca^{2+}. Moreover, the DAG and PS domains induced by Ca^{2+} always colocalized in the same area. Without Ca^{2+}, DAG domains were absent even in the presence of all the other factors.

Under the conditions used for these experiments, the enrichment of PS domains induced by either the MARCKS

Table 1: Effect of Varying the Concentration of the MARCKS Peptide on the Components of the Domains and the Activity of PKC[a]

MARCKS (μM)	mean radiance value for whole vesicles				mean radiance value of domains				max enrichment in domains (fold of enrichment)				domain size (%)	activity (%)
	MARCKS	DAG	PS	PKC	MARCKS	DAG	PS	PKC	MARCKS	DAG	PS	PKC		
0	0	49	95	21	0	154	280	70	0	5.6	9.0	6.1	7	0
1	9	49	95	22	22	157	280	70	8.1	5.8	9.1	6.3	8	31
2	15	49	95	22	36	157	280	70	8.3	5.6	9.5	6.5	10	58
4	24	49	95	22	58	157	280	70	8.3	5.7	9.2	6.4	11	72
8	28	49	95	22	68	157	280	70	8.5	5.9	9.4	6.9	12	100

[a] The vesicles were composed of 10% DOPS, 5% DAG, and 85% egg PC in PKC assay buffer with the indicated amounts of the MARCKS peptide, 0.5 μM PKC, and 300 μM Ca^{2+}. All the values were determined by averaging the results from 55 vesicles. The fold of enrichment in the domains was calculated using the highest radiance values in the domains over the average radiance values of the vesicles. The measurements of the domains were done in two separate triple-labeling experiments as described in Figure 1G,H.

peptide, PKC, or Ca^{2+} individually was examined for a population of vesicles. For each condition, the average enrichment of PS in the domains was found to be 6.6-fold, 4.3-fold, and 2.9-fold, respectively. The enrichment of a particular molecule in a domain was defined as the highest radiance value in the domain over the average radiance value of the vesicle. When Ca^{2+} and the MARCKS peptide were added to the vesicles at the same time, the PS domains were still 6.6-fold enriched, which was the same as the domains induced by the MARCKS peptide alone. When Ca^{2+} and PKC were added together to the vesicles, the PS domain was 9.4-fold enriched, which was higher than the one induced by either PKC or Ca^{2+} alone. This was equivalent to the domains produced when the MARCKS peptide, Ca^{2+}, and PKC were all added to the vesicles at the same time, as described below.

Formation of Domains with PS, DAG, Ca^{2+}, Substrate, and PKC. The results shown in Figure 1 indicated that Ca^{2+}, the MARCKS peptide, and PKC were all capable of inducing the formation of PS domains. To completely understand how domains influence the reaction catalyzed by PKC, it is necessary to evaluate the effects of all five components (three activators, the substrate, and the enzyme). Are these components separated in different domains or located in the same domains? To answer this question, triple-labeling experiments were carried out using vesicles (molar ratio of DOPS:DAG:egg PC = 10:5:85) in the complete PKC assay mixture (10 mM Tris, pH 7.0, 10 mM $MgCl_2$, 50 μM AMP-PNP, 8 μM MARCKS peptide, 0.3 mM $CaCl_2$, and 0.5 μM PKC). The nonhydrolyzable analog AMP-PNP was used in place of ATP to determine the initial distribution of the components. Figure 1G shows images obtained from the same vesicle but viewed for NBD-PS (Figure 1G, left), rhodamine−PKC (Figure 1G, middle), and dansyl-DAG (Figure 1G, right). The results indicated that PS, PKC, and DAG all colocalized in the same domain. Since Ca^{2+} was the only factor that induced the formation of DAG domains, Ca^{2+} had to be present in the domains as well. Figure 1H shows images of another vesicle captured in a different triple-labeling experiment. The images of NBD-PS (Figure 1H, left), rhodamine−PKC (Figure 1H, middle), and acrylodan−MARCKS peptide (Figure 1H, right) of this vesicle show the formation of a single domain enriched in PS, PKC, and the MARCKS peptide. Combining the information from the two triple-labeling experiments, it can be concluded that all the components necessary to activate PKC and carry out the reaction, *i.e.*, PS, DAG, the MARCKS peptide, Ca^{2+}, and PKC, are organized into the same domains.

Importance of the MARCKS Peptide in Forming Domains. The MARCKS peptide was varied from 0 to 8 μM in egg PC vesicles containing 5% DAG and 10% DOPS in the PKC assay mixture with 0.5 μM PKC and 300 μM Ca^{2+}. Several parameters were evaluated in these vesicles, and the results are shown in Table 1. The mean radiance values of the acrylodan−MARCKS peptide, dansyl-DAG, NBD-PS, and rhodamine−PKC were measured for the entire vesicle and in the domains. Because of the overlap of the excitation and emission spectra of acrylodan and dansyl, the measurements were done in two separate triple-labeling experiments as described for Figure 1G and 1H. A domain was arbitrarily defined as a region with radiance value greater than 150% of the average radiance value of the vesicle. The greatest enrichment of these labeled components in the domains was also measured and expressed as the ratio of the highest radiance value in the domain over the average radiance value of the vesicle. Finally, the size of the MARCKS peptide domains was measured in these experiments and defined as the number of pixels in the domains divided by number of pixels in the whole vesicle. The average results for a population of vesicles (sample size 55) was used for all the data shown in the tables. The PKC activity was also examined under each corresponding condition and compared with the activity in vesicles containing 10% DOPS, 5% DAG, 85% egg PC, 0.3 mM $CaCl_2$, 0.5 μM PKC, and 8 μM MARCKS peptide (100% activity).

As shown in Table 1, the PKC activity increased when the concentration of the MARCKS peptide was increased in the reaction mixture. The binding of the substrate to the whole vesicles and in the domains increased simultaneously, while the binding of PKC to the whole vesicles and in the domains remained unchanged. 5% DAG and 10% DOPS were always present in the vesicles. Interestingly, the concentrations of these two lipid activators in the domains were also unchanged as well as the enrichment of all the components in the domains when the concentration of the MARCKS peptide was varied in the mixture. The data indicated that the binding of the substrate to the whole vesicles and the amounts of the substrate in the domains were important for PKC activity.

Polylysine displaces the MARCKS peptide from the domains to nondomain areas of the vesicles, and this has provided a valuable method for distinguishing between the importance of binding of the substrate to the vesicles and the concentration of the substrate into domains for the phosphorylation by PKC (Yang & Glaser, 1995). Similar experiments were carried out in the present study, keeping the concentration of all the components constant but adding

Table 2: Effect of Polylysine on the Components of the Domains and the Activity of PKC[a]

polylysine (μM)	mean radiance value for whole vesicles				mean radiance value of domains				max enrichment in domains (fold of enrichment)				domain size (%)	activity (%)
	MARCKS	DAG	PS	PKC	MARCKS	DAG	PS	PKC	MARCKS	DAG	PS	PKC		
0	28	49	95	22	68	157	280	70	8.5	5.9	9.4	6.9	12	100
10	27	52	105	23	54	146	285	70	6.8	5.6	9.5	6.9	12	80
20	28	51	101	23	45	152	283	71	5.6	5.7	9.4	7.0	12	70
25	28	52	99	21	39	153	281	72	4.8	5.7	9.4	7.0	12	61
40	27	50	102	22	32	156	280	71	4.1	5.7	9.4	7.1	12	50

[a] The vesicles were composed of 10% DOPS, 5% DAG, and 85% egg PC in PKC assay buffer with 8 μM MARCKS peptide, 0.5 μM PKC, 300 μM Ca^{2+}, and the indicated amounts of polylysine. All the values were determined by averaging the results from 55 vesicles. The fold of enrichment in the domains was calculated using the highest radiance values in the domains over the average radiance values of the vesicles. The measurements of the domains were done in two separate triple-labeling experiments as described in Figure 1G,H.

Table 3: Effect of Ionic Strength on the Components of the Domains[a]

ionic strength (mM)	Ca^{2+}	DAG	mean radicance value for whole vesicles				mean radicance value of domains				max enrichment in domains (fold of enrichment)				domain size (%)	activity (%)
			MARCKS	DAG	PS	PKC	MARCKS	DAG	PS	PKC	MARCKS	DAG	PS	PKC		
40	−	−	28	−	95	2	68	−	167	7	8.2	−	6.6	6.1	11	9
40	+	+	28	49	95	22	68	157	280	70	8.5	5.9	9.4	6.9	12	100
140	−	−	19	−	95	−	35	−	156	−	5.2	−	5.3	−	12	0
140	+	+	18	49	94	22	36	112	168	42	5.6	4.5	6.5	5.2	12	50

[a] The vesicles were composed of 10% DOPS, 85% egg PC, and 5% DAG (or no DAG) in PKC assay buffer with 8 μM MARCKS peptide, 0.5 μM PKC, and 300 μM Ca^{2+} (or no Ca^{2+}). The experiments were carried out in the presence of either 100 mM NaCl (total ionic strength 140 mM) or no NaCl (total ionic strength 40 mM). All the values were determined by averaging the results from 55 vesicles. The fold of enrichment in the domains was calculated using the highest radiance values in the domains over the average radiance values of the vesicles. The measurements of the domains were done in two separate triple-labeling experiments as described in Figure 1G,H. (−) absent. (+) present.

different concentrations of polylysine. The results are shown in Table 2. The total binding of the MARCKS peptide and PKC to the whole vesicles, as measured by the mean radiance values of acrylodan−MARCKS peptide and rhodamine−PKC in the entire vesicles, was not changed by the addition of polylysine. Again, DAG and PS were always maintained at 5% and 10% in the vesicles. In the domains, the amounts and the enrichment of DAG, PS, and PKC did not change; only the amounts and the enrichment of the MARCKS peptide changed. PKC activity decreased proportionally to the reduction in the amount and enrichment of the MARCKS peptide in the domains when more polylysine was added. Because polylysine was also capable of forming domains with PS (Yang & Glaser, 1995), it competed with the MARCKS peptide in the PS domains. Thus, the MARCKS peptide was displaced from the PS domains to the nondomain areas of vesicles by polylysine. Interestingly, the amount of PKC in the domains was not affected by polylysine. The results from the polylysine experiment clearly indicated that the amount of the substrate in the domains with PKC, rather than the binding of the substrate to the vesicles, was critical for the rate of the reaction.

Except for the PKC activity assay, all the quantitation experiments were carried out using AMP-PNP instead of ATP to determine the initial amounts of the MARCKS peptide in the vesicles and in the domains. The total binding of the substrate to the vesicles was determined by the mean radiance value of acrylodan in the unnormalized whole vesicles. This was 14 for the population of vesicles using ATP and 28 for the population of vesicles using AMP-PNP. This result confirms the results of Kim et al. (1994) that once the MARCKS peptide was phosphorylated, it dissociated from the vesicles.

Effect of Ionic Strength on the Components in the Domains. In order to use conditions closer to the physiological ionic strength, 100 mM NaCl was added to the PKC assay mixture, bringing the total ionic strength to approximately 140 mM. As shown in Table 3, when the ionic strength was increased in the solution containing vesicles with both DAG and Ca^{2+}, the mean radiance value of the MARCKS peptide in the domains decreased from 68 to 36, and the enrichment of the MARCKS peptide in the domains was reduced from 8.5-fold to 5.6-fold. This corresponded to a decrease in the PKC activity of 50%. In addition to the decrease in the MARCKS peptide in the domains, the amount of PKC was also reduced (the mean radiance value of rhodamine−PKC in the domains decreased from 70 to 42). This effect was not observed when polylysine was added (Table 2). Therefore, at the higher ionic strength, the reduced PKC activity could be attributed to a reduction in two components, the substrate and the enzyme.

The total binding of PKC to the whole vesicles containing DAG and Ca^{2+} was not changed by increasing the ionic strength in contrast to the behavior of the MARCKS peptide (Table 3). The reason for the change of PKC in the domains, rather than the total binding of PKC to the vesicles, could partially reflect the fact that the higher ionic strength also reduced the enrichment of DAG in the domains. The lower level of DAG would result in a lower amount of PKC in the domains.

It is interesting to note that, in the lower ionic strength solution, PKC bound to the vesicles very weakly without DAG and Ca^{2+}, and in the higher ionic solution, PKC neither bound to the vesicles nor formed domains. The latter situation probably reflects the *in vivo* situation because PKC is found in the cytoplasm before it is activated. Substrates similar to the MARCKS peptide could be the first component to form domains with PS in membranes, and then PKC would translocate to the domains when the concentrations of DAG and Ca^{2+} increased.

Effects of Ca^{2+} and DAG on PKC Binding and Domain Formation. The concentrations of Ca^{2+} and DAG were

Table 4: Effect of Ca^{2+} Concentration on the Binding of PKC and the Formation of Domains[a]

Ca^{2+}	mean radiance value for whole vesicles				mean radiance value of domains				max enrichment in domains (fold of enrichment)				domain	activity
(μM)	MARCKS	DAG	PS	PKC	MARCKS	DAG	PS	PKC	MARCKS	DAG	PS	PKC	size (%)	(%)
0	28	49	95	5	68	49[b]	166	16	8.0	1.0[b]	6.6	6.1	12	36
1	28	49	95	11	68	92	197	35	8.2	3.4	7.4	6.7	12	57
10	28	49	95	18	68	114	219	57	8.1	4.3	8.1	6.2	12	73
300	28	49	95	22	68	157	280	70	8.5	5.9	9.4	6.9	12	100

[a] The vesicles were composed of 10% DOPS, 5% DAG, and 85% egg PC in PKC assay buffer with 8 μM MARCKS peptide, 0.5 μM PKC, and the indicated amounts of Ca^{2+}. All the values were determined by averaging the results from 55 vesicles. The fold of enrichment in the domains was calculated using the highest radiance values in the domains over the average radiance values of the vesicles. The measurements of the domains were done in two separate triple-labeling experiments as described in Figure 1G,H. [b] No domains were observed and the distribution of DAG was uniform.

Table 5: Effect of the Concentration of DAG on the Binding of PKC and the Formation of Domains[a]

DAG	mean radiance value for whole vesicles				mean radiance value of domains				max encirhment in domains (fold of enrichment)				domain	activity
(mol %)	MARCKS	DAG	PS	PKC	MARCKS	DAG	PS	PKC	MARCKS	DAG	PS	PKC	size (%)	(%)
0.0	28	−	95	3	68	−	219	9	8.2	−	8.3	6.2	11	12
1.5	28	49[b]	95	11	68	92[b]	219	35	8.1	3.3	8.1	6.5	12	51
3.5	28	114[b]	95	14	68	238[b]	219	44	8.3	3.8	8.2	6.7	12	66
5.0	28	163[b]	95	18	68	380[b]	219	57	8.1	4.3	8.1	6.2	12	73

[a] The vesicles were composed of 10% DOPS, 85% egg PC, and the indicated amounts of DAG in PKC assay buffer with 8 μM MARCKS peptide, 0.5 μM PKC, and 10 μM Ca^{2+}. All the values were determined by averaging the results from 55 vesicles. The fold of enrichment in the domains was calculated using the highest radiance values in the domains over the average radiance values of the vesicles. The measurements of the domains were done in two separate triple-labeling experiments as described in Figure 1G,H. [b] The vesicles were always labeled with 1.5% dansyl-DAG. The radiance values in the table were corrected as if there was 100% labeled DAG present in the vesicles. (−) absent.

Table 6: Effect of Different Concentrations of PS on the Formation of Domains and the Activity of PKC[a]

PS	mean radiance value for whole vesicles				mean radiance value of domains				max enrichment in domains (fold of enrichment)				domain	activity
(mol %)	MARCKS	DAG	PS	PKC	MARCKS	DAG	PS	PKC	MARCKS	DAG	PS	PKC	size (%)	(%)
0	2	49	–	–	–	–	–	–	–	–	–	–	–	2
2	8	49	630[b]	22	25	157	1860[b]	70	8.2	5.8	9.4	6.3	1	5
4	20	49	1260[b]	22	60	157	3730[b]	70	8.0	5.8	9.3	6.4	5	40
10	27	49	3160[b]	22	69	157	9330[b]	70	8.5	5.9	9.4	6.9	12	100
20	30	49	6330[b]	23	72	156	18660[b]	71	8.4	5.8	9.3	6.8	18	110

[a] The vesicles were composed of 5% DAG, 85% egg PC, and the indicated amounts of PS in PKC assay buffer with 8 μM MARCKS peptide, 0.5 μM PKC, and 300 μM Ca^{2+}. All the values were determined by averaging the results from 55 vesicles. The fold of enrichment in the domains was calculated using the highest radiance values in the domains over the average radiance values of the vesicles. The measurements of the domains were done in two separate triple-labeling experiments as described in Figure 1G,H. [b] The vesicles were always labeled with 0.3% NBD-PS. The radiance values in the table were corrected as if there was 100% labeled PS in the vesicles. (−) absent.

varied in order to understand their effects on the formation of domains and the binding of PKC. Tables 4 and 5 show that when the concentration of either Ca^{2+} or DAG was increased, both the binding of PKC to the vesicles and the amounts of PKC in the domains increased. Without Ca^{2+}, the distribution of DAG in vesicles was uniform. Increasing the Ca^{2+} concentration caused DAG to partition into the domains and also increased the amounts of PS and PKC in the domains but did not affect the amounts of the MARCKS peptide in the domains (Table 4). Increasing the DAG concentration in the vesicles caused increased amounts of DAG and PKC in the domains, but did not affect the amount of the MARCKS peptide or the amount of PS in the domains (Table 5). The mean radiance values of PKC in the domains increased from 16 to 70 when the Ca^{2+} concentration was increased from 0 to 300 μM and the DAG concentration was maintained at 5% in the vesicles. When DAG was increased from 0 to 5% in the vesicles and Ca^{2+} was maintained at 10 μM, the mean radiance values of PKC in domains increased from 9 to 57 (Table 5). There was a proportional increase in PKC binding to the vesicles as well as the domains. The increase in PKC activity always corresponded well with the increased amounts of PKC in the domains and with the higher binding of PKC to whole vesicles. Since the mean radiance values of the MARCKS peptide in whole vesicles and in the domains remained the same in these experiments, it was obvious that the increase in PKC activity resulted directly from the increased binding of PKC to the domains and vesicles.

Effects of PS on the Formation of Domains. Increasing the PS concentration in the vesicles had no significant effect on the enrichment of all the components in the domains (Table 6). However, the sizes of the domains changed dramatically when the concentration of PS was increased and the rate of the PKC reaction increased proportionally to the size of the domains. The total binding of PKC to the vesicles was basically unchanged under these conditions, but the binding of the MARCKS peptide to the vesicles increased. PKC did not bind to vesicles without PS (vesicles made up of 100 mol % PC). At the lowest concentration of PS used in these experiments (2 mol %), all the PKC in the reaction mixture bound to the vesicles under these conditions.

The size and the enrichment of PS domains were also examined before and after the addition of PKC without changing the other conditions. The results showed that the addition of PKC caused the size of PS domains to decrease and the enrichment of the domains to increase (data not shown). For example, when vesicles containing 10% DOPS were used, the average size of the PS domains was 18% of the whole vesicle before the addition of PKC, but it was reduced to 12% upon the addition of PKC. Concurrently, the enrichment of the PS in the domains increased from 6.6-fold to 9.4-fold. Similarly, in the 10% DOPS vesicles, the enrichment of the MARCKS peptide in the domains increased from 4.8-fold to 8.5-fold, and DAG in the domains increased from 3.6-fold to 5.9-fold as determined on a population of vesicles. The sizes of the domains enriched with PKC, DAG, the MARCKS peptide, and PS were always comparable.

Compositional Analysis of the Domains. Fluorescence digital imaging microscopy permits quantitative analysis of the concentration of a fluorescent molecule in the domains (Luan et al., 1995). For the purpose of these experiments, domains are defined as all areas in vesicles with radiance values 1.5 times higher than the average radiance values of the images. The average radiance values in the domains of a population of vesicles were measured and used for the calculations. When PKC was activated in vesicles composed of 10 mol % DOPS, 5 mol % DAG, and 85 mol % egg PC in the complete PKC assay buffer, the domains contained 32 mol % PS, 15 mol % DAG, and 53 mol % PC. That is, on average, PS and DAG in the domains were enriched approximately 3 times and PC was depleted about 1.5 times. In addition, the domains contained 1 mol of PKC and 5.2 mol of the MARCKS peptide per 100 mol of lipids.

Many domains found in the vesicles had an area of approximately 40 × 40 pixels, which is equivalent to 8×10^6 nm^2. The average area occupied by one phospholipid is approximately 0.68 nm^2 (McInsoth & Simon, 1986). Therefore, in an average domain there are about 1.18×10^7 lipids in the outer leaflet of vesicles (assuming DAG would occupy the same area as a phospholipid) composed of 0.62 million PC molecules, 0.38 million PS molecules, 0.18 million DAG molecules, 0.05 million MARCKS peptide, and 0.01 million PKC molecules.

Model for the Activation of PKC by Membrane Domains. A general model for the activation of PKC is proposed in Figure 2 on the basis of what is already known about PKC plus the inclusion of domain formation suggested by the results of this study. First, domains containing two components, the MARCKS protein and PS, are formed. This depends on the nature of the substrate, of course, but it is interesting to note that many PKC substrates are basic. If other substrates do not form domains themselves with PS, they would have to bind to the domains formed by PKC at some time in order for the reaction to take place. The second step occurs when extracellular stimuli activate a plasma membrane receptor that in turn activates an isoform of phospholipase C. This results in the breakdown of phosphatidylinositol 4,5-bisphosphate to give IP_3 and DAG (Rana & Hokin, 1990). The increased concentration of IP_3 leads to an increased concentration of cytoplasmic Ca^{2+} that binds to the domains. In the next step, the Ca^{2+} in the domains induces DAG molecules to partition into the domains to form a four-component domain (substrate, PS, DAG and Ca^{2+}).

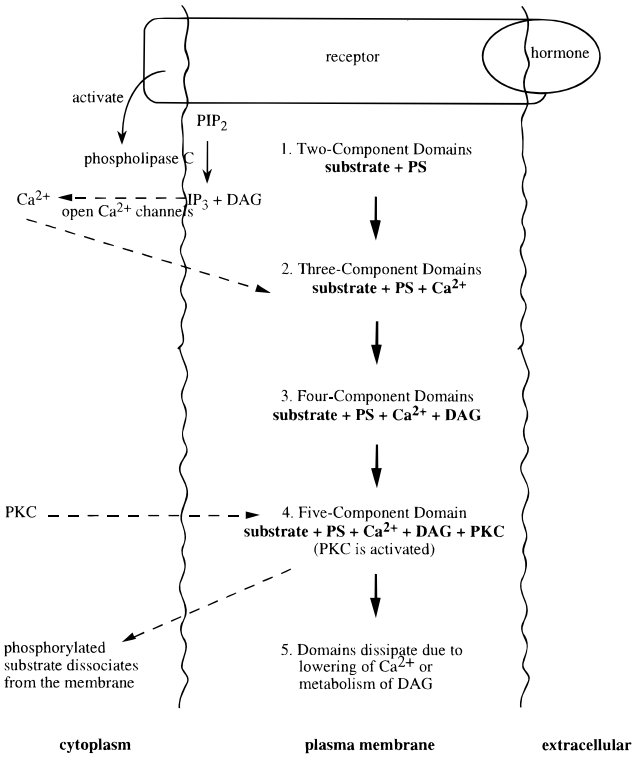

FIGURE 2: A model for the activation of PKC by membrane domains. First, the substrate (in this case, the MARCKS protein) induces the formation of PS domains in the plasma membrane to form two-component domains (substrate and PS) while inactive PKC is located in the cytoplasm. Second, when phospholipase C is activated, the increased Ca^{2+} in the cytoplasm binds to the domains, and third, it induces DAG to partition into the domains to form four-component domains (substrate, PS, DAG, and Ca^{2+}). Fourth, the presence of DAG and Ca^{2+} in the domains greatly increases the affinity of PKC for the domains; PKC binds to the domains and becomes activated (five-component domains). After the substrate is phosphorylated, the substrate dissociates from the domains and the domains dissipate due to lowering of the calcium concentration or metabolism of DAG.

In the fourth step, the domains of DAG, and Ca^{2+} greatly increase the affinity of PKC for the PS domains. Thus, the cytoplasmic PKC translocates to the domains and becomes fully activated. After the reaction occurs, the phosphorylated MARCKS peptide dissociates from the domains. Either new substrate binds to the domains or the domains dissipate as the final step due to metabolism of DAG and/or the lowering of the cytoplasmic calcium concentration.

DISCUSSION

A previous study found that Ca^{2+} and the MARCKS peptide were capable of inducing the formation of PS domains (Yang & Glaser, 1995). The experiments in this paper show that PKC alone induced the formation of PS domains, but this did not occur when 100 mM NaCl was added if DAG and Ca^{2+} were not present in the vesicles (Table 3). If DAG and Ca^{2+} were present, the high ionic strength could no longer block the formation of PKC and PS domains. The amount of PKC in the domains increased when more Ca^{2+} and DAG were added to the vesicles (Tables 4 and 5). When PKC was activated, a domain enriched in all the three activators (PS, DAG, and Ca^{2+}), the substrate (the MARCKS peptide), and the enzyme (PKC) formed in the vesicles.

The total binding of the MARCKS peptide to whole vesicles was not changed when polylysine was added, but

the substrate was displaced from the domain area to the nondomain areas of the vesicles (Table 2). The other components in the domains remained almost unchanged in this experiment. The PKC activity decreased proportionally to the reduction in the MARCKS peptide in the domains. This experiment, in particular, illustrates the importance of the partitioning of the MARCKS peptide into the domains for PKC activity.

To accurately evaluate the initial composition of the domains without any reaction taking place, advantage was taken of the ATP analog, AMP-PNP (Yount et al., 1971). With ATP in the complete reaction mixture, the MARCKS peptide dissociated from vesicles. This was demonstrated by Kim et al. (1994), who found that phosphorylation reversed the electrostatic association between the MARCKS peptide and vesicles composed of PS and PC. Other studies have reported that phosphorylation causes a translocation of the intact MARCKS protein from the membrane to the cytoplasm and this may represent a simple electrostatic switch mechanism (McLaughlin & Aderem, 1995). In addition, the MARCKS protein has been found in clusters in some cells, and it associates with PKC in nascent phagosomes (Allen & Aderem, 1995).

Besides the amount of the MARCKS peptide in the domains, the experiments in Tables 4 and 5 show that the binding of PKC to the vesicles and into domains was also important for PKC activity. The present study showed that Ca^{2+} was the only factor to cause the formation of DAG domains. Increasing either Ca^{2+} or DAG alone did not fully activate the enzyme. It seemed, therefore, that Ca^{2+} and DAG together were responsible for causing the optimal translocation of PKC to the domains and for the full activation of the enzyme. In all these experiments, a higher PKC activity was always accompanied by increased amounts of PKC in the domains. Epand and Bottego (1988) showed that DAG lowered the hexagonal phase transition more with PS/PE mixtures in the absence of Ca^{2+} than in its presence. This suggests that with Ca^{2+} the DAG is mixing less with PE and more with PS, consistent with the role of Ca^{2+} in forming DAG domains as observed here.

It has been found that the activation of PKC was highly cooperative with respect to PS in detergent micelles (Hannun & Bell, 1990; Newton & Koshland, 1990). Orr and Newton (1992a,b) reported that the activity and binding of PKC reached a plateau when >12 molecules of PS were included in micelles. The dependence of PKC binding on the mole percent of PS in vesicles is also highly sigmoidal (Mosior & Epand, 1993). In this study, PKC activity increased with increasing PS concentration in the vesicles (Table 6). In this case the increase in PS concentrations caused the size of the domains to increase, and this was proportional to the increase in the activity. The enrichment of all the components remained the same. The amount of the MARCKS peptide in the domains and in the vesicles increased as well, but this was a smaller effect as compared with the increase in domain size and the PKC activity.

A micelle made with lipids composed of 10 mol % PS and 5 mol % DAG in Triton X-100 contained approximately 16 molecules of PS and 8 molecules of DAG at 25 °C (Newton, 1993). It was reported by Hannun and Bell (1985) that one monomer PKC binds to one micelle. The results presented here demonstrated that when PKC was activated in vesicles formed with 10% DOPS, 5% DAG, and 85% egg PC at 0.3 mM Ca^{2+} and 8 μM MARCKS peptide, the domains contained 32 mol % PS, 15 mol % DAG, 53 mol % PC, 1 mol % PKC (1 mol of PKC/100 mol of lipids), and 5.2 mol % of the MARCKS peptide (5.2 mol of the MARCKS peptide/100 mol of lipids).

In these experiments, 300 μM calcium was routinely used, which is a saturating concentration for PKC activity. However, weak PS and DAG domains were visualized in the presence of approximately 1 μM Ca^{2+} without addition of other components. Further experiments are necessary to determine the effects of Ca^{2+} on domain formation in other circumstances. The lowest DAG found to form domain was approximately 0.3 mol % in the vesicles. Vesicles with lower amounts of DAG did not give adequate signal to produce good images.

In all the experiments done in this study, the enrichment of the MARCKS peptide and PKC in the domains did not change very much. The enrichment of the MARCKS peptide in the domains was affected by the addition of polylysine and high ionic strength, and the enrichment of PKC in the domains was only affected by high ionic strength. These results suggested that the enrichment of the domains with the MARCKS peptide and PKC was similar in most of the cases.

According to the results in this study, a model for the activation of PKC by membrane domains is proposed (Figure 2). Physiological ionic strength prevents the formation of membrane domains with PKC and PS in the absence of Ca^{2+} and DAG, so PKC stays in the cytoplasm before being activated as demonstrated in Table 3. The substrate (in this case, the MARCKS protein) forms domains with PS in the membranes because it has a strong ability to induce PS domains. When hormone binds to the external receptors, phospholipase C becomes activated, producing IP_3 and DAG (Rana & Hokin, 1990). IP_3 opens Ca^{2+} channels, and the increased Ca^{2+} in the cytoplasm induces DAG to partition into the domains (PS and substrate). Other pathways are possible, of course, since DAG can be formed by hydrolysis of lipids other than PIP_2, for example. Once DAG and Ca^{2+} are enriched in the domains, the affinity of PKC for the domains is greatly increased, so PKC binds to the domains and becomes activated. PKC phosphorylates the substrate in the domains and the product of the reaction, the phosphorylated MARCKS, leaves the domains.

The principal function of domains in these vesicles is to increase the effective concentration of the activators, the enzyme, and the substrate in the same location. The reaction would occur in the absence of domains if the concentration of the components was sufficiently high. Diacylglycerol and phorbol esters will cause conventional PKC to bind to neutral vesicles in the absence of Ca^{2+} and PS, for example (Mosior & Newton, 1996). Also, novel isoforms would not require Ca^{2+}-induced DAG domains. The presence of all the components at high concentrations in domains optimizes the process, however. In cellular membranes, where many other components are present as well, domains could also increase the specificity of the interactions necessary for the reaction and provide a novel level of regulation. By causing a component to partition either into or out of a domain, the reaction could be closely regulated.

In summary, the formation of domains enriched with PKC, PS, DAG, Ca^{2+}, and substrate in vesicles was visualized *via* fluorescence digital imaging microscopy. By concentrating

these components that are necessary to activate PKC and carry out the reaction into domains, the efficiency of the process is increased. This concept may reflect a general feature of biological membrane and provide another level of control to regulate membrane reactions (Thompson et al., 1995). In general, it seems reasonable that the organization of membrane components, e.g., components of signal transduction pathways, into functional domains, would lead to greater efficiency and specificity in carrying out membrane processes.

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