The C2 domains of conventional protein kinase C (PKC) have been implicated in their Ca$^{2+}$-dependent membrane binding. The C2 domain of PKC-$\alpha$ contains several Ca$^{2+}$ ligands that bind multiple Ca$^{2+}$ ions and other putative membrane binding residues. To understand the roles of individual Ca$^{2+}$ ligands and protein-bound Ca$^{2+}$ ions in the membrane binding and activation of PKC-$\alpha$, we mutated five putative Ca$^{2+}$ ligands (D187N, D193N, D246N, D248N, and D254N) and measured the effects of mutations on vesicle binding, enzyme activity, and monolayer penetration of PKC-$\alpha$. Altered properties of these mutants indicate that individual Ca$^{2+}$ ions and their ligands have different roles in the membrane binding and activation of PKC-$\alpha$. The binding of Ca$^{2+}$ to Asp$^{187}$, Asp$^{193}$, and Asp$^{246}$ of PKC-$\alpha$ is important for the initial binding of protein to membrane surfaces. On the other hand, the binding of another Ca$^{2+}$ to Asp$^{248}$, Asp$^{254}$, and Asp$^{255}$ induces the conformational change of PKC-$\alpha$, which in turn triggers its membrane penetration and activation. Among these Ca$^{2+}$ ligands, Asp$^{246}$ was shown to be most essential for both membrane binding and activation of PKC-$\alpha$, presumably due to its coordination to multiple Ca$^{2+}$ ions. Furthermore, to identify the residues in the C2 domain that are involved in membrane binding of PKC-$\alpha$, we mutated four putative membrane binding residues (Trp$^{245}$, Trp$^{247}$, Arg$^{249}$, and Arg$^{252}$). Membrane binding and enzymatic properties of two double-site mutants (W245A/W247A and R249A/R252A) indicate that Arg$^{249}$ and Arg$^{252}$ are involved in electrostatic interactions of PKC-$\alpha$ with anionic membranes, whereas Trp$^{245}$ and Trp$^{247}$ participate in its penetration into membranes and resulting hydrophobic interactions. Taken together, these studies provide the first experimental evidence for the role of C2 domain of conventional PKC as a membrane docking unit as well as a module that triggers conformational changes to activate the protein.

The protein kinase C (PKC)$^1$ family is a set of serine/threonine kinases that transduce the myriad of signals activating cellular functions and proliferation (1–3). More than 10 members of the PKC family have been identified by molecular cloning. Based on common structural features, PKCs are generally classified into three groups; conventional PKC ($\alpha$, $\beta$, $\beta\iota$, and $\gamma$ subtypes), novel PKC ($\delta$, $\epsilon$, $\eta$, and $\theta$ subtypes), and atypical PKC ($\zeta$ and $\iota$ subtypes). Conventional PKCs are activated by the Ca$^{2+}$-dependent translocation to the membrane containing phosphatidyl serine (PS) and diacylglycerol (DG). It has been proposed that the C2 domain of conventional PKCs is involved in this Ca$^{2+}$-dependent membrane binding activity (4). On the other hand, novel PKCs and atypical PKCs that have either a modified or no C2 domain can be activated in a Ca$^{2+}$-independent way (5, 6). In addition to PKC, the C2 domain has been found in a wide variety of proteins that are involved in diverse cellular functions (7). Sequence alignment of C2 domains of these proteins suggests that all known C2 domains exhibit either type I or type II topology, differing slightly in their $\beta$-strand connectivity (7). The C2 domains of conventional PKCs and synaptotagmins have type I topology and show significant sequence homology (see Fig. 1A). High resolution crystal structures have been determined for the isolated C2 domains of synaptotagmin, phospholipase C-$\gamma$, and cytosolic phospholipase $A_2$ (8−10). Despite noticeable variations in primary structures, all of these proteins have highly homologous tertiary structural folds consisting of eight antiparallel $\beta$-strands and connecting loops (Fig. 1B). These structures of C2 domains have defined much of Ca$^{2+}$-ligands in the Ca$^{2+}$ binding sites, which consist of three Ca$^{2+}$ binding loops dubbed calcium binding region 1 (CBR1), CBR2, and CBR3. Multiple Ca$^{2+}$ ions have been located within these sites, and the binding of these Ca$^{2+}$ ions shows positive cooperativity (11). A recent NMR study assigned two Ca$^{2+}$ ions (CA1 and CA2) bound to the C2 domain of a conventional PKC, PKC-$\beta$ (12). A putative coordination pattern of the two Ca$^{2+}$ ions, based on this study and the tertiary structure of homologous synaptotagmin C2A domain, is illustrated in Fig. 1C. At present, the roles of individual Ca$^{2+}$ ligands and Ca$^{2+}$ ions bound to these ligands in the membrane binding and activation of conventional PKC are not fully understood. Furthermore, other residues in the C2 domain that are involved in membrane binding of conventional PKC have not been identified. To address these questions, we mutated several residues in the C2 domains of PKC-$\alpha$, including five putative Ca$^{2+}$ ligands and four putative membrane binding residues. Membrane binding affinity, enzyme activity, and membrane penetrating power of these mutants demonstrate that individual Ca$^{2+}$ ions and their ligands have distinct roles in the membrane binding and activation of PKC-$\alpha$. These studies also identify the C2 domain residues of PKC-$\alpha$ that are involved in its electrostatic and hydrophobic interactions with membranes.

**EXPERIMENTAL PROCEDURES**

**Materials—**1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; CBR, calcium binding region; DG, diacylglycerol; PG, phosphatidyl glycerol; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; PS, phosphatidyl serine.
Membrane and Cytochemical Analysis

Mutagenesis—Baculovirus transfer vectors encoding the cDNA of PKC-α with appropriate C2 domain mutations were generated by the overlap extension polymerase chain reaction (16) using pVL1392-PKC-α plasmid (17) as a template. Briefly, appropriate complementary synthetic oligonucleotides introducing the desired mutation and two other primers at the beginning of the PKC-α gene and around theNeoI site inside the PKC-α gene were used as primers for polymerase chain reactions, which were performed in a DNA thermal cycler (Perkin-Elmer) using Pfu DNA polymerase (Stratagene). The method consisted of two steps. In the first step, two DNA fragments overlapping at the mutation site were generated and purified on an agarose gel. Then, these two fragments were combined to generate the fusion product, which was further amplified by polymerase chain reaction. The product with the desired mutation was then used to prepare a ligated construct with the desired C2 domain mutation, which was digested with NcoI, dephosphorylated with alkaline phosphatase to prevent self-ligation, and purified on an agarose gel. The mutagenesis was verified by DNA sequencing of the PKC-α gene using a Sequenase 2.0 kit (Amersham Pharmacia Biotech).

Expression of PKC-α and Mutants in Baculovirus-infected Sf9 Cells—Wild type PKC-α and mutants were expressed in baculovirus-infected Sf9 cells (Invitrogen, La Jolla, CA). Transfection of Sf9 cells was performed using BaculoGoldTM Transfection Kit from Pharmingen (San Diego, CA). After transfection, endotoxins were removed from plasmid DNA using LPS extraction kit (Qiagen, Valencia, CA). Cells were incubated for 4 days at 27 °C, and the supernatant was collected and used to infect more cells for the amplification of virus. After three cycles of amplification, high-titer virus stock solution was obtained. Sf9 cells were maintained as monolayer cultures in TMN-FH medium (Invitrogen) containing 10% fetal bovine serum (Life Technologies, Inc.). For protein expression, 3 × 10^6 cells/ml in 500-ml suspension culture was incubated with the multiplicity of infection (MOI) of 10, and then cultured for 3 days at 27 °C. For harvesting, cells were centrifuged at 1000 × g for 10 min, washed once with Tris-HCl buffer, pH 7.5, and resuspended in 25 ml of extraction buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 50 µM µl peptin, 1% Triton X-100, and 0.2 mM phenylmethylsulfonyl fluoride. The suspension was homogenized in a hand-held homogenizer (Polytron) and the homogenate was centrifuged at 50,000 × g for 40 min. The supernatant was collected and used to infect more cells for the amplification of virus. After three cycles of amplification, high-titer virus stock solution was obtained. Sf9 cells were maintained as monolayer cultures in TMN-FH medium (Invitrogen) containing 10% fetal bovine serum (Life Technologies, Inc.). For protein expression, cells were grown to 2 × 10^6 cells/ml in 500-ml suspension culture and infected with the multiplicity of infection (MOI) of 10, and then cultured for 3 days at 27 °C. For harvesting, cells were centrifuged at 1000 × g for 10 min, washed once with Tris-HCl buffer, pH 7.5, and resuspended in 25 ml of extraction buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 50 µM µl peptin, 1% Triton X-100, and 0.2 mM phenylmethylsulfonyl fluoride. The suspension was homogenized in a hand-held homogenizer (Polytron) and the homogenate was centrifuged at 500,000 × g for 1 hr. The supernatant was loaded onto a 100-ml Q-Sepharose Fast Flow column (Amersham Pharmacia Biotech). After washing with 100 ml of Buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol), the column was eluted with 200 ml of a linear salt gradient from 0.5 to 5 M KCl in Buffer A. Active PKC fractions were pooled, adjusted to 2 mM KCl, and loaded onto a 10 ml Poros PE column (Boehringer Mannheim) with a flow rate of 4 ml/min. A linear salt gradient from 2 to 0 M KCl in Buffer A (total volume, 60 ml) was applied. Active PKC fractions were concentrated and desalted in an Ultrafree-15 centrifugal filter device (Millipore) and stored in Buffer A containing 50% glycerol at −20 °C. Protein concentration was determined by the bicinchoninic acid method (Pierce).

Determination of PKC Activity—Activity of PKC was assayed by measuring the initial rate of [γ-32P]ATP incorporation from [γ-32P]ATP (50 µM, 0.6 µCi/tube) into the histone III-SS (400 µg/ml). The reaction mixture contained large unilamellar vesicles (0.2 mM), 5 mM MgCl2, 12 mM PKC, and various concentrations of CaCl2 (see under “Results”) in 50 µl of 20 mM HEPES, pH 7.0. Protein kinase activity was determined by [γ-32P]ATP incorporation into histone III-SS. The reaction mixture was incubated at room temperature for a given period of incubation (e.g.5 min for histone) and the reaction was stopped by the addition of 50 µM of an aqueous phosphoric acid solution after a given period of incubation. The reaction was started by adding MgCl2 to the mixture and quenched by adding 50 µl of 1% aqueous phosphoric acid solution after a given period of incubation (e.g.5 min for histone) at room temperature. Seventy-five-µl aliquots of quenched reaction mixtures were spotted on P-81 ion-exchange papers (Whatman), washed four times with 1% aqueous phosphoric acid solution, and washed once with 95% aqueous ethanol. Papers were transferred into scintillation vials containing 4 ml of scintillation fluid (Sigma), and radioactivity was measured by liquid scintillation counting. The linearity of the time dependence of the background-subtracted radioactivity was checked by monitoring the degree of phosphorylation at regular intervals.

Protein Kinase C-Vesicle Binding—The binding of PKC to phospholipid vesicles was measured by a centrifugation assay using sucrose-loaded large unilamellar vesicles (100 nm in diameter) (19). Sucrose-loaded vesicles were prepared as described elsewhere (20). Briefly, the lipid solution was added to a redi-mixed flask, and organic solvent was removed by rotary evaporation. The lipid film was suspended in 20 mM HEPES buffer, pH 7.0, containing 0.2 mM sucrose and vortexed vigorously. Unilamellar vesicles were prepared by multiple extrusion through a 0.1 µm polycarbonate filter (Millipore) in a Liposofast microextruder (Avestin, Ottawa, Ontario, Canada). The vesicle solution was diluted 5 times with 20 mM HEPES buffer, pH 7.0, containing 0.1 mM CaCl2 and centrifuged at 100,000 × g for 30 min at 25 °C. The supernatant was removed, and the lipid pellet was resuspended in the same buffer solution. The final concentration of vesicle solution was determined by measuring the radioactivity of a trace of [γH]POPC (typically 0.1 mol %) included in all phospholipid mixtures. For binding experiments, PKC (approximately 12 µg) was incubated for 15 min with sucrose-loaded vesicles and CaCl2 (final concentration, 10 mM). Then, the reaction was quenched with 150 µl of 20 mM HEPES (pH 7.0) containing 100 mM KCl. Bovine serum albumin was added to minimize the loss of protein due to nonspecific adsorption to tube walls. Vesicles were pelleted at 100,000 × g for 30 min using a Servall RC-M120EX microcentrifuge. Aliquots of supernatants were used for protein determination by PKC activity assay using protamine sulfate as a substrate. The fraction of bound enzyme was plotted against mol percentage of anionic lipid in vesicles or against free Ca2+ concentration.

Monolayer Measurements—Surface pressure (π) of solution in a circular Teflon trough was measured using a du Nouy ring attached to a computer-controlled Cahn electrobalance (Model C-32) as described previously (17, 21, 22). The trough (4 cm in diameter × 1 cm deep) has a magnetic stir bar and a small angle through the wall to allow an addition of protein solution. Five to 10 microliters of phospholipid solution in ethanol/hexane (1.9 v/v) or chloroform was spread onto 10 ml of subphase (20 mM HEPES, pH 7.0 containing either 0.1 or 0.5 mM of free Ca2+) to form a monolayer with a given initial surface pressure (πo). The subphase was continuously stirred at 60 rpm with a magnetic stir bar. Once the surface pressure reached a stable level, the monolayer was allowed to equilibrate (after 10 min) and the protein solution (typically 50 µl) was injected to the subphase, and the change in surface pressure (Δπ) was measured as a function of time at 23 °C. Typically, the Δπ value reached a maximum after 20 min. The maximal Δπ value depended on the protein concentration at the low concentration range and reached a saturation when the protein concentration was higher than 1 µM. Protein concentrations were therefore maintained below 1.5 µM/ml to ensure that the observed Δπ represented a maximal value. The critical surface pressure (πc) was determined by extrapolating the Δπ versus πo plot to the x axis.

Equilibrium Dialysis Measurements—Equilibrium dialysis was carried out at room temperature using a MEGA TM System microdialyzer (Pierce) with separated sample chambers. Dialysis membranes with 3500 molecular weight cut-off were used. Prior to calcium binding measurements, protein solutions were concentrated in Ultrafree-4 centrifugal filter units (Millipore) and recovered with 20 mM HEPES, pH 7.0 to remove EGTA and EDTA present in the storage buffer. Twenty-five microliters of PKC-α and selected mutant solutions (final concentration, 10–15 µM) in individual sample chambers were dialyzed against 30 ml of 20 mM HEPES buffer, pH 7.0, containing 0.1 mM CaCl2, 0.5 mM DTT, and 0.1 mM 4CaCl2 (specific activity of 7 Ci/mmol). Controls contained 25 µl of 20 mM HEPES, pH 7.0, instead of a protein solution. After equilibration for 19 hr, free and total Ca2+ concentrations were determined by counting the radioactivity of 5-µl aliquots from control and protein chambers, respectively, from which the radioactivity of bound Ca2+ was calculated.

RESULTS

Design and Physical Properties of PKC-α Mutants—According to the model structure of the C2 domain of PKC-α shown in Fig. 1C, five Ca2+-binding aspartyl residues can be classified into three groups; CB1R ligands, which primarily coordinate CA1 (Asp187 and Asp189), CB2R ligands, which mainly coordi-
nate CA2 (Asp\textsuperscript{248} and Asp\textsuperscript{254}), and Asp\textsuperscript{246}, which coordinates both Ca\textsuperscript{2+} ions. In addition to Asp\textsuperscript{246}, Asp\textsuperscript{187} and Asp\textsuperscript{248} could also be involved in partial coordination to the other Ca\textsuperscript{2+}. Based on this assignment, the mutations of Asp\textsuperscript{193} and Asp\textsuperscript{254} would only affect the binding of CA1 and CA2, respectively, whereas the mutations of Asp\textsuperscript{187} and Asp\textsuperscript{248} would have effects on the binding of both Ca\textsuperscript{2+} ions. Thus, it is possible to systematically analyze the roles of the two Ca\textsuperscript{2+} ions in the membrane binding and activation of PKC-\textalpha by selectively mutating their ligands and separately measuring the effects of mutations on membrane binding and activation. From crystal structures of C2 domains of phospholipase C-\textbeta1 and cytosolic phospholipase A\textbeta, it has been proposed that CBR1 and CBR3 are involved in binding to membranes (10, 23): more specifically, CBR3 in membrane penetration and CBR1 in interfacial contact with the lipid head group. There are two relatively conserved tryptophans (Trp\textsuperscript{245} and Trp\textsuperscript{247}) in the CBR3 of PKC-\textalpha: they are absolutely conserved among conventional PKCs and substituted for by either aromatic or hydrophobic residues in other proteins (7). Given the importance of tryptophans in membrane-protein interactions (24–26), we reasoned that the two tryptophans might be involved in penetration into membranes. Also present in CBR3 are two surface-exposed arginines that are relatively conserved among topology I C2 domains (7) and might be involved in electrostatic interactions between PKC-\textalpha and anionic phospholipids, such as PS. To assess the roles of these residues, we generated two double-site mutants, W245A/W247A and R249A/R252A. Unlike CBR3, CBR1 of PKC-\textalpha contains neither conserved hydrophobic nor ionic residues. Because all nine mutated residues are located in loop regions, the above mutations were not expected to cause deleterious conformational changes. Indeed, all seven mutants were expressed in baculovirus-infected insect cells as well as wild type, indicating comparable thermodynamic stability and a lack of gross conformational changes. Furthermore, all mutants exhibited full membrane binding affinity and enzyme activity at saturating Ca\textsuperscript{2+}, PS, and DG concentrations (see below), again demonstrating that the mutations did not significantly disrupt its tertiary structural fold.

Properties of Ca\textsuperscript{2+} Ligand Mutants—To systematically analyze the effects of mutations on the Ca\textsuperscript{2+}-dependent membrane binding and activation of PKC-\textalpha, we measured the following five properties: Ca\textsuperscript{2+} dependence of vesicle binding, PS content dependence of vesicle binding, Ca\textsuperscript{2+} dependence of enzyme activity, PS content dependence of enzyme activity, and monolayer penetration. We previously showed (17) that PKC-\textalpha displayed full vesicle binding affinity if the POPS content was $>20$ mol % in POPC/POPS vesicles containing 2.5 mol % DG under the conditions employed in these studies (see also Fig. 3). We therefore used POPC/POPS/DG (67.5:30:2.5) vesicles to measure the Ca\textsuperscript{2+} dependence of vesicle binding for wild type and mutants. The binding of Ca\textsuperscript{2+} ions to the isolated C2 domain was shown to be consistent with the cooperative Hill model (11). The concentration of Ca\textsuperscript{2+} giving rise to half-maximal binding (or activity) $([\text{Ca}^{2+}]_{1/2})$ was thus determined from curve fitting of data to a Hill equation,

$$y = a \left( \frac{[\text{Ca}^{2+}]^h}{[\text{Ca}^{2+}]^h + [\text{Ca}^{2+}]^D} \right)$$

(Eq. 1)

where $y$, $a$, $h$, and $[\text{Ca}^{2+}]$ are relative binding (or activity),
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FIG. 2. Ca<sup>2+</sup> dependence of vesicle binding of PKC-α and Ca<sup>2+</sup>-ligand mutants. Proteins (12 μM) include wild type (○), D187N ( ●), D193N ( ■), D246N ( ▲), D248N ( △), and D254N ( ▲). Total lipid concentration of POPC/POPS/DG (67.5:30:2.5) vesicles was 0.1 mM. Solid lines represent theoretical curves constructed from parameters determined from the nonlinear least squares fit using Equation 1. Theoretical curves were not generated for those mutants the Ca<sup>2+</sup> values were not large enough to have any physical meaning (data not shown). We then measured the dependence of vesicle binding and activity, indicating that Ca<sup>2+</sup>-dependent vesicle binding and activation are coupled processes. In contrast, all mutants but D193N required much higher Ca<sup>2+</sup> concentrations for half-maximal activity than for half-maximal vesicle binding, suggesting that all Ca<sup>2+</sup> ligands except Asp<sup>187</sup> participate in coordination to a Ca<sup>2+</sup>-ion(s) that is involved in not only the vesicle binding but also the activation of PKC-α. Most notably, mutants of Asp<sup>187</sup> and Asp<sup>246</sup>, which are implicated in coordination to both Ca<sup>2+</sup> ions, resulted in drastic increases in [Ca<sup>2+</sup>]<sub>1/2</sub> (see Table 1). This might be due to the combination of reduced vesicle binding and activation. Less drastic but significant decreases in activity were observed for mutants of Asp<sup>246</sup> and Asp<sup>254</sup>, which mainly coordinate to CA2. Between the two, D248N required higher Ca<sup>2+</sup> than D254N for the same degree of activity, suggesting the relative closeness of Asp<sup>246</sup> to CA2. The dependences of PKC activity of mutants on the PS content of vesicles (Fig. 5) also exhibited a similar pattern. Taken together, these results

Table I: Properties of C2 domain mutants of PKC-α

| PKC-α          | Vesicle binding | Enzyme activity | Monolayer |
|----------------|----------------|-----------------|-----------|
|                | [Ca<sup>2+</sup>]<sub>1/2</sub> | [PS]<sub>1/2</sub> | [Ca<sup>2+</sup>]<sub>1/2</sub> | [PS]<sub>1/2</sub> | π<sub>c</sub> |
| Wild type      | 2.2 ± 0.3       | 15° ± ND        | D187N      | 20° ± 5                        | 38 |
| D187N          | 200 ± 10        | 40° ± ND        | D246N      | 60° ± 20                       | 23 |
| D193N          | 180 ± 10        | 40° ± ND        | D248N      | >1 μM                          | ND |
| D246N          | >1 μM           | 65° ± ND        | D248N      | >1 μM                          | 55° |
| D248N          | 30 ± 3          | 33° ± ND        | D254N      | 55° ± 30                       | 24 |
| D254N          | 60 ± 4          | 30° ± ND        | W245A/W247A| 40° ± 30                       | 25° |
| W245A/W247A    | 500 ± 50        | ND              | R249A/R252A| 20° ± 0.7                      | 36 |

* Measured in the presence of 0.1 mM Ca<sup>2+</sup> (see Fig. 3).
* Measured in the presence of 0.4 mM Ca<sup>2+</sup> (see Figs. 5 and 9).
* ND, not determined.

Next, we measured the PKC activity of wild type and mutants as a function of free Ca<sup>2+</sup> concentration and the PS content in vesicles. If the role of Ca<sup>2+</sup> ions is primarily to bring PKC-α molecules to membranes, the relative enzyme activity of PKC-α mutants would directly reflect their relative vesicle binding affinity. When PKC activity of mutants was measured using POPC/POPS/DG (67.5:30:2.5) vesicles and histone in the presence of varying concentrations of Ca<sup>2+</sup>, however, an unexpected pattern was observed (Fig. 4). Wild type PKC-α showed comparable Ca<sup>2+</sup>-dependences for vesicle binding and activity, indicating that Ca<sup>2+</sup>-dependent vesicle binding and activation are coupled processes. In contrast, all mutants but D193N required much higher Ca<sup>2+</sup> concentrations for half-maximal activity than for half-maximal vesicle binding, suggesting that all Ca<sup>2+</sup> ligands except Asp<sup>187</sup> participate in coordination to a Ca<sup>2+</sup>-ion(s) that is involved in not only the vesicle binding but also the activation of PKC-α. Most notably, mutants of Asp<sup>187</sup> and Asp<sup>246</sup>, which are implicated in coordination to both Ca<sup>2+</sup> ions, resulted in drastic increases in [Ca<sup>2+</sup>]<sub>1/2</sub> (see Table 1). This might be due to the combination of reduced vesicle binding and activation. Less drastic but significant decreases in activity were observed for mutants of Asp<sup>246</sup> and Asp<sup>254</sup>, which mainly coordinate to CA2. Between the two, D248N required higher Ca<sup>2+</sup> than D254N for the same degree of activity, suggesting the relative closeness of Asp<sup>246</sup> to CA2. The dependences of PKC activity of mutants on the PS content of vesicles (Fig. 5) also exhibited a similar pattern. Taken together, these results
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Fig. 4. Ca^{2+} dependence of enzyme activities of PKC-α and Ca^{2+} ligand mutants. Proteins include wild type (○), D187N (□), D193N (●), D246N (▲), D248N (△), and D254N (▲). Total lipid concentration and PKC concentration were 0.2 mM and 12 nM, respectively, in 20 mM HEPES, pH 7.0, containing 0.1 M KCl, 5 mM MgCl2, histone III-SS (400 μg/ml), and varying concentrations of Ca^{2+}. Each data point represents an average of two experiments. The absolute value of maximal activity was 0.30 nmol/μg·min. Solid lines represent theoretical curves constructed from parameters determined from the nonlinear least squares fit using Equation 1.

Fig. 5. Dependence of activities of PKC-α and Ca^{2+} ligand mutants toward histone on the PS content in POPC/POPS vesicles containing 1 mol % DG. Proteins include wild type (○), D187N (□), D193N (●), D246N (▲), D248N (△), and D254N (▲). Ca^{2+} concentration was 0.4 mM, which was higher than that employed for vesicle binding measurements (0.1 mM). Other experimental conditions were the same as in Fig. 4.

suggested that CA2 and its ligands (mostly CBR3 ligands) are essential for the activation of PKC-α although CA2 and some of its ligands (Asp^{248} and Asp^{254}) are not critically involved in membrane binding of protein. This in turn points to distinct roles of the two Ca^{2+} ions and their ligands in the function and regulation of PKC-α.

Our recent study showed that Ca^{2+} promotes the penetration of PKC-α into membranes containing PS, which eventually results in PKC activation (17). If two Ca^{2+} ions indeed have distinct roles, as indicated above, the mutants of their ligands might have different effects on the membrane penetration of PKC-α. To test this notion, we measured the interaction of PKC-α and selected mutants with phospholipid monolayers. Lipid monolayers have proven to be a sensitive tool for measuring lipid-protein interactions (27,28). In this system, the penetration of a protein into a phospholipid monolayer at the air-water interface can be sensitively monitored at constant area or at constant surface pressure. In these studies, a phospholipid monolayer of a given initial surface pressure πo was spread at constant area, and the change in surface pressure (∆π) was monitored after the injection of the protein into the subphase. Those proteins of which the actions involve the partial or full penetration of membranes have an ability to penetrate into the phospholipid monolayer with πo comparable to or higher than that of biological membranes (approximately 31 dyn/cm) (29–32), and vice versa. In general, ∆π is inversely proportional to πo of the phospholipid monolayer, and an extrapolation of the ∆π versus πo plot yields the critical surface pressure (πc) (28), which specifies an upper limit of πo of a monolayer that a protein can penetrate into. Therefore, πc should be above 31 dyn/cm if the protein is able to penetrate into the membrane under a physiological condition. Fig. 6 shows the ∆π versus πo plot for wild type, D187N, D193N, and D248N. As reported previously, PKC-α could penetrate POPC/POPS (5:5) monolayers even when πc > 35 dyn/cm (Table I), demonstrating its ability to penetrate biological membranes. Note that these monolayer measurements were performed under the condition in which wild type and D193N were fully active, whereas D187N and D248N were only partially active (see Figs. 4 and 5). Under this condition, a CBR1 ligand (CA1 ligand) mutant D193N, which exhibited comparable decreases in vesicle binding and activity, showed a slight reduction in monolayer penetrating power compared with wild type. In contrast, two mutants, D187N and D248N, of which the enzyme activities were reduced much more than their vesicle binding affinities were, showed much lower penetrating power; as a result, they would not be able to penetrate into biological membranes under the condition in which wild type and D193N could (see Table I for their πc values). Thus, these results strongly support the notion that the binding of CA2 induces conformational changes of PKC-α, which in turn triggers its membrane penetration and activation. On the other hand, the binding of CA1 might simply anchor the protein to membrane surfaces.

Properties of R249A/R252A—To evaluate the contributions of Arg^{249} and Arg^{252} to the membrane binding and activation of PKC-α, several properties of R249A/R252A were measured. We first measured the Ca^{2+} dependence of its binding to vesicles of different compositions. When the binding to POPC/POPS/DG (67.5:30:2.5) vesicles was measured, wild type and R249A/R252A did not show any significant difference in Ca^{2+} dependence (Fig. 7), implying that the two arginines are not directly
involved in the vesicle binding. It has been shown, however, that DG greatly enhances the hydrophobic interactions between PKC and membranes, thereby rendering the relative contribution from electrostatic interactions less important (17, 33). We therefore measured the Ca\(^{2+}\) dependence of vesicle binding while varying the DG content in the vesicles. As shown in Fig. 7, the smaller the DG content was, the larger the difference in Ca\(^{2+}\) requirement for binding between wild type and R249A/R252A was. In the absence of DG, apparent [Ca\(^{2+}\)]\(_{1/2}\) values were 40 \(\mu\)M for wild type and \(>1\) mM for the mutant. Similar results were obtained for the Ca\(^{2+}\) dependence of PKC activity determined in the presence of POPC/POPS/DG vesicles and histone (Fig. 8). Again, the difference in Ca\(^{2+}\) requirement increased with the decrease in DG content in vesicles. These results thus all point to the importance of Arg\(^249\) and Arg\(^252\) in electrostatic interactions between PKC-\(\alpha\) and anionic membrane surfaces. This notion was further supported by two additional sets of data. First, we measured the dependences of vesicle binding and enzyme activity of wild type and R249A/R252A on the PS content of POPC/POPS/DG vesicles at a fixed Ca\(^{2+}\) concentration. As shown in Fig. 9, R249A/R252A required a higher PS content than wild type to achieve the same degree of vesicle binding and activity. Also, the difference in PS dependence between wild type and R249A/R252A was more pronounced with the decrease of DG content (data not shown). Second, we measured the Ca\(^{2+}\) dependences of binding of PKC-\(\alpha\) and R249A/R252A to POPC/POPS/DG vesicles. It has been shown that PKC-\(\alpha\) binds, albeit less tightly, to phosphatidyl glycerol-containing vesicles via mainly nonspecific electrostatic interactions even in the presence of DG (17, 34). As shown in Fig. 10, R249A/R252A showed a higher Ca\(^{2+}\) requirement than wild type in both the presence and absence of DG. Note that wild type and R249A/R252A exhibited indistinguishable Ca\(^{2+}\) dependence when binding to POPC/POPS/DG (67.5:30:2.5) vesicles. Thus, these results again indicated that Arg\(^249\) and Arg\(^252\) make significant contributions to electrostatic interactions of PKC-\(\alpha\) with anionic membranes. Furthermore, the finding that R249A/R252A had reduced affinity for both PS and phosphatidyl glycerol-containing vesicles suggests that Arg\(^249\) and Arg\(^252\) are not a part of the specific PS binding site. This notion was also consistent with monolayer penetration behaviors of R249A/R252A. Our previous study showed that PKC-\(\alpha\) could selectively penetrate into PS-containing monolayers. If Arg\(^249\) and Arg\(^252\) form a specific PS binding site, R249A/R252A would then show much reduced monolayer penetration into PS-containing monolayers. As shown in Fig. 11, R249A/R252A had essentially the same monolayer penetration power as wild type. Taken all together, these data show that Arg\(^249\) and Arg\(^252\) are involved in nonspecific electrostatic interactions with anionic phospholipids.

Properties of W245A/W247A—Several properties of W245A/W247A were measured to evaluate the contributions of Trp\(^245\) and Trp\(^247\) to the membrane binding and activation of PKC-\(\alpha\). First, the Ca\(^{2+}\) dependences of vesicle binding and activity were measured and compared with that of wild type. As shown in Fig. 12, W245A/W247A had much higher Ca\(^{2+}\) requirement for vesicle binding than wild type (see also Table 1), which indicated much reduced membrane binding affinity. A comparable effect was seen with PKC activity measurements, indi-
cating that the decreased activity mainly derived from the reduced membrane affinity. Because the two tryptophans are located within CBR3, we measured the intrinsic Ca\(^{2+}\) binding affinities of wild type and W245A/W247A in the absence of vesicles to preclude the possibility that the high Ca\(^{2+}\) requirements for the membrane binding and activation of W245A/W247A were due to the local disruption of Ca\(^{2+}\) binding loops and the consequent decrease in intrinsic Ca\(^{2+}\) affinity. Conventional PKCs and isolated C2 domains can bind Ca\(^{2+}\) ions in the absence of phospholipids, albeit with much reduced affinity (11, 35). Although accurate determination of calcium dissociation constant was not performed due to the requirement for a large amount of protein and radiolabeled Ca\(^{2+}\), the Ca\(^{2+}\) binding results illustrated in Fig. 13 demonstrate that wild type and W245A/W247A have comparable intrinsic Ca\(^{2+}\) affinity, whereas D187N has much reduced one. Thus, properties of W245A/W247A shown in Fig. 12 should be mainly due to reduced membrane binding affinity of this mutant. Furthermore, we measured the dependences of vesicle binding and enzyme activity of wild type and W245A/W247A on the PS content of POPC/POPS/DG vesicles at a fixed Ca\(^{2+}\) concentration (Fig. 9). Consistent with its other properties, W245A/W247A required much higher PS than wild type to achieve half-maximal vesicle binding and activity; again, the vesicle affinity and the enzyme activity were reduced to comparable degrees. W245A/W247A was, however, able to display full wild type activity at higher PS contents (>60 mol %), indicating that the decrease in its activity was not due to a deleterious gross conformation change. Finally, we measured the monolayer penetrating ability of W245A/W247A to find out if the tryptophans are involved in membrane penetration of PKC-\(\alpha\). Fig. 11 shows that Trp 245 and Trp 247 play an important role in the membrane penetration of PKC-\(\alpha\). Thus, these results corroborate the notion that the CBR3 of PKC-\(\alpha\) containing Trp 245 and Trp 247 is involved in membrane penetration.
This report describes a systematic structure-function analysis on the C2 domain of PKC-α. In particular, it represents the first investigation to dissect the roles of different Ca\(^{2+}\) ions and their ligands in the membrane binding and function of C2 domain-containing protein. The C2 domain has been found in a wide variety of proteins and shown to be involved in Ca\(^{2+}\) signaling for some of these proteins (7). The ability of the C2 domain to bind phospholipid vesicles in a Ca\(^{2+}\)-dependent manner has been demonstrated by functional expression and characterization of recombinant C2 domain fragments of synaptotagmin (36-37) and cytosolic phospholipase A\(_2\) (7, 11, 38). The structural studies of C2 domains of synaptotagmin, phospholipase C-51, and cytosolic phospholipase A\(_2\) revealed the presence of three potential Ca\(^{2+}\) binding sites but all three proteins bind two Ca\(^{2+}\) ions in two of these sites. These studies were analyzed using the model structure of PKC-α C2 domain with two bound Ca\(^{2+}\) ions. This model is based on sequence homology to synaptotagmin (see Fig. 1A) and a NMR titration study of PKC-β-Ca\(^{2+}\) binding (12). It is thus possible that the actual conformation of Ca\(^{2+}\) binding loops of PKC-α might be slightly different from the model structure shown in Fig. 1, B and C, and that a different number of Ca\(^{2+}\) ions (e.g. three instead of two) might bind to the Ca\(^{2+}\) binding loops. This uncertainty will be resolved by determining the high resolution structure of PKC-α C2 domain complexed with Ca\(^{2+}\) ions. Nevertheless, the conclusions from these structure-function studies as to differential roles of Ca\(^{2+}\) binding ligands/Ca\(^{2+}\) ions and the involvement of Arg\(_{240}\)/Arg\(_{252}\) and Trp\(_{245}\)/Trp\(_{247}\) in electrostatic and hydrophobic membrane binding, respectively, will not be significantly affected by minor discrepancy between model and true structures.

**Differential Roles of Ca\(^{2+}\) Binding Ligands and Ca\(^{2+}\) Ions**—At least three roles have been proposed for Ca\(^{2+}\) ions in the regulation of proteins containing the C2 domain. One possible role is to alter the surface electrostatic potential of the C2 domain. This possibility has been ruled out by a recent mutagenesis study of PKC-β\(_{1}\), C2 domain (39). The second postulated role is that Ca\(^{2+}\) ions provide a bridge between PKC and membranes by coordinating to both protein and anionic phospholipids. This is similar to the role of Ca\(^{2+}\) ions proposed for annexins (40, 41). Finally, Ca\(^{2+}\) ions can induce a conformational change of protein, which in turn triggers interactions of PKC with membranes. The last mechanism has been controversial due to conflicting observations from different structural studies. For instance, a NMR study of PKC-β revealed no significant conformational change induced by Ca\(^{2+}\) binding (12). On the other hand, the coordination of multiple Ca\(^{2+}\) ions severely disrupted the crystal of the C2 domain of synaptotagmin (8). A recent study of the isolated C2 domain of cytosolic phospholipase A\(_2\) showed that two Ca\(^{2+}\) ions bind to the C2 domain with positive cooperativity, which induces intradomain conformational changes and drives the membrane interactions (11). Similarly, our results indicate that Ca\(^{2+}\) ions not only anchor the protein to membrane surfaces but also induce conformational changes resulting in PKC activation. Most importantly, all evidence indicates that individual Ca\(^{2+}\) ions are differentially involved in these processes. Differential roles of two Ca\(^{2+}\) ions and their ligands are clearly illustrated in unparalled Ca\(^{2+}\) (and PS) dependence of vesicle binding and that of enzyme activity seen for most Ca\(^{2+}\) ligand mutants (D187N, D246N, D248N, and D254N). For D193N, the decreased enzyme activity is directly correlated with its reduced membrane affinity. Because Asp\(_{193}\) would only coordinate CA1, this finding supports the notion that CA1 is involved mainly in initial membrane anchoring of protein. This notion is also consistent with monolayer data showing that D193N can penetrate POPC/POPS monolayers almost as well as wild type. Other four mutants show much more pronounced decreases in activity than expected from their membrane affinity. It is interesting to find that all of these ligands are postulated to coordinate CA2. Clearly, Asp\(_{246}\) is the most essential ligand for both Ca\(^{2+}\)-dependent vesicle binding and activation of PKC-α. Drastically reduced membrane affinity and enzyme activity of this mutant is not due to deleterious conformational changes as it shows the activity toward histone comparable to that of wild type under a certain condition; e.g. in the presence of POP/DG (97.5:2.5) vesicles and 0.6 mM Ca\(^{2+}\) (data not shown). In the crystal structure of synaptotagmin, carboxylates of Asp\(_{172}\) (corresponding to Asp\(_{187}\) of PKC-α) and Asp\(_{178}\) (Asp\(_{190}\) of PKC-α) are slightly closer to CA1 (2.63 and 2.79 Å, respectively) than that of Asp\(_{230}\) (Asp\(_{246}\) of PKC-α) (2.99 Å) (8); yet D246N has lower vesicle affinity than D187N and D193N. Thus, it is likely that the critical role of Asp\(_{246}\) in vesicle binding derives from its ability to coordinate both Ca\(^{2+}\) ions tightly. Similarly, its essential role in activation might originate from its ability to coordinate both Ca\(^{2+}\) ions and, presumably, its proximity to CA2. Overall, properties of mutants of the CA2 ligands can be best accounted for by assuming that CA2 is involved in PKC activation. It has been generally proposed that the activation of conventional PKC involves conformational changes of protein, including the removal of the pseudosubstrate region from the active site of PKC (42). Our recent study showed that the penetration of PKC-α into PS-containing membranes is a part of these conformational changes and is also essential for its interactions with DG (17). Thus, much reduced monolayer penetration ability of D187N and D248N indicates that the binding of CA2 to PKC-α leads to its activation by triggering the membrane penetration of protein. Taken all together, our results indicate that CA1 is primarily involved in initial membrane anchoring, whereas CA2 is more directly involved in conformational changes. It should be noted that the deactivation of CA2 ligands also significantly impaired the membrane binding of PKC-α. Thus, the distinction between the two Ca\(^{2+}\) ions is not that obvious as far as the membrane anchoring role is concerned. As for inducing conformational changes, however, all evidence indicates that CA1 plays no direct role. It is likely that the anchoring role of CA1 (and CA2) is achieved by the formation of Ca\(^{2+}\) bridge between PKC and phospholipid head group(s). It is less clear, however, how CA2 would induce conformational changes of PKC-α. Further studies are necessary to address these questions.

**Arse\(_{249}\)/Arg\(_{252}\) and Trp\(_{245}\)/Trp\(_{247}\)—**Because the C2 domain is responsible for the Ca\(^{2+}\)-dependent membrane binding of many proteins, it is reasonable to postulate that the domain contains essential membrane binding residues. For most peripheral membrane-binding proteins, both electrostatic and hydrophobic interactions play roles in their membrane binding, although their relative contributions vary with the type of proteins (see, for example, Refs. 24, 43, and 44). The C2 domain of conventional PKC contains several putative membrane binding residues. Recent mutagenesis studies of PKC-β,3 showed that cationic residues in β3 and β4 strands and a loop connecting β5 and β6 strands (see Fig. 1A) are not involved in membrane binding (39, 45). Our studies positively identify those residues that are involved in electrostatic interactions (Arse\(_{249}\)/Arg\(_{252}\)) and hydrophobic interactions (Trp\(_{245}\)/Trp\(_{247}\)). These studies thus provide the first experimental evidence for the notion that the C2 domain of conventional PKC is directly involved in membrane binding. Both W245A/W247A and R249A/R252A show the similar Ca\(^{2+}\) (and PS) dependences for membrane binding and for enzyme activity, indicating that
these residues are involved primarily in membrane binding but not in subsequent activation steps. These properties of mutants are due neither to the deleterious gross conformational change of protein, which would abolish enzyme activity, nor to local conformational perturbation of calcium binding loops, which would lower intrinsic calcium affinity. It has been shown that the membrane binding and activation of conventional PKC requires the binding of a large number of PS molecules (and other anionic phospholipids) to PKC (34, 46, 47). Despite relatively high PS specificity of PKC, the presence of PS-specific binding site(s) has been disputed based on the ability of certain synthetic phospholipids to simulate the effects of PS (48). In any event, the binding of multiple PS molecules (and other anionic phospholipids) would require the presence of PS binding site(s) on the surface of PKC molecule because such binding could not be mediated by PKC-bound Ca\(^{2+}\) ions alone. Our data show that Arg\(^{249}\) and Arg\(^{252}\) form a part of binding sites for anionic phospholipids. It is evident from the similar phosphatidylglycerol and PS dependences of R249A/R252A that this site is not specific for PS. The crystal structure of synaptotagmin shows that Arg\(^{233}\) and Lys\(^{236}\), which correspond to Arg\(^{249}\) and Arg\(^{252}\) of PKC-α, respectively, form a cationic patch on the surface of CR3 that has been proposed to make direct contact with membranes (10, 23). Thus, Arg\(^{249}\) and Arg\(^{252}\) are properly located for their putative role as a nonspecific binding site for anionic phospholipids. A question still remains as to whether or not a separate PS-specific site(s) exists in another part of PKC molecule, the answer of which awaits further structure-function studies.

Drastically reduced vesicle affinity and enzyme activity of W245A/W247A underscore the importance of these residues in the membrane binding of PKC-α. In particular, its significantly lower monolayer-penetrating power compared with wild type indicates that the tryptophans are involved in membrane penetration of PKC-α and resulting interactions with the hydrophobic interior of membranes. This penetration is, however, different from the CA2-induced penetration, which results in PKC activation, in that the W245A/W247A mutation shows no further reducing effect on PKC activity than expected from the decrease in vesicle affinity. Unlike Arg\(^{249}\) and Arg\(^{252}\), Tyr\(^{249}\) and Phe\(^{251}\) of synaptotagmin, which correspond to Trp\(^{245}\) and Trp\(^{247}\) of PKC-α, are not fully surface-exposed. Thus, the penetration by Trp\(^{245}\) and Trp\(^{247}\) appears to be a part of conformational changes of PKC-α induced by CA2 binding. This particular conformational change does not directly lead to PKC activation but enhances the membrane binding of PKC-α. Judging from the extent of reduction in vesicle affinity, Trp\(^{245}\) and Trp\(^{247}\) make significant contribution to total membrane binding energy of PKC-α. Evaluation of contributions of individual membrane binding modules of PKC-α to electrostatic and hydrophobic membrane-protein interactions will require further systematic structure-function analyses.

In summary, these studies clearly indicate distinct roles of calcium ions bound to the C2 domain of PKC-α in its membrane binding and activation and identify residues that are directly involved in electrostatic and hydrophobic membrane binding, thereby defining the role of C2 domain as a membrane docking unit of PKC-α and as a module that triggers conformational changes to activate the protein. Two calcium ions and cationic Arg\(^{249}\)/Arg\(^{252}\) of the C2 domain all contribute to the initial membrane binding of PKC-α, which is mainly electrostatic in nature. Although the sequence of events involved in membrane binding and subsequent activation of PKC-α is not fully understood, the formation of the complex of protein-calcium ions-phospholipids induces conformational changes and membrane penetration of PKC-α, which not only strengthens its membrane binding (largely hydrophobic in nature) but also lead to enzyme activation. Because minor but definite structural and functional differences of isolated C2 domain fragments have been found, it is premature to speculate whether or not the regulation of PKC-α by its C2 domain might represent a general mechanism of Ca\(^{2+}\) signaling by the C2 domain. These studies pave the way toward a better understanding of mechanism of C2 domain-mediated Ca\(^{2+}\) signaling for other membrane-binding proteins.