

# Regulation of Protein Kinase C $\beta$ II by Its C2 Domain<sup>†</sup>

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**ABSTRACT:** The C2 domain serves as a membrane-targeting module in a diverse group of proteins that includes the conventional protein kinase Cs. This work examines the mechanism by which the C2 domain targets protein kinase C to membranes. Molecular modeling identified two highly-charged surfaces on the C2 domain of protein kinase C  $\beta$ II: the  $\text{Ca}^{2+}$  binding site which contains five aspartates and a basic face positioned behind the  $\text{Ca}^{2+}$  site that contains seven lysine residues. Both surfaces were mutated to assess their role in  $\text{Ca}^{2+}$ -dependent membrane binding. Surprisingly, removal of four positive charges on the basic face had no effect on protein kinase C's lipid or  $\text{Ca}^{2+}$  sensitivity, revealing that the basic face does not provide determinants involved in lipid binding, nor is it positioned close enough to the membrane to enhance nonspecific recruitment by its electropositive face. In contrast, replacement of two negative charges with two positive charges in the  $\text{Ca}^{2+}$  binding site decreased protein kinase C's affinity both for  $\text{Ca}^{2+}$  and for anionic lipids by several orders of magnitude. The dramatic reduction in electronegative potential resulting from this mutation did not increase protein kinase C's affinity for acidic membranes in the absence of  $\text{Ca}^{2+}$ , revealing that simple charge neutralization does not account for how  $\text{Ca}^{2+}$  increases protein kinase C's affinity for anionic membranes. Our data suggest that (1) the membrane interaction surface of the C2 domain is localized to the  $\text{Ca}^{2+}$ -binding site, with the positive face positioned away from the membrane, and (2) the  $\text{Ca}^{2+}$  site does not serve as a simple electrostatic switch.

The second messenger  $\text{Ca}^{2+}$  regulates the structure and function of an array of cellular proteins, either indirectly by altering the properties of sensors such as calmodulin or directly by altering the properties of enzymes, transporters, and structural proteins (1). This ubiquitous second messenger plays a particularly prominent role in regulating events at the membrane, including signal transduction, fusion and secretory events, and membrane permeability.

One function of  $\text{Ca}^{2+}$  is in triggering the membrane translocation of amphitropic proteins. For some proteins, such as the annexins, X-ray crystallographic studies have revealed that  $\text{Ca}^{2+}$  forms a bridge between determinants on the protein and phospholipid head groups (2). For others, such as the visual protein recoverin,  $\text{Ca}^{2+}$  binding to a specific EF-hand site results in a conformational change that exposes a hydrophobic myristoyl anchor (3). However, one of the most common  $\text{Ca}^{2+}$  switches mediating membrane translocation may be the C2 motif originally identified in the  $\text{Ca}^{2+}$ -regulated (conventional) protein kinase Cs (4).

The C2 motif is found in over a dozen classes of proteins, including amphitropic ones such as cytosolic phospholipase A2, phospholipase C $\gamma$ , and the GTPase-activating protein GAP, as well as in transmembrane proteins such as the synaptotagmins (5–7). These proteins share the property of interacting with acidic membranes in a  $\text{Ca}^{2+}$ -regulated manner; expression of single C2 domains and deletion mutagenesis have confirmed that the C2 domain mediates

this membrane interaction (8). The C2 domain also mediates protein–protein interactions; for example, the first C2 domain of synaptotagmin binds syntaxin in a  $\text{Ca}^{2+}$ -dependent manner (9). Elucidation of the crystal structure of a C2 domain from synaptotagmin revealed a novel  $\text{Ca}^{2+}$ -binding motif: an aspartate-lined mouth formed by loops comprising sequences at the amino and carboxyl termini of a  $\beta$ -sandwich (10). NMR and crystallographic data from structures analyzed in the presence of >10 mM  $\text{Ca}^{2+}$  (11) or  $\text{Sm}^{3+}$  indicate that the pocket coordinates two metal ions for the C2 domains studied. Despite elegant structural studies of the C2 domain, the mechanism by which  $\text{Ca}^{2+}$  binding increases the domain's affinity for anionic lipids is unknown.

The protein kinase Cs have served as a paradigm for amphitropic proteins that reversibly bind membranes. This family of enzymes transduces the myriad of signals initiated by phospholipid hydrolysis (12). The function of conventional protein kinase Cs is regulated by two membrane-targeting domains, the C1 domain which binds diacylglycerol and is present in all protein kinase C isozymes and the C2 domain, which is present only in the conventional and novel protein kinase Cs (13). Each domain forms a discrete structural and functional unit and is found separately in a number of other signaling proteins (14). Protein kinase C can be recruited to membranes by either domain alone, but both domains must be membrane-bound for the high-affinity interaction that results in activation. This high-affinity membrane interaction is accompanied by a conformational change that removes an autoinhibitory (pseudosubstrate) domain from the active site. Curiously, the novel protein kinase Cs are not regulated by  $\text{Ca}^{2+}$ , yet they contain a C2 domain (15); examination of their sequence reveals that, although residues that maintain the fold of the  $\beta$ -sandwich are present, key residues involved in  $\text{Ca}^{2+}$  coordination are

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absent (14). Elucidation of the structure of the C2 domain now provides invaluable opportunities to dissect the mechanism by which the C2 domain mediates protein kinase C's binding to anionic lipids.

In this paper, we identify determinants on the C2 domain that are involved in  $\text{Ca}^{2+}$ -dependent binding of protein kinase C to phosphatidylserine-containing membranes. Specifically, we show that mutation of the aspartate-lined  $\text{Ca}^{2+}$  site decreases protein kinase C  $\beta$ II's affinity both for  $\text{Ca}^{2+}$  and for phosphatidylserine but does not change the enzyme's affinity for membranes in the absence of  $\text{Ca}^{2+}$ . In contrast, mutation of the highly basic  $\beta$ -sheet behind the  $\text{Ca}^{2+}$  site has no effect on protein kinase C's interaction with  $\text{Ca}^{2+}$  or membranes. These data reveal that the C2 domain interfaces with acidic lipids via the loops that form the aspartate-lined mouth and not via the basic  $\beta$ -sheet behind this mouth. They also show that neutralization of the charge in the  $\text{Ca}^{2+}$  site is not the primary mechanism by which  $\text{Ca}^{2+}$  regulates the function of the C2 domain.

## MATERIALS AND METHODS

Bovine brain L- $\alpha$ -phosphatidylserine, 1-palmitoyl-2-oleoylphosphatidylserine, 1-palmitoyl-2-oleoylphosphatidylcholine, and *sn*-1,2-dioleoylglycerol were purchased from Avanti Polar Lipids, Inc. Dithiothreitol (DTT),<sup>1</sup> *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (HEPES), [ethylenebis-(oxyethylenenitrilo)]tetraacetic acid (EGTA), protamine sulfate, trypsin (type XIII from bovine pancreas, 10 units  $\mu\text{g}^{-1}$ ), and ATP were from Sigma Chemical Co. [ $\gamma$ - $^{32}\text{P}$ ]ATP (3000 Ci  $\text{mmol}^{-1}$ ) and [ $^3\text{H}$ ]dipalmitoylphosphatidylcholine (DPPC, 42 Ci  $\text{mmol}^{-1}$ ) were from Du Pont-New England Nuclear, and calcium chloride (analytical grade) was purchased from J. T. Baker, Inc. Triton X-100 [10% (w/v) aqueous solution] and chemiluminescence SuperSignal substrates were from Pierce. Lipofectin reagent was purchased from Gibco BRL and Baculogold DNA from Pharmingen. Peroxidase-conjugated goat anti-rabbit antibodies and bovine serum albumin were obtained from Boehringer Mannheim Biochemicals. A protein kinase C-selective peptide [Ac-FKKSFKL-NH<sub>2</sub> (16)] was synthesized by the Indiana University Biochemistry Biotechnology Facility. All other chemicals were reagent grade. A polyclonal antibody against the bacterially-expressed catalytic domain of protein kinase C  $\beta$ II was a gift from A. Flint and D. E. Koshland, Jr.

**Model.** The structure of residues 182–257 of protein kinase C  $\beta$  was modeled on the basis of the crystal structure of the C2A domain of synaptotagmin (10) using the program Homology (Biosym software). An electrostatic potential map was constructed using the finite difference solution to the nonlinear Poisson Boltzmann equation (17). The internal dielectric of the protein was set to 2, and that of water was set to 78;  $\text{pK}_a$  calculations were carried out at pH 7. Calculations were performed with the simulation program UHBD (18), and Figure 1B was prepared using the program GRASP (19).

**Mutagenesis.** A baculovirus transfer vector encoding the cDNA sequence of PKC  $\beta$ II with the double mutation D248R/D254R or the quadruple mutation K197A/K199A/

K211A/ K213A was made by polymerase chain reaction using protein kinase C  $\beta$ II in the pBS vector (pbluePKC) as the template. The sense primer for the D248R/D254R mutant corresponding to the sequence around the codons for Asp 248 and Asp 254 was synthesized and contained the nucleic acid changes needed to encode the desired mutations. The sense primer was TAGAGATCTGGGATTGGCGC-CTGACCAGCAGGAATCGCTTCATGGGATCTC and contained a *Bgl*II site. The antisense primer was CCTTGG-TACCTTGGCCAATC and contained a *Kpn*I restriction site. The polymerase chain reaction product and the pbluePKC template were partially digested with *Bgl*II and *Kpn*I, and the products were gel-purified and ligated together. The quadruple mutant was made using K197A/K199A PKC as the template for two PCR. The sense primer for the first reaction was TCTGGTACCTATGGACCCC, and the antisense primer contained the changes needed to encode the K211A/K213A mutations (GGAGCATTTGATAGTCGCG-GTGCCTGCTTGCTCTC). The second reaction mixture contained the sense primer which encoded the mutation (sense primer corresponding to antisense in the first reaction) and an antisense primer with the sequence CCCAGATCTC-TACGGACAGT. The products of these two polymerase chain reactions were then pooled, and PCR was repeated with the two outside primers (sense of the first and antisense of the second) which contained a *Kpn*I site and a *Bgl*II site, respectively. The product from this reaction and the pbluePKC template were digested with *Kpn*I and *Bgl*II and ligated together (see above). The mutant protein kinase C genes were then subcloned into either pcDNA3, a mammalian expression vector, using *Not*I and *Eco*RV, or the pVL1393 (Invitrogen) baculovirus transfer vector using *Xba*I and *Sma*I (see below). All mutants were verified by DNA sequencing. Similar data were obtained for protein kinase C mutants expressed in mammalian (COS-7) or insect cells.

**Expression of Mutant Protein Kinase C in Sf21 Cells.** Sf21 insect cells were cotransfected with the baculoviral transfer vectors encoding the protein kinase C mutants and linearized wild-type baculovirus DNA (Baculogold-Pharmingen) by liposome-mediated transfection. Isolated recombinant baculovirus was obtained by plaque purification and amplified by two rounds of propagation in insect cells as described in a manual from Pharmingen for the Baculogold expression system (Pharmingen, San Diego, CA). Sf21 insect cells were then infected with high-titer ( $1 \times 10^8$  pfu  $\text{mL}^{-1}$ ) baculovirus encoding wild-type protein kinase C or its mutants. The cells were harvested after 3 days at 27 °C and lysed by homogenization in buffer containing 50 mM HEPES (pH 7.4), 0.2% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 85  $\mu\text{M}$  leupeptin, 2 mM benzamidine, and 0.2 mM phenylmethanesulfonyl fluoride (lysis buffer). A portion of the cell lysate was retained, and the remainder was centrifuged at 100000g for 20 min at 4 °C (TLA 120.2, Beckman). The pellet was resuspended in lysis buffer, and lysate, supernatant (detergent-soluble), and pellet (detergent-insoluble) fractions were diluted 2-fold in glycerol and stored at  $-20$  °C. All experiments were performed using the high-speed, detergent-soluble supernatants.

**Electrophoresis and Western Blots.** Aliquots of the cell lysate, the detergent-soluble supernatant, or the detergent-insoluble pellet from Sf21 cells expressing wild-type protein kinase C  $\beta$ II, the D248R/D254R mutant, or the 4K/4A mutant were separated by SDS–polyacrylamide gel elec-

<sup>1</sup> Abbreviations: DG, diacylglycerol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PAGE, polyacrylamide gel electrophoresis; PS, phosphatidylserine; SDS, sodium dodecyl sulfate.

trophoresis (7% acrylamide). The proteins were transferred to PVDF (Immobilon-P, Millipore) and probed with an antibody to the catalytic domain of protein kinase C  $\beta$ II followed by labeling with peroxidase-conjugated secondary antibodies. Labeling was detected using chemiluminescence.

**Lipid.** Sonicated dispersions of phosphatidylserine (1.4 mM) and diacylglycerol (38  $\mu$ M) (PS/DG) or Triton X-100 (1.0% w/v) mixed micelles containing phosphatidylserine (15 mol %) and diacylglycerol (5 mol %) were prepared as described (20). For the membrane binding experiments, sucrose-loaded large unilamellar vesicles containing trace [ $^3$ H]DPPC were prepared as described (21). Briefly, mixtures of phosphatidylcholine (0–95 mol %), phosphatidylserine (0–95 mol %), and diacylglycerol (5 mol %) containing trace [ $^3$ H]DPPC were prepared by extrusion as described (21). Stock phospholipid concentrations were determined by phosphate assay (22). Concentrations of lipids after extrusion were calculated on the basis of radioactivity.

**Proteolysis.** Protein kinase C's sensitivity to trypsin was determined by incubating the Sf21 cell supernatants containing 10–20 ng of wild-type or mutant protein kinase Cs in a total volume of 90  $\mu$ L [containing 20 mM HEPES, 0.4 mM  $\text{Ca}^{2+}$ , with or without brain phosphatidylserine (250  $\mu$ M), and diacylglycerol (7  $\mu$ M)] in the presence of 0–20 units  $\text{mL}^{-1}$  trypsin, as indicated in the legend to Figure 3. Proteolysis was carried out for 10 min at 30  $^{\circ}\text{C}$  and stopped by addition of 30  $\mu$ L of SDS sample buffer. Samples were analyzed by SDS–PAGE (9% polyacrylamide) followed by Western blot analysis using antibodies that recognize the catalytic domain of protein kinase C  $\beta$ II.

**Protein Kinase C Activity Assay.** Protein kinase C activity in the detergent-soluble fraction of insect cells was assayed by measuring the rate of phosphorylation of a protein kinase C-selective peptide in the presence or absence of brain phosphatidylserine, diacylglycerol, and  $\text{Ca}^{2+}$ , as described (20). In some experiments, protamine sulfate served as the substrate. The reaction mixture contained 50  $\mu$ M synthetic peptide or 1 mg  $\text{mL}^{-1}$  protamine sulfate in 20 mM HEPES (pH 7.4), 1 mM DTT, 100  $\mu$ M [ $\gamma$ - $^{32}\text{P}$ ]ATP, 5 mM  $\text{MgCl}_2$ , and either 0.5 mM  $\text{Ca}^{2+}$  and lipid (see above) or 0.5 mM EGTA in a final volume of 80  $\mu$ L. Samples were incubated at 30  $^{\circ}\text{C}$  for 4–6 min; the reactions were quenched by the addition of 25  $\mu$ L of a solution containing 0.1 M ATP and 0.1 M EDTA (pH 8–9) and analyzed as described (20).

**Protein Kinase C Membrane Binding Assay.** The binding of protein kinase C to sucrose-loaded vesicles was measured as described by Rebecchi et al. (23) and adopted for protein kinase C (24). Briefly, protein kinase C (15–20  $\mu$ L of the detergent-soluble supernatant containing wild-type or mutant protein kinase C) was incubated with vesicles (100  $\mu$ M total lipid; composition described in the figure legends) in the presence of 100 nM to 1 mM free  $\text{Ca}^{2+}$  in buffer containing 20 mM HEPES (pH 7.4), 0.3 mg  $\text{mL}^{-1}$  BSA, 5 mM  $\text{MgCl}_2$ , 1 mM DTT, and 100 mM KCl for 5 min at 22  $^{\circ}\text{C}$ . Vesicle-bound enzyme was separated from free enzyme by centrifugation of the vesicle/enzyme mixture at 100000g for 30 min at 25  $^{\circ}\text{C}$ . Protein kinase C activity toward protamine sulfate was assayed under identical conditions for both the supernatant and pellet, and the vesicle-associated kinase activity was calculated as described (24, 25).

**Data Analysis.** The apparent association constant for binding of protein kinase C to vesicles was calculated as the fraction of protein kinase C bound to membranes divided

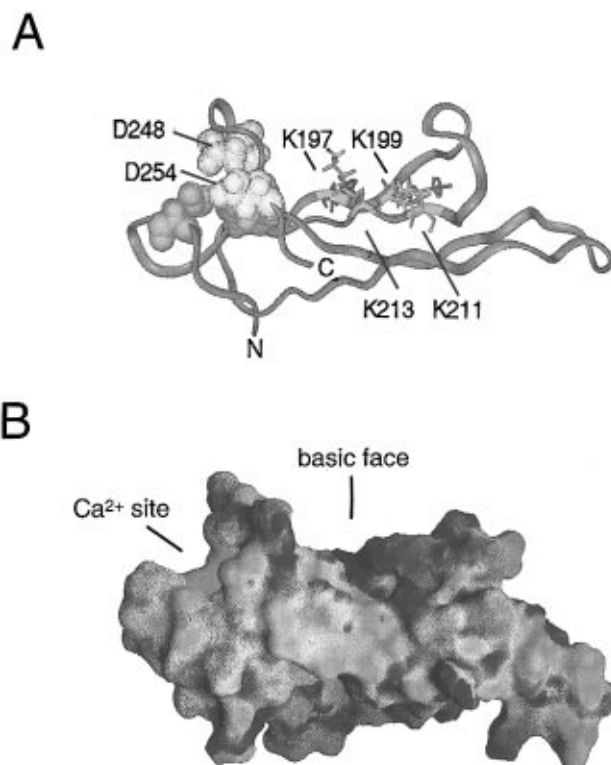


FIGURE 1: Modeled structure of residues 182–257 of the C2 domain of protein kinase C  $\beta$ II based on the crystal structure of the C2 domain of synaptotagmin. (A) Ribbon diagram showing the five aspartates in the  $\text{Ca}^{2+}$  binding site in a space-filling representation: Asp 187, Asp 193, and Asp 246 in pink and the aspartates mutated to arginine (Asp 248 and Asp 254) in yellow. Also indicated are the four lysine residues on the basic  $\beta$ -sheet behind the  $\text{Ca}^{2+}$  site that were mutated to Ala (Lys 197, 199, 211, and 213 in an orange, stick representation). (B) Surface electrostatic potential map showing electronegative potential in red ( $\geq -5K_B T/e$ ) and electropositive potential in blue ( $\leq 5K_B T/e$ ).

by the product of the fraction of protein kinase C remaining in the supernatant and the total lipid (24); the membrane interaction is reversible and follows the law of mass action (25). The dependence of protein kinase C membrane binding or activity on the phosphatidylserine content of vesicles or micelles was analyzed by a nonlinear least-squares fit of the data to a modified Hill equation as described (26).

**Free Calcium Determinations.** Concentrations of free  $\text{Ca}^{2+}$  were calculated using a program provided by Claude Klee (27) that takes into account pH and  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^+$ ,  $\text{Na}^+$ , EGTA, EDTA, and ATP concentrations.

## RESULTS

Figure 1A shows a modeled structure of the C2 domain of protein kinase C  $\beta$  based on the crystal structure of synaptotagmin's C2A domain (10). This domain forms a  $\beta$ -sheet-rich domain with opposite ends of the strand coming together to form an aspartate-lined mouth. In protein kinase C  $\beta$ , these correspond to Asp 187, 193, and 246 in the lower jaw (Figure 1A, pink space-filling representation) and Asp 248 and Asp 254 in the upper jaw (yellow space-filling representation). These aspartates are conserved in the conventional protein kinase Cs, which bind  $\text{Ca}^{2+}$ , but not in the novel protein kinase Cs which are not regulated by  $\text{Ca}^{2+}$ . The aspartate-lined mouth is an ideal candidate for a metal binding site, and in support of this, the C2 domains of synaptotagmin and phospholipase C $\delta$  have recently been

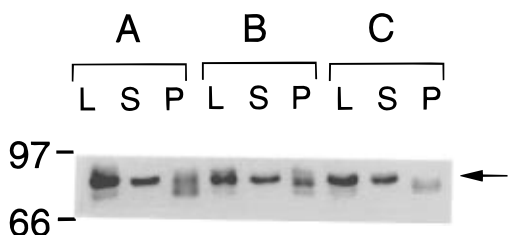


FIGURE 2: Baculovirus expression of C2 domain mutants. Western blot of the whole cell lysate (L), detergent-soluble supernatant (S), and detergent-insoluble pellet (P) of wild-type protein kinase C (A), the D248R/D254R mutant (B), or the K197A/K199A/K211A/K213A mutant (C) from Sf21 cells. The blot was probed with a polyclonal antibody against the catalytic domain of protein kinase C  $\beta$ II. Each lane contained a sample from approximately  $5 \times 10^5$  cells.

shown to coordinate two metal ions (11, 28). On the back face of the  $\text{Ca}^{2+}$  site is a basic  $\beta$ -sheet that is conserved in many C2 domains (contains Lys 197, 199, 211, and 213 highlighted in orange in Figure 1A, as well as nonhighlighted Lys 205, 209, and 216). The electrostatic potential map in Figure 1B illustrates the two highly charged surfaces on the C2 domain: an aspartate-lined groove on one side which is backed by a basic surface.

In order to elucidate how the C2 domain serves as a membrane-targeting module, the role of the two charged faces of the C2 domain in regulating protein kinase C's membrane interaction was addressed. Specifically, we tested how mutation of acidic or basic residues in these faces affected protein kinase C's affinity for  $\text{Ca}^{2+}$  and phospholipid. First, two aspartates in the acidic  $\text{Ca}^{2+}$  binding site were mutated to arginine to make the double mutant D248R/D254R; these aspartates are positioned in the upper jaw of the putative  $\text{Ca}^{2+}$  binding site (Figure 1A, yellow). These mutations essentially neutralized the charge in the  $\text{Ca}^{2+}$  site, allowing us to test the hypothesis that  $\text{Ca}^{2+}$  acts as an electrostatic switch. Second, four lysines in the basic face were simultaneously mutated to alanine to make the quadruple mutant K197A/K199A/K211A/K213A (Figure 1A, orange residues). Mutants were expressed in insect cells using the baculovirus expression system.

The Western blot in Figure 2 shows that wild-type protein kinase C  $\beta$ II and the two mutants were expressed at comparable levels in Sf21 cells. Whole cell lysate (L) of wild-type protein kinase C contained three bands which we have previously shown to correspond to the fully phosphorylated mature protein that has an apparent molecular mass of 80 kDa, and two partially-phosphorylated precursor forms that have apparent molecular masses of 78 and 76 kDa (29). As previously reported, the mature, fully-phosphorylated wild-type protein partitioned almost exclusively in the detergent-soluble supernatant (S) while the precursor forms partitioned in the detergent-insoluble pellet (P) (29). Similar to the wild-type enzyme, faster-migrating forms of both mutants partitioned in the detergent-insoluble fraction, whereas species comigrating with the fully-phosphorylated mature wild-type enzyme partitioned in the detergent-soluble fraction. A faint additional band with a slower electrophoretic mobility was observed in the samples of both the mutants, suggesting additional phosphorylation on a small fraction (<10%) of the mutant enzymes. Importantly, the major band, indicated with an arrow, had the same electrophoretic mobility as the wild-type enzyme. Phosphatase

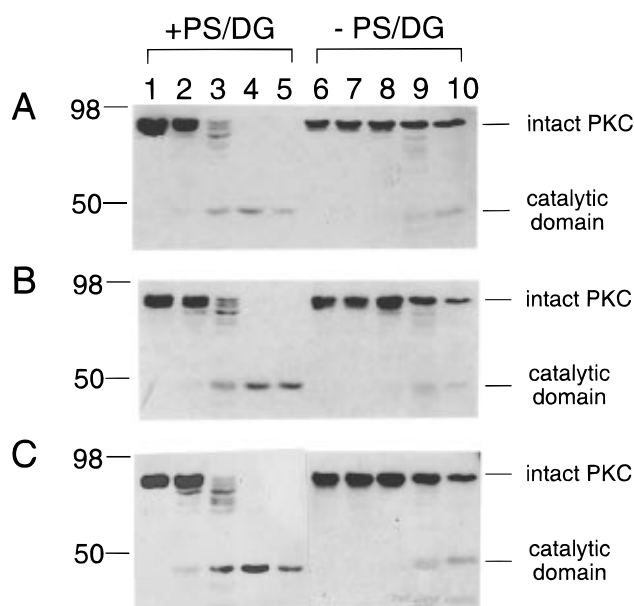


FIGURE 3: Mutation of the  $\text{Ca}^{2+}$  binding region or the basic face of the C2 domain does not affect protein kinase C's lipid-induced conformational change. Western blots showing the detergent-soluble fraction from cells expressing wild-type protein kinase C  $\beta$ II (A), the D248R/D254R mutant (B), or the K197A/K199A/K211A/K213A mutant (C) after treatment with 0 (lanes 1 and 6), 0.02 unit  $\text{mL}^{-1}$  (lanes 2 and 7), 0.2 unit  $\text{mL}^{-1}$  (lanes 3 and 8), 2 units  $\text{mL}^{-1}$  (lanes 4 and 9), or 20 units  $\text{mL}^{-1}$  (lanes 5 and 10) trypsin in the presence (lanes 1–5) or absence (lanes 6–10) of lipid (250  $\mu\text{M}$  phosphatidylserine and 7  $\mu\text{M}$  diacylglycerol; PS/DG) containing 0.4 mM  $\text{Ca}^{2+}$ . Blots were probed with a polyclonal antibody that recognizes the catalytic domain of protein kinase C, described in the legend to Figure 2. Molecular mass markers are indicated on the left.

treatment of mutant and wild-type protein kinase C resulted in proteins that migrated with the same apparent molecular mass, revealing that the altered charge in the C2 domain did not affect protein kinase C's electrophoretic mobility (data not shown). Thus, mutation of the C2 domain did not significantly alter protein kinase C's phosphorylation state or subcellular distribution.

To determine if the C2 domain mutants displayed the same conformational change as wild-type protein kinase C upon membrane binding, their trypsin sensitivity was examined in the presence or absence of lipid (Figure 3). The hinge separating the regulatory and catalytic moieties of wild-type protein kinase C becomes over 1 order of magnitude more sensitive to proteolysis by trypsin upon membrane binding (26), and this increased proteolytic sensitivity serves as an effective diagnostic of the "membrane-bound conformation" of protein kinase C (30). Figure 3 shows that wild-type and the mutant protein kinase Cs displayed the same sensitivity to trypsin, either in the presence (lanes 1–5) or in the absence (lanes 6–10) of phosphatidylserine and diacylglycerol membranes (for example, the catalytic domain is first apparent in lane 3 for all protein kinase Cs). Importantly, proteolytic sensitivity decreased similarly for both wild-type and mutant protein kinase Cs in the absence of lipid; approximately 100-fold more trypsin was required to catalyze the same amount of proteolysis in the absence of lipid than in the presence [compare lanes 3 (0.2 unit  $\text{mL}^{-1}$  trypsin) in the presence of lipid and lane 10 (20 units  $\text{mL}^{-1}$  trypsin) in the absence of lipid]. The stability of the proteolyzed kinase core (labeled catalytic domain in Figure 3) and the regulatory

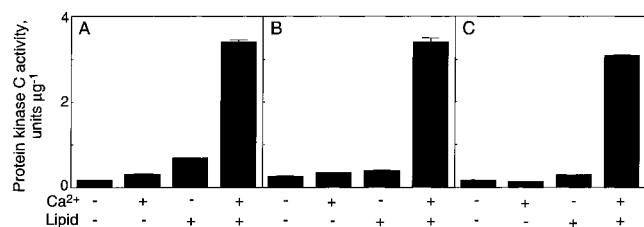


FIGURE 4: Mutation of the  $\text{Ca}^{2+}$  binding region or the basic face of the C2 domain does not impair catalysis. Protein kinase C activity in the detergent-soluble supernatant from insect cells expressing wild-type protein kinase C  $\beta\text{II}$  (A), the D248R/D254R mutant (B), or the K197A/K199A/K211A/K213A mutant (C) was measured in the presence of 0.5 mM  $\text{Ca}^{2+}$  or 0.5 mM EGTA with or without sonicated dispersions of phosphatidylserine (140  $\mu\text{M}$ ) and diacylglycerol (3.8  $\mu\text{M}$ ), using the protein kinase C-selective peptide (50  $\mu\text{M}$ ) as the substrate (standard assay procedure, see Materials and Methods for details). One unit is defined as 1 nmol of phosphate incorporated per minute onto the peptide. The amount of protein kinase C was determined by Western blot analysis relative to the known amounts of pure protein kinase C  $\beta\text{II}$ . Data are expressed as the mean  $\pm$  SD for one representative experiment performed in triplicate.

domain (as assessed using regulatory domain antibodies; data not shown) was similar for wild-type protein kinase C and both mutants. These data reveal that mutation of the C2 domain did not detectably affect exposure of the hinge that accompanies membrane binding, or the stability of the catalytic or regulatory domains.

Figure 4 shows that wild-type protein kinase C  $\beta\text{II}$  (A), the D248R/D254R mutant (B), and the quadruple K197A/K199A/K211A/K213A mutant (C) had similar specific activities when measured under standard assay conditions. These conditions provide saturating  $\text{Ca}^{2+}$  (0.5 mM), phosphatidylserine (140  $\mu\text{M}$ ), and diacylglycerol (3.8  $\mu\text{M}$ ) for wild-type protein kinase C. No significant activity was observed in the absence of  $\text{Ca}^{2+}$  or lipid, revealing that activity of the mutants depended on the presence of both  $\text{Ca}^{2+}$  and lipid. Thus, the mutations in the C2 domain did not impair catalysis. In addition, the thermal stability of both mutants was similar to that of wild-type protein kinase C (data not shown).

In order to address whether mutation of the C2 domain had any effect on protein kinase C's interaction with lipid cofactors, we measured the effectiveness of different lipid presentations in activating the mutants. Specifically, we explored whether Triton X-100/lipid mixed micelles (10 mol % phosphatidylserine and 5 mol % diacylglycerol) or large unilamellar vesicles (LUVs, containing 40 mol % phosphatidylserine, 5 mol % diacylglycerol, and 55 mol % phosphatidylcholine) could activate protein kinase C. The lower mole fraction phosphatidylserine in these presentations resulted in significantly weaker binding of protein kinase C to the micelle or vesicle surface compared with the binding to the multilamellar phosphatidylserine/diacylglycerol vesicles in Figure 4 (e.g. refs 24 and 31). In the presence of saturating  $\text{Ca}^{2+}$  (solid bars), all three lipid presentations activated wild-type protein kinase C (Figure 5A). The degree of activation by LUVs and mixed micelles was  $69 \pm 8$  and  $87 \pm 9\%$ , respectively, of the activation stimulated by the multilamellar phosphatidylserine/diacylglycerol dispersions. All three lipid presentations also activated the quadruple Lys-to-Ala mutant (C); LUVs supported  $58 \pm 3$  and  $80 \pm 6\%$ , respectively, of the activity stimulated by phosphatidylserine/diacylglycerol dispersions. Thus, within the error of the assays, the lipid

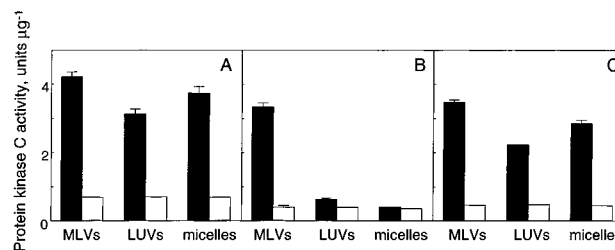


FIGURE 5: The  $\text{Ca}^{2+}$  binding mutant is sensitive to lipid presentation. Protein kinase C activity in the detergent-soluble fraction of cells expressing wild-type protein kinase C  $\beta\text{II}$  (A), the D248R/D254R mutant (B), or the K197A/K199A/K211A/K213A mutant (C) was measured in the presence (solid bars) or absence (open bars) of 0.5 mM  $\text{Ca}^{2+}$  and the indicated lipid presentation: multilamellar vesicles containing phosphatidylserine (140  $\mu\text{M}$ ) and diacylglycerol (3.8  $\mu\text{M}$ ) (MLVs); large unilamellar vesicles (LUVs) containing 40 mol % phosphatidylserine, 55 mol % phosphatidylcholine, and 5 mol % diacylglycerol; and Triton X-100 mixed micelles containing 15 mol % phosphatidylserine and 5 mol % diacylglycerol (micelles). The data represent the weighted average of two separate experiments performed in triplicate.

sensitivity of the quadruple mutant was similar to that of wild-type protein kinase C. In striking contrast, the D248R/D254R mutant was not significantly activated by either LUVs or mixed micelles; these lipid systems supported  $8 \pm 7$  and  $1 \pm 1\%$ , respectively, of the activity supported by phosphatidylserine/diacylglycerol sonicated dispersions. This result provided the first indication that the lipid sensitivity of the  $\text{Ca}^{2+}$  site mutant, but not the basic face mutant, was impaired.

To further probe whether the quadruple mutant's lipid affinity was the same as that of wild-type protein kinase C, as suggested from the activity assays above, we measured the enzyme's affinity for vesicles in the presence or absence of diacylglycerol. Figure 6A shows that the apparent membrane affinity of wild-type protein kinase C and the quadruple basic face mutant was similar both in the presence of diacylglycerol [ $(5 \pm 2) \times 10^4$  and  $(7 \pm 2) \times 10^4 \text{ M}^{-1}$ , respectively] and in the absence of diacylglycerol [ $(1.2 \pm 0.5) \times 10^3$  and  $(1.0 \pm 0.5) \times 10^3 \text{ M}^{-1}$ , respectively]. Thus, mutation of the basic face of the C2 domain had no significant effect on protein kinase C's low-affinity interaction with phosphatidylserine that occurs in the absence of diacylglycerol, nor did it affect the diacylglycerol-dependent high-affinity interaction.

As one additional measure of the quadruple basic face mutant's lipid interaction, we measured its phosphatidylserine dependence for activation. Figure 6B reveals that the phosphatidylserine dependence for activation of the quadruple mutant was similar to that for activation of the wild-type enzyme; the phosphatidylserine concentration for half-maximal activation was  $8.0 \pm 0.2$  mol % for the wild-type enzyme and  $9.1 \pm 0.1$  mol % for the mutant. Thus, removal of four positive charges on the  $\beta$ -strand behind the  $\text{Ca}^{2+}$  site does not affect protein kinase C's phosphatidylserine dependence for activation.

Given the apparent difference in the D248R/D254R mutant's sensitivity to lipid presentation, we explored the effect of this mutation on protein kinase C's affinity for phosphatidylserine. Figure 7 shows the binding and activity of protein kinase C incubated with large unilamellar vesicles containing 0–95 mol % phosphatidylserine in the presence of saturating (0.2 mM)  $\text{Ca}^{2+}$  and diacylglycerol (5 mol %). The binding data in Figure 7A show that concentrations of

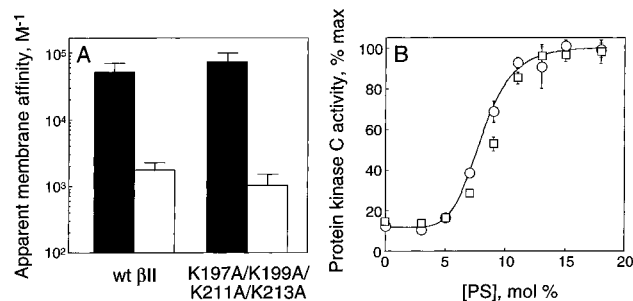


FIGURE 6: Mutation of the basic face of the C2 domain does not change protein kinase C's phosphatidylserine dependence. (A) The binding of wild-type protein kinase C or the K197A/K199A/K211A/K213A mutant to large unilamellar vesicles composed of 40 mol % phosphatidylserine, 5 mol % diacylglycerol, and 55 mol % phosphatidylcholine (solid bars) or 40 mol % phosphatidylserine and 60 mol % phosphatidylcholine (open bars) was measured in the presence of 0.4 mM  $\text{Ca}^{2+}$  as described in Materials and Methods. Data are expressed as the apparent membrane affinity, determined from the ratio of free protein kinase C to bound protein kinase C divided by the total lipid concentration (24, 25). Data represent the mean  $\pm$  SD of two separate experiments. (B) Protein kinase C activity of wild-type enzyme ( $\circ$ ) or the K197A/K199A/K211A/K213A mutant ( $\square$ ) was measured as a function of phosphatidylserine concentration in Triton X-100 mixed micelles containing 5 mol % diacylglycerol and the indicated percentage of phosphatidylserine, in the presence of 0.5 mM  $\text{Ca}^{2+}$ . The data are presented as the percentage of maximal activity (calculated using a modified Hill equation) and are expressed as the mean  $\pm$  SD of a representative experiment performed in triplicate. The curve is that predicted for wild-type data using the Hill equation (26).

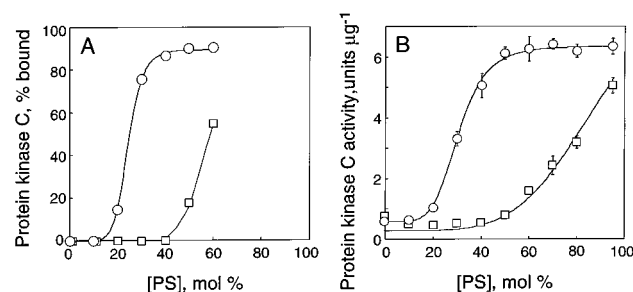


FIGURE 7: Mutation of the  $\text{Ca}^{2+}$  binding domain alters the phosphatidylserine-dependent binding and activation of protein kinase C. (A) The binding of wild-type protein kinase C ( $\circ$ ) or the D248R/D254R mutant ( $\square$ ) to large unilamellar phosphatidylcholine vesicles containing 5 mol % diacylglycerol and an increasing mole percentage of phosphatidylserine (100  $\mu\text{M}$  total lipid) was measured in the presence of 0.2 mM free  $\text{Ca}^{2+}$ . The amount of vesicle-associated enzyme was determined by monitoring the activity toward the cofactor-independent substrate, protamine sulfate, as described in Materials and Methods. (B) Protein kinase C activity toward the protein kinase C-selective peptide was measured under the same conditions as those used for the binding experiment except that the protein kinase C-selective peptide (50  $\mu\text{M}$ ) and 100  $\mu\text{M}$  ATP were present. Curves shown for the wild-type data in panels A and B are those predicted from a modified Hill equation (26).

phosphatidylserine approximately 4 times higher were required to promote half-maximal membrane binding of the D248R/D254R mutant compared with that of the wild type; 60 mol % phosphatidylserine was required for half-maximal binding of the mutant compared with 25 mol % for the wild type. It was not possible to measure binding at higher mole fractions of lipid because the relatively high concentration of  $\text{Ca}^{2+}$  resulted in a slow time-dependent aggregation and precipitation of the vesicles. We were, however, able to measure activation since no detectable aggregation was observed during the 10 min required for activity measurements (see below).

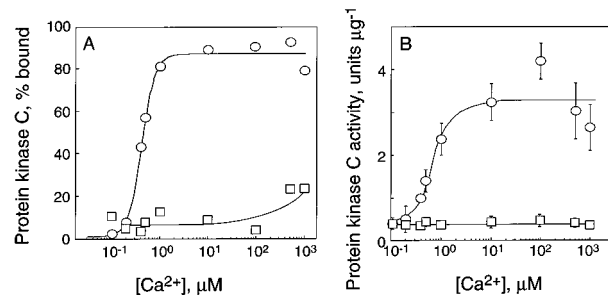


FIGURE 8: Mutation of the calcium binding domain alters the calcium-dependent binding and activation of protein kinase C. (A) The binding of wild-type protein kinase C ( $\circ$ ) or the D248R/D254R mutant ( $\square$ ) to large unilamellar vesicles (100  $\mu\text{M}$  total lipid) containing 5 mol % diacylglycerol, 40 mol % phosphatidylserine, and 55 mol % phosphatidylcholine was measured as a function of free  $\text{Ca}^{2+}$  concentration. The amount of vesicle-associated enzyme was determined by monitoring the activity toward the cofactor-independent substrate, protamine sulfate, as described in Materials and Methods. The curve shown for the wild-type data is that predicted from a modified Hill equation (26). (B) Protein kinase C activity toward the protein kinase C-selective peptide was measured under the same conditions as those used for the binding experiment except that the protein kinase C-selective peptide (50  $\mu\text{M}$ ) and 100  $\mu\text{M}$  ATP were present.

Figure 7B shows the activity accompanying membrane binding of the D248R/D254R mutant compared with that of wild-type protein kinase C. As described previously, in the presence of 5 mol % diacylglycerol, the phosphatidylserine dependence for activation was similar to that of binding for the wild-type enzyme (31). As observed for binding, activation of the mutant required considerably more phosphatidylserine than that required by the wild-type protein. The activity of the D248R/D254R mutant increased with an increasing mole percentage of phosphatidylserine up to the highest concentration tested, 95 mol % phosphatidylserine. At this concentration, the activity of the mutant was almost equal to the maximal activity of the wild-type enzyme (Figure 7B). Note that the assay conditions in the presence of 95 mol % phosphatidylserine are similar to those in Figure 3 except that the mixtures in Figure 3 contained sonicated dispersions of lipid (rather than sucrose-loaded large unilamellar vesicles), the lipid concentration was 145  $\mu\text{M}$  (rather than 100  $\mu\text{M}$ ), and assays were performed at slightly lower ionic strength.

The effect of the D248R/D254R mutation on protein kinase C's  $\text{Ca}^{2+}$  regulation was examined in Figure 8. Figure 8A shows that 0.4  $\mu\text{M}$   $\text{Ca}^{2+}$  promoted half-maximal membrane binding of the wild-type enzyme to vesicles containing 40 mol % phosphatidylserine and 5 mol % diacylglycerol (remaining lipid phosphatidylcholine). In striking contrast, no significant membrane binding of the D248R/D254R mutant was detected until  $\text{Ca}^{2+}$  concentrations were raised to 1 mM. These data are consistent with those in Figure 7A, where no significant binding to vesicles containing 40 mol % phosphatidylserine was observed in the presence of 0.3 mM  $\text{Ca}^{2+}$ . No detectable activity of the mutant was observed, even in the presence of 1 mM  $\text{Ca}^{2+}$ . Vesicle aggregation prevented measurements at higher  $\text{Ca}^{2+}$  concentrations.

Finally, we addressed whether mutation of the  $\text{Ca}^{2+}$  binding site altered the diacylglycerol-induced specificity for phosphatidylserine that is a hallmark of protein kinase C's membrane interaction. We have previously shown that diacylglycerol causes a dramatic increase in protein kinase

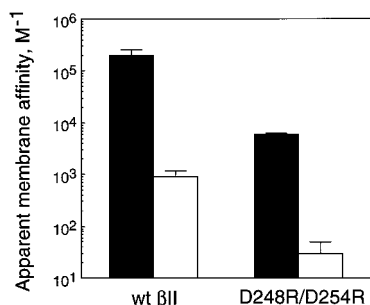


FIGURE 9: Mutation of the  $\text{Ca}^{2+}$  binding site does not alter protein kinase C's diacylglycerol-induced specificity for phosphatidylserine. The binding of wild-type protein kinase C or the D248R/D254R mutant to large unilamellar phosphatidylcholine vesicles containing 50 mol % phosphatidylserine in the presence (solid bars) or absence (open bars) of 5 mol % diacylglycerol was measured in the presence of 300  $\mu\text{M}$  free  $\text{Ca}^{2+}$  (see the legend to Figure 8). Data are expressed as the mean  $\pm$  SEM of one experiment performed in sextuplet.

C's affinity for surfaces containing phosphatidylserine, but not other anionic lipids (31). To address whether mutation of the  $\text{Ca}^{2+}$  site destroyed this selectivity for phosphatidylserine, we asked whether diacylglycerol caused the same relative increase in the affinity of the D248R/D254R mutant for phosphatidylserine as occurs for wild-type protein kinase C. Given the low affinity of the mutant for phosphatidylserine, the binding to vesicles containing 50 mol % phosphatidylserine was examined. Figure 9 shows that 5 mol % diacylglycerol caused a 220-fold increase in the affinity of wild-type protein kinase C for membranes containing 50 mol % phosphatidylserine; specifically, the apparent membrane affinity increased from  $(9 \pm 3) \times 10^2 \text{ M}^{-1}$  in the absence of diacylglycerol (open columns) to  $(2.0 \pm 0.6) \times 10^5 \text{ M}^{-1}$  in the presence of diacylglycerol (solid columns). Diacylglycerol induced the same increase in membrane affinity for the D248R/D254R mutant. The second messenger caused the apparent membrane affinity of the mutant to increase from  $30 \pm 20$  to  $(5.8 \pm 0.5) \times 10^3 \text{ M}^{-1}$ . Thus, although the mutant bound membranes with approximately 30 times lower affinity than the wild-type enzyme, this membrane interaction was subject to the same regulation by diacylglycerol as that of the wild-type enzyme. These data reveal that the  $\text{Ca}^{2+}$  site does not contain determinants that mediate the diacylglycerol-induced selectivity for phosphatidylserine.

## DISCUSSION

The surface electrostatic potential of a modeled structure of the C2 domain of protein kinase C  $\beta$  reveals two charged surfaces: the aspartate-lined  $\text{Ca}^{2+}$ -binding cavity and a basic surface behind the  $\text{Ca}^{2+}$  site. To address the mechanism by which  $\text{Ca}^{2+}$  recruits conventional protein kinase C to membranes, the charge of these two surfaces was reduced and the effect on protein kinase C's  $\text{Ca}^{2+}$  and lipid regulation examined. Binding and enzymological analyses of mutants in these two regions established the following. (1)  $\text{Ca}^{2+}$  regulates protein kinase C's membrane affinity in a manner that does not involve simple charge neutralization of the  $\text{Ca}^{2+}$  site; replacement of two of the five aspartates in the  $\text{Ca}^{2+}$  binding site with arginine did not increase protein kinase C's membrane affinity in the absence of  $\text{Ca}^{2+}$ . (2) The basic  $\beta$ -sheet does not bind phosphatidylserine or influence protein kinase C's membrane interaction either in the presence or in the absence of diacylglycerol.

**Mechanism of  $\text{Ca}^{2+}$  Regulation.** NMR and crystallographic data have localized the  $\text{Ca}^{2+}$  site in the C2 domain to the aspartate-lined mouth formed by loops of a  $\beta$ -strand-rich motif (11, 28). NMR analysis of the first C2 domain of synaptotagmin in the presence or absence of  $\text{Ca}^{2+}$  revealed recently that  $\text{Ca}^{2+}$  binding stabilizes the domain without inducing significant conformational changes (11). One possibility is that  $\text{Ca}^{2+}$  mediates the function of the C2 domain by altering surface properties of the domain, similar to how phorbol esters regulate the C1 domain of protein kinase C by altering the surface hydrophobicity of the domain in the absence of conformational changes (32). Alternatively,  $\text{Ca}^{2+}$  binding could provide a bridge to interact with anionic lipids or other coordinating ligands on protein partners. A third explanation could be that  $\text{Ca}^{2+}$  re-adjusts the position of basic residues in the C2 domain to better align them for interaction with acidic lipids or other proteins.

The possibility that the altered electronegative potential resulting from  $\text{Ca}^{2+}$  binding serves as an electrostatic switch was first proposed by Rizo and co-workers (33). Surface electrostatic maps of the first C2 domain of synaptotagmin revealed a zwitterionic surface formed by the five conserved aspartates in the  $\text{Ca}^{2+}$  site and a number of basic residues on the periphery of the site. Binding of  $\text{Ca}^{2+}$  was shown to convert the surface to one that is primarily electropositive. This led to the suggestion that  $\text{Ca}^{2+}$  serves as an electrostatic switch and that the altered surface potential resulting from  $\text{Ca}^{2+}$  binding mediates the macromolecular interactions of the C2 domain (33).

Here, we show that the mechanism of  $\text{Ca}^{2+}$  regulation of the C2 domain of protein kinase C  $\beta$ II is not via an electrostatic switch; mutation of two aspartates to arginine, converting the electrostatic potential of the  $\text{Ca}^{2+}$  site to one that is electropositive, does not promote the binding of protein kinase C to anionic membranes in the absence of  $\text{Ca}^{2+}$ . In contrast, this mutation results in a protein that still requires  $\text{Ca}^{2+}$  for binding to anionic membranes. These data reveal that the function of the aspartate-lined mouth is not to inhibit membrane juxtaposition because of its electronegative potential, with  $\text{Ca}^{2+}$  binding releasing this constraint.

A second possibility is that  $\text{Ca}^{2+}$  serves as a bridge for binding anionic phospholipids (or protein partners), as occurs for the annexins (2). NMR data reveal incomplete coordination of one of the two  $\text{Ca}^{2+}$  bound to the second C2 domain of synaptotagmin, suggesting sites of interaction with other molecules (11). Although this is an attractive model for the regulation of the protein kinase Cs, the ability of the novel protein kinase C  $\epsilon$  to bind acidic membranes in the complete absence of  $\text{Ca}^{2+}$  would argue against this. The C2 domain of protein kinase C  $\epsilon$  lacks most of the aspartates involved in  $\text{Ca}^{2+}$  coordination (one aspartate is replaced with arginine, one with glycine, and one with cysteine, with two aspartates conserved). Nonetheless, this protein kinase C interacts with phospholipid by a mechanism apparently similar to that of the conventional protein kinase Cs; specifically, the enzyme binds anionic lipids with low affinity in the absence of diacylglycerol, with this second messenger inducing a dramatic increase in affinity for phosphatidylserine (34). If  $\text{Ca}^{2+}$  serves as a bridge with phosphatidylserine, the novel protein kinase Cs would not be expected to display the same lipid regulation as the conventional protein kinase Cs.

A third possibility is that  $\text{Ca}^{2+}$  causes subtle rearrangements of side chains involved in binding lipid. For example,

some of the basic residues on the periphery of the  $\text{Ca}^{2+}$  site may be better positioned to bind anionic lipids when  $\text{Ca}^{2+}$  occupies the  $\text{Ca}^{2+}$  site. In this regard, mutation of some of the basic residues on the periphery of the  $\text{Ca}^{2+}$  site in the C2A domain of synaptotagmin decreased this domain's interaction with syntaxin (11). The possibility that residues adjacent to the  $\text{Ca}^{2+}$  site bestow specificity in macromolecular interactions of various C2 domains is supported by their lack of conservation.

In summary, the above data suggest that the mechanism of recruiting protein kinase C to membranes via the C2 domain does not result from simple neutralization of the acidic cluster in the  $\text{Ca}^{2+}$  site, nor does it result exclusively from  $\text{Ca}^{2+}$  bridging. Rather, both the ability to bind calcium and the charge of the site may be involved in allowing membrane binding, with specific side chains around the  $\text{Ca}^{2+}$  site possibly allowing for specificity in C2 domain interactions.

**Basic Surface.** An unexpected finding from this research is that the highly basic  $\beta$ -strand behind the  $\text{Ca}^{2+}$  binding site has no significant effect on any parameter of protein kinase C regulation tested. We originally constructed two double lysine-to-alanine mutants (K197A/K199A and K211A/K213A). Because neither affected protein kinase C's lipid regulation (data not shown), we constructed a quadruple mutant in which four of seven positive charges in the  $\beta$ -strands encompassing residues 196–218 of protein kinase C  $\beta$  were mutated to alanine. This dramatic reduction in electrostatic potential on the top face of the C2 domain had no effect on the nonspecific binding of protein kinase C to anionic lipids that occurs in the absence of diacylglycerol, nor did it have any effect on the specific binding to phosphatidylserine that occurs in the presence of diacylglycerol. Thus, this surface does not contain determinants that interact with anionic lipids. It is also unlikely to be oriented close to the membrane because positive charges on this domain do not facilitate protein kinase C's interaction with phosphatidylserine. The finding that this surface does not contribute to protein kinase C's membrane interaction is consistent with the C2 domain approaching the membrane "jaws first", as proposed by Hurley and co-workers for the C2 domain of phospholipase  $\text{C}\delta$  (28).

The basic face is also unlikely to be involved in intramolecular protein interactions since removal of four positive charges did not affect the protein's thermal stability, catalytic rate, or proteolytic sensitivity. Given the fact that the corresponding surface of many C2 domains is basic (7), it may provide a recognition surface for intermolecular protein–protein interactions. In this regard, Mochly-Rosen and co-workers found that a peptide based on sequences on this face of the C2 domain competed with protein kinase C  $\beta$  for binding to the anchoring protein, RACK1 (35).

**Lipid Specificity of Protein Kinase C.** Protein kinase C stands out from other C2 domain-containing proteins in its selective interaction with phosphatidylserine-containing membranes that occurs in the presence, but not in the absence, of diacylglycerol. In the absence of diacylglycerol, protein kinase C binds anionic membranes with no selectivity for the head group beyond the requirement for negative charge. This property is shared with other C2 domain-containing proteins (8). However, diacylglycerol causes a dramatic and selective increase in protein kinase C's affinity for phosphatidylserine-containing membranes or micelles. For ex-

ample, 5 mol % diacylglycerol causes a 250-fold increase in binding affinity for mixed micelles containing 10 mol % phosphatidyl-L-serine, but only a 10-fold increase for mixed micelles containing 10 mol % phosphatidyl-D-serine or other monovalent anionic lipids such as phosphatidylglycerol (31). Clearly, part of the increase in affinity induced by diacylglycerol results from the additional membrane anchor provided by the C1 domain. However, the additional 1 order of magnitude increase that occurs only when phosphatidylserine is present suggests that determinants on the protein could selectively bind phosphatidylserine. Here, we show that potential phosphatidylserine-interacting determinants do not reside in the region of the C2 domain that is targeted to membranes. Although disruption of the  $\text{Ca}^{2+}$  site dramatically decreased protein kinase C's membrane affinity, it had no effect on the ability of diacylglycerol to induce a 2 orders of magnitude increase in the mutant enzyme's affinity for phosphatidylserine-containing membranes. Whether phosphatidylserine-binding determinants reside elsewhere on the protein, or whether phosphatidylserine is able to uniquely position diacylglycerol for optimal binding to protein kinase C, is an open question.

**Conclusions.** The insensitivity of membrane binding to dramatic reduction in the surface charge of the basic face of the C2 domain suggests that this surface is positioned sufficiently far from the membrane that it does not contribute to electrostatic attraction to acidic membranes. Nor does this surface appear to be involved in intramolecular protein interactions, since the stability and proteolytic sensitivity of the quadruple Lys mutant are similar to those of the wild-type enzyme. Rather, critical determinants that mediate protein kinase C's interaction with anionic membranes appear to be localized to the  $\text{Ca}^{2+}$  site of the C2 domain. This site is critical for allowing both  $\text{Ca}^{2+}$  binding and phospholipid binding; mutation of the site dramatically reduces phospholipid affinity as well as  $\text{Ca}^{2+}$  affinity. The foregoing data also reveal that  $\text{Ca}^{2+}$  binding to this site increases protein kinase C's membrane affinity by a mechanism that cannot be accounted for by a simple electrostatic switch. Whether specific residues surrounding the  $\text{Ca}^{2+}$  site contribute to specificity in C2 domain macromolecular interactions remains to be explored. It is interesting to note that the loops of the C2 domain that form the  $\text{Ca}^{2+}$  site may be multifunctional; not only do they contain determinants for binding phospholipid, as determined above for protein kinase C, but NMR data suggest that they bind syntaxin in the C2A domain of synaptotagmin (33).

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