THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 41, Issue of October 10, pp. 25959–25967, 1997

Printed in U.S.A.

Ca^{2+} Differentially Regulates Conventional Protein Kinase Cs' Membrane Interaction and Activation*

(Received for publication, May 20, 1997, and in revised form, July 23, 1997)

Lisa M. Keranen and Alexandra C. Newton‡

From the Department of Pharmacology, University of California at San Diego, La Jolla, California 92093-0640

The regulation of conventional protein kinase Cs by Ca^{2+} was examined by determining how this cation affects the enzyme's 1) membrane binding and catalytic function and 2) conformation. In the first part, we show that significantly lower concentrations of Ca^{2+} are required to effect half-maximal membrane binding than to half-maximally activate the enzyme. The disparity between binding and activation kinetics is most striking for protein kinase C βII, where the concentration of Ca^{2+} promoting half-maximal membrane binding is approximately 40-fold higher than the apparent K_m for Ca^{2+} for activation. In addition, the Ca^{2+} requirement for activation of protein kinase C βII is an order of magnitude greater than that for the alternatively spliced protein kinase C βI; these isozymes differ only in 50 amino acids at the carboxyl terminus, revealing that residues in the carboxyl terminus influence the enzyme's Ca^{2+} regulation. In the second part, we use proteases as conformational probes to show that Ca^{2+}-dependent membrane binding and Ca^{2+}-dependent activation involve two distinct sets of structural changes in protein kinase C βII. Three separate domains spanning the entire protein participate in these conformational changes, suggesting significant interdomain interactions. A highly localized hinge motion between the regulatory and catalytic halves of the protein accompanies membrane binding; release of the carboxyl terminus accompanies the low affinity membrane binding mediated by concentrations of Ca^{2+} too low to promote catalysis; and exposure of the amino-terminal pseudosubstrate and masking of the carboxyl terminus accompany catalysis. In summary, these data reveal that structural determinants unique to each isozyme of protein kinase C dictate the enzyme's Ca^{2+}-dependent affinity for acidic membranes and show that, surprisingly, some of these determinants are in the carboxyl terminus of the enzyme, distal from the Ca^{2+}-binding site in the amino-terminal regulatory domain.

Protein kinase C is a multi-domain family of enzymes that transduces the myriad of signals resulting in phospholipid hydrolysis (1–3). The amino-terminal regulatory half of the conventional isozymes comprises three functionally distinct domains as follows: 1) an intramolecular inhibitory domain, the pseudosubstrate, that occupies the active site when the enzyme is inactive; 2) two cysteine-rich domains (C1) that are each capable of binding diacylglycerol or phorbol esters; and 3) the C2 domain that binds Ca^{2+} and acidic phospholipids. The C1 and C2 domains function as discrete membrane-targeting motifs (4); binding of ligand to either domain alone can promote protein kinase C translocation to membranes, but both domains must be membrane-bound for the high affinity interaction resulting in maximal activation (3). The carboxyl-terminal half of the protein comprises the conserved kinase core, followed by a carboxyl-terminal region whose sequence varies among isozymes.

Understanding the mechanism by which Ca^{2+} first recruits protein kinase C to membranes and then activates its kinase activity is essential to understanding how calcium mobilization regulates the function of protein kinase C *in vivo*. Although the relationship between Ca^{2+} effects on binding and activity has not been established, much progress has been made toward describing the effects of the cation on protein kinase C activation. Since the initial reports that lipid-stimulated activation of protein kinase C requires Ca^{2+} (5), biochemical analyses from several laboratories have established that Ca^{2+} decreases the concentration of negatively charged phospholipids required for maximal activation (6, 7) or membrane binding (8, 9). It is also well established that the Ca^{2+}-mediated increase in affinity for acidic membranes is considerably more pronounced in the presence of diacylglycerol or phorbol esters (10). The molecular mechanism for this synergy was recently found to result not from allosteric interactions between the binding sites for these two ligands but rather because each cofactor, by separate mechanisms, increases protein kinase C's affinity for phosphatidylserine (9, 11, 12). Thus, considerably less diacylglycerol is required to activate protein kinase C as the Ca^{2+} concentration is raised, and vice versa, because both Ca^{2+} and diacylglycerol cause tighter binding to phosphatidylserine (and it is this tight binding that results in release of the autoinhibitory pseudosubstrate from the active site (13)). The dissociation constant of Ca^{2+} from protein kinase C has recently been estimated to be 3 mM in the absence of lipid and 0.7 μM from the protein kinase C-lipid complex (11). Furthermore, the enzyme's membrane affinity has been shown to vary linearly with Ca^{2+} concentration from the low micromolar to the submillimolar concentration range of this cation (11).

The stoichiometry of the protein kinase C-Ca^{2+} complex has been reported to be 1 (11, 14) to 1 or 2, depending on the isozyme (15), and to up to 8 (8). However, NMR and crystallographic data from C2 domains analyzed in the presence of >10 mM Ca^{2+} (16) or Sm^{3+} (17) indicate that the Ca^{2+}-binding pocket coordinates two metal ions for the C2 domains studied; the actual affinities for each cation and whether both are bound in the presence of lipid remains to be established. Despite great advances in elucidating how Ca^{2+} is accommodated in the protein kinase C structure, understanding how this cation affects the function of protein kinase C awaits further biochemical characterization.

* This work was supported by National Institutes of Health Grant GM 43154 and a National Science Foundation Young Investigator Award. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 619-534-4527; Fax: 619-534-6020; E-mail: anewton@ucsd.edu.

This contribution examines the role of Ca^{2+} in regulating the membrane binding and catalytic function of protein kinase C and how these functional changes correlate with structural changes. Specifically, we show that 1) membrane binding and activation of conventional protein kinase Cs display different Ca^{2+} requirements, with activation requiring higher concentrations of the cation than membrane binding, and 2) distinct conformational changes accompany Ca^{2+}-dependent membrane binding and Ca^{2+}-dependent activation. In addition, we show that determinants in the carboxyl terminus modulate protein kinase C's affinity for Ca^{2+}.

MATERIALS AND METHODS

Bovine brain L-α-phosphatidylserine, *sn*-1-palmitoyl-2-oleoylphosphatidylserine (POPS),[1] and *sn*-1-palmitoyl-2-oleoylphosphatidylcholine (POPC) were obtained from Avanti Polar Lipids, Inc. [^{3}H]L-α-Dipalmitoylphosphatidylcholine (30–60 Ci mmol^{-1}) and [γ-^{32}P]ATP (3000 Ci mmol^{-1}) were from NEN Life Science Products. Triton X-100 (10%, w/v, aqueous solution) was from Pierce. *sn*-1,2-Dioleoylglycerol, trypsin from bovine pancreas (1.22×10^4 BAEE units mg^{-1}), HEPES, dithiothreitol (DTT), EGTA, trypsin inhibitor (soybean), and protamine sulfate were supplied by Sigma. Thermolysin, elastase, chymotrypsin, endoproteinases Arg-C, Asp-N, Glu-C, and Lys-C, bovine serum albumin, and alkaline phosphatase-conjugated and peroxidase-conjugated goat anti-rabbit IgG were obtained from Boehringer Mannheim. Nitrocellulose (Schleicher & Schuell) was from Midwest Scientific. Calcium chloride (analytical grade) was purchased from J. T. Baker, Inc. A protein kinase C-selective peptide substrate (Ac-FKK**S**FKL-NH_2; (18)) was synthesized by the Indiana University Biochemistry Biotechnology Facility. All other chemicals were reagent grade. Unless otherwise noted, experiments were performed using 20 mM HEPES, pH 7.5, at 22 °C (HEPES buffer). Protein sequencing was performed by the Harvard Microchemistry Facility.

Antibodies—Polyclonal antibodies against bacterially expressed catalytic domain and bacterially expressed regulatory domain of protein kinase C βII were a gift of Drs. Andrew Flint and Daniel Koshland, Jr.; a polyclonal antibody against a peptide comprising residues 645–673 of protein kinase C βII was generously provided by Lilly, and one against residues 19–32 of protein kinase C βII was from the laboratory of Dr. David S. Williams.

Protein Kinase C—Protein kinase C α, βI, or βII were expressed in Sf21 insect cells (Invitrogen) by infection with recombinant baculovirus (generous gifts from Robert Bell, Peter Parker, and Daniel Koshland Jr., respectively) and purified to homogeneity as described (13). The enzymes were stored at −20 °C in 10 mM Tris buffer, pH 7.5 at 4 °C, 150 mM KCl, 0.5 mM DTT, 0.5 mM EDTA, 0.5 mM EGTA, and 50% glycerol.

Lipid—Sucrose-loaded vesicles (40 mol % POPS, 55 mol % POPC, and 5 mol % diacylglycerol) were prepared as described (19). Triton X-100:lipid mixed micelles of composition 0–15 mol % brain phosphatidylserine and 5 mol % diacylglycerol were prepared as described by Newton and Koshland (20). Phospholipid concentrations in chloroform were determined by assay for phosphate concentration (21). Lipids were diluted 10-fold into the assay mixture corresponding to 0.1% Triton X-100 and approximately 100–300 μM lipid for assays using micelles or 100 μM lipid for assays using vesicles.

Protein Kinase C Activity Assay—Protein kinase C (typically 3 nM) activity was assayed by measuring the initial rate of [^{32}P]phosphate incorporation from [γ-^{32}P]ATP (50 μM; 3000 Ci mmol^{-1}) into saturating amounts of a protein kinase C-selective peptide (50 μg ml^{-1}) or protamine sulfate (0.2 mg ml^{-1}) as described (13). Reactions were allowed to proceed for 6 min at 30 °C. The concentrations of $CaCl_2$ and lipid present in the assays are indicated in the figure legends.

Centrifugation Assay for Protein Kinase C Membrane Binding—Protein kinase C (3–30 nM) was incubated with sucrose-loaded vesicles (100 μM lipid; 40 mol % POPS, 5 mol % diacylglycerol, 55 mol % POPC) and various Ca^{2+} concentrations in the presence of 20 mM HEPES buffer, 0.8 mM DTT, 0.3 mg ml^{-1} bovine serum albumin, 500 μM EGTA, 60 mM KCl, and in the presence or absence of 5 mM $MgCl_2$. Membrane binding was measured by determining the fraction of protein kinase C bound to sucrose-loaded vesicles after centrifugation for 30 min at 100,000 × *g*, 22 °C. The amount of protein kinase C in the supernatant or associated with the vesicles was measured either by assaying kinase activity toward protamine sulfate as described above or by Western blot analysis of supernatant and pellet samples.

Trypsin Sensitivity Assay for Protein Kinase C Membrane Binding—This assay takes advantage of the large increase in trypsin sensitivity of protein kinase C's hinge region that accompanies membrane binding (22). For comparison of the two binding assay methods, protein kinase C (30 nM) was incubated under conditions described above for the centrifugation assay. Samples were treated with trypsin (0.12 units ml^{-1}; 10 min at 30 °C), and proteolysis was quenched by addition of 0.25 volume of SDS-PAGE sample buffer. Samples were analyzed by SDS-PAGE (8% polyacrylamide) followed by electrophoretic transfer to nitrocellulose membranes and Western blotting with protein kinase C antibodies against the carboxyl terminus of βII. Quantitative analysis of proteolysis was carried out by densitometric scanning of the intact protein kinase C bands with a scanning densitometer (Molecular Dynamics). Percent of protein kinase C bound to membranes was correlated to the amount of proteolyzed protein as described (22).

Proteolysis—Protein kinase C (approximately 30 nM) was incubated at 30 °C in the presence of HEPES, DTT (1 mM final concentration), $CaCl_2$ (1 nM to 5 mM final concentration), Triton X-100 (0.1% w/v final concentration) mixed micelles containing 15 mol % brain phosphatidylserine and 5 mol % diacylglycerol and proteases (concentrations and incubation times as indicated in the figure legends). Proteins were separated by SDS-PAGE on 7 or 9% polyacrylamide gels and visualized by silver staining, or in some cases, proteins were electrophoretically transferred to nitrocellulose and labeled with antibodies to protein kinase C via incubation with alkaline phosphatase-conjugated IgG and detection by the formation of the insoluble product of 5-bromo-4-chloroindoyl phosphate hydrolysis.

Data Analysis—The dependence of protein kinase C binding or activity on the lipid composition of vesicles (or micelles) or the Ca^{2+} concentration was analyzed by a nonlinear least-squares fit to a modified Hill equation as described (20). The apparent association constant, K_a, for binding of protein kinase C to vesicles was calculated as the fraction of protein kinase C bound to membranes divided by the product of the fraction of protein kinase C remaining in the supernatant and the total lipid concentration (19).

Free [Ca^{2+}] Determinations—Concentrations of free Ca^{2+} were calculated using a computer program kindly provided by Dr. Claude Klee (23) that takes into account pH, Ca^{2+}, Mg^{2+}, K^+, Na^+, EGTA, EDTA, and ATP concentrations. Binding constants used in this program were those given by Fabiato and Fabiato (24).

RESULTS

Ca^{2+} Dependence for Membrane Binding Detected by Two Independent Assays—The Ca^{2+} dependence for the binding of protein kinase C βII to large unilamellar vesicles (100 μM lipid) containing phosphatidylserine (40 mol %) and diacylglycerol (5 mol %) is presented in Fig. 1. Binding was detected three ways, using two independent assays, to ensure that the measured kinetics reflected properties of the protein-cofactor interaction rather than the assay system. One assay was conformation-based (○); it detects the marked increase in proteolytic sensitivity of protein kinase C's hinge region that accompanies membrane binding (approximately 100-fold increase for protein kinase C βII) (22). The second assay involved physical separation of membrane-bound protein kinase C from free protein kinase C by centrifugation (19). In the latter, the amount of membrane-bound protein kinase C was determined either from Western blot analysis (□) or activity assays using the cofactor-independent substrate protamine (△). The only compositional difference between the two assays was the presence of trypsin in the conformation-based assay. The centrifugation assay has the advantage of being a direct measure of the membrane association, whereas the conformation-based assay has the advantage of not involving separation of free enzyme from vesicles and thus avoids potential perturbation of the equilibrium between the bound and free enzyme.

Importantly, the centrifugation assay (□, △) and conformation-based assay (○) yielded similar results; under the conditions of the experiment, half-maximal binding to vesicles was

[1] The abbreviations used are: POPS, *sn*-1-palmitoyl-2-oleoylphosphatidylserine; BAEE, N^{α}-benzoyl-L-arginine ethyl ester; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; POPC, *sn*-1-palmitoyl-2-oleoylphosphatidylcholine.

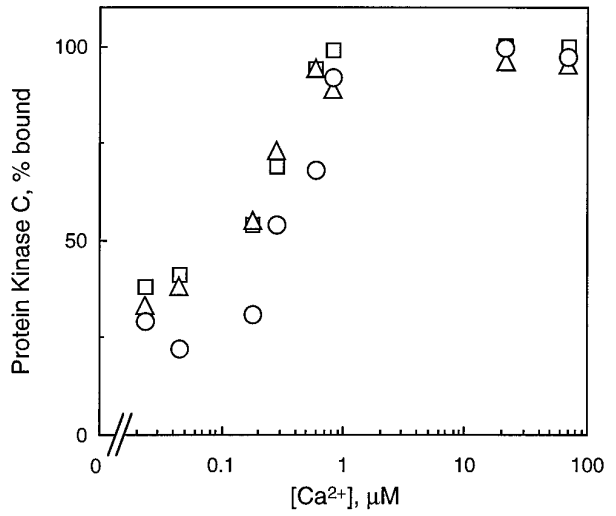

FIG. 1. **The Ca^{2+} dependence of protein kinase C βII binding to model membranes is the same using two assay methods.** The binding of protein kinase C βII (30 nM) to vesicles (100 μM lipid) containing 40 mol % POPS, 5 mol % diacylglycerol, and 55 mol % POPC was measured in the presence of various Ca^{2+} concentrations by pelleting the vesicle-bound enzyme (□, △) or by proteolyzing with trypsin (○), as described under "Materials and Methods." The amount of membrane-bound protein kinase C was determined from Western blot analysis (□) or activity assays (△). Binding measurements were performed in buffer containing 500 μM EGTA; Ca^{2+} was added to yield the indicated concentrations of free Ca^{2+}. Data are representative of two separate experiments.

mediated by approximately 300 nM Ca^{2+}. At this free Ca^{2+} concentration, the enzyme's apparent membrane affinity was 10^4 M^{-1}. This affinity is comparable to that reported for the interaction of protein kinase C from rat brain with vesicles containing slightly more phosphatidylserine (50 mol %) but less diacylglycerol (1 mol %) in the presence of 1 μM Ca^{2+} (11).

Ca^{2+} Regulation of Three Protein Kinase C Isozymes' Membrane Affinity and Activation—Fig. 2*A* compares the Ca^{2+} dependence for membrane binding of three conventional protein kinase Cs as follows: α (○), βII (□), and the alternatively spliced βI (△) which differs from βII in the last 50 residues at the carboxyl terminus. The Ca^{2+} requirement for membrane binding differed modestly for these three isozymes; under the conditions of the assay, the concentrations of Ca^{2+} mediating half-maximal binding ranged from 0.3 to 1.0 μM for the three isozymes (Table I). Note that the concentration of Ca^{2+} resulting in half-maximal membrane binding for βII was approximately 3-fold higher in Fig. 2*A* (and Table I) than that in Fig. 1; this resulted from the presence of 5 mM Mg^{2+} in the assay in Fig. 2, which decreases the membrane surface potential and hence protein kinase C's membrane affinity (19). Importantly, the apparent Ca^{2+} affinity of protein kinase C βI was 3-fold higher than the apparent Ca^{2+} affinity of protein kinase C βII, despite the two having identical Ca^{2+}-binding sites in the C2 domain. Furthermore, protein kinase C βI displayed a significantly higher level of Ca^{2+}-independent membrane binding than the other two isozymes: 40% of the protein kinase C βI associated with vesicles in the absence of Ca^{2+}, compared with 15 and 10% for protein kinase C α and βII, respectively. These results reveal that the very carboxyl-terminal residues of protein kinase C influence its Ca^{2+}-dependent membrane interaction.

The Ca^{2+} requirement for activation differed significantly among isozymes, and, for two isozymes, this requirement was markedly different from the Ca^{2+} requirement for membrane binding (Fig. 2*B*). The Ca^{2+} dependence for both activation and binding was similar for protein kinase C α; however, both protein kinase Cs' βI and βII required over an order of magnitude higher concentrations of Ca^{2+} for half-maximal activation compared with that required for half-maximal membrane binding (Table I). Conditions for the membrane binding and activity assays were identical except that bovine serum albumin (0.3 mg ml^{-1}) was included in the binding assay (its presence in activity assays did not affect results (data not shown)) and ATP and peptide substrate were present in the activity assay (their presence in binding assays does not significantly affect protein kinase Cs' interaction with vesicles[2]). Most strikingly, the alternatively spliced β isozymes displayed a difference in their Ca^{2+} requirements for activation that was even greater than the difference in their requirements for membrane binding; the apparent K_m for Ca^{2+} (for activity) was approximately 10 × higher for protein kinase C βII compared with protein kinase C βI.

The Ca^{2+} dependence for activation was found to be sensitive to the total lipid concentration. Analysis of the data in Fig. 3 revealed that increasing the lipid concentration 10-fold resulted in a 10-fold drop in the apparent K_m (from approximately 30 μM in the presence of 100 μM lipid to approximately 3 μM in the presence of 1 mM lipid). This is consistent with binding studies by Mosior and Epand (19) showing that the interaction of protein kinase C with membranes obeys the mass action law; altering either the total lipid concentration or the total enzyme concentration affects the amount of protein kinase C that partitions with the membrane. Thus, reported K_m values for Ca^{2+} for protein kinase C are specific to particular assay conditions.

Ca^{2+}-induced Conformational Changes—The finding that protein kinase C requires different Ca^{2+} concentrations for membrane binding and activation led us to explore whether distinct conformational changes accompany the Ca^{2+}-dependent membrane interaction and the Ca^{2+}-dependent activation. In a first set of experiments, we examined whether the proteolytic sensitivity of protein kinase C βII was altered with increasing Ca^{2+} concentrations when in solution or when membrane-bound. Fig. 4 shows that the trypsin sensitivity of protein kinase C βII, in the absence of lipid cofactors, was the same in the presence of 100 nM to 5 mM Ca^{2+}. Under the conditions of the assay, the enzyme was partially proteolyzed to generate the catalytic domain (approximately 45 kDa) and regulatory half (approximately 35 kDa; does not stain well by silver) (*lanes 2–9*). The similar degree of cleavage in *lanes 2–9* reveals that the intrinsic activity of trypsin is not affected by altering the Ca^{2+} concentration between 100 nM and 5 mM Ca^{2+} (also established using BAEE as substrate, not shown).

In contrast to the cleavage in the absence of lipid, incubation of protein kinase C βII with phosphatidylserine:diacylglycerol mixed micelles resulted in pronounced differences in the enzyme's proteolytic sensitivity as the Ca^{2+} concentration was raised. Two sets of changes in proteolytic rates or proteolytic fragments produced were observed. First, at Ca^{2+} concentrations sufficient to effect membrane binding, but not activation, the enzyme was markedly more sensitive to proteolysis at the hinge region as assessed by the increased generation of the catalytic domain (compare *lane 11* (100 nM Ca^{2+}; no binding) with *lanes 12* and *13* (1 and 10 μM Ca^{2+}; under the conditions of this assay, half-maximal binding required 1 μM Ca^{2+})). In the presence of Ca^{2+} concentrations too low to effect membrane binding (100 nM Ca^{2+}), the enzyme was proteolyzed at a similar rate as in the absence of lipid (note that the trypsin concentration in *lanes 11–18* (plus lipid) was 10-fold lower than in *lanes 2–9* (no lipid)). This change in proteolytic sensitivity is the well-characterized exposure of the hinge region upon mem-

[2] M. Mosior and A. C. Newton, unpublished results.

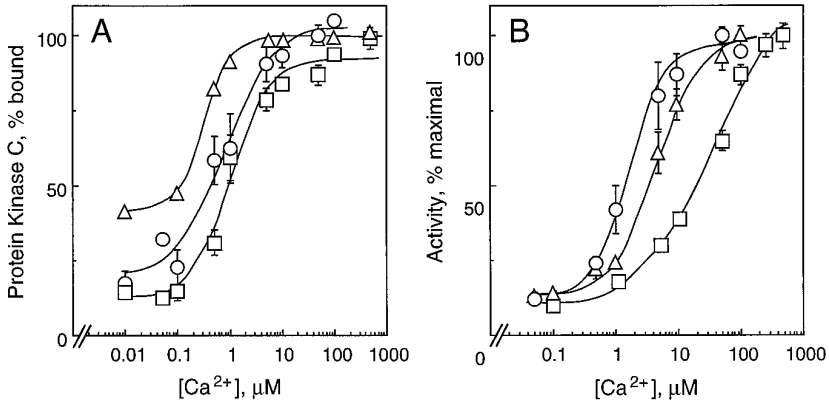

FIG. 2. **Three Ca^{2+}-dependent isozymes of protein kinase C display similar Ca^{2+} requirements for binding to membranes but an order of magnitude difference in Ca^{2+} requirements for activity.** *A*, protein kinase C (3 nM) α (○), βI (△), or βII (□) binding to vesicles (100 μM lipid) was measured in the presence of 5 mM Mg^{2+} and various Ca^{2+} concentrations using the centrifugation assay as described under "Materials and Methods." Data represent the average ± S.E. of the mean of 4, 2, and 7 separate experiments for α, βI, and βII, respectively. *B*, activity of protein kinase C (3 nM) toward selective peptide was determined under identical conditions to those described in *A* except no bovine serum albumin was present, and ATP and peptide substrate were present. Data show the average ± S.D. of two experiments performed in sextuplet.

TABLE I
Comparison of Ca^{2+} requirements for various protein kinase C isozymes

Isozyme	Binding	Activity
	μM	μM
α	0.8 ± 0.2	1.5 ± 0.4
βII	1.0 ± 0.2	38 ± 14
βI	0.31 ± 0.02	4.4 ± 0.6

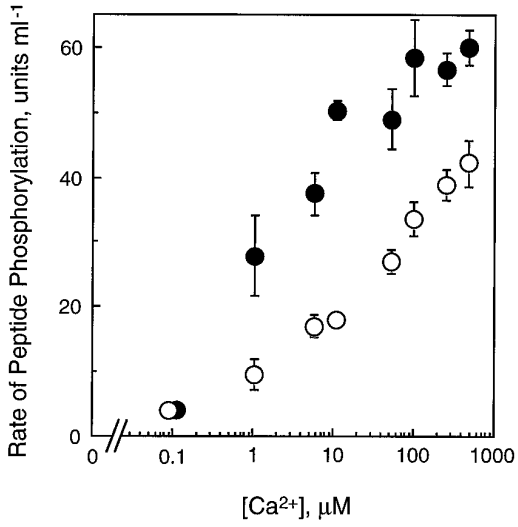

FIG. 3. **The Ca^{2+} requirement for protein kinase C βII activity is dependent on the lipid concentration.** Protein kinase C activity toward selective peptide was measured in the presence of vesicles (100 μM (○) or 1 mM (●) lipid) containing 40:5:55 mol % POPS:diacylglycerol: POPC. Data represent the average ± S.D. of triplicate measurements. Data points at 0.1 μM Ca^{2+} are slightly offset for clarity.

brane binding (22). In addition, a novel 70-kDa intermediate (*asterisk*) became more pronounced in lanes where protein kinase C was bound to lipid but not active (*e.g. lanes 12* and *13*); a slightly faster migrating band (68 kDa) was barely visible below this band. A second set of changes in proteolytic sensitivity occurred as the Ca^{2+} concentration was raised to activating levels (*e.g.* above 500 μM Ca^{2+}); the 70-kDa intermediate became less apparent and a 78-kDa intermediate (*arrow*) became apparent. Qualitatively similar results were obtained whether protein kinase C was incubated with mixed micelles (Fig. 4) or vesicles (data not shown).

Antibodies Detect Cleavage at Carboxyl or Amino Terminus of Protein Kinase C—To better understand the Ca^{2+}-induced changes in protein kinase C's structure, a series of polyclonal antibodies generated against different determinants on protein kinase C were used to identify proteolytic fragments. Fig. 5*A* characterizes the epitope of each antibody. Protein kinase C βII was proteolyzed so that the native protein (*80 kDa*) and 78- (*arrow*), 70- (*asterisk*), and 68-kDa intermediates were trapped (the 78- and 68-kDa forms are more apparent when endoproteinase Arg-C is used; all these fragments co-migrate with the bands generated by trypsin cleavage). Samples were analyzed by SDS-PAGE and stained with silver (*panel 1*) or labeled with four antibodies (*panels 2–5*). The 78-kDa fragment (*arrow*) has been previously characterized and results from activation-dependent cleavage at Arg-19 in the pseudosubstrate of protein kinase C βII (13). This fragment was recognized by all antibodies except one generated against the bacterially expressed regulatory domain of protein kinase C: thus, the epitope for this antibody (anti-regulatory) lies in the first 18 residues of protein kinase C βII. The 70-kDa intermediate (*asterisk*) was recognized by all antibodies except an antibody generated against the carboxyl terminus of protein kinase C βII (residues 645–673); this revealed that the 70-kDa form was proteolyzed at the carboxyl terminus. Antibodies generated against bacterially expressed catalytic domain (anti-catalytic) and the peptide sequence from the pseudosubstrate of protein kinase C βII (residues 19–32; anti-pseudosubstrate) recognized native and amino- or carboxyl-terminally cleaved protein kinase C equally well. Neither the anti-regulatory or anti-carboxyl-terminal domain antibodies labeled the 68-kDa fragment, indicating that it was truncated at both termini. Thus, one antibody recognized the first 18 residues of protein kinase C βII (anti-regulatory), one recognized the pseudosubstrate of protein kinase C (anti-pseudosubstrate), one recognized the catalytic domain (anti-catalytic), and one recognized the extreme carboxyl terminus of the protein (anti-carboxyl terminus).

Ca^{2+} Binding Induces Three Structural Changes in Protein Kinase C—The four antibodies, with epitopes spanning the length of protein kinase C, were used to characterize the tryptic fragments described in Fig. 4. Protein kinase C was partially proteolyzed by trypsin under three conditions, shown in Fig. 5*B*, when it was 1) soluble (*lane 2*), 2) bound to phosphatidylserine:diacylglycerol mixed micelles in the presence of 1 μM Ca^{2+} (*lane 3*) and thus inactive, or 3) bound to phosphatidylserine:diacylglycerol mixed micelles in the presence of 200 μM

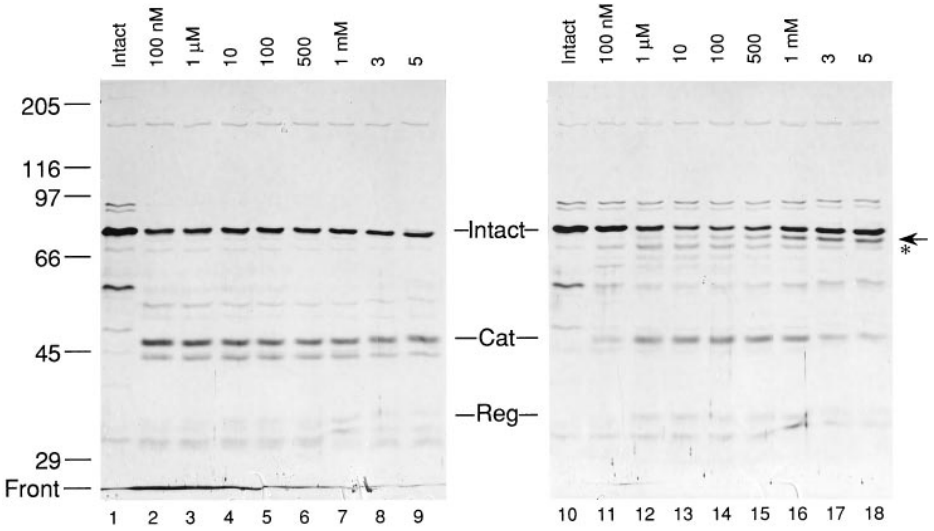

FIG. 4. **Effect of Ca^{2+} on the trypsin sensitivity of protein kinase C.** Silver-stained gels of protein kinase C βII proteolyzed with trypsin as a function of increasing Ca^{2+} concentrations, in the presence or absence of lipid. Protein kinase C βII (35 nM) was incubated in the absence (*lanes 2–9*) or presence (*lanes 11–18*) of Triton X-100 (0.1%) mixed micelles containing 15 mol % phosphatidylserine and 5 mol % diacylglycerol and the indicated concentrations of Ca^{2+}; the enzyme was then treated with trypsin (3.7 units ml^{-1} in the absence of lipid; 0.37 units ml^{-1} in the presence of lipid) for 20 min at 30 °C. Native protein kinase C that was not treated with trypsin is shown in *lanes 1* and *10*. Samples were analyzed on 9% polyacrylamide gels. *Arrow* indicates the 78-kDa intermediate; *asterisk* indicates 70-kDa intermediate.

Ca^{2+} (*lane 4*) and thus active. Labeling with the catalytic domain antibody (*lower left panel*) shows the 70-kDa fragment was most pronounced when protein kinase C was lipid-bound but inactive (*lane 3*). The carboxyl-terminal antibody did not recognize this fragment (*top left panel*) but the regulatory antibody that recognizes the first 18 amino acids of the protein did (*bottom right panel*). Thus, Ca^{2+} concentrations sufficient for membrane binding but not activation cause a conformational change that exposes the carboxyl terminus to proteolysis. The conformational changes occurring at activating Ca^{2+} concentrations were characterized by generation of the 78-kDa fragment, which results from exposure of the pseudosubstrate and cleavage at Arg-19 (13) (Fig. 5*B, lane 4*; labeled by anti-carboxyl-terminal, anti-catalytic, and anti-pseudosubstrate antibodies, but not the anti-regulatory antibody recognizing the first 18 amino acids; see also Fig. 5*A*).

The proteolytic pattern resulting from inactivating and activating Ca^{2+} concentrations was explored in more detail in the time courses of trypsinolysis in Fig. 6*A*. Protein kinase C was incubated with 1 nM Ca^{2+} and no lipid (soluble conformation), with 1 μM Ca^{2+} and phosphatidylserine:diacylglycerol mixed micelles (lipid-bound but inactive), or with 200 μM Ca^{2+} and phosphatidylserine:diacylglycerol mixed micelles (lipid-bound and active) and treated with trypsin for 0–30 min. Under the conditions of the assay, soluble protein kinase C was relatively resistant to proteolysis, whereas the lipid-bound enzyme was markedly sensitive to proteolysis at the hinge to generate the catalytic domain (apparent in Fig. 6*A* starting at 1 min time point in the presence of 1 and 200 μM Ca^{2+}). Importantly, the rate of disappearance of the native (80 kDa) protein kinase C was similar in the presence of 1 and 200 μM Ca^{2+}, consistent with the hinge exposure reflecting the lipid-bound conformation of protein kinase C and being independent of the active state of the enzyme. The 70-kDa fragment resulting from carboxyl-terminal cleavage was specific to the incubation with 1 μM Ca^{2+}; in addition, a truncated catalytic domain was apparent (this catalytic domain fragment was proteolyzed at the carboxyl terminus because it was not labeled with anti-carboxyl-terminal antibodies (data not shown)). Cleavage at the pseudosubstrate (*N-nicked PKC* in Fig. 6*A*) but not at the carboxyl terminus characterized the proteolytic pattern in the presence of activating Ca^{2+} concentrations (*lane 14*). At this activating Ca^{2+} concentration, the cleaved catalytic domain was considerably more stable than at the non-activating concentration (after 5 min of proteolysis in the presence of 1 μM Ca^{2+}, the catalytic core is almost completely proteolyzed at the

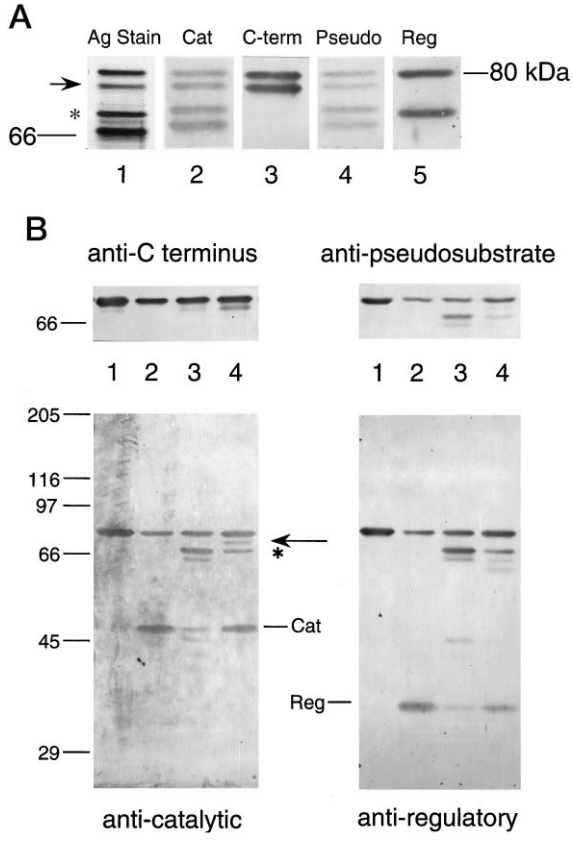

FIG. 5. **The identification of proteolytic fragments of protein kinase C.** *A*, protein kinase C βII (30 nM) was digested with endoproteinase Arg-C (4 units ml^{-1}) in the presence of 200 μM Ca^{2+} and mixed micelles of the composition described in Fig. 5. The proteolytic digests were analyzed by silver (*Ag*) staining (*lane 1*) or by Western blotting with antibodies generated against the catalytic (*Cat*) domain (*lane 2*), the carboxyl terminus (*C-term*) (*lane 3*), the pseudosubstrate (*Pseudo*) (*lane 4*), or the regulatory (*Reg*) domain (*lane 5*) of protein kinase C. The four bands stained by the antibodies correspond to the four bands stained by silver. *B*, protein kinase C βII was proteolyzed with trypsin in the absence of lipid and the presence of 1 μM Ca^{2+} (*lane 2*), in the presence of phosphatidylserine (15 mol %):diacylglycerol (5 mol %):Triton X-100 (0.1% w/v) mixed micelles, and 1 μM Ca^{2+} (*lane 3*), or in the presence of mixed micelles and 200 μM Ca^{2+} (*lane 4*). Trypsin concentrations were 60 units ml^{-1} for *lane 2* and 0.6 units ml^{-1} for *lanes 3* and *4*. *Lane 1* contains untreated protein kinase C. Samples were analyzed by Western blot analysis using the antibodies described in *A*. Indicated are the 78-kDa fragment, *arrow*; the 70-kDa fragment, *asterisk*; the 45-kDa catalytic domain, *Cat*; the 35-kDa regulatory domain, *Reg*.

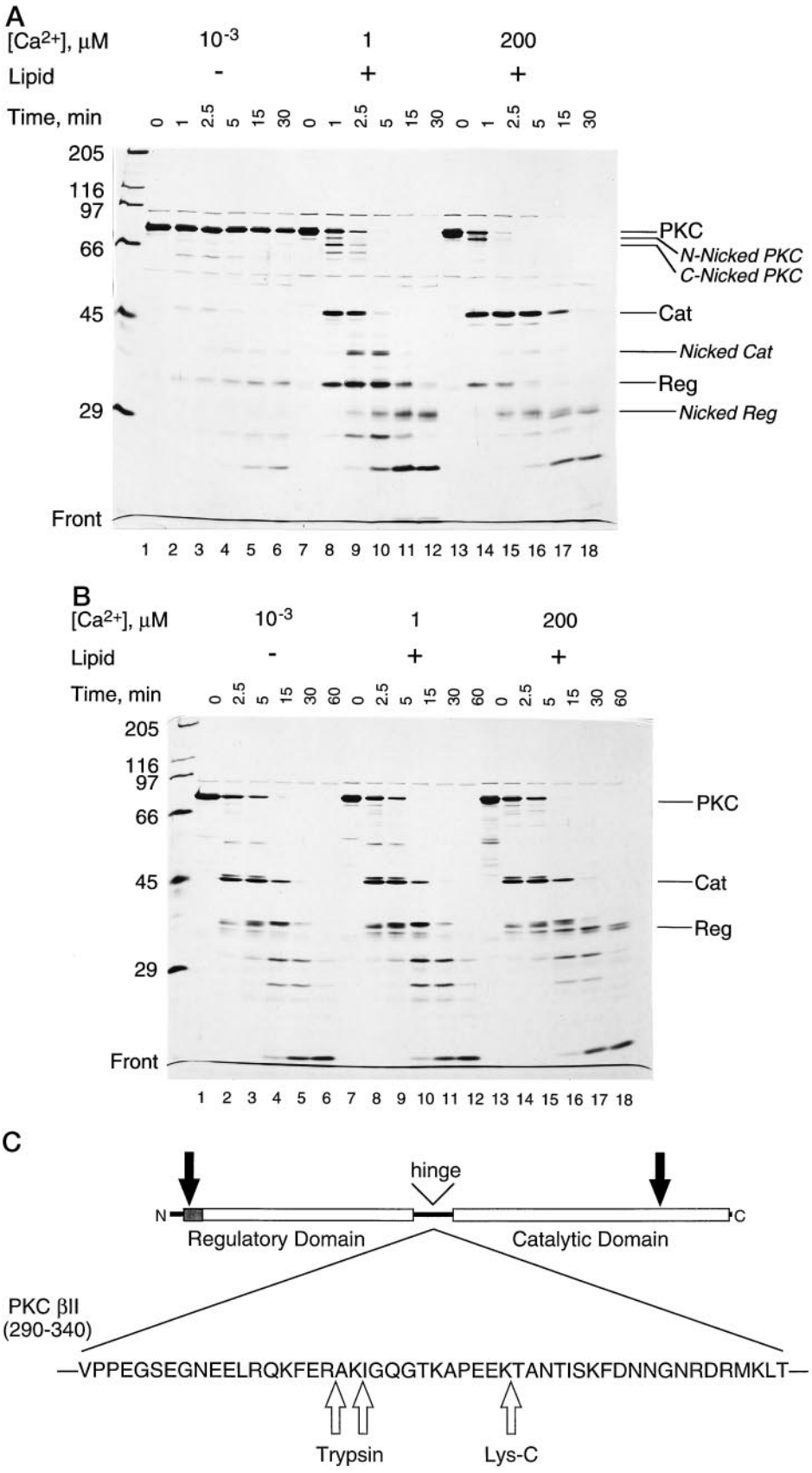

FIG. 6. **Ca^{2+} affects the proteolytic sensitivity of three separate regions of protein kinase C.** Protein kinase C (*PKC*) βII (30 nM) was incubated in the presence of Triton X-100:lipid mixed micelles (15 mol % phosphatidylserine and 5 mol % diacylglycerol in 0.1% Triton X-100) and the indicated concentrations of Ca^{2+} and treated with trypsin (0.12 units ml^{-1}) (*A*) or endoproteinase Lys-C (0.24 units ml^{-1}) (*B*) for the indicated times. *N-Nicked* refers to 78-kDa fragment cleaved at amino-terminal pseudosubstrate; *C-Nicked* refers to 70-kDa fragment cleaved at carboxyl terminus; *Cat*, to 45-kDa catalytic domain; *Nicked Cat*, to catalytic domain cleaved at carboxyl terminus; *Reg*, to 35-kDa regulatory domain, and *Nicked Reg*, to regulatory domain cleaved at pseudosubstrate. *C*, schematic of the primary structure of protein kinase C βII showing the trypsin and Lys-C cleavage sites in the hinge identified by amino-terminal sequencing (*open arrows*) and the trypsin cleavage sites at both termini of the protein localized by antibody labeling (*filled arrows*).

carboxyl terminus; 200 μM Ca^{2+} protects this fragment from additional proteolysis).

Hinge Conformational Change Is Highly Localized—Fig. 6*B* shows the sensitivity of protein kinase C to endoproteinase Lys-C for soluble, lipid-bound but inactive, and lipid-bound and active protein kinase C. In marked contrast to the cleavage by trypsin, Lys-C did not discriminate between the different forms of protein kinase C; proteolysis at the hinge to generate the catalytic and regulatory domains occurred at the same rate for all three conditions. To localize the site of the conformational

TABLE II
Proteolytic sensitivity of protein kinase C

Protease	Fragments generated (approximate size)	Increased proteolytic rate upon membrane binding[a]	Sensitivity to Mg^{2+}	Detected Ca^{2+}-induced changes
	kDa			
Arg-C	78[b]		ND[c]	+
Asp-N	70	>10 ×	ND	−
Chymotrypsin	70, 45, 35	~4 ×	+	−
Elastase	78,[b] 70, 55, 45, 35, 33, 30	~100 ×	ND	+
Glu-C	50, 30		−	−
Lys-C	45, 35		−	−
Thermolysin	70, 45, 35	~4 ×	+	−
Trypsin	78,[b] 70, 48, 40, 35, 30	~100 ×	+	+

[a] Proteolysis of 80-kDa band was measured.
[b] Generation of this fragment is dependent on activation.
[c] ND, not determined.

change in the hinge, the catalytic domains generated by trypsin or Lys-C cleavage were subjected to amino-terminal sequencing. Fig. 6*C* shows that trypsin cleaves at two sites, Arg-307 and Lys-309, which are amino-terminal to the single Lys-C site at Lys-320. Thus, the region around residue 320 is equally accessible in all three conformations of protein kinase C (soluble, membrane-bound with or without activity), whereas a region removed by only a dozen residues is masked in the soluble conformation and undergoes a dramatic increase in exposure upon membrane binding.

Other Proteases as Conformational Probes—Table II summarizes the results of treating protein kinase C with a number of proteases. Proteases fell into three classes as follows: those such as elastase, trypsin, and to a lesser extent chymotrypsin and thermolysin that preferentially cleaved the membrane-bound conformation of protein kinase C at the hinge; those such as endoproteinase Glu-C and endoproteinase Lys-C that cleaved at the hinge independently of the membrane-bound conformation; and those such as endoproteinase Arg-C and endoproteinase Asp-N that did not cleave significantly at the hinge. Trypsin, elastase, and endoproteinase Arg-C were unique in being able to detect Ca^{2+}-induced conformational changes occurring upon activation of membrane-bound protein kinase C. Curiously, Mg^{2+} inhibited the proteolysis at the hinge catalyzed by proteases that detected the hinge conformational change attendant to membrane binding (chymotrypsin, thermolysin, and trypsin) but did not inhibit the hinge proteolysis catalyzed by enzymes that did not discriminate between soluble and membrane-bound protein kinase C (endoproteinases Glu-C and Lys-C). Mg^{2+} did not inhibit the intrinsic activity of trypsin, assessed using BAEE as a substrate (data not shown), suggesting that Mg^{2+} may be interacting with the protein in such a manner to alter the hinge accessibility.

DISCUSSION

The foregoing data establish that 1) Ca^{2+}-dependent membrane binding and activation of conventional protein kinase Cs are differentially regulated by Ca^{2+}, and 2) distinct conformational changes accompany these two events for the isozyme examined, protein kinase C βII. Kinetic studies reveal that the Ca^{2+} requirements for membrane binding and activation of conventional protein kinase Cs differ, with activation lagging behind membrane binding. Proteolytic sensitivity experiments with protein kinase C βII reveal that one set of conformational changes accompanies the membrane binding mediated by Ca^{2+} concentrations too low to activate the enzyme, and a second set of conformational changes accompanies activation resulting from higher Ca^{2+} concentrations.

Ca^{2+} Differentially Regulates Membrane Binding and Activation of Protein Kinase C—Kinetic analyses reveal that higher concentrations of Ca^{2+} are required to effect activation of protein kinase C compared with membrane binding. This difference is particularly striking for protein kinase C βII, where approximately 40-fold greater Ca^{2+} concentrations are required to half-maximally activate the enzyme compared with the concentrations promoting half-maximal binding to membranes. This difference is only 2-fold for protein kinase C α and approximately 10-fold for protein kinase C βI. Importantly, the Ca^{2+} requirement for binding is similar, within a factor of 3, for protein kinase C α, βI, and βII; what differs most significantly between the isozymes is the Ca^{2+} requirement for activation. Thus, all three isozymes bind to membranes with similar affinity; however, some isozymes require Ca^{2+} concentrations that cause a much tighter binding to be catalytically competent. One explanation for the different Ca^{2+} requirements for membrane binding and activation is that protein kinase C binds two Ca^{2+} ions with differing affinity and different consequences on the structure (see below) and function of the protein; in this regard, the Ca^{2+} site in the C2 domains of synaptotagmin and phospholipase Cδ have been shown to accommodate two metal ions (16, 17).

Activation of protein kinase C requires removal of a basic autoinhibitory domain, the pseudosubstrate, from the substrate-binding cavity in the kinase core (3). One possibility to account for the discrepancy in Ca^{2+} concentrations required for binding and activation is that the tighter membrane binding induced by higher Ca^{2+} concentrations provides the energy to release the pseudosubstrate. For example, an extra kcal mol^{-1} in binding energy is gained by a 10-fold increase in binding affinity. Alternatively, tighter membrane binding may position protein kinase C closer to the membrane, perhaps promoting domain movements that might facilitate removal of the pseudosubstrate (for example, interaction with a basic surface in the regulatory region could neutralize the patch of acidic residues on the surface of the substrate-binding cavity that holds the pseudosubstrate in place (26)).

Modulation of Protein Kinase C's Ca^{2+} Affinity by Carboxyl-terminal Interactions—An unexpected finding from this work is that the Ca^{2+} requirements for activation and, to a lesser extent, membrane binding differ between the two alternatively spliced protein kinase C β isozymes. Notably, these isozymes have identical regulatory domains and differ only in the kinase domain; the carboxyl-terminal 52 residues of βII are replaced by 50 residues in protein kinase C βI, 24 of which are identical to those in protein kinase C βII (25). Thus, the Ca^{2+}-binding site in the C2 domain must be influenced by interactions with the carboxyl terminus of the kinase core. The carboxyl terminus has recently been shown to be critical to protein kinase C's regulation: phosphorylation at this region influences the enzyme's subcellular localization (27, 28); this region has been reported to promote the binding of protein kinase C βII but not

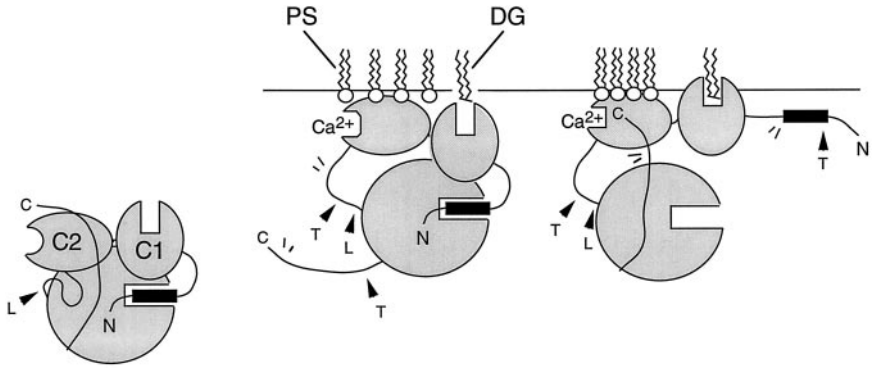

FIG. 7. **Model for possible Ca^{2+}-dependent conformational changes that occur upon membrane binding and activation.** Soluble, inactive protein kinase C with its amino-terminal pseudosubstrate occupying the active site of the catalytic domain is relatively resistant to proteolysis (*left panel*). Low concentrations of Ca^{2+} promote a weak membrane interaction, but not activation, that is accompanied by two conformational changes as follows: exposure of a highly localized region in the hinge separating the kinase domain from the regulatory half of the protein, and exposure of a domain approximately 10-kDa from the carboxyl terminus; both conformational changes are detected by the increased sensitivity to trypsin (indicated *T*; *middle panel*). Higher concentrations of Ca^{2+} result in activation, concomitant with two additional conformational changes as follows: the carboxyl terminus becomes masked again, and the amino-terminal pseudosubstrate becomes exposed (*right panel*). The endoproteinase Lys-C site in the hinge (indicated *L*) is equally exposed in all three conformations of protein kinase C. Membrane attachment is mediated by phosphatidylserine (*PS*) binding to the C2 domain and diacylglycerol (*DG*) binding to the C1 domain. The carboxyl terminus, which modulates Ca^{2+} binding, is depicted interfacing with the C2 domain; alternatively, the carboxyl terminus could indirectly influence the conformation of the Ca^{2+}-binding site in the C2 domain.

βI to actin (29), and deletion of this region prevents expression of mature, active protein kinase C (30). The finding that the Ca^{2+} requirement for activation is significantly greater than that for binding in protein kinase C βII compared with βI suggests that this carboxyl-terminal interaction may be more critical in allowing catalysis than in modulating membrane binding.

A second line of evidence supports the regulation of protein kinase C's Ca^{2+} interaction by carboxyl-terminal interactions. All protein kinase C isozymes contain two conserved phosphorylation sites on their carboxyl terminus (27); we recently showed that phosphorylation at one of these positions (Ser-660 in protein kinase C βII) increases protein kinase C's affinity for Ca^{2+} by an order of magnitude (28). Whether the carboxyl terminus interacts directly with the C2 domain, perhaps providing coordination sites for Ca^{2+}, or whether it indirectly modulates the conformation of the Ca^{2+}-binding site remains to be established.

Distinct Conformational Changes Accompany Ca^{2+}-dependent Membrane Binding and Activation—Using proteases as conformational probes, three regions of protein kinase C βII were identified that undergo structural changes representative of membrane binding and of activation: 1) the hinge, which becomes exposed whenever protein kinase C binds lipid, independently of the active conformation, 2) the carboxyl terminus, which becomes exposed only when protein kinase C is bound to lipid in its inactive conformation, and 3) the pseudosubstrate, which becomes exposed when protein kinase C is active.

The increased proteolytic sensitivity of the hinge accompanying membrane binding was originally noted by Nishizuka and co-workers (31) and forms the basis for the membrane binding assay (22) illustrated in Fig. 1. An unexpected finding from the present contribution is that the region in the hinge that becomes proteolytically labile upon membrane binding is highly localized; Arg-307 and Lys-309 are protected from proteolysis by trypsin when protein kinase C is in solution and become 2 orders of magnitude more sensitive to proteolysis upon membrane binding. In sharp contrast, Lys-320 is equally accessible to proteolysis by endoproteinase Lys-C whether protein kinase C is soluble or membrane-bound. This finding suggests a highly localized "hinge" motion, as depicted in Fig. 7. The increased proteolytic sensitivity of membrane-bound protein kinase C to elastase, chymotrypsin, and thermolysin suggests that the sites of cleavage of these proteases are localized near the trypsin site. An alternative explanation is that these proteases recognize interaction sites elsewhere on protein kinase C that are better exposed when protein kinase C is membrane-bound, so that proteolysis of the hinge is accelerated without sequences in this region being more exposed.

Importantly, the hinge conformational change does not result in activation of protein kinase C; membrane-bound but inactive protein kinase C has an exposed hinge, and this exposure is not influenced by the activation induced by increasing Ca^{2+} concentrations. Consistent with this, binding of protein kinase C to non-activating acidic membranes (*e.g.* containing phosphatidylserine but no diacylglycerol or containing acidic phospholipids other than phosphatidylserine (9)) also is accompanied by exposure of the hinge (7). Conversely, the active conformation of protein kinase C does not require hinge exposure; we showed previously that cofactor-independent activation by protamine sulfate does not expose the hinge of protein kinase C (26). Thus, the hinge exposure results from protein kinase C's interaction with phospholipid, independently of the activation state of the kinase. One possible mechanism is that tethering of protein kinase C to acidic membranes via the C2 domain promotes the hinge conformational change; this domain, which contains the binding sites for Ca^{2+} and acidic phospholipids, is adjacent to the hinge region (3).

The exposure of the carboxyl terminus to proteolysis is particularly interesting in that it occurs only when protein kinase C is membrane-bound but inactive; *i.e.* concentrations of Ca^{2+} that promote membrane binding but not activation result in exposure of the carboxyl terminus, but higher concentrations of Ca^{2+} that promote activation result in masking of the carboxyl terminus to proteolysis. One possibility is that activation promotes a domain movement that masks the carboxyl terminus, for example release of the pseudosubstrate from the active site might mask the carboxyl-terminal cleavage site. A second is that tight membrane binding of protein kinase C prevents access of trypsin to the carboxyl terminus. A third possibility is that the carboxyl terminus is a flexible region of the protein that transiently "opens up" as protein kinase C makes its initial low affinity membrane interaction (a conformation that can be trapped by having non-activating Ca^{2+} concentrations present in *in vitro* assays) but closes up again when the enzyme binds tightly to membranes and adopts the active conforma-

tion. Several lines of evidence suggest that the carboxyl terminus of protein kinase C is flexible. Modeling studies of protein kinase C's carboxyl terminus, based on the crystal structure of protein kinase A, suggest that it is a random coil which, by analogy to protein kinase A, wraps around the top of the kinase domain (26). However, the ability to autophosphorylate at residues in the carboxyl terminus suggests a flexible coil that can access the active site (27, 32). The proteolytic sensitivity is also consistent with a flexible loop or hinge.

The third conformational change detected by proteases involves the activation-dependent exposure of the pseudosubstrate (13). We have previously shown that Arg-19, the first residue in the pseudosubstrate of protein kinase C βII, becomes sensitive to proteolysis only when protein kinase C is active and that this exposure is independent of how protein kinase C is activated; activation by its second messengers, by cofactor-independent activators such as protamine sulfate or by short chained phosphatidylcholines, is accompanied by pseudosubstrate exposure (13, 26). In this contribution, we show that membrane binding alone is not sufficient for pseudosubstrate release and that activating concentrations of Ca^{2+} are required to release the pseudosubstrate.

Conclusions—Fig. 7 presents a possible model illustrating the low affinity membrane interaction that does not result in activation, and the high affinity membrane interaction that does result in activation. Potential Ca^{2+}-dependent conformational changes that accompany each interaction are also illustrated and explained in the legend. This model assumes that the increased proteolytic sensitivity of defined regions of protein kinase C that accompany membrane binding and activation result from conformational changes. As discussed above, protease accessibility could be affected as a result of other interactions. For example, juxtaposition of protein kinase C with the membrane could prevent trypsin from accessing the carboxyl terminus.

In summary, these data reveal that Ca^{2+}-dependent membrane binding is not sufficient to activate protein kinase C and that the discrepancy between Ca^{2+} requirements for membrane binding and activation varies depending on isoform, and, significantly, varies considerably between two isozymes that differ only in the carboxyl terminus. In addition, membrane binding and activation of protein kinase C βII appear to be accompanied by two distinct sets of conformational changes involving the amino terminus, the hinge, and the carboxyl terminus. The movement of three separate domains spanning the entire protein suggests complex interplay between the membrane-targeting domains, the kinase core, and the carboxyl terminus. The finding that Ca^{2+} differentially regulates protein kinase C βI and βII reveals, in particular, that the carboxyl terminus modulates Ca^{2+} binding; the carboxyl terminus may thus provide a mechanism for differential Ca^{2+} regulation of two very closely related isozymes that are often both present in the same cell type.

Acknowledgments—We thank Andrew Flint and Daniel Koshland for a generous gift of the catalytic and regulatory domain antibodies, Lilly for the carboxyl-terminal antibodies, and David Williams for the pseudosubstrate antibodies. We thank Sherry Cai for protein purification, and Calbiochem and Avanti Polar Lipids for participating in the National Science Foundation Young Investigator Program.

REFERENCES

1. Nishizuka, Y. (1992) *Science* **258,** 607–614
2. Nishizuka, Y. (1995) *FASEB J.* **9,** 484–496
3. Newton, A. C. (1997) *Curr. Opin. Cell Biol.* **9,** 161–167
4. Newton, A. C. (1995) *Curr. Biol.* **5,** 973–976
5. Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., and Nishizuka, Y. (1979) *J. Biol. Chem.* **254,** 3692–3695
6. Hannun, Y. A., Loomis, C. R., and Bell, R. M. (1986) *J. Biol. Chem.* **261,** 7184–7190
7. Orr, J. W., and Newton, A. C. (1992) *Biochemistry* **31,** 4667–4673
8. Bazzi, M. D., and Nelsestuen, G. L. (1990) *Biochemistry* **29,** 7624–7630
9. Newton, A. C., and Keranen, L. M. (1994) *Biochemistry* **33,** 6651–6658
10. Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T., and Nishizuka, Y. (1979) *Biochem. Biophys. Res. Commun.* **91,** 1218–1224
11. Mosior, M., and Epand, R. M. (1994) *J. Biol. Chem.* **269,** 13798–13805
12. Mosior, M., and Newton, A. C. (1996) *Biochemistry* **35,** 1612–1623
13. Orr, J. W., Keranen, L. M., and Newton, A. C. (1992) *J. Biol. Chem.* **267,** 15263–15266
14. Luo, J.-H., and Weinstein, I. B. (1993) *J. Biol. Chem.* **268,** 23580–23584
15. Maurer, M. C., Sando, J. J., and Grisham, C. M. (1992) *Biochemistry* **31,** 7714–7721
16. Shao, X., Davletov, B. A., Sutton, R. B., Südhof, T. C., and Rizo, J. (1996) *Science* **273,** 248–251
17. Grobler, J. A., Essen, L.-O., Williams, R., and Hurley, J. H. (1996) *Nat. Struct. Biol.* **3,** 788–795
18. Chakravarthy, B. R., Bussey, A., Whitfield, J. F., Sikorska, M., Williams, R. E., and Durkin, J. P. (1991) *Anal. Biochem.* **196,** 144–150
19. Mosior, M., and Epand, R. M. (1993) *Biochemistry* **32,** 66–75
20. Newton, A. C., and Koshland, D. E., Jr. (1989) *J. Biol. Chem.* **264,** 14909–14915
21. Bartlett, G. R. (1959) *J. Biol. Chem.* **234,** 466–468
22. Orr, J. W., and Newton, A. C. (1992) *Biochemistry* **31,** 4661–4667
23. Jean, T., and Klee, C. B. (1986) *J. Biol. Chem.* **261,** 16414–16420
24. Fabiato, A., and Fabiato, F. (1979) *J. Physiol.* (*Lond.*) **75,** 463–505
25. Ono, Y., Kikkawa, U., Ogita, K., Fujii, T., Kurokawa, T., Asaoka, Y., Sekiguchi, K., Ase, K., Igarashi, K., and Nishizuka, Y. (1987) *Science* **236,** 1116–1120
26. Orr, J. W., and Newton, A. C. (1994) *J. Biol. Chem.* **269,** 8383–8387
27. Keranen, L. M., Dutil, E. M., and Newton, A. C. (1995) *Curr. Biol.* **5,** 1394–1403
28. Edwards, A. S., and Newton, A. C. (1997) *J. Biol. Chem.* **272,** 18382–18390
29. Blobe, G. C., Stribling, D. S., Fabbro, D., Stabel, S., and Hannun, Y. A. (1996) *J. Biol. Chem.* **271,** 15823–15830
30. Amadeo, L. S., Parissenti, A. M., and Reidel, H. (1993) *Receptors and Channels* **1,** 1–9
31. Kishimoto, A., Kajikawa, N., Shiota, M., and Nishizuka, Y. (1983) *J. Biol. Chem.* **258,** 1156–1164
32. Flint, A. J., Paladini, R. D., and Koshland, D. E., Jr. (1990) *Science* **249,** 408–411