The lipid activators of protein kinase C, phosphatidylserine and diacylglycerol, induce a reversible conformational change that exposes the auto-inhibitory pseudosubstrate domain of the enzyme. The pseudosubstrate domain of \( \beta\)-II protein kinase C is cleaved after the first residue, arginine 19, by the endoproteinase Arg-C only when the kinase is bound to the activating lipid phosphatidylserine. Exposure of this residue is markedly enhanced by diacylglycerol. In contrast, the pseudosubstrate domain is not cleaved in the absence of lipids, when protein kinase C is bound to non-activating acidic lipids, when the kinase has autophosphorylated on the amino terminus, or after dilution of the activating lipids. This work reveals specificity in the interaction of protein kinase C with phosphatidylserine since only this phospholipid causes the specific conformational change detected in the regulatory domain of the enzyme, and demonstrates that allosteric regulators expose the intramolecular auto-inhibitory domain of a kinase.

Allosterically regulated protein kinases have been proposed to have a stretch of peptide that binds to the active site, thus maintaining the kinase in an inactive state by steric inhibition (1, 2). Binding of activators is thought to result in a conformational change that displaces the auto-inhibitory domain to allow access of substrates. Auto-inhibitory domains are structurally similar to consensus phosphorylation site sequences for each kinase except that frequently a non-phosphorylatable residue, hence the nomenclature "pseudosubstrate" domain. Since Corbin and co-workers (3) proposed that a substrate-like domain regulates the cAMP-dependent kinase, pseudosubstrate motifs have been identified in a variety of kinases, including the cGMP-dependent kinase, Ca\(^{2+}\)/calmodulin-dependent kinase, myosin light chain kinase, S6 kinase, and protein kinase C, as well as the phosphatase calcineurin (1, 3, 4). Despite considerable biochemical, genetic and molecular biological evidence that auto-inhibition represents a general mechanism in allosterically regulated enzymes, an activator-induced conformational change that releases an intramolecular pseudosubstrate domain has not been demonstrated directly.

Phosphorylation by protein kinase C plays a central role in signal transduction (5). The enzyme is a family of kinases that binds to the plasma membrane in response to receptor-mediated generation of diacylglycerol and, for conventional isoforms, Ca\(^{2+}\) (6). Membrane-association is mediated by a highly cooperative and specific binding of multiple phosphatidylinerine molecules, an interaction that is allosterically regulated by diacylglycerol (7, 8). Enzymatic activity displays a strict requirement for sn-1,2-diacylglycerol and \( \alpha\)-phosphatidylinerine (6), supporting the existence of specific binding sites for each of these lipids. The enzyme also binds non-activating acidic lipids through an electrostatic interaction that is insensitive to diacylglycerol (8).

All known isoforms of protein kinase C, including yeast protein kinase C, photoreceptor-specific Drosophila protein kinase C, and the 10 mammalian isoforms contain a pseudosubstrate sequence near their amino terminus (6, 9, 10). This domain comprises residues 19–31 (RFARKGALRQKNV) in the Ca\(^{2+}\)-dependent \( \beta\)-II rat isoform (11). An autoregulatory role for this sequence is supported by the ability of peptides corresponding to the pseudosubstrate domain to inhibit protein kinase C, by the ability of an antibody against this domain to activate protein kinase C in the absence of its regulators, and by the increased activator-independent activity of mutants in this domain (11–14).

In this report we show that phosphatidylinerine, but not non-activating acidic lipids, induces a specific and reversible conformational change that exposes the pseudosubstrate domain of protein kinase C. Diacylglycerol enhances the exposure of the pseudosubstrate domain but only in the presence of phosphatidylinerine.

**MATERIALS AND METHODS**

Bovine brain \( \alpha\)-phosphatidylinerine (PS),\(^\dagger\) sn-1,2-dioleoylglycerol (DG), sn-1,2-dioleoylphosphatidic acid (PA), bovine liver phosphatidylinositol (PI), and sn-1,2-dioleoylphosphatidylglycerol (PG) were obtained from Avanti Polar Lipids, Inc. Triton X-100 (10% (w/v) aqueous solution) was obtained from Pierce Chemical Co. Q-Sepharose Fast Flow and phenyl-Superose HR 5/5 (10% (w/v) aqueous solution) was obtained from Du Pont-New England Nuclear. Endoproteinase Arg-C was obtained from Boehringer Mannheim, and tetraethionate l-5′-adenylidimidophosphosphate (AMP-PNP) was from Sigma. A protein kinase C-selective peptide (FKKSFKL-NH\(_2\); Ref. 15) was synthesized by the Indiana University Biochemistry Biotechnology Facility. All other chemicals were reagent-grade. Unless otherwise noted experiments were performed using 20 mM Tris, pH 7.5, at 30 °C (Tris buffer).

**Protein Kinase C**—Protein kinase C \( \beta\)-II was expressed in insect cells (SF-21; Invitrogen) by infection with recombinant baculovirus (generous gift from Daniel Koshland, Jr., University of California, Berkeley) and purified to homogeneity by lysis of infected cells and sequential chromatography of the cytosolic fraction on Q-Sepharose

\(\dagger\)The abbreviations used are: PS, phosphatidylinerine; AMP-PNP, tetraethionate l-5′-adenylidimidophosphosphate; DG, dioleoylglycerol; DTT, dithiothreitol; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PA, phosphatidic acid; PAGE, polyacrylamide gel electrophoresis; PG, phosphatidylinerine; PI, phosphatidylinositol; SDS, sodium dodecyl sulfate.
and phenyl-Superose. The enzyme was stored at −20 °C in 10 mM Tris buffer, pH 7.5, at 4 °C, 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM dithiothreitol (DTT), and 50% glycerol. Lипид—Тритон X-100-липидные микреллы были описаны как (7). Все фосфолипидные концентрации в цилюлоидном или ацетонитрилном растворе были определены нефосфолипидным анализом для фосфолипидного концентрации (16). Lипиды в микреллах (например, 70 μg) были растворены в объеме 448 μl (шесть реакционных объемов) при 30 °C в присутствии Tris буфера (10 mM конечная концентрация, pH 7.5, 30 °C), DTT (1 mM конечная концентрация), CaCl₂ (750 μM конечная концентрация), и Triton X-100 (0.1% (w/v) конечная концентрация) с добавлением 0 или 15 мол % Triton X-100 (0.1% (w/v) конечная концентрация) микреллах концентрация, с добавлением 0 или 5 мол % DG (см. Fig. 1 легенду). Активность реакции (one reaction volume) была заново добавлена 5 μл сульфат-полиакриламид мембраны гель электрофорезиса (SDS-PAGE) буфер (8% SDS, 40% глицерина, 0.008% бромфенолное, 20% β-меркаптоэтанол, 0.25 М Tris, pH 6.8) за 3 min post-incubation на 30 °C (0-min time point, intact protein kinase C). After 6 мин at 30 °C, 96 μl of endoproteinase Arg-C (5 units ml⁻¹) were added to the reaction mixture. Aliquots (80 μl, one reaction volume) were removed into SDS-PAGE sample buffer (25 μl) at the indicated times. Proteins were separated by SDS-PAGE (9% polyacrylamide) and visualized by silver staining (18). No significant proteolysis was observed when endoproteinase Arg-C was omitted from the reaction mixture. The activity of Arg-C was unaffected by the concentrations of Triton X-100 employed.

**Protein Kinase C Activity Assay**—Activity of protein kinase C was assayed by measuring the initial rate of [32P]ATP incorporation from [γ-32P]ATP into saturating amounts of a protein kinase C-selective peptide (FKKSFKL-NH₂, 50 μg ml⁻¹ in assay; Ref. 15), as described by Orr and Newton (7) except that peptide replaced histone. Reaction mixtures contained 750 μM CaCl₂, and Triton X-100 (0.1%, w/v) mixed micelles of the compositions described in the legend of Fig. 1 or 0.2 mM EGTA and 0.2 mM EDTA.

**Reversible Conformational Change Assay**—Protein kinase C (approximately 70 μg) was incubated in a volume of 364 μl (seven reaction volumes) at 30 °C in the presence of Tris buffer (10 mM final concentration, pH 7.5, 30 °C), DTT (1 mM final concentration), and CaCl₂ (750 μM final concentration), to which 28 μl of 1% (w/v) Triton X-100 micelles containing 10 μl % PS and 5 μl % DG was added. After a 2-min incubation at 30 °C, 56 μl of 1: H₂O, 2) 1% (w/ v) Triton X-100 micelles containing 10 μl % PS and 5 μl % DG, or 3) 1% (w/v) Triton X-100 micelles were added. Samples were incubated at 30 °C for 4 min during which time a 64-μl aliquot (one reaction volume) was removed into 25 μl of SDS-PAGE sample buffer (0-min time point, intact protein kinase C). Proteolysis was initiated by addition of 96 μl of endoproteinase Arg-C (5 units ml⁻¹) followed by incubation at 30 °C. Thus, the samples contained final concentrations as follows: 0.05% (w/v) Triton X-100 mixed micelles containing 10 μl % PS and 5 μl % DG, 2) 1% (w/v) Triton X-100 mixed micelles containing 10 μl % PS and 5 μl % DG, or 3) 1% (w/v) Triton X-100 mixed micelles containing 3.3 μl % PS and 1.7 mol % DG, respectively. Aliquots (80 μl, one reaction volume) were removed into SDS-PAGE sample buffer (25 μl) at the indicated times. Proteins were separated by SDS-PAGE on 9% gels and visualized by silver staining as described above. Activity was measured under the same conditions, except that approximately 10-fold less enzyme was used, by addition of the protein kinase C-selective peptide and [γ-32P]ATP (as described above) instead of Arg-C. **Proteolysis of Autophosphorylated Protein Kinase C**—Protein kinase C (approximately 1 μg) was incubated for 30 min, 30 °C, in a volume of 364 μl (seven reaction volumes) containing Tris buffer (10 mM final concentration, pH 7.5 at 30 °C), DTT (1 mM final concentration), CaCl₂ (500 μM final concentration), Triton X-100 (0.1% (w/ v) final concentration) mixed micelles containing 15 mol % PS and 5 mol % DG, MgCl₂ (15 mM final concentration), and [γ-32P]ATP (20 μM final concentration). 3000 Ci mm⁻¹ or a non-hydrolysable analogue of ATP, AMP-PNP (20 μM final concentration). 76-μl aliquots (one reaction volume) were removed into 20 μl of SDS-PAGE sample buffer (0-min time point, intact protein kinase C), followed by immediate addition of 32 μl of solution containing Arg-C (31 units ml⁻¹). Aliquots of 80 μl (one reaction volume) were removed into 20 μl of SDS-PAGE sample buffer at the indicated times. Proteins were separated by SDS-PAGE (7.5% polyacrylamide), and visualized by silver staining. Radioactive samples were further analyzed by autoradiography (Kodak X-Onmat film). Under the conditions of the assays, approximately 1 mol of phosphate/mol of protein kinase C was incorporated after a 90-min incubation with [γ-32P]ATP.

**RESULTS AND DISCUSSION**

The silver-stained gel in Fig. 1A shows that the endoproteinase Arg-C cleaved native 80-kDa β II protein kinase C to yield a 78-kDa "nicked" form of the enzyme. Prolonged treatment with Arg-C resulted in disappearance of protein kinase C, with no additional intermediate fragments apparent. Fig. 1B shows that protein kinase C not bound to micelles, in the presence or absence of diacylglycerol, was not cleaved after arginine to yield the 78-kDa form of protein kinase C (Fig. 1B, no lipid and DG lanes). Neither was the lipid-bound conformation of protein kinase C sufficient to expose the proteolytically labile arginine; protein kinase C bound to micelles containing the non-activating acidic lipids phosphatidic acid, phosphatidylglycerol, or phosphatidylinositol was not cleaved to yield a nicked form (Fig. 1B, PA, PG, and PI lanes). In contrast, protein kinase C bound to phosphatidylserine was cleaved to the 78-kDa form, although the degree of cleavage was significantly less than that observed in the presence of both activating lipids, phosphatidylserine and diacylglycerol (Fig. 1A). The rate of overall proteolysis of the

**FIG. 1.** Time course of endoproteinase Arg-C cleavage of protein kinase C incubated in the presence of activating or non-activating lipids. A, silver-stained gel showing generation of the "nicked" form of protein kinase C by Arg-C in the presence of phosphatidylserine and diacylglycerol detergent:lipid mixed micelles, as described under "Materials and Methods." B, silver-stained gel of protein kinase C cleaved in the presence of mixed micelles containing 0 or 15 mol % brain phosphatidylserine (PS), sn-1,2-dioleoylphosphatidic acid (PA), sn-1,2-dioleoylphosphatidylglycerol (PG), or liver phosphatidylinositol (PI), and 5 mol % DG where indicated. Only the 80-kDa protein kinase C and 78-kDa cleavage product (if any) are shown. Results are representative of at least two separate experiments.

**FIG. 2.** Phosphatidylserine- and diacylglycerol-dependent activation of protein kinase C. Activity of protein kinase C (substrate phosphorylation) was assayed by measuring the initial rate of [32P]phosphate incorporation from [γ-32P]ATP into saturating amounts of a protein kinase C-selective peptide (FKKSFKL-NH₂), and visualized by silver staining. Radioactive samples were further analyzed by autoradiography (Kodak X-Onmat film). Under the conditions of the assays, approximately 1 mol of phosphate/mol of protein kinase C was incorporated after a 90-min incubation with [γ-32P]ATP.
Exposure of Protein Kinase C Pseudosubstrate Domain by Lipid

FIG. 3. Reversibility of conformational change that exposes the pseudosubstrate domain of protein kinase C. A, protein kinase C was incubated with mixed micelles under conditions promoting quantitative binding and activity (top panel). An aliquot of this sample was then incubated with pure Triton X-100 micelles to dilute the lipid to non-activating concentrations (bottom right panel) or with lipid mixed micelles of the same composition as in the top panel (bottom left panel), as described under "Materials and Methods." The susceptibility to proteolysis by Arg-C was measured as in Fig. 1. B, the activity of protein kinase C incubated under the conditions described in A was assessed by monitoring the Ca\textsuperscript{2+}-dependent phosphorylation of the protein kinase C-selective peptide described in the legend of Fig. 2. Data are expressed as mean ± S.E. for two separate triplicate experiments.

FIG. 4. Effect of autophosphorylation of protein kinase C on proteolysis by Arg-C. A, silver-stained gel of autophosphorylated (+ [\textsuperscript{32}P]ATP) and control (+ AMP-PNP) protein kinase C treated with Arg-C. B, autoradiogram of the gel in A containing the [\textsuperscript{32}P]phosphorylated protein kinase C. Results are representative of three separate experiments.

intact protein kinase C was similar for soluble and micelle-bound enzyme. This is in marked contrast to the trypsin-catalyzed cleavage at the hinge region, which is 10-100-fold faster for membrane-bound protein kinase C (7).

Diacylglycerol was not sufficient to expose the labile arginine in lipid-bound protein kinase C, since protein kinase C quantitatively bound to micelles containing phosphatidic acid and diacylglycerol was not cleaved to the 78-kDa form (Fig. 1B, PA/DG lane). In contrast, Arg-C catalyzed a small, but detectable, amount of cleavage to the 78-kDa form when protein kinase C was incubated with diacylglycerol and phosphatidylglycerol or phosphatidylinositol (Fig. 1B, PG/DG and PI/DG lanes). These two lipids, unlike phosphatidic acid, are able to substitute for some of the multiple molecules of phosphatidylserine necessary for enzymatic activity (19, 20).

Fig. 2 shows that protein kinase C activity was absolutely dependent on phosphatidylserine and diacylglycerol, the lipids that resulted in maximal exposure of the labile arginine. This specificity is consistent with work of Lee and Bell (21) demonstrating a strict requirement for the L-serine headgroup in activating the enzyme.

The location of the Arg-C-sensitive site was determined by NH\textsubscript{2}-terminal sequencing of approximately 20 pmol of the 78-kDa nicked form of protein kinase C (22). The sequence FARK was obtained, corresponding to residues 20-23 of \beta-II protein kinase C (23). This is consistent with the 2-kDa decrease in molecular weight of the intact enzyme and indicates that Arg-C cleaves after the first residue in the pseudosubstrate domain, Arg-19. Trace cleavage after Arg-22 to yield the sequence KXAL, also in the pseudosubstrate domain, was obtained and accounted for approximately 25% of the sequenced residues. No cleavage after Arg-27 was detected, suggesting that only the first few residues of the pseudosubstrate domain are accessible to proteases, which is consistent with the inability of endoproteinase Lys-C to cleave Lys-23 to yield a nicked form of protein kinase C (data not shown).

In order to test the reversibility of the activator-induced conformational change, protein kinase C was bound to phosphatidylserine:diacylglycerol:Triton X-100 mixed micelles (10:5:85; mol%: 0.05% detergent) under conditions where the pseudosubstrate domain was exposed to Arg-C (Fig. 3A, upper gel) and the enzyme catalytically active (Fig. 3B). Addition of 2-fold more detergent:lipid mixed micelles of the same composition did not alter the cleavage pattern (Fig. 3A, lower gel) or activity (Fig. 3B), indicating that tripling the number of micelles (0.15% Triton X-100), at a constant lipid composition, did not alter the exposure of the pseudosubstrate domain. However, addition of pure Triton X-100 micelles to dilute the relative PS and DG concentrations in the micelle 3-fold to a non-activating level (Fig. 3B) resulted in masking...
of Arg-19, consistent with reversal of the activator-induced conformational change (Fig. 3A, lower right gel). Addition of excess concentrations of a peptide comprising residues 19–31 of the pseudosubstrate domain, with a Ser at position 25 (80 μM; 400-times above the \( K_m \) for phosphorylation by protein kinase C; Ref. 11) to activated protein kinase C did not maintain the pseudosubstrate domain in its exposed conformation upon dilution of the activating lipid to non-activating concentrations (not shown). Thus, the intramolecular pseudosubstrate domain has a significantly higher probability of binding the active site than the intermolecular substrate peptide and suggests a mechanism for remarkable sensitivity in the regulation of protein kinase C activity by phosphatidylserine and diacylglycerol.

Autophosphorylation of protein kinase C protected Arg-19 from proteolysis (Fig. 4). This resistance to proteolysis did not appear to result from the presence of ATP in the active site, since the non-hydrolyzable ATP analogue AMP-PNP did not protect Arg-19 from proteolysis. Under the conditions of our assays, >80% of the phosphates were incorporated on the amino-terminal regulatory domain as determined by proteolysis with trypsin (not shown). Since the two major autophosphorylation sites on the regulatory domain of β-II protein kinase C are at Ser-16 and Thr-17 (24), phosphorylation of one or both of these residues protects Arg-19 from proteolysis. Protection could arise because the phosphates sterically or electrostatically block access of the protease or because of an altered conformation of the pseudosubstrate domain.

The phosphatidylserine- and diacylglycerol-dependent nicked form was also generated upon limited trypsinolysis of protein kinase C bound to small unilamellar vesicles. Furthermore, the nicked form was generated by trypsinolysis of rat brain Type I and Type III isozymes (data not shown), indicating that this mechanism may be common to the conventional Ca\(^{2+}\)-dependent protein kinase C isozymes (6). Whether this is true for the Ca\(^{2+}\)-independent isozymes remains to be elucidated. Similarly, it remains to be determined whether activation of the enzyme by short-chained phosphatidylcholines (25) or fatty acids (26) involves exposure of the pseudosubstrate domain.

Fig. 5 illustrates a model consistent with the foregoing data. The pseudosubstrate domain is masked when protein kinase C is bound to non-activating membranes. Binding of phosphatidylserine and diacylglycerol displaces the pseudosubstrate domain, presumably allowing access of substrates to the active site since exposure of Arg-19 correlates with activity.

An attractive possibility is that diacylglycerol increases the affinity of protein kinase C for phosphatidylserine by exposing the pseudosubstrate domain, thus providing additional stabilization of the membrane interaction. In this regard, Mosior and McLaughlin (27) have proposed that the interaction of basic residues in the pseudosubstrate domain with acidic membranes provides 6 kcal mol\(^{-1}\) of stabilization energy. Pseudosubstrate-mediated stabilization would explain why diacylglycerol has no effect on the affinity of protein kinase C for non-activating acidic lipids (8), since the pseudosubstrate domain is masked in the absence of phosphatidylserine. If the pseudosubstrate-mediated stabilization occurs through electrostatic interactions, this may account for the ability of monovalent acidic lipids to reduce the number of phosphatidylserine molecules required for membrane binding and activity (8, 19). Thus, specific binding sites for phosphatidylserine may exist in the regulatory domain of the enzyme, while electrostatic stabilization may be provided by interaction of the exposed pseudosubstrate domain with acidic lipids.

In conclusion, we have shown that the allosteric activators of protein kinase C induce a reversible conformational change that exposes the pseudosubstrate domain, consistent with displacement of the domain from the active site. This work provides the first direct indication that lipid activators regulate protein kinase C function by causing a conformational change that exposes an intramolecular auto-inhibitory domain. It also strongly supports the hypothesis that protein kinase C has specific binding sites for phosphatidylserine since this lipid exposes the pseudosubstrate domain.

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