Protein Kinase C Acylation by Palmitoyl Coenzyme A Facilitates Its Translocation to Membranes†

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ABSTRACT: Protein kinase C (PKC) translocation to specific subcellular membrane loci after cellular stimulation is mediated, in part, through its interaction with diacylglycerol, phosphatidylserine, and calcium. Herein, we present multiple lines of evidence which demonstrate that purified rabbit brain PKC undergoes specific acylation with palmitoyl CoA that facilitates its interaction with membrane bilayers. First, incubation of purified rabbit brain PKC with [^{14}C]palmitoyl CoA (5 μM) resulted in the radiolabeling of an 80 kDa band demonstrated by SDS−PAGE and autoradiography, while incubation of PKC with other acyl CoA molecular species (e.g., [^{3}H]myristoyl CoA or [^{14}C]arachidonoyl CoA), fatty acids (e.g., [^{14}C]-palmitic and [^{14}C]arachidonic acid), or [^{14}C]diacylglycerol did not result in the incorporation of radiolabel. Second, multiple extractions of [^{14}C]palmitoyl CoA-treated PKC with butanol did not remove the radiolabeled moiety from the 80 kDa PKC band. Third, incubation of the [^{14}C]palmitoyl CoA-radiolabeled PKC moiety with neutral hydroxylamine, hydrochloric acid, or sodium hydroxide released incorporated radiolabel which identified the association between PKC and palmitic acid as a covalent thioester linkage. Fourth, formation of the [^{14}C]palmitoyl CoA−radiolabeled PKC adduct could be prevented by pretreatment of PKC with either dithiobis(nitrobenzoic acid) or *N*-ethylmaleimide. Fifth, limited trypsinolysis of palmitoylated PKC demonstrated that palmitic acid was exclusively present in the regulatory fragment of PKC without detectable amounts of palmitic acid associated with the catalytic fragment. Sixth, palmitoylated PKC was resolved from its nonpalmitoylated counterpart by Mono Q chromatography, and palmitoylated PKC preferentially associated with cellular membranes while nonpalmitoylated PKC did not. Both palmitoylated and nonpalmitoylated PKC were activated by phosphatidylserine, diacylglycerol, and calcium ion. Collectively, these results demonstrate the acylation of PKC by palmitoyl CoA and identify a novel mechanism which may facilitate the interaction of PKC with biologic membranes.

Activation of protein kinase C is critical in such diverse biological processes as cellular growth, differentiation, chemotaxis, and synaptic plasticity (*1*−*4*). The biochemical mechanisms responsible for activation of protein kinase C are complex, but include the calcium-dependent interaction of the regulatory domain of protein kinase C with phosphatidylserine and diacylglycerols in cellular membranes which results in alterations of its tertiary structure leading to the exposure of a previously cryptic kinase domain (*5*−*7*). During cellular activation, the translocation of protein kinase C from cytosol to membrane-associated compartments represents a critical step in the sequence of biochemical events leading to its activation in stimulated cells, but the molecular events surrounding the interaction of protein kinase C with membrane bilayers remain incompletely understood.

In general, two major types of biochemical mechanisms are responsible for facilitating the translocation of an endogenous cytosolic protein to specific membrane-associated compartments during cellular activation. One strategy exploits protein−protein interactions (e.g., interaction of RACKS proteins with protein kinase C) (*8*). A second strategy exploits lipid−lipid interactions induced by covalent modification of cytosolic proteins with lipids. An example of the second strategy is the specific post-translational reversible acylation of cysteine residues in target proteins by palmitoyl CoA resulting in a palmitoylated polypeptide which creates a lipophilic domain which is primed for interacting with cellular membranes (*9*−*12*). The palmitoylation of cellular proteins is both rapid and reversible and thus has been implicated as an important biochemical mechanism regulating the interaction of cellular proteins with membrane constituents. Accordingly, we considered the possibility that one mechanism contributing to protein kinase

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C translocation during cellular activation was one that proceeded through the de novo generation of lipophilic domains in protein kinase C mediated by protein palmitoylation. In initial experiments, we demonstrated the rapid incorporation of palmitoyl CoA into partially purified protein kinase C from rabbit brain. Remarkably, palmitoyl CoA was specifically, and covalently, incorporated into protein kinase C through a covalent thioester bond in a reaction which formally represents the acylation of protein kinase C. Moreover, palmitoylated protein kinase C associates with cellular membranes in a manner which is separate and distinct from that of its nonpalmitoylated counterpart, suggesting the potential importance of protein kinase C acylation and deacylation in modulating the interaction of protein kinase C with its membrane-associated targets.

EXPERIMENTAL PROCEDURES

Purification of Rabbit Brain Protein Kinase C. Protein kinase C was purified from rabbit brain as previously described (*13*, *14*). Briefly, protein kinase C was purified from the cerebrums of 15 New Zealand white male rabbits by sequential DE-52, threonine sepharose and phenyl Sepharose column chromatography as reported previously (*13*, *14*). These chromatographic procedures resulted in a protein kinase C preparation that demonstrated a single intense band after SDS−PAGE and silver staining which was comprised of three protein kinase C isozymes (i.e., α, *β*, and *γ* isozymes of protein kinase C) as determined by immunoblotting with antibodies directed against individual protein kinase C isozymes. The presence of the α, *β*, and *γ* isozymes in rabbit brain is consistent with the results of others (*15*, *16*).

Chemical Characterization of Protein Kinase C Acylation. In experiments in which the covalent modification of protein kinase C by lipids was examined, purified protein kinase C (1−2 μg) from rabbit brain was incubated with selected concentrations of either [^{14}C]palmitoyl CoA (40 mCi/mmol), [^{3}H]palmitoyl CoA (40 mCi/mmol), [^{14}C]arachidonoyl CoA (40 mCi/mmol), [^{3}H]myristoyl CoA (40 mCi/mmol), [^{14}C]-palmitic acid (40 mCi/mmol), [^{14}C]arachidonic acid (40 mCi/mmol), or 1-stearoyl-2-[^{14}C]arachidonoyl-*sn*-glycerol (40 mCi/mmol) in 100 μL of 100 mM Tris buffer (pH 7.4) containing 10 mM EGTA at 30 °C. After the indicated times, incubations were terminated by the addition of 100 μL of SDS−PAGE sample buffer (in the absence of *β*-mercaptoethanol) followed by boiling. Reaction products were immediately subjected to SDS−PAGE, and radiolabeled protein kinase C was detected by either autoradiography utilizing Enlightening (NEN) or phosphorimaging utilizing a Molecular Dynamics phosphorimager. Protein kinase C radiolabeling was quantitated on gels analyzed by phosphorimaging utilizing ImageQuant software (Molecular Dynamics). To facilitate the quantitation of protein kinase C radiolabeling, gels were prepared with selected amounts of [^{14}C]BSA standards loaded into adjacent lanes to provide a standard curve for internal calibration of radiolabel associated with protein kinase C. In selected experiments, the protein kinase C mass was determined by quantitative amino acid analysis to calculate the stoichiometry of incorporated palmitic acid to protein kinase C mass.

The covalent binding of palmitate to protein kinase C was determined by exhaustive butanol extraction of protein kinase C (1−2 μg) previously acylated with 5 μM [^{14}C]palmitoyl CoA (40 mCi/mmol) for 60 min at 30 °C. Following incubation with palmitoyl CoA, 20 μg of bovine serum albumin was added as a carrier protein and the reaction mixture (100 μL) was extracted five times with 200 μL of butanol (saturated with water), resulting in the removal of noncovalently bound [^{14}C]palmitoyl CoA. The aqueous phase (∼100 μL) was then mixed with 100 μL of SDS−PAGE sample buffer and subjected to autoradiography following electrophoresis. Alternatively, following incubations with 5 μM [^{14}C]palmitoyl CoA, palmitoylated protein kinase C in 100 μL was mixed with 100 μL of either Tris buffer (20 mM, pH 7.4) (control), 2 N HCl, 2 N NaOH, or neutral 2 M hydroxylamine in Tris buffer (20 mM, pH 7.4) and the mixture incubated for 1 h at 30 °C (*13*, *14*). Following each of these chemical treatments, samples were extensively dialyzed against 20 mL of SDS sample buffer (three separate dialyses for 2 h per dialysis) (*17*). In selected experiments, protein kinase C was preincubated with 300 μM dithiobis(nitrobenzoic acid) (DTNB) and 300 μM *N*-ethylmaleimide for 5 min at 30 °C prior to the addition of 5 μM [^{14}C]palmitoyl CoA and an additional incubation for 1 h at 30 °C followed by SDS−PAGE and autoradiography (*18*). In other experiments, [^{14}C]palmitoyl CoA-labeled protein kinase C was sequentially subjected to SDS−PAGE, in-gel treatment with either 1 M neutral hydroxylamine or water for 1 h, and analysis by autoradiography (*17*).

Limited Trypsinolysis of Protein Kinase C: Identification of the Palmitoylated Protein Kinase C Domain Following Incubations with Palmitoyl CoA. To identify the protein kinase C domain that is palmitoylated utilizing palmitoyl CoA as an acyl donor, limited trypsinolysis was exploited which cleaves protein kinase C into a 50 kDa catalytic fragment and a 32 kDa regulatory fragment (*19*). For these studies purified rabbit brain protein kinase C (1.5 μg) was first palmitoylated with 5 μM [^{14}C]palmitoyl CoA (vide supra) and subsequently subjected to trypsinolysis for 2 min with 1 milliunit of TPCK−trypsin (Worthington) at 30 °C in a total volume of 0.1 mL. Trypsinolysis was rapidly terminated by the addition of SDS−PAGE sample buffer followed by immediate boiling and gel electrophoresis. The extent of trypsinolysis was monitored by SDS−PAGE followed by silver staining, and the conditions employed were determined to be optimal for limited trypsinolysis resulting in the fragmentation into predicted molecular mass components (*19*). Radiolabel associated with the catalytic and regulatory fragments of protein kinase C was assessed by electrophoresis and subsequent autoradiography.

Mono Q FPLC Purification of Palmitoylated and Nonpalmitoylated Protein Kinase C. Palmitoylated protein kinase C was chromatographically resolved from nonpalmitoylated protein kinase C by Mono Q (HR 5/5) chromatography utilizing a Pharmacia FPLC system. Approximately 160 μg of purified protein kinase C was subjected to palmitoylation by incubating [^{14}C]palmitoyl CoA (5 μM, 40 mCi/mmol) in 8 mL of buffer at 30 °C for 60 min as described above. After incubation, the palmitoylation reaction mixture was directly loaded onto a Mono Q column equilibrated with 100 mL of buffer A [20 mM Tris buffer (pH 7.5) comprised of 0.5 mM EGTA and 0.5 mM EDTA] utilizing a 10 mL superloop. After the column was loaded at a flow rate of 1 mL/min, nonpalmitoylated and palmi-

toylated protein kinase C were resolved utilizing a gradient from 0 to 500 mM NaCl in buffer A over the course 60 min at a flow rate of 1 mL/min. Column fractions were collected every 1.5 min, and UV absorbance was monitored at 280 nm. Column fractions were immediately assayed for protein kinase C activity utilizing the histone phosphorylation assay in the presence of 10 μM calcium with phosphatidylserine and 1,2-diacyl-*sn*-glycerol as activators (*14*). The elution of palmitoylated protein kinase C from the Mono Q column was monitored by liquid scintillation spectrophotometry of aliquots from each fraction and was confirmed by SDS−PAGE and subsequent autoradiography of each fraction. Individual isozymes of palmitoylated and nonpalmitoylated protein kinase C purified by Mono Q chromatography were identified by Western blot analysis utilizing anti-α, anti-β, and anti-γ protein kinase C from Boehringer-Mannheim.

Translocation of Palmitoylated Protein Kinase C to Sf9 Cell Membranes. To compare the translocation of palmitoylated and nonpalmitoylated protein kinase C to cell membranes, Sf9 cell microsomal membranes were utilized because of their relatively low endogenous protein kinase C content in comparison to mammalian cell lines. For the preparation of microsomal membranes from Sf9 cells, cells were first lysed by repetitive freezing−thawing in homogenization buffer comprised of 0.3 M sucrose, 50 mM Tris·HCl (pH 7.4), 2 mM EGTA, and 2 mM EDTA. Sf9 cell homogenates were centrifuged at 10000g_{max} for 10 min, and the resultant supernatant was then centrifuged at 100000g_{max} for 1 h. The pellet (microsomal membranes) was resuspended in homogenization buffer. Either palmitoylated protein kinase C or nonpalmitoylated protein kinase C (1 μg of each) purified by Mono Q chromatography was incubated with Sf9 cell microsomal membranes (50 μg) in the presence of 10 mM EGTA and 100 mM Tris (pH 7.4) for 5 min at 30 °C. Incubations were terminated by chilling samples to 4 °C and immediate ultracentrifugation at 100000g_{max} for 1 h. Samples from the 100000g_{max} pellets (resuspended in homogenization buffer) and supernatants were immediately boiled in the presence of SDS−PAGE sample buffer and subjected to electrophoresis. Following SDS−PAGE, the translocation of protein kinase C to microsomal membranes was determined by Western blot analysis of the supernatants and pellets utilizing a mouse monoclonal antibody which recognizes the α, β, and γ isozymes of protein kinase C (MC5 antibody from Santa Cruz).

Materials. [^{14}C]Palmitoyl CoA, [^{14}C]palmitic acid, [^{14}C]-arachidonic acid, and [^{14}C]arachidonoyl CoA were purchased from NEN. 1-Stearoyl-2-[^{14}C]arachidonoyl-*sn*-glycerol was purchased from Amersham. [^{3}H]Palmitoyl CoA and [^{3}H]-myristoyl CoA were purchased from American Radiolabeled Chemicals. Reagents for SDS−PAGE and chemiluminescence detection of immunoblots were obtained from Bio-Rad and Tropix, respectively. AH-Sepharose, phenyl Sepharose, and Mono Q columns were purchased from Pharmacia. AH-Sepharose was coupled with threonine in the presence of 1-alkyl-3-(3-dimethylaminopropyl)carbodiimide for the preparation of threonine Sepharose as described previously (*13*). DE-52 resin was purchased from Whatman. Phosphatidylserine and 1,2-dioleoyl-*sn*-glycerol were purchased from Avanti Polar Lipids and Sigma, respectively. Most other reagents were purchased from Sigma Chemical and Fisher Scientific. Mouse laminin and *Escherichia coli* DnaJ protein were purchased from Collaborative Biomedical Products and Stressgen, respectively.

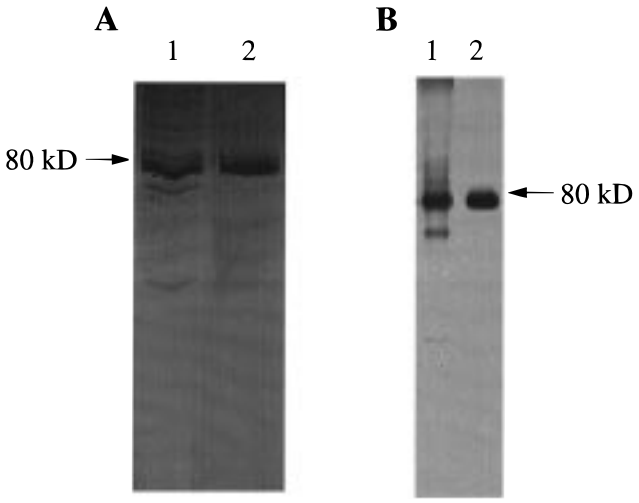

FIGURE 1: Radiolabeling of threonine sepharose-purified and homogeneously purified preparations of protein kinase C by [^{14}C]-palmitoyl CoA. Rabbit brain protein kinase C was prepared as described in Experimental Procedures. Protein kinase C prepared through the threonine Sepharose step (lane 1) as well as protein kinase C that was purified to homogeneity (purified by sequential DE-52, threonine Sepharose, and phenyl Sepharose chromatography) (lane 2) was incubated in buffer containing 5 μM [^{14}C]-palmitoyl CoA for 30 min as described in Experimental Procedures. Reactions were terminated by the addition of β-mercaptoethanol-free SDS−PAGE sample buffer followed by boiling. Samples were subjected to SDS−PAGE utilizing 10 to 15% gradient gels which were subsequently silver stained (A). Radiolabeling of protein kinase C by [^{14}C]palmitoyl CoA was determined by autoradiography following SDS−PAGE utilizing 10% gels (B).

RESULTS

Since protein kinase C is translocated to specific subcellular membrane compartments during cellular activation (*20*−*23*), we examined the possibility that protein kinase C was posttranslationally modified by palmitoylation. In initial experiments, we demonstrated that coincubation of [1-^{14}C]-palmitoyl CoA with partially purified protein kinase C resulted in the covalent labeling of an 80 kDa protein band (Figure 1). Remarkably, the incorporation of radiolabeled [1-^{14}C]palmitoyl CoA into the 80 kDa band remained prominent in incubations utilizing homogeneous preparations of protein kinase C which yielded a single intense 80 kDa band after SDS−PAGE and subsequent silver staining of the gel (Figure 1A, lane 2). These results demonstrate that the incorporation of radiolabel from [1-^{14}C]palmitoyl CoA into the 80 kDa band likely required only protein kinase C itself and did not apparently result from other acyl transferases or transacylases catalyzing the acylation of protein kinase C, similar to the traditionally envisaged post-translational modification of cellular proteins with acyl CoA species. However, it should be pointed out that proteins that could not be detected with silver staining in this apparently homogeneous preparation of protein kinase C could potentially be catalyzing the incorporation of palmitate residues into protein kinase C. Subsequent analyses of protein kinase C palmitoylation were performed utilizing these apparently homogeneous preparations of rabbit brain protein kinase C.

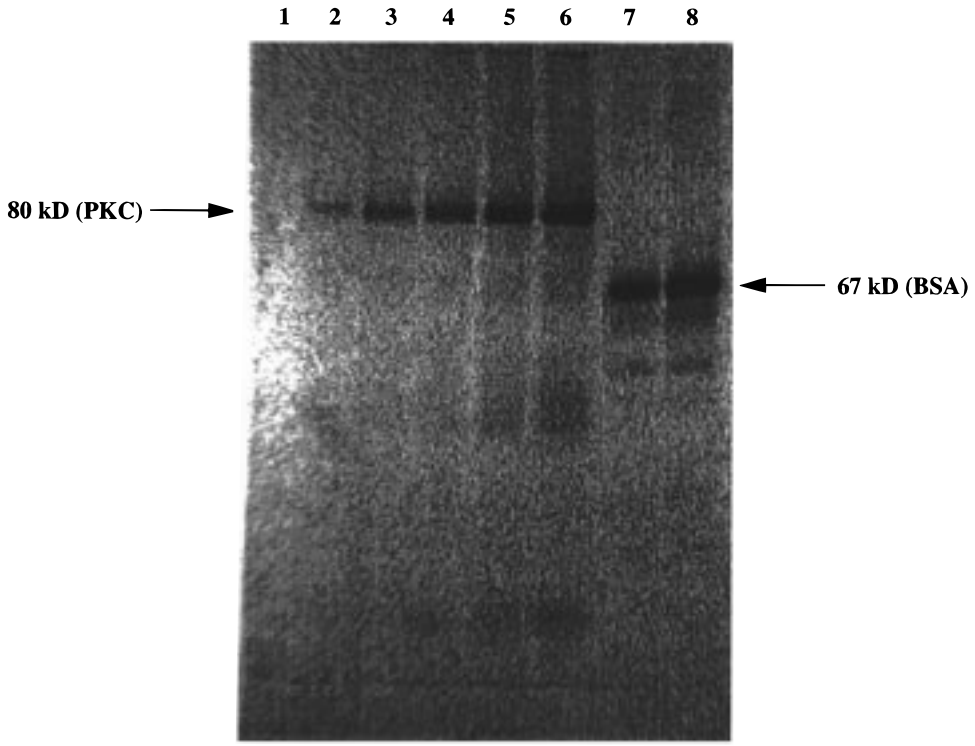

FIGURE 2: Temporal course of [^{14}C]palmitoyl CoA radiolabeling of rabbit brain protein kinase C. Purified rabbit brain protein kinase C was incubated in buffer containing 5 μM [^{14}C]palmitoyl CoA for either 0, 10, 30, 60, 120, or 180 min (lanes 1−6, respectively) as described in Experimental Procedures. Reactions were terminated by the addition of β-mercaptoethanol-free SDS−PAGE sample buffer followed by boiling. Lanes 7 and 8 were loaded with 1000 and 3000 cpm [^{14}C]BSA, respectively. Radiolabeling of protein kinase C by [^{14}C]palmitoyl CoA was determined by autoradiography following SDS−PAGE and was quantified utilizing a phosphorimager.

Next, purified rabbit brain protein kinase C was incubated with [1-^{14}C]palmitoyl CoA (5 μM) for selected time intervals in the presence of 10 mM EGTA at 30 °C (Figure 2). These experiments demonstrated that radiolabeling of protein kinase C by palmitoyl CoA is rapid and reaches near steady state levels at ≈120 min. Utilizing [^{14}C]BSA run on SDS−PAGE as external standards, the mass of radiolabel derived from the amount of [1-^{14}C]palmitoyl CoA incorporated into protein kinase C was quantified (Figure 2). Utilizing the mass of protein kinase C which was assessed by quantitative amino acid analysis, the specific activity of radiolabel, and the amount of radioactivity incorporated into protein kinase C following incubation for 180 min with palmitoyl CoA, the stoichiometry of incorporated [1-^{14}C]palmitoyl CoA molecules per molecule of protein kinase C was calculated to be at least 7:1 after radiolabeling for 180 min (Figure 2). It should be pointed out that it is possible that the stoichiometry of palmitoylation may be higher than 7:1 since these calculations do not take into account the possibility that purified protein kinase C is already partially palmitoylated. Taken together, these results demonstrate that protein kinase C is radiolabeled by palmitoyl CoA rapidly (within 10 min), reaching a stoichiometry of at least 7 palmitoyl CoA molecules per protein kinase C molecule at near steady state conditions, and that this process occurs without the apparent obligatory participation of accessory proteins (i.e., protein kinase C can acylate itself on multiple amino acid residues).

Protein kinase C radiolabeling with palmitoyl CoA was chemically characterized by conventional techniques to identify the chemical nature of the association of palmitoyl CoA (or its products) with protein kinase C. First, palmitate is covalently bound to protein kinase C since exhaustive

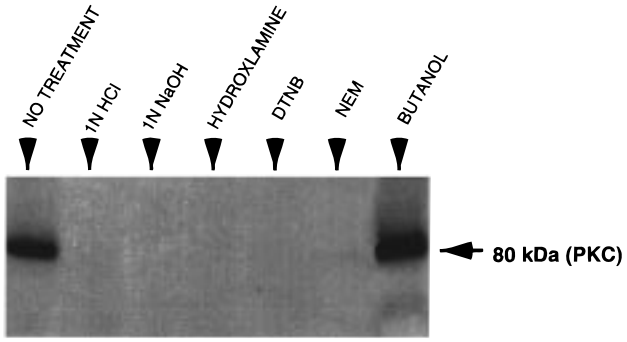

FIGURE 3: Chemical characterization of the covalent adduct of palmitoyl CoA and protein kinase C. Protein kinase C was palmitoylated by incubating it with 5 μM [^{14}C]palmitoyl CoA for 1 h at 30 °C as described in Experimental Procedures. Following palmitoylation, reaction mixtures were either prepared for SDS−PAGE (no treatment), exhaustively extracted with butanol (butanol), treated with 1 N HCl (1 N HCl), treated with 1 N NaOH (1 N NaOH), or treated with 1 N neutral hydroxylamine (hydroxylamine) as described in Experimental Procedures. Alternatively, protein kinase C was pretreated with either 300 μM dithiobis(nitrobenzoic acid) (DTNB) or 300 μM *N*-ethylmaleimide (NEM), as indicated prior to incubations with 5 μM [^{14}C]palmitoyl CoA as described in Experimental Procedures. Following either pre- or postpalmitoylation chemical treatments, samples were prepared for SDS−PAGE and subsequent autoradiography as described in Experimental Procedures.

butanol extraction to strip away noncovalently bound lipid following protein kinase C acylation with 5 μM [^{14}C]-palmitoyl CoA failed to diminish protein kinase C radiolabeling (Figure 3). Second, treatment of [^{14}C]palmitoyl CoA-labeled protein kinase C with either neutral hydroxylamine (1 M), base (1 M NaOH), or acid (1 M HCl) removed

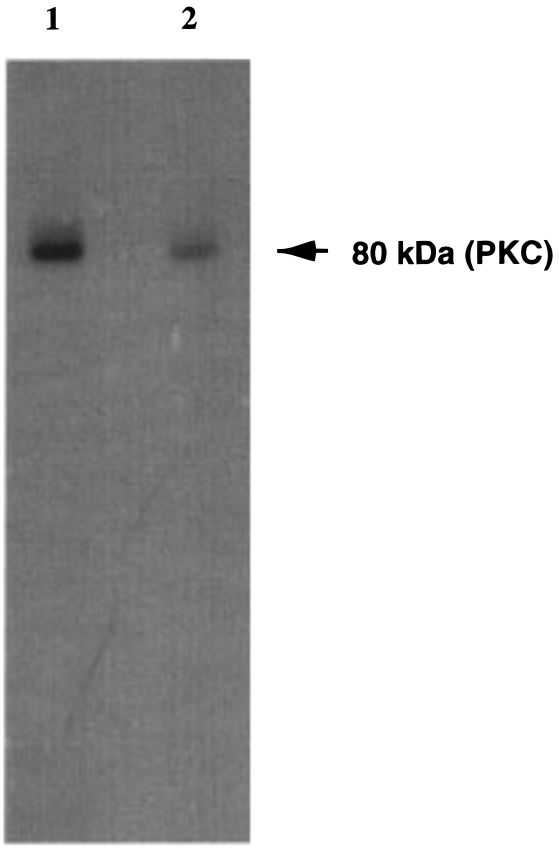

FIGURE 4: In situ hydroxylamine treatment of [^{14}C]palmitoyl CoA-labeled protein kinase C. Protein kinase C was palmitoylated by incubating it with 5 μM [^{14}C]palmitoyl CoA for 1 h at 30 °C as described in Experimental Procedures. Following palmitoylation, reaction mixtures were subjected to SDS−PAGE and proteins were fixed in the gels prior to additional treatment of the gels. Gels were then incubated at 24 °C for 1 h with either water or 1 N neutral hydroxylamine (lanes 1 and 2, respectively). Following treatments, gels were analyzed by autoradiography as described in Experimental Procedures.

radiolabel from palmitoylated protein kinase C (Figure 3). This removal of palmitate from protein kinase C by acid, base, and hydroxylamine suggests protein kinase C is palmitoylated by palmitoyl CoA by the formation of a thioester bond. Third, neutral hydroxylamine treatment of polyacrylamide gels containing radiolabeled protein kinase C resulted in the removal of the majority of radioactivity from [^{14}C]palmitoyl CoA-labeled protein kinase C embedded in the gel (Figure 4). Fourth, pretreatment of protein kinase C with either of the sulfhydryl-reactive reagents, dithiobis-(nitrobenzoic acid) (300 μM) or *N*-ethylmaleimide (300 μM), prevented protein kinase C acylation by 5 μM [^{14}C]palmitoyl CoA (Figure 3). Although pretreatment of protein kinase C with dithiobis(nitrobenzoic acid) and *N*-ethylmaleimide may potentially prevent palmitoylation by inhibiting a putative autocatalytic domain in the protein kinase C molecule, these data in conjunction with the ability of hydroxylamine to remove palmitate from protein kinase C after palmitoylation suggest that dithiobis(nitrobenzoic acid) and *N*-ethylmaleimide likely block potential reactive sulfhydryl residues that are potential targets for acylation. Taken together, these studies demonstrate that protein kinase C purified from rabbit brain is covalently acylated with palmitate through a thioester bond in a reaction which utilizes palmitoyl CoA as the fatty acid donor in the acylation of protein kinase C.

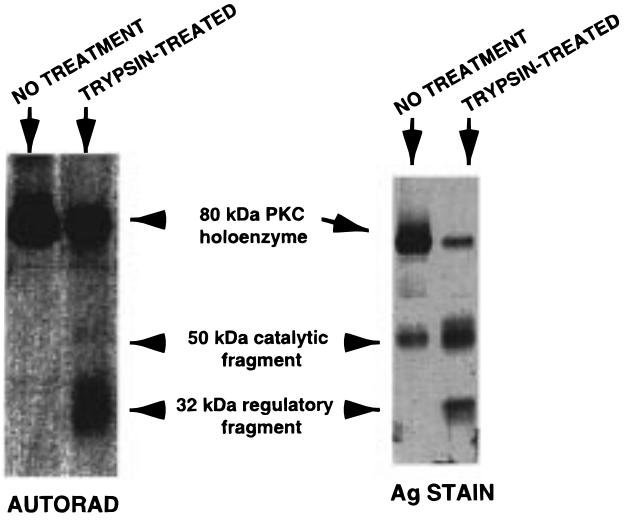

FIGURE 5: Identification of specific protein kinase C palmitoylation of the regulatory domain. Protein kinase C was palmitoylated with 5 μM [^{14}C]palmitoyl CoA and was subsequently treated with 1 milliunit of TPCK trypsin for 2 min as described in Experimental Procedures. The cleavage of protein kinase C into its catalytic (32 kDa) and regulatory (50 kDa) fragments was determined by SDS−PAGE followed by silver staining, and the incorporation of palmitoyl CoA into these fragments was determined by autoradiography of SDS−PAGE-treated samples.

Since three cysteine residues and one histidine residue provide the structural elements of the zinc fingers in the regulatory domain of protein kinase C (*19*), further studies were directed at determining the relative amounts of acylation in the regulatory and catalytic domains. For these studies, limited trypsinolysis of protein kinase C was employed which results in cleavage of the protein kinase C holoenzyme into its catalytic and regulatory fragments. Silver staining of SDS−PAGE gels of palmitoylated protein kinase C confirmed that trypsin treatment resulted in the generation of a catalytic (50 kDa) and regulatory fragment (32 kDa) of protein kinase C without further significant degradation under the conditions employed (Figure 5). Remarkably, autoradiography of the SDS−PAGE gels revealed that only the regulatory fragment is acylated (Figure 5). The selective acylation of the regulatory fragment as compared to that of the catalytic fragment, in conjunction with the 7:1 stoichiometry of palmitate residues to protein kinase C, suggests that each molecule of the regulatory fragment is palmitoylated with as many as seven palmitate residues. These results, taken together with the identification of the covalent binding of palmitate to protein kinase C through a thioester bond, suggest that protein kinase C palmitoylation may occur at or near the critical polypeptide domains that regulate protein kinase C activity such as the cysteine residues present in the multiple zinc finger regions of the regulatory domain.

Since protein kinase C is activated by several structurally distinct lipids, further studies were designed to determine the specificity of acylation of protein kinase C by palmitoyl CoA in comparison to that of selected lipid activators of protein kinase C (e.g., 1,2-diacyl-*sn*-glycerol and arachidonic acid). Neither of the radiolabeled lipid activators, [^{14}C]-arachidonic acid and 1-stearoyl-2-[^{14}C]arachidonoyl-*sn*-glycerol, was effective as an acyl donor of protein kinase C utilizing assay conditions identical to those used for palmitoyl CoA radiolabeling (Figure 6). Additionally, neither [^{14}C]-

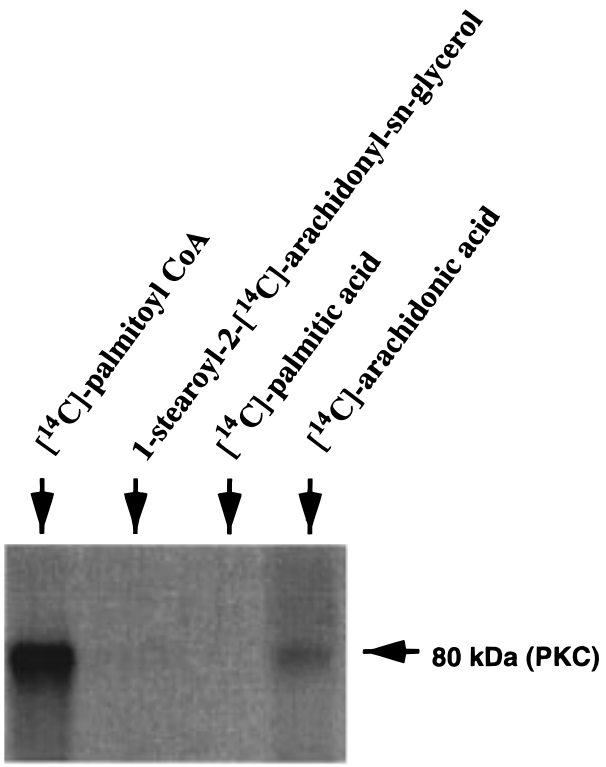

FIGURE 6: Conventional lipid activators of protein kinase C do not acylate protein kinase C. Purified rabbit brain protein kinase C was incubated with either 5 μM [^{14}C]palmitoyl CoA (40 mCi/mmol), 5 μM [^{14}C]arachidonic acid (40 mCi/mmol), 5 μM 1-stearoyl-2-[^{14}C]arachidonyl-*sn*-glycerol (40 mCi/mmol), or 5 μM [^{14}C]palmitic acid (40 mCi/mmol) in reaction buffer comprised of 10 mM EGTA and 100 mM Tris·HCl (pH 7.5) for 1 h at 30 °C as described in Experimental Procedures. Incubations were terminated by the addition of SDS−PAGE sample buffer followed by boiling. The incorporation of radiolabel into protein kinase C was detected by autoradiography of SDS−PAGE-treated samples as described in Experimental Procedures.

arachidonic acid nor 1-stearoyl-2-[^{14}C]arachidonoyl-*sn*-glycerol radiolabeled protein kinase C in the presence of calcium and phosphatidylserine (data not shown). Furthermore, the ineffective acylation of protein kinase C by palmitic acid demonstrates the importance of an activated carbonyl in palmitic acid (e.g., its CoA derivative) for effective protein kinase C palmitoylation (Figure 6). Also, protein kinase C palmitoylation by 5 μM palmitoyl CoA was not altered by the addition of either phosphatidylserine alone or phosphatidylserine and diacylglycerol (Figure 7).

Since purified protein kinase C is acylated by palmitoyl CoA through either an apparent autoacylation process or a nonenzymic thioesterification process, the molecular species specificity of this reaction was explored. The specificity of the acylation of protein kinase C by palmitoyl CoA was underscored by the demonstration that neither myristoyl CoA (5 μM) nor arachidonoyl CoA (5 μM) was an effective substrate for the acylation of protein kinase C (Figure 8). The protein specificity of protein kinase C acylation was demonstrated by virtue of the fact that neither bovine serum albumin, hexokinase, laminin, nor DnaJ protein was acylated by palmitoyl CoA under identical conditions (Figure 9). It should also be noted that both laminin and DnaJ protein possess zinc finger domains (*24, 25*). Thus, the palmitoylation of protein kinase C by palmitoyl CoA is specific with regard to both the acyl CoA donor and the protein acceptor.

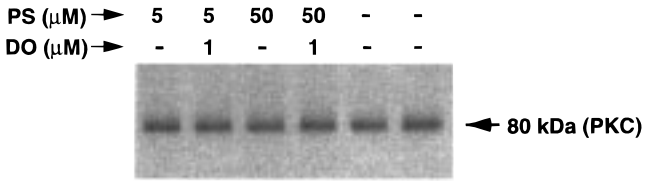

FIGURE 7: Radiolabeling of protein kinase C by [^{14}C]palmitoyl CoA in the presence of phosphatidylserine and diacylglycerol. Purified rabbit brain protein kinase C (1.6 μg) was incubated with 5 μM [^{14}C]palmitoyl CoA (40 mCi/mmol) for 1 h at 30 °C in the presence and absence of the indicated concentrations of phosphatidylserine (PS) or 1,2-dioleoyl-*sn*-glycerol (DO) in reaction buffer comprised of 10 mM EGTA and 100 mM Tris·HCl (pH 7.5). Phosphatidylserine and diacylglycerol were added to this reaction mixture as liposomes that were prepared as sonicates (*14*). Incubations were terminated by the addition of SDS−PAGE sample buffer followed by boiling. The incorporation of radiolabel into protein kinase C was detected by autoradiography of SDS−PAGE-treated samples as described in Experimental Procedures.

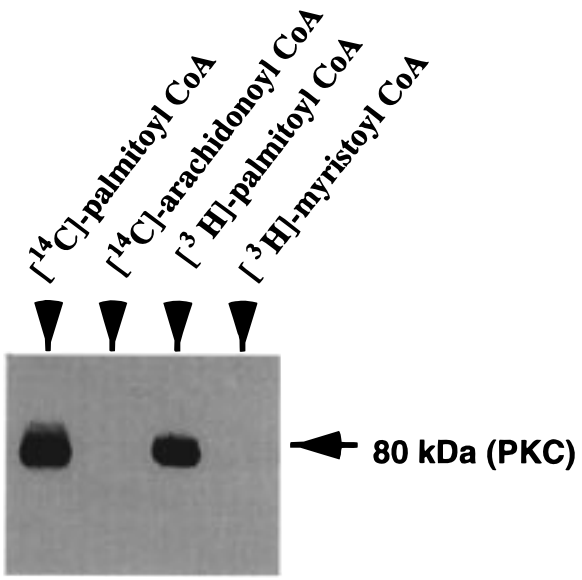

FIGURE 8: Protein kinase C is selectively acylated by palmitoyl CoA in comparison to other acyl CoA molecular species. Purified rabbit brain protein kinase C was incubated with either 5 μM [^{14}C]-palmitoyl CoA (40 mCi/mmol), 5 μM [^{14}C]arachidonoyl CoA (40 mCi/mmol), 5 μM [^{3}H]palmitoyl CoA (40 mCi/mmol), or 5 μM [^{3}H]myristoyl CoA (40 mCi/mmol) in reaction buffer comprised of 10 mM EGTA and 100 mM Tris (pH 7.5) for 1 h at 30 °C as described in Experimental Procedures. Incubations were terminated by the addition of SDS−PAGE sample buffer followed by boiling. The incorporation of radiolabel into protein kinase C was detected by autoradiography of SDS−PAGE-treated samples.

To identify functional differences between palmitoylated and nonpalmitoylated protein kinase C, palmitoylated protein kinase C was resolved from its nonpalmitoylated protein kinase C counterpart. Initial experiments examined multiple stationary phases, including threonine Sepharose, phenyl Sepharose, and high-performance hydroxyapatite chromatographies which were each unsuccessful in resolving palmitoylated and nonpalmitoylated protein kinase C. In contrast, Mono Q anion exchange chromatography resolved palmitoylated protein kinase C from its nonpalmitoylated counterpart (Figure 10). For example, after incubation of protein kinase C with 5 μM [1-^{14}C]palmitoyl CoA for 60 min at 30 °C, two peaks of UV-absorbing material each containing protein kinase C activity were resolved by Mono Q chromatography. Sequential SDS−PAGE and autoradiography

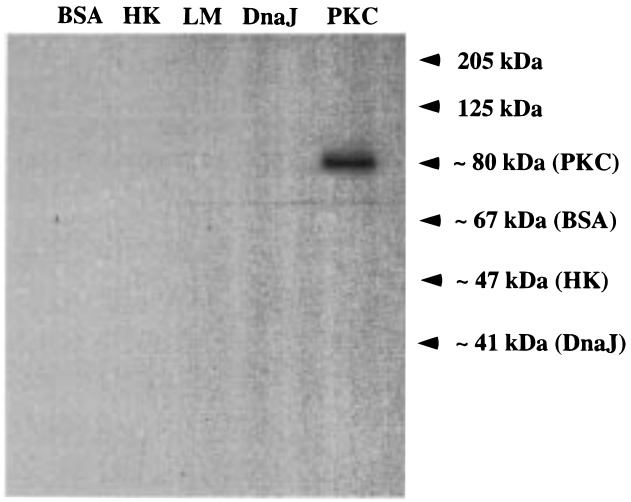

FIGURE 9: Protein kinase C is specifically palmitoylated by palmitoyl CoA. Either purified rabbit brain protein kinase C (PKC, 1 μg), *E. coli* DnaJ protein (DnaJ, 1 μg), purified laminin (LM, 1 μg), hexokinase (HK, 1 μg), or bovine serum albumin (BSA, 1 μg) was incubated with 5 μM [^{14}C]palmitoyl CoA (40 mCi/mmol) in reaction buffer comprised of 10 mM EGTA and 100 mM Tris (pH 7.5) for 1 h at 30 °C. Incubations were terminated by the addition of SDS−PAGE sample buffer followed by boiling. Following SDS−PAGE treatment of samples, the incorporation of palmitoyl CoA into these proteins was determined by autoradiography.

demonstrated that only the second peak was palmitoylated (see the inset of Figure 10). Both palmitoylated and nonpalmitoylated protein kinase C that were resolved after Mono Q purification required phosphatidylserine and diacylglycerol for maximal activity (Figure 11). Thus, although multiple palmitate residues are covalently attached to the regulatory domain, this domain can still interact with diacylglycerol and phosphatidylserine, resulting in protein kinase C activation. To determine if there is specificity in the acylation of individual protein kinase C isozymes present in rabbit brain, Western blot analysis of each peak separated by Mono Q chromatography was employed. Both nonpalmitoylated and palmitoylated protein kinase C purified by Mono Q chromatography contained similar amounts of the α, β, and γ protein kinase C isozymes, demonstrating the absence of isozyme selectivity in the autoacylation process among the isozymes examined (data not shown).

To explore the possibility that palmitoylated protein kinase C is translocated to membrane domains, FPLC-purified palmitoylated protein kinase C and nonpalmitoylated protein kinase C were used in membrane translocation assays. For these experiments, palmitoylated and nonpalmitoylated protein kinase C were incubated with Sf9 cell membranes and subsequently centrifuged to separate membranes from supernatant. Only palmitoylated protein kinase C translocated to membranes, while nonpalmitoylated protein kinase C preferentially remained in the supernatant (Figure 12A). The translocation of palmitoylated protein kinase C was substantiated by both Western blot analysis and autoradiography after SDS−PAGE (Figure 12A,B). If we assume that protein kinase C is palmitoylated homogeneously in the experiments shown in Figure 2, it is likely that the palmitoylated protein

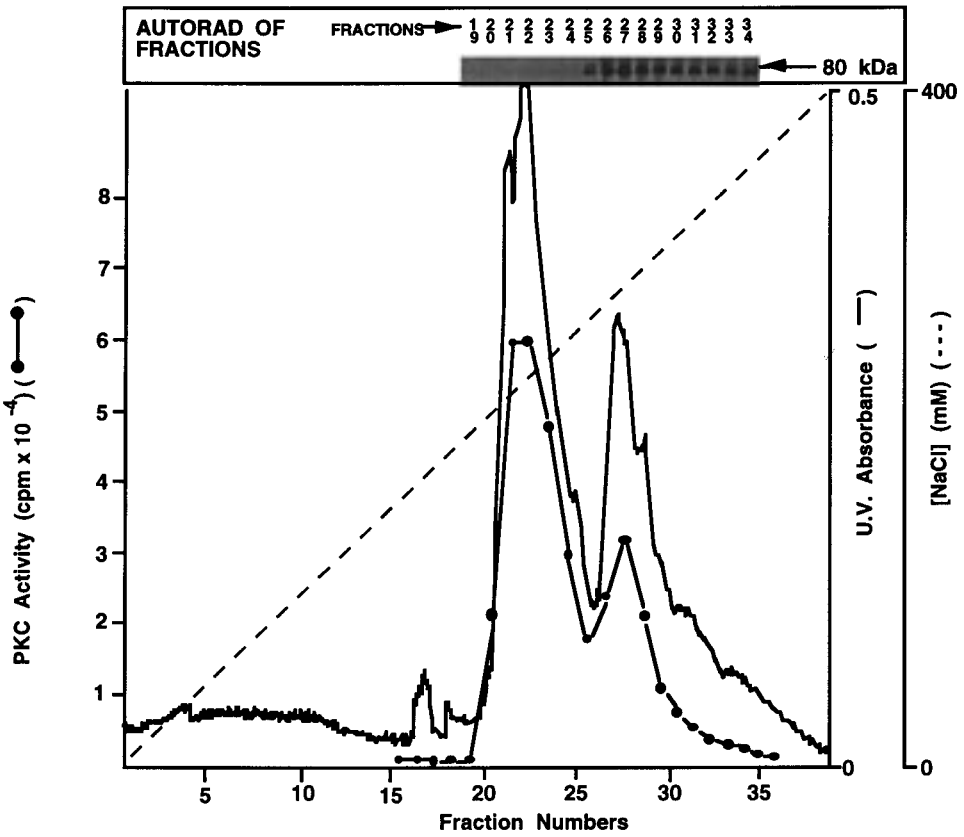

FIGURE 10: Mono Q FPLC purification of palmitoylated protein kinase C. Purified rabbit brain protein kinase C (160 μg in 5 mL) was incubated with 5 μM [^{14}C]palmitoyl CoA in reaction buffer comprised of 10 mM EGTA and 100 mM Tris (pH 7.5) for 1 h at 30 °C. Following incubation, reaction mixtures were immediately cooled to 4 °C prior to Mono Q chromatography as described in detail in Experimental Procedures. Each fraction was assayed for protein kinase C activity and radioactivity. Fractions containing radioactivity were subjected to SDS−PAGE followed by autoradiography (inset).

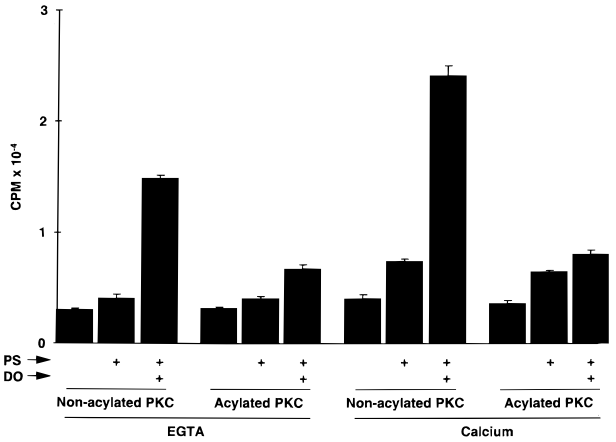

FIGURE 11: Activation of palmitoylated and nonpalmitoylated protein kinase C by phosphatidylserine and diacylglycerol. Purified rabbit brain protein kinase C (160 μg in 5 mL) was incubated with 5 μM [^{14}C]palmitoyl CoA in reaction buffer comprised of 10 mM EGTA and 100 mM Tris (pH 7.5) for 1 h at 30 °C and subsequently subjected to Mono Q chromatography as described in Figure 10. Mono Q-purified, palmitoylated protein kinase C and Mono Q-purified, nonpalmitoylated protein kinase C were assayed for kinase activity in the presence of either 200 μM $CaCl_2$ or 5 mM EGTA as indicated. Kinase activity was measured in the presence or absence of the lipid activators, phosphatidylserine (40 μg/mL, PS) and 1,2-dioleoyl-*sn*-glycerol (10 μg/mL, DO), as previously described (*14*). Kinase activity was measured by the incorporation of ^{32}P from [γ-^{32}P]ATP into histones, and values represent the mean plus the standard error of the mean for four independent determinations.

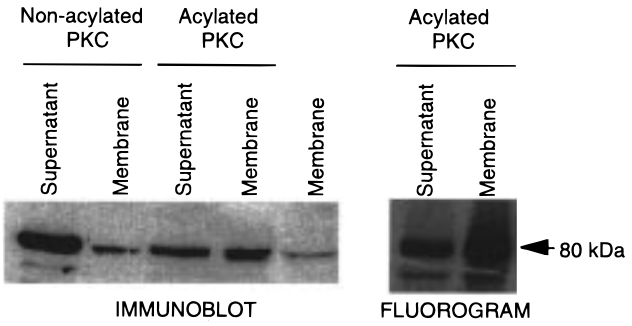

FIGURE 12: Translocation of palmitoylated protein kinase C to Sf9 cell membranes. Purified rabbit brain protein kinase C (160 μg in 5 mL) was incubated with 5 μM [^{14}C]palmitoyl CoA in reaction buffer comprised of 10 mM EGTA and 100 mM Tris (pH 7.5) for 1 h at 30 °C and subsequently subjected to Mono Q chromatography as described in Figure 10. Either Mono Q-purified, palmitoylated protein kinase C (acylated PKC) or Mono Q-purified, nonpalmitoylated protein kinase C (nonacylated PKC) was incubated with purified Sf9 cell membranes for 10 min at 30 °C as described in Experimental Procedures. Following incubation, membranes were pelleted by ultracentrifugation and membranes as well as supernatant fractions were immediately boiled in SDS−PAGE sample buffer. Additionally purified Sf9 cell membranes that were not incubated with either palmitoylated or nonpalmitoylated protein kinase C were also analyzed to determine the background signal (indicated as membranes with no addition of protein kinase C). Samples were subjected to electrophoresis followed by either immunoblotting or autoradiography. Immunoblots were probed with anti-PKC (MC5, Santa Cruz) as the primary antibody and developed utilizing the Tropix chemiluminescence system.

kinase C used in these translocation experiments is palmitoylated with a ~4:1 stoichiometry of palmitate residues per molecule of protein kinase C. It is conceivable that only one or two of these four residues are responsible for the association of this palmitoylated form of protein kinase C with membrane domains. Thus, these results demonstrate that palmitoylated protein kinase C is translocated to membranes and suggest that the acylation of protein kinase C by palmitoyl CoA may represent an important mechanism for the translocation of protein kinase C isozymes during cellular activation.

DISCUSSION

Several biochemical mechanisms have been previously proposed for protein kinase C translocation to membrane domains. These mechanisms include the docking of protein kinase C to membranes through a multicoordinate system of four phosphatidylserine molecules, one diacylglycerol molecule, and one calcium molecule (*26*) as well as the targeting of protein kinase C to specific membrane-associated receptor proteins (e.g., RACK proteins) (*8*). Another mechanism that could mediate protein kinase C translocation is the covalent modification of the protein kinase C polypeptide with a long-chain fatty acid. The potential physiological relevance of protein kinase C palmitoylation is demonstrated by the acylation of protein kinase C by physiologically relevant amounts of palmitoyl CoA (*27*, *28*) and the translocation of palmitoylated (as opposed to nonpalmitoylated) protein kinase C to membranes. Furthermore, it is likely that protein kinase C palmitoylation and hormone-mediated diacylglycerol production act in concert to mediate the translocation and activation of protein kinase C to specific membrane pools.

The demonstration that protein kinase C acylation occurs in the regulatory domain through as many as seven thioester covalent modifications suggests that this modification could be localized to the multiple cysteine residues that are members of the zinc finger regions within the regulatory domain. Protein kinase C isozymes contain two zinc finger clusters (e.g., cysteine-rich domain I, CRD-I, and cysteine-rich domain II, CRD-II) with each cluster containing two zinc fingers (*29*). Each of these four zinc fingers contains three cysteines and one histidine. The function of these zinc fingers is not fully understood; however, their crystalline structure has revealed that they form a configuration which can bind diacylglycerols and thereby promote the activation and translocation of protein kinase C (*29*). The precise role for each of the zinc finger in these processes is not clear. However, recent studies employing mutational substitutions suggest that the zinc fingers have nonequivalent roles in the ability to mediate protein kinase C translocation (*30*). In light of the complex nature and biochemical role of the zinc fingers, it is likely that palmitoylation in the zinc finger region could further facilitate the role of this region in the translocation process. The demonstration that palmitoylation does not alter the diacylglycerol and phosphatidylserine requirement for maximal protein kinase C activity suggests either that not all of the cysteines that are palmitoylated are involved in the activation of the holoenzyme or alternatively that cysteines that are involved in the activation of protein kinase C are still functional even when thioesterified with palmitate residues.

The demonstration that protein kinase C was selectively acylated utilizing palmitoyl CoA as an acyl donor utilizing partially purified preparations as well as homogeneously purified preparations of protein kinase C suggests that

acylation occurs through either autoacylation or nonenzymatic transthioesterification. The selective acylation of protein kinase C utilizing palmitoyl CoA is similar to that previously described for rhodopsin which undergoes a nonenzymatic transthioesterification which is selective for palmitoyl CoA as the acyl donor (*31*, *32*). We speculate that the biologic regulation of protein kinase C palmitoylation may be mediated by specific enzymes responsible for the removal of the thioesterified fatty acyl residues as proposed for other proteins (cf. ref *10*).

Several mechanisms may be responsible for the regulation of protein kinase C palmitoylation that could involve either changes in the cellular palmitoyl CoA concentration or alternatively the activation of a deacylase responsible for the removal of the acyl moieties. Multiple physiological and pathophysiological perturbations result in profound increases in intracellular acyl CoA mass, including myocardial ischemia, diabetes, and fasting states (*33*, *34*). Accordingly, increases in palmitoyl CoA content could lead to increased palmitoylation of protein kinase C and the sequential translocation of protein kinase C to membranes, phosphorylation of membrane-associated proteins, and changes in cellular physiology. Alternatively, regulation of protein deacylase activity could regulate the palmitoylation state of protein kinase C. Since the results herein demonstrate a potential new biochemical mechanism for protein kinase C translocation, the identification of the precise regulatory mechanisms that regulate the level of palmitoylation of protein kinase C may prove important in understanding the mechanisms which modulate protein kinase C activity and cellular function.

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