A Putative Phosphatidylycerine Binding Motif Is Not Involved in the Lipid Regulation of Protein Kinase C*

Joanne E. Johnson‡§, Amelia S. Edwards‡§, and Alexandra C. Newton‡§

From the Departments of ¶Pharmacology and $Chemistry and Biochemistry, University of California at San Diego, La Jolla, California 92037-0640

Protein kinase C is specifically regulated by diacylglycerol and the amino phospholipid, phosphatidylycerine. The molecular basis for the phosphatidylycerine sensitivity was recently proposed to arise from the presence of a putative phosphatidylycerine binding motif, FXXFKLXXXKXR, localized in the C2 domain of protein kinase C (Igarashi, K., Kaneda, M., Yamaji, A., Saida, T. C., Kikkawa, U., Ono, U., Inoue, K., and Umeda, M. (1995) J. Biol. Chem. 270, 29075–29078). To determine whether this motif mediates the interaction of protein kinase C with phosphatidylycerine, the carboxyl-terminal basic residues were mutated to Ala in protein kinase C II (K236A and R238A), and the phosphatidylycerine regulation of the mutant enzyme was examined. Membrane binding and activity measurements revealed that the phosphatidylycerine regulation for the mutant protein was indistinguishable from that of wild-type protein kinase C. Specifically, neither the apparent membrane affinity for phosphatidylycerine-containing membranes in the presence or absence of diacylglycerol nor the phosphatidylycerine-dependence for activation was affected by removal of the conserved basic residues at the carboxyl terminus of the consensus sequence. In addition, a synthetic peptide corresponding to the amino terminus of the consensus sequence (FTFNVK) had no effect on the concentration of phosphatidylycerine resulting in half-maximal activation of protein kinase C. These results reveal that the carboxyl-terminal basic residues in the consensus motif FXXFKLXXXKXR are not responsible for the phosphatidylycerine selectivity of protein kinase C and that, furthermore, the region of the C2 domain containing this motif is not involved in the membrane binding of protein kinase C.

The protein kinase C family of serine/threonine kinases transduces the multitude of extracellular signals that result in generation of the lipid second messenger, diacylglycerol (1, 2). This lipid causes protein kinase C to translocate from the cytosol to, typically, the plasma membrane where it becomes activated by an additional interaction with phosphatidylycerine. Membrane translocation is mediated by two membrane-targeting modules, the C1 and the C2 domains. Each comprises a functional module present separately in a number of otherwise unrelated proteins (3). The C1 domain of conventional and novel protein kinase Cs binds membranes via a high affinity interaction with diacylglycerol or its analog, phorbol esters. The C2 domain is responsible for the calcium-dependent binding to anionic lipids for a number of amphitropic proteins, including the conventional protein kinase Cs. Although protein kinase C can be recruited to membranes by either domain alone, maximal activation of the enzyme requires membrane binding of both the C1 and C2 domains. The resulting membrane interaction is of sufficiently high affinity to provide the energy to break the interaction between the autoinhibitory pseudosubstrate and the active site.

An intriguing property of protein kinase C lipid regulation is the remarkable selectivity of the enzyme’s for phosphatidylycerine. Shortly after the enzyme’s discovery, Nishizuka and co-workers (4) discovered that the “membrane-associated factor” that activated protein kinase C was phosphatidylycerine. Extensive enzymological studies by Bell and workers (5) in the mid-1980s revealed strict specificity for the l-serine headgroup in activating the enzyme, with alterations in the stereochemistry of the headgroup, distance between carboxyl and amine groups, or removal of any functional group resulting in phospholipids unable to significantly activate protein kinase C.

Binding measurements revealed that this selective recognition of phosphatidylycerine occurs only in the presence of diacylglycerol (6–8). In the absence of diacylglycerol, protein kinase C binds all anionic lipids with equal affinity; this interaction is driven primarily by electrostatic forces and depends only on the net charge of the lipid rather than its structure (8). However, the presence of diacylglycerol causes a dramatic increase in the affinity of protein kinase C for phosphatidylycerine-containing surfaces (mixed micelles or membranes) relative to surfaces containing other anionic lipids. For example, diacylglycerol was shown to cause a 250-fold increase in the affinity of protein kinase C for mixed micelles containing phosphatidyl-l-serine compared with only a 10-fold increase in affinity for micelles containing phosphatidyl-d-serine (8). This remarkable selectivity for phosphatidylycerine appears to be unique to protein kinase C and is not a common feature of other C2 domain-containing proteins (9).

A mechanism mediating phosphatidylycerine specificity was proposed recently based on identification of a putative phosphatidylycerine-binding motif. The motif was localized to a consensus sequence (FXXFKLXXXKXR) found in the C2 domain of protein kinase C (residues 227–238 of protein kinase C β); it was identified based on the ability of anti-idiotypic antibodies raised against the combining site of a phosphatidylycerine-specific antibody to bind protein kinase C or peptides based on the C2 domain (10). The amino-terminal hydrophobic residues of the consensus sequence (see Fig. 1A) are conserved in most known C2 domains, including those in proteins that bind membranes with no selectivity for phosphatidylycerine (9). Thus, these residues are unlikely to dictate phosphatidylycerine specificity and may be more important in maintaining the charac-

http://www.jbc.org/content/272/49/30787.full.pdf
teristic β-strand structure of the C2 domain. In contrast, the three basic residues in the carboxyl-half of the consensus sequence are commonly found in proteins that specifically interact with phosphatidylserine, such as the protein kinase Cs and phosphatidylserine decarboxylase (11, 12), and are therefore more likely to comprise a phosphatidylserine-specific binding site.

This contribution investigates the role of the putative phosphatidylserine-binding motif in the lipid regulation of protein kinase C βII. Mutation of the conserved carboxyl-terminal basic residues, in addition to competition studies with a peptide based on the amino-terminal sequence of the motif, reveals that this motif does not regulate the specific interaction of protein kinase C with phosphatidylserine or its nonspecific interaction with anionic lipids. Thus, the FFXLKKXXXKR consensus motif does not provide determinants that bind phosphatidylserine, nor is it involved in the lipid regulation of protein kinase C.

EXPERIMENTAL PROCEDURES

Materials

1-Palmitoyl-2-oleoylphosphatidylserine, 1-palmitoyl-2-oleoylphosphatidylcholine, 1-palmitoyl-2-oleoylphosphatidylglycerol and sn-1,2-dioleoylglycerol were purchased from Avanti Polar Lipids. Triton X-100 and peroxidex-conjugated anti-rabbit IgG antibodies were from Calbiochem. The protein kinase C-selective peptide substrate (Ac-FKKSFKL-NH2) was kindly synthesized by Dr. E. Komives, UCSF. The phosphatidylserine-binding motif peptide (FTFNVK) was provided by Dr. D. Daleke, Indiana University. ATP and phosphatase were from Sigma, and chemiluminescence reagents were from Pierce. Polyvinylidene difluoride membrane was pur-}

phenylmethylsulfonyl fluoride at 4 °C. The lysate was centrifuged at 100,000 × g for 20 min at 4 °C to separate the detergent-soluble fraction (supernatant) from the detergent-insoluble fraction (pellet). The detergent-insoluble pellet was resuspended in lysis buffer by sonication and analyzed by gel electrophoresis. The detergent-soluble supernatant was used as the source of protein kinase C for activity and binding measurements. Qualitatively similar activation kinetics and membrane affinities were observed for wild-type protein kinase C βII in the detergent-soluble fraction and for homogeneously pure protein kinase C βII (data not shown). Detergent-soluble and resuspended particulate fractions were stored at −20 °C in 50% glycerol.

Western Blot Analysis of Expressed Protein Kinase C—The distribution of protein kinase C in the detergent-soluble and detergent-insoluble fractions was analyzed by Western blot analysis. Samples of the extracts were separated on SDS-polyacrylamide gels (7% acrylamide) and transferred to polyvinylidene difluoride membrane. Blots were probed with antibodies reactive against the catalytic domain of protein kinase C βII, and labeling was detected by chemiluminescence after incubation with peroxidase-conjugated secondary antibodies. Antibody staining was quantified by densitometric analysis of blots (Molecular Dynamics scanner, ImageQuant software) and compared with standard amounts of purified protein kinase C βII that had been electrophoresed on the same gel.

Lipids—Sucrose-loaded large unilamellar vesicles containing trace (1% DPPC) were prepared by drying mixtures of lipids in chloroform and evaporating a stream of nitrogen, followed by evaporation under vacuum, suspension of lipids in 20 mM HEPES, pH 7.5, 170 mM sucrose, and then 5 freeze-thaw cycles followed by extrusion using a Liposofast microex-}

Phospholipid concentration in chloroform stocks was determined by measuring free phosphate (17), and purity of lipids was checked by thin layer chromatography.

Protein Kinase C Activity Assay—Protein kinase C activity toward a synthetic peptide (Ac-FKKSFKL-NH2) was analyzed as described previously (13). The standard reaction contained 20 mM HEPES, pH 7.5, 2 mM DTT, 5 mM MgCl2, 100 μM ATP, 50 μM peptide substrate, and either 0.5 mM EGTA (non-activating conditions) or 0.5 mM CaCl2 and sonicated diaphragm sarcoplasmic reticulum (140 μM) and myosin light chain kinase (4 μM). For some experiments, assays contained Triton X-100, lipid mixed micelles (0.1% Triton X-100) of the compositions noted in the legends to Figs. 4 and 6. For peptide competition studies, a peptide stock (20 mM in ME2SO) was diluted to 1 mM in 20 mM HEPES, pH 7.5, and added to the assay mixture to yield a final concentration of 280 μM peptide and 1.5% (v/v) ME2SO. 1 unit of activity is defined as 1 nmol of phosphate transferred per minute at 30 °C.

Protein Kinase C Membrane-binding Assay—The membrane affinity of protein kinase C was determined by measuring the binding to sucrose-loaded vesicles, as described (7, 15, 18). Briefly, protein kinase C in the detergent-soluble fraction of cells (0.8 nM protein kinase C, containing less than 0.001% Triton X-100 in the binding mixture) was incubated with sucrose-loaded vesicles (100 μM lipid) in the presence of 0.3 mM CaCl2, 0.3 mg ml−1 BSA, 100 mM KCl, 1 mM DTT, 5 mM MgCl2, 20 mM HEPES, pH 7.5. Membrane-bound protein kinase C was separated from free enzyme by centrifugation at 100,000 × g for 30 min at 25 °C. The fraction of sedimented vesicles was determined from radioactivity; the fraction of protein kinase C that sedi-}

RESULTS AND DISCUSSION

Expression and Activity of Mutant Protein Kinase C—To test the role of the putative phosphatidylserine-binding motif, FFXLKKXXXKR, in the regulation of protein kinase C, we investigated the effect of mutating the two carboxyl-terminal basic residues on the lipid regulation of protein kinase C. Specifically, Lys-236 and Arg-238 in protein kinase C βII were
mutated to Ala to form the K236A/R238A mutant. Basic residues at one or both corresponding positions are generally found in the C2 domain of proteins that selectively recognize phosphatidylserine, such as the conventional protein kinase Cβ and the yeast phosphatidylserine decarboxylase 2 but not in C2 domains of proteins that do not display phosphatidylserine specificity (Fig. 1A). This motif is also present in phosphatidylserine-regulated proteins that do not appear to have a C2 specificity (Fig. 1). This motif is also present in phosphatidylserine decarboxylase type 1 from yeast (11), phosphatidylserine decarboxylase type 2 from yeast (12), phosphatidylserine decarboxylase from Chinese hamster ovary cells (13), synaptotagmin (14), and basic residues conserved in all C2 domains are in boldface, and basic residues conserved in the proposed motif are boxed.

Residues conserved in all C2 domains are in boldface, and basic residues conserved in the proposed motif are boxed. Asterisks indicate residues mutated in this study (Lys-236 and Arg-238 in protein kinase CβII). Sequences shown are from protein kinase Cα (PKCα, residues 224 to 243; rat brain; Ref. 22), protein kinase Cβ (PKCβ, residues 224 to 243; rat brain; Refs. 23 and 24), protein kinase Cγ (PKCγ, residues 224 to 243; rat brain; Refs. 23 and 24), protein kinase Cε (PKCe, residues 66 to 85; rat brain; Ref. 25), phosphatidylserine decarboxylase from Chinese hamster ovary cells (PSD-CHO, residues 348 to 367; Ref. 11), phosphatidylserine decarboxylase type 1 from yeast (PSD1-yeast, residues 472 to 491; Refs. 26 and 27) and type 2 from yeast (PSD2-yeast, residues 558 to 577; Ref. 12); phosphatidylserine-specific phospholipase A2 (PS-PLA2, residues 319 to 338; Ref. 28), synaptotagmin 1 (residues 472 to 491; Refs. 29 and 30). B, modeled structure of the C2 domain of protein kinase Cβ (21) based on the crystal structure of the C2A domain of synaptotagmin 3 (31). The five conserved aspartates in the Ca2+ binding site are shown in space-filling representation. Conserved residues of the putative phosphatidylserine binding motif are shown in stick representation. Residues mutated in this study are labeled.

Fig. 1. A, alignment of primary sequences of the indicated proteins with the sequence of the putative phosphatidylserine-binding motif. B, modeled structure of the C2 domain of protein kinase CβII. Sequences shown are from protein kinase Cα (PKCα, residues 224 to 243; rat brain; Ref. 22), protein kinase Cβ (PKCβ, residues 224 to 243; rat brain; Refs. 23 and 24), protein kinase Cγ (PKCγ, residues 224 to 243; rat brain; Refs. 23 and 24), protein kinase Cε (PKCe, residues 66 to 85; rat brain; Ref. 25), phosphatidylserine decarboxylase from Chinese hamster ovary cells (PSD-CHO, residues 348 to 367; Ref. 11), phosphatidylserine decarboxylase type 1 from yeast (PSD1-yeast, residues 472 to 491; Refs. 26 and 27) and type 2 from yeast (PSD2-yeast, residues 558 to 577; Ref. 12); phosphatidylserine-specific phospholipase A2 (PS-PLA2, residues 319 to 338; Ref. 28), synaptotagmin 1 (residues 472 to 491; Refs. 29 and 30). B, modeled structure of the C2 domain of protein kinase Cβ (21) based on the crystal structure of the C2A domain of synaptotagmin 3 (31). The five conserved aspartates in the Ca2+ binding site are shown in space-filling representation. Conserved residues of the putative phosphatidylserine binding motif are shown in stick representation. Residues mutated in this study are labeled.

**Mutation of Lys-236 and Arg-238 does not affect the expression, post-translational phosphorylation, or subcellular partitioning of protein kinase CβII.** Western blot of whole cell lysate (L), detergent-soluble supernatant (S), or detergent-insoluble pellet (P) from insect cells infected with either wild-type (lanes 1–3) or K236A/R238A (lanes 4–6) protein kinase CβII recombinant baculovirus; blot was probed with an antibody against the catalytic domain of protein kinase CβII. Each lane contains sample from approximately 1 × 106 cells. Position of molecular weight markers is shown on left; arrow indicates the position of mature, fully phosphorylated protein kinase CβII.

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Fig. 2. Mutation of Lys-236 and Arg-238 does not affect the expression, post-translational phosphorylation, or subcellular partitioning of protein kinase CβII. Western blot of whole cell lysate (L), detergent-soluble supernatant (S), or detergent-insoluble pellet (P) from insect cells infected with either wild-type (lanes 1–3) or K236A/R238A (lanes 4–6) protein kinase CβII recombinant baculovirus; blot was probed with an antibody against the catalytic domain of protein kinase CβII. Each lane contains sample from approximately 1 × 106 cells. Position of molecular weight markers is shown on left; arrow indicates the position of mature, fully phosphorylated protein kinase CβII.

**Phosphatidylserine Dependence of Mutant Protein Kinase Cβ**—As a first step to determine whether Lys-236 and Arg-238 modulate the lipid interaction of protein kinase C, we examined the phosphatidylserine dependence for activation of the mutant and wild-type enzyme. Fig. 4 shows that the phosphatidylserine-dependence for activation of the K236A/R238A mutant, measured in the presence of Triton X-100, lipid mixed micelles containing 5 mol % diacetylgluceral, was the same as that of wild-type enzyme. Specifically, the mutant (open circles) and wild-type (solid circles) enzymes were half-maximally activated by 9.0 ± 0.6 and 9.5 ± 0.6 mol % phosphatidylserine, respectively. Lowering the Ca2+ concentration from 0.5 mM (Fig. 4) to 1 μM increased the concentration of phosphatidylserine required for half-maximal activation, as reported previously (6), but did not cause any differences in the phosphatidylserine
the absence of diacylglycerol (Fig. 4, data not shown). No activity was observed for either protein in regulation of the mutant compared with wild-type enzyme kinase C and represent the weighted average as units of activity (see “Experimental Procedures”) per nmol of protein kinase C was quantified by Western blot analysis. Data are expressed described under “Experimental Procedures.” The amount of protein kinase C was measured in the presence of 0.5 mM Ca²⁺ or in the absence of cofactors and presence of 0.5 mM EGTA (−), as described under “Experimental Procedures.” The amount of protein kinase C was quantified by Western blot analysis. Data are expressed as units of activity (see “Experimental Procedures”) per nmol of protein kinase C and represent the weighted average ± S.D. of the data obtained from three separate infections.

**FIG. 3.** Specific activity of wild-type and K236A/R238A protein kinase C βII. Activity toward phosphorylation of the protein kinase C-selective peptide in the detergent-soluble fraction of cells expressing wild-type (solid bars) or K236A/R238A (open bars) protein kinase C βII was measured in the presence of 0.5 mM Ca²⁺ and sonicated dispersions of phosphatidylserine (140 μM) and diacylglycerol (4 μM) membranes (+) or in the absence of cofactors and presence of 0.5 mM EGTA (−), as described under “Experimental Procedures.” The amount of protein kinase C was quantified by Western blot analysis. Data are expressed as units of activity (see “Experimental Procedures”) per nmol of protein kinase C and represent the weighted average ± S.D. of the data obtained from three separate infections.

**FIG. 4.** Mutation of K236A and R238A does not affect the phosphatidylserine dependence of protein kinase C βII for activation. Protein kinase C activity in the detergent-soluble fraction of cells expressing wild-type (●, ■) or K236A/R238A (▲, □) protein kinase C βII was measured in the presence of 0.5 mM Ca²⁺ and Triton X-100 (0.1%) mixed micelles containing 0–14 mol % phosphatidylserine and 0 mol % (squares) or 5 mol % (circles) diacylglycerol, as described under “Experimental Procedures.” Data are normalized to the 100% value predicted from analysis of the wild-type data using a modified Hill equation (see “Experimental Procedures”) and represent the average ± S.D. of triplicate measurements. The curve shown is that predicted for the wild-type enzyme from the modified Hill equation.

regulation of the mutant compared with wild-type enzyme (data not shown). No activity was observed for either protein in the absence of diacylglycerol (Fig. 4, squares) nor if phosphatidylserine was replaced with another anionic lipid, phosphatidylglycerol (data not shown), as previously reported for wild-type protein kinase C (8). The indistinguishable kinetics of phosphatidylserine-dependent activation of the mutant and wild-type protein kinase C reveal that residues Lys-236 and Arg-238 are not involved in the activation of protein kinase C by phosphatidylserine.

**Membrane Binding of Mutant Protein Kinase C**—Although the above results establish that there is no effect of the K236A/R238A mutation on protein kinase C's activation by phosphatidylserine, we explored whether the mutation altered the affinity of protein kinase C for membranes. In particular, we tested whether the mutation altered protein kinase C's low-affinity interaction with acidic lipids that occurs in the absence of diacylglycerol and whether the mutation altered protein kinase C's high-affinity interaction with phosphatidylserine-containing membranes that occurs in the presence of diacylglycerol.

**FIG. 5.** Mutation of K236A and R238A does not affect the membrane affinity of protein kinase C. The binding of wild-type (solid bars) or K236A/R238A (open bars) protein kinase C to large unilamellar vesicles containing 40 mol % phosphatidylserine or 40 mol % phosphatidylglycerol, and 0 or 5 mol % diacylglycerol, was measured in the presence of 0.3 mM Ca²⁺. The apparent membrane binding affinity was calculated from the amount of protein kinase C that was free and the amount that was vesicle-associated, as described (15). Data are expressed as the mean ± range of duplicate measurements.
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The activity of pure protein kinase C βII was measured in the presence of 0.5 mM Ca\(^2+\) and Triton X-100 (0.1%) mixed micelles containing 0–12 mol % phosphatidylserine and 5 mol % diacylglycerol, either in the absence (○) or presence (●) of 280 μM hexapeptide FTFNVK. 10 mol % phosphatidylserine corresponds to 180μM. Data represent the average ± S.D. of triplicate measurements. The curve shown is that predicted for the data obtained in the absence of peptide from a modified Hill equation (see Materials and Methods).

We have previously shown that membrane binding of wild-type protein kinase C results in a 100-fold increase in the proteolytic sensitivity of the hinge separating the regulatory and catalytic moieties of the enzyme (16). Examination of the proteolytic sensitivity of the mutant revealed that 1) membrane binding induced the same increase in proteolytic sensitivity of the hinge as occurs for wild-type enzyme, and 2) the stability of the mutant was similar to that of wild-type enzyme (data not shown). Thus, the mutation of the putative phosphatidylserine-binding motif did not affect protein kinase C’s conformation or stability as detected using proteases.

Competition for Phosphatidylserine by a Consensus Motif Peptide—To investigate the possibility that the amino-terminal half of the putative phosphatidylserine-binding motif (FXFNLK) alone may function as a phosphatidylserine-binding site, we tested whether a synthetic peptide based on this sequence (FTFNVK) could compete with protein kinase C for phosphatidylserine. Fig. 6 shows that this peptide had no significant effect on protein kinase C’s affinity for phosphatidylserine-containing membranes and only 5-fold, from 1.6 × 10⁷ M⁻¹ to 8.7 × 10⁶ M⁻¹, for phosphatidylglycerol-containing membranes. These data reveal that the basic residues in the putative phosphatidylserine motif have no effect on 1) the nonspecific interaction of protein kinase C with anionic lipids that occurs in the absence of diacylglycerol, or 2) the specific interaction of protein kinase C with phosphatidylserine that occurs in the presence of diacylglycerol.

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Fig. 6. A peptide based on the putative phosphatidylserine-binding motif does not affect the phosphatidylserine regulation of protein kinase C βII. The activity of pure protein kinase C βII was measured in the presence of 0.5 mM Ca\(^2+\) and Triton X-100 (0.1%) mixed micelles containing 0–12 mol % phosphatidylserine and 5 mol % diacylglycerol, either in the absence (○) or presence (●) of 280 μM hexapeptide FTFNVK. 10 mol % phosphatidylserine corresponds to 180 μM. Data represent the average ± S.D. of triplicate measurements. The curve shown is that predicted for the data obtained in the absence of peptide from a modified Hill equation (see Materials and Methods).

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