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## RESEARCH ARTICLE

# ENHANCEMNET OF PKCε ACTIVITY THROUGH SERINE AUTOPHOSPHORYLATION

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**ABSTRACT**

The linoleic acid derivative 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA), with cyclopropane rings instead of *cis*-double bonds, serves as a selective activator of PKCε. The present study investigated whether PKCε, activated by DCP-LA, phosphorylates PKCε itself and how phosphorylated PKCε affects its own activity. DCP-LA phosphorylated PKCε at the serine residues, but not the threonine residues, in PC-12 cells, which is abolished the PKC inhibitor GF109203X. The plasmids for wild-type and mutant PKCε were transfected into MSTO-211H human malignant mesothelioma cells with very little expression of the PKCε mRNA. DCP-LA enhanced PKCε activity approximately 7 folds in MSTO-211H cells transfected with the wild-type rat PKCε plasmid as compared with that in non-transfected control cells. Such effect was not obtained with cells transfected with the plasmid for mutant rat PKCε replacing Ser234, Ser316, Ser368, or Ser729 by Ala. Taken together, these results indicate that PKCε, activated by DCP-LA, enhances its own activity through serine autophosphorylation

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## INTRODUCTION

PKC isozymes are classified into conventional PKCs such as PKCα, -βI, -βII, and -γ, novel PKCs such as PKCδ, -ε, -η, -θ, and -μ, and atypical PKCs such as PKCλ/ι for mouse/human, -ζ, and -ν. PKCs are activated through several pathways linked to phospholipase C (PLC), phospholipase $A_2$ ($PLA_2$), phospholipase D (PLD), and phosphatidylcholine-specific PLC (Isakov, 1993; Nishizuka, 1992; Nishizuka, 1995). PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol 1,4,5-trisphosphate ($IP_3$), the latter activating $IP_3$ receptors to release $Ca^{2+}$ from intracellular calcium stores, and conventional PKCs are activated by diacylglycerol and $Ca^{2+}$ (Nishizuka, 1992; Nishizuka, 1995). Phosphatidylcholine-specific PLC produces diacylglycerol by hydrolysis of phosphatidylcholine, thereby activating PKC (Isakov, 1993). *cis*- Unsaturated free fatty acids such as arachidonic, oleic, linoleic, linolenic, and docosahexaenoic acid, that are produced by $PLA_2$-catalyzed hydrolysis of phosphatidylcholine, activate novel PKCs in a $Ca^{2+}$-independent manner (Nishizuka, 1992; Nishizuka, 1995). The free fatty acids, alternatively, are implicated in synergistic activation of conventional PKCs or sustained activation of conventional PKCs activated (Nishizuka, 1992; Nishizuka, 1995). The linoleic acid derivative 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA), with cyclopropane rings instead of *cis*-double bonds, activates PKCε selectively (Tanaka and Nishizaki, 2003; Kanno *et al*., 2006). DCP-LA binds to the phosphatidylserine binding/associating sites Arg50 and Ile89 in the C2-like domain of PKCε at the carboxyl-terminal end and the cyclopropane rings, respectively, which are distinct from the phorbol 12-myristate 13-acetate (PMA) binding site in the C1 domain (Kanno *et al*., 2015). PKCε consists of the regulatory domain including the C2-like domain and the C1A/1B domain, the V3 domain (hinge domain), and the kinase (catalytic) domain including the C3 domain, the V4 domain, the C4 domain, and the V5 domain (Steinberg, 2008). PKCε as well as other PKC isozymes contain several serine/threonine phosphorylation sites. Little, however, is known about the functional role of PKCε autophosphorylation. To address this question, the present study examined how phosphorylated PKCε affects the PKCε activity. The results show that PKCε, activated by DCP-LA, enhances the its own activity through serine autophosphorylation.

## MATERIALS AND METHODS

**Cell culture:** PC-12 cells, that were obtained from RIKEN Cell Bank (Tsukuba, Japan), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 10% (v/v) heat-

inactivated horse serum, penicillin (100 U/mL), and streptomycin (0.1 mg/mL) in a humidified atmosphere of 5% $CO_2$ and 95% air at 37 °C. PC-12 cells were differentiated by treatment with nerve growth factor (100 ng/mL) for 5 days.
Human malignant mesothelioma cell lines MSTO-211H and NCIH-28 cells were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 0.003% L-glutamine, penicillin (100 U/mL), and streptomycin (0.1 mg/mL) in a humidified atmosphere of 5% $CO_2$ and 95% air at 37 °C.

**Immunoprecipitation and Western Blotting:** PC-12 cells, treated with dimethyl sulfoxide (DMSO) or DCP-LA (100 nM) for 10 min, were homogenized by sonication in TBS-T [150 mM NaCl, 0.1% (v/v) Tween-20 and 20 mM Tris, pH 7.5] containing 1% (v/v) phosphatase inhibitor cocktail and subsequently, homogenates were centrifuged at 3,000 rpm for 5 min at 4 °C. The supernatants (200 μg of protein) were incubated with an antibody against PKCε (BD Biosciences, San Jose, CA, USA) overnight at 4 °C. Then, 20 μL of protein G sepharose (GE healthcare, Piscataway, NJ, USA) was added to the extracts and incubated for 60 min at 4 °C. Pellets were washed three times with TBS-T and dissolved in 30 μL of a sodium dodecyl sulfate (SDS) sample buffer [0.2 mM Tris, 0.05% (w/v) SDS, and 20% (v/v) glycerol, pH 6.8]. After boiling for 5 min, proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a TGX gel (BioRad, Hercules, CA, USA) and then transferred to polyvinylidene difluoride membranes. Blotting membranes were blocked with TBS-T containing 5% (w/v) bovine serum albumin and subsequently incubated with an anti-phospho-Ser (pSer) antibody (QIAGEN, Hilden, Germany) or an anti-phospho-Thr (pThr) antibody (QIAGEN). After washing, membranes were reacted with a horseradish peroxidase-conjugated goat anti-mouse IgG antibody. Immunoreactivity was detected with an ECL kit (GE Healthcare) and visualized using a chemiluminescence detection system (GE Healthcare). Protein concentrations for each sample were determined with a BCA protein assay kit (Pierce, Rockford, IL, USA).

**Real-time reverse transcription-polymerase chain reaction (RT-PCR):** Real-time RT-PCR was carried-out by the method previously described (Tsuchiya *et al*., 2012). Briefly, total RNAs from MSTO-211H and NCIH-28 cells were purified using a Sepasol-RNA I Super kit (Nacalai, Kyoto, Japan) and treated with RNase-free DNase I (2 units) for 30 min at 37 °C to remove genomic DNAs. RNAs purified was incubated in a RT buffer containing random primers, dNTP, and Multiscribe Reverse Transcriptase for 10 min at 25 °C and in turn, for 120 min at 37 °C to synthesize the first-strand cDNA. Real-time RT-PCR was performed using a SYBR Green Realtime PCR Master Mix (Takara Bio, Otsu, Japan) and the Applied Biosystems 7900 real-time PCR detection system (ABI, Foster City, CA). Thermal cycling conditions were as follows: first step, 94 °C for 4 min; the ensuing 40 cycles, 94 °C for 1 s, 65 °C for 15 s, and 72 °C for 30 s. The mRNA quantity for each PKC isozyme was calculated from the standard curve made by amplifying different amount of the GAPDH mRNA. Primers used for real-time RT-PCR are shown in Table I.

**Construction of the plasmid for PKCε and transfection:** Nucleotide sequence coding for wild-type rat PKCε was cloned into pcDNA6/V5-His A vector (Invitrogen)at the KpnI-XhoI site.

For mutant rat PKCε the serine residues at 234, 316, 368, and 729 were replaced by alanine (mS234A, mS316A, mS368A, and mS729A, respectively). The plasmids for wild-type and mutant PKCε were constructed, and transfected into MSTO-211H cells using a Lipofectamine reagent (Invitrogen). Cells were used for experiments 48 h after transfection.

***In situ* PKC assay**

PKC activity in cells was assayed by the method as previously described (Kanno *et al*., 2006). Cells were treated with DMSO or DCP-LA (100 nM) at 37 °C for 10 min in an extracellular solution [137 mM NaCl, 5.4 mM KCl, 10 mM $MgCl_2$, 5 mM ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 0.3 mM $Na_2HPO_4$, 0.4 mM $K_2HPO_4$, and 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, pH 7.2]. Then, cells were rinsed with 100 μL of $Ca^{2+}$-free phosphate-buffered saline (PBS) and incubated at 30 °C for 15 min in 50 μL of the extracellular solution containing 50 μg/mL digitonin, 25 mM glycerol 2-phosphate, 200 μM ATP, and 100 μM synthetic PKC substrate peptide (Pyr-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu) (Peptide Institute Inc., Osaka, Japan). The supernatants were collected and boiled at 100 °C for 5 min to terminate the reaction. Aliquot of the solution (20 μL) was loaded onto a reversed phase high performance liquid chromatography (HPLC) (LC-10ATvp, Shimadzu Co., Kyoto, Japan). A substrate peptide peak and a new product peak were detected at an absorbance of 214 nm (SPD-10Avp UV-VIS detector, Shimadzu Co., Kyoto, Japan). It was confirmed that each peak corresponds to non-phosphorylated and phosphorylated substrate peptide in the analysis of matrix-assisted laser desorption ionization time of flight mass spectrometry (Voyager DE-STR, PE Biosystems Inc., Foster City, USA). Molecular weights were calibrated from the two standard spectrums, bradykinin (MW 1060.2) and neurotensin (MW 1672.9). Areas for non-phosphorylated and phosphorylated PKC substrate peptide were measured (total area corresponds to the concentration of PKC substrate peptide used here). The quantity of phosphorylated substrate peptide (pmol/min/cell protein weight) was calculated and used as an index of PKC activity.

**Statistical analysis:** Statistical analysis was carried out using analysis of variance (ANOVA) followed by a Bonferonni correction.

## RESULTS

**PKCε phosphorylates PKCε itself at the serine residues:** DCP-LA is recognized to activate PKCε selectively (Kanno *et al*., 2006). The signal intensity for pSer-PKCε in the immunoprecipitants from PC-12 cells treated with DCP-LA using an anti-PKCε antibody was significantly enhanced as compared with that in the immunoprecipitants from untreated control cells (Figure 1A). The effect of DCP-LA was clearly inhibited by the PKC inhibitor GF109203X (Figure 1A). In contrast, DCP-LA had no effect on the signal intensity for pThr-PKCε (Figure 1B). Collectively, these results indicate that DCP-LA activates PKCε, to phosphorylate its own PKCε or each other's PKCε at the serine residues, but not the threonine residues.

**PKCε enhances its activity through its own serine phosphorylation:** The next attempt was to understand the effect of PKCε phosphorylation on the PKCε activity.

**Table 1.** Primers used for real-time RT-PCR.

| | | |
|---|---|---|
| *PKCα* | Sense | GCCGTATGGAAAATCTGTGGACTGG |
| | Anti-sense | TGGGCTTGAATGGTGGCTGGAT |
| *PKCβI* | Sense | TTTGAAGGGGAGGATGAAGATGAACTC |
| | Anti-sense | TGAAGAGTTTATCAGTGGGGGTCAGTTC |
| *PKCβII* | Sense | TTTGAAGGGGAGGATGAAGATGAACTC |
| | Anti-sense | TGAATGACAGAAATGAAGGACGGAGAT |
| *PKCγ* | Sense | GCAGCCCCACTTCACCCCC |
| | Anti-sense | CCAGAAATCCCCAGAGCACAGCA |
| *PKCδ* | Sense | CAACAGTGGGACCTACGGCAAGAT |
| | Anti-sense | CCTGTAAATGATGCCCTTGCTGTGTAG |
| *PKCε* | Sense | ATGCCCCACAAGTTCGGTATCCAC |
| | Anti-sense | GGTGCTCCTCTCCTCGGTTGTCA |
| *PKCι* | Sense | TAGATGAGGAAGGAGACCCGTGTACAGTAT |
| | Anti-sense | CTTTCCCTGGTGTTCATTGCCTCTT |
| *PKCθ* | Sense | GGACTGGCACGGCAAGGACTC |
| | Anti-sense | GCAGAGATGGTCTTTCTTTGTTCAGTTCA |
| *PKCζ* | Sense | GCCTCCAGTAGACGACAAGAACGAGG |
| | Anti-sense | CGTAGAACCTGGCGTGCTCCTCA |
| *PKCη* | Sense | CTATGCGAAGGCGAGTCCACCAG |
| | Anti-sense | TCCTTGTCGCATTATTCCCCAGAG |
| *PKCμ* | Sense | AGTGCCCCTGATGAGCCCCTT |
| | Anti-sense | CCCACTGTTCCTTTCACTATCATTGTCA |
| *PKCν* | Sense | ACTCTCTGCCCGACTCTCTAATGGAAG |
| | Anti-sense | CCACAGCCTTCACATTTCAGTCCTTG |

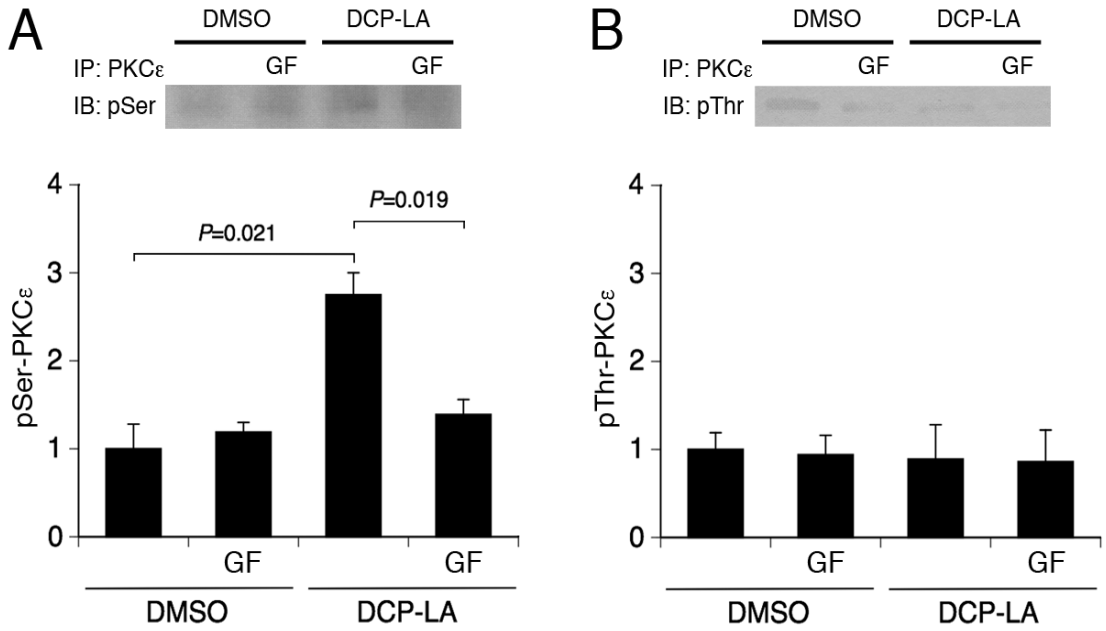


**Figure 1. PKCε, activated by DCP-LA, phosphorylates PKCε itself at the serine residues. Lysates from PC-12 cells treated with DMSO or DCP-LA (100 nM) for 10 min in the presence and absence of GF109203X (GF) (100 nM), were immunoprecipitated with an anti-PKCε antibody, followed by Western blotting using antibodies against phospho-serine (pSer) and phosphor-threonine (pThr). IP, immunoprecipitation; IB, immunoblot. In the graphs, each column represents the mean (± SEM) signal intensity for pSer-PKCε or pThr-PKCε relative to that for cells treated with DMSO in the absence of GF109203X (n=4 independent experiments). *P* values, ANOVA followed by a Bonferroni correction**

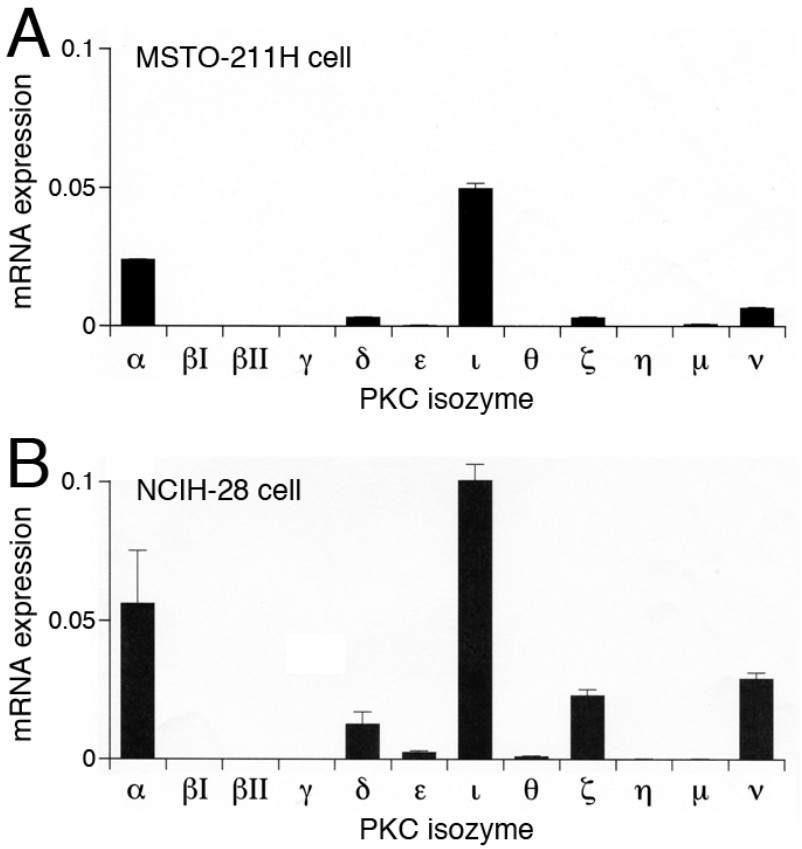


**Figure 2. Expression of the PKC isozyme mRNAs in human malignant mesothelioma cell lines. Real-time RT-PCR was carried out in MSTO-211H and NCIH-28 cells. In the graphs, each point represents the mean (± SEM) mRNA quantity (n=4 independent experiments).**

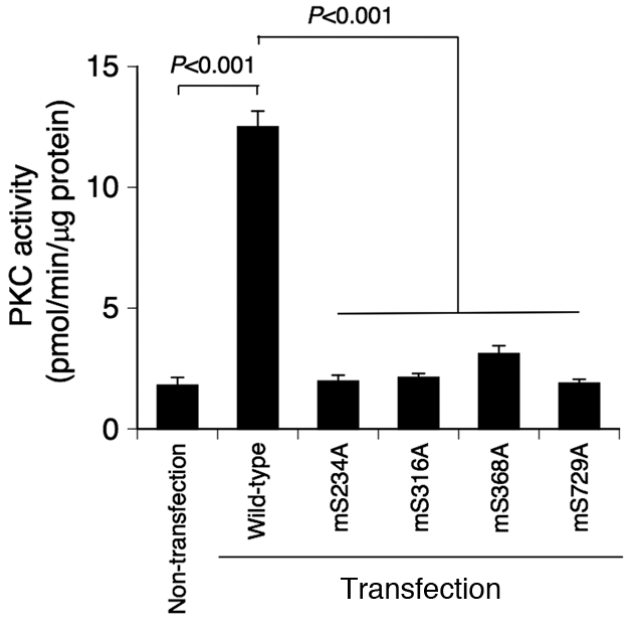


**Figure 3. PKC activity. MSTO-211H cells, non-transfected and transfected with the wild-type and mutant rat PKCε plasmids as indicated, were treated with DCP-LA (100 nM) for 10 min, and PKC activity was assayed. In the graph, each column represents the mean (± SEM) PKC activity (n=6 independent experiments). *P* values, ANOVA followed by a Bonferroni correction.**

In the real-time RT-PCR, the PKCε mRNA was very little expressed in MSTO-211H human malignant mesothelioma cells (Figure 2A), while expression of the PKCε mRNA was detected at the low levels in NCIH-28 cells (Figure 2B). DCP-LA enhanced the PKC activity in MSTO-211H cells transfected with the wild-type rat PKCε plasmid to more than 7 folds than that in non-transfected control cells (Figure 3).

In contrast, no enhancement of the PKC activity was obtained with cells transfected with the plasmid for mutant rat PKCε replacing Ser234, Ser316, Ser368, or Ser729 by Ala (Figure 3). Overall, these results indicate that PKCε, activated by DCP-LA, phosphorylates PKCε itself at the serine residues, to enhance the PKCε activity.

## DISCUSSION

As is the case with other PKC isozymes, PKCε is inactivated in the closed conformation at the hinge domain, and when the closed conformation is opened by binding phosphatidylserine, diacylglycerol, or free fatty acids, PKCε is activated. The linoleic acid derivative DCP-LA activates PKCε selectively by binding to the phosphatidylserine binding/associating sites in the C2-like domain (Kanno *et al*., 2006; Kanno *et al*., 2015). PKC contains the phosphorylation sites on PKC itself (Steinberg, 2008). In the present study, DCP-LA significantly increased serine phosphorylation of PKCε, that is abolished by the PKC inhibitor GF109203X. This indicates that PKCε, activated by DCP-LA, phosphorylates PKCε at the serine residues by itself or each other. Intriguingly, DCP-LA had no effect on threonine phosphorylation of PKCε. This explains that PKCε preferentially phosphorylates its own serine residues. DCP-LA markedly enhanced the PKCε activity in human MSTO-211H cells with very poor expression of PKCε by transfecting with the wild-type rat PKCε plasmid. DCP-LA, however, had no effect on the PKC activity in MSTO-211H cells transfected with the plasmid for the mutant rat PKCε mS234A, mS316A, mS368A, or mS729A. These results indicate that the PKCε activity is enhanced through its own serine phosphorylation. PKCε-Ser729 belongs to the hydrophobic motif located in the V5 domain (Steinberg, 2008). The V5 domain is critical for the PKCε catalytic activity and function; ATP binding pocket is formed between the kinase domain and the V5 domain (Steinberg, 2008). PKCε-Ser729 phosphorylation is supposed to sustain the PKCε activity by dissociating Ser/Thr protein phosphatase. The present results suggest that other serine phosphorylation sites PKCε-Ser234 in the C2-like domain, PKCε-Ser316 in the C1A/1B domain, and PKCε-Ser368 in the C1A/1B domain as well as PKCε-Ser729 in the V5 domain regulate the PKCε catalytic activity.

### Conclusion

The results of the present study show that PKCε, activated by DCP-LA, phosphorylates its own serine residues, to enhance the PKCε activity. This may extend our understanding about the mechanism of PKCε activation.

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