

The linoleic acid derivative DCP-LA selectively activates PKC- ϵ , possibly binding to the phosphatidylserine binding site

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Abstract This study examined the effect of 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA), a newly synthesized linoleic acid derivative with cyclopropane rings instead of *cis*-double bonds, on protein kinase C (PKC) activity. In the *in situ* PKC assay with reverse-phase high-performance liquid chromatography, DCP-LA significantly activated PKC in PC-12 cells in a concentration-dependent (10 nM–100 μ M) manner, with the maximal effect at 100 nM, and the DCP-LA effect was blocked by GF109203X, a PKC inhibitor, or a selective inhibitor peptide of the novel PKC isozyme PKC- ϵ . Furthermore, DCP-LA activated PKC in HEK-293 cells that was inhibited by the small, interfering RNA against PKC- ϵ . In the cell-free PKC assay, of the nine isozymes examined here, DCP-LA most strongly activated PKC- ϵ , with >7-fold potency over other PKC isozymes, in the absence of dioleoyl-phosphatidylserine and 1,2-dioleoyl-*sn*-glycerol; instead, the DCP-LA action was inhibited by dioleoyl-phosphatidylserine. DCP-LA also activated PKC- γ , a conventional PKC, but to a much lesser extent compared with that for PKC- ϵ , by a mechanism distinct from PKC- ϵ activation. Thus, DCP-LA serves as a selective activator of PKC- ϵ , possibly by binding to the phosphatidylserine binding site on PKC- ϵ . These results may provide fresh insight into lipid signaling in PKC activation.—Kanno, T., H. Yamamoto, T. Yaguchi, R. Hi, T. Mukasa, H. Fujikawa, T. Nagata, S. Yamamoto, A. Tanaka, and T. Nishizaki. **The linoleic acid derivative DCP-LA selectively activates PKC- ϵ , possibly binding to the phosphatidylserine binding site.** *J. Lipid Res.* 2006. 47: 1146–1156.

Supplementary key words 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid • protein kinase C- ϵ • protein kinase C- γ

Protein kinase C (PKC) is linked to lipid signaling pathways and participates in a wide range of signal transduction pathways. PKC isozymes are classified as conventional

PKCs, such as PKC- α , - β I, - β II, and - γ ; novel PKCs, such as PKC- δ , - ϵ , - η , - θ , and - μ ; and atypical PKCs, such as PKC- λ / ι for mouse/human, - ζ , and - ν . PKCs are activated via several pathways mediated by phospholipase C, phospholipase A₂, phospholipase D, and phosphatidylcholine-specific phospholipase C (1–3). Phospholipase C hydrolyzes phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol 1,4,5-trisphosphate, the latter activating inositol 1,4,5-trisphosphate receptors to release Ca²⁺ from intracellular calcium stores, and conventional PKCs are activated by diacylglycerol and intracellular Ca²⁺ increase (1, 2). Phosphatidylcholine-specific phospholipase C produces diacylglycerol by hydrolysis of phosphatidylcholine, thereby activating PKC (3). *Cis*-unsaturated free fatty acids, such as arachidonic, oleic, linoleic, linolenic, and docosahexaenoic acid, that are produced by phospholipase A₂-catalyzed hydrolysis of phosphatidylcholine activate novel PKCs in a Ca²⁺-independent manner (1, 2). The free fatty acids, alternatively, may synergistically activate conventional PKCs or sustain the activity of conventional PKCs (1, 2).

We have shown that *cis*-unsaturated free fatty acids induce a long-lasting facilitation of hippocampal synaptic transmission, as a result of the enhancing activity of nicotinic acetylcholine (ACh) receptors, in a PKC-dependent manner (4–9). A similar effect was obtained with 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA; former name, FR236924), a newly synthesized linoleic acid derivative with cyclopropane rings instead of *cis*-double bonds (10, 11) (**Fig. 1**); however, there has been no direct evidence for PKC activation induced by DCP-LA.

Abbreviations: ACh, acetylcholine; CaMKII, Ca²⁺/calmodulin-dependent protein kinase II; DCP-LA, 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid; dNTP, deoxynucleoside triphosphate; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MW, molecular weight; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; siRNA, small interfering RNA.

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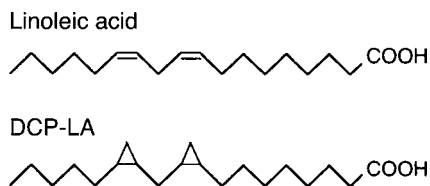


Fig. 1. Chemical structure of 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA).

To address this point, we performed an *in situ* PKC assay using PC-12 cells, a rat pheochromocytoma cell line, and HEK-293 cells, a human embryonic kidney cell line, and a cell-free PKC assay with reverse-phase HPLC. We show here that DCP-LA is capable of activating PKC- ϵ , a novel PKC, in the absence of phosphatidylserine and diacylglycerol, with the highest potency among the nine PKC isozymes examined here, possibly by binding to the phosphatidylserine binding site on PKC- ϵ . DCP-LA also activates PKC- γ , a conventional PKC, without phosphatidylserine and diacylglycerol, but the potency is much weaker than that for PKC- ϵ , and dioleoyl-phosphatidylserine enhances PKC- γ activation induced by DCP-LA. These results may represent a new regulatory pathway for PKC activation linked to lipid signals.

MATERIALS AND METHODS

Materials

Synthetic PKC substrate peptide (Pyr-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu) [molecular weight (MW) 1,374] was purchased from Peptide Institute, Inc. (Osaka, Japan). Autocamide-2 (Lys-Lys-Ala-Leu-Arg-Arg-Gln-Glu-Thr-Val-Asp-Ala-Leu) (MW, 1,527.8), a substrate peptide for Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) (MW, 771.9), a substrate peptide for protein kinase A (PKA), and PKC isozymes were from Calbiochem (San Diego, CA). PC-12 cells and HEK-293 cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan).

RT-PCR

Total RNAs of PC-12 cells or HEK-293 cells were purified by an acid/guanidine/thiocyanate/chloroform extraction method using the Sepasol-RNA I Super kit (Nacalai Tesque, Kyoto, Japan). After purification, total RNAs were treated with RNase-free DNase I (2 units) at 37°C for 30 min to remove genomic DNAs, and 10 μ g of RNAs was resuspended in water. Then, random primers, deoxynucleoside triphosphate (dNTP), 10 \times RT buffer, and Multiscribe Reverse Transcriptase (Applied Biosystems) were added to an RNA solution and incubated at 25°C for 10 min followed by 37°C for 120 min to synthesize the first-strand cDNA. Subsequently, 2 μ l of the reaction solution was diluted with water and mixed with 10 \times PCR buffer, dNTPs, MgCl₂, oligonucleotide, dimethyl sulfoxide [final concentration, 5% (v/v)], and 1 unit of Taq polymerase (Fermentas, St. Leon-Roth, Germany) (final volume, 20 μ l). For PC-12 cells, polymerase chain reaction was carried out with a GeneAmp PCR system model 9600 DNA thermal cycler (Applied Biosystems, Indianapolis, IN) programmed 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. For HEK-293 cells, 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 for PKC- α , - δ , - ϵ , - ι , - θ , - ζ , - η , - μ , and - ν ; or first step, 94°C for 4 min; the ensuing 40 cycles, 94°C for 1 s, 61°C for 15 s, and 72°C for 30 s for PKC- β I, - β II, and - γ . PCR products were electrophoretically separated on a 2% (w/v) agarose gel in 1 \times Tris-borate-EDTA buffer, stained with ethidium bromide, and detected with an ultraviolet illuminator (ATTO, Tokyo, Japan).

For PC-12 cells, primers used for RT-PCR were as follows: 5'-ATCCAACCGCCATTCAAGCCC-3' and 5'-TTGGGATTGGGTGGGGGAAGA-3' for PKC- α (accession number X07286); 5'-GCAAGTGGCTAATGACCAAAACACCC-3' and 5'-TGAAGCATTTTGGTATCGGACACAGTT-3' for PKC- β I (accession number M19007); 5'-GCAAAGGGCTAATGACCAAAACACCC-3' and 5'-TTGAGCTTGAATGACAGAAATGAAGGAC-3' for PKC- β II (accession number M13706); 5'-ACTTTCTCACACAACCTTCATTCCACCTTTC-3' and 5'-GCTCCTCCTCATCTTCCCCATCAA-3' for PKC- γ (accession number X07287); 5'-TGTCCTTTTGGGGTCTCTCTATG-3' and 5'-CCCAGCCAACAGGAGCCAGACT-3' for PKC- δ (accession number BC076505); 5'-GTTGTGGATCCCGAAGCTTGTGGG-3' and 5'-CTGAAAGCTTTCATGACCAAGAACCC-3' for PKC- ϵ (accession number NM_017171); 5'-CTCAACAAGGACCCAAAGGAACGA-3' and 5'-GCACTAAATGGCAGTAAGCAGAAATCAGA-3' for PKC- λ (accession number AB020615); 5'-CACAAATGCTGGGAAAGGAAGTT-3' and 5'-GAAGAACCCCAAAGGACCACAGT-3' for PKC- θ (accession number XM_341553); 5'-TCCTCCTGGTGGGTTGAAGAAA-3' and 5'-CGCCCAGCCATCATCTCAAACATA-3' for PKC- ζ (accession number NM_022507); 5'-TCGGGAAGGTGATGCTCGCC-3' and 5'-GTCCGCACAGCATCTCATAAAGCAA-3' for PKC- η (accession number NM_031085); 5'-GCATCAGCAGACCCTTCCCTCAG-3' and 5'-TCCACACTGTAGCGTTTTCTCATTTTCA-3' for PKC- μ (accession number AB020616); and 5'-TCGTGTGCTCCATGTTTATCAGAAGTT-3' and 5'-GGACGGGATTCTCTTACTTGGCTCTT-3' for PKC- ν (accession number XM_233808).

For HEK-293 cells, primers used for RT-PCR were as follows: 5'-GCCGTATGAAAATCTGTGGACTGG-3' and 5'-TGGGCTTGAA-TGGTGGCTGGAT-3' for PKC- α (accession number NM_002737); 5'-TTTGAAGGGGAGGATGAAGATGAATC-3' and 5'-TGAA-GAGTTTATCAGTGGGGGTCAGTTC-3' for PKC- β I (accession number NM_212535); 5'-TTTGAAGGGGAGGATGAAGATGAACTC-3' and 5'-TGAATGACAGAAATGAAGGACGGAGAT-3' for PKC- β II (accession number NM_002738); 5'-GCAGCCCCACTTCACCCCC-3' and 5'-CCAGAAATCCCGAGCAGCAGCA-3' for PKC- γ (accession number NM_002739); 5'-CAACAGTGGGACCTACGGCAAGAT-3' and 5'-CCTGTAAATGATGCCCTTGCTGTGTAG-3' for PKC- δ (accession number NM_006254); 5'-ATGCC-CACAAGTTCCGTATCCAC-3' and 5'-GGTGCTCTCTCCTCG-TTGTGCA-3' for PKC- ϵ (accession number NM_005400); 5'-TAG-ATGAGGAAGGAGACCCGTGTACAGTAT-3' and 5'-CTTTCCT-GGTGTTTCATTGCCTCTT-3' for PKC- ι (accession number NM_002740); 5'-GGACTGGCAGCGCAAGGACTC-3' and 5'-GCAGA-GATGGTCTTCTTTGTTTCAGTTCA-3' for PKC- θ (accession number NM_006257); 5'-GCCTCCAGTAGACGACAAGAAGC-AGG-3' and 5'-CGTAGAACCTGGCGTCTCCTCA-3' for PKC- ζ (accession number NM_002744); 5'-CTATGCGAAGGCGAGTCC-ACCAG-3' and 5'-TCCTTGTCGATTATCCCCAGAG-3' for PKC- η (accession number NM_006255); 5'-AGTGCCCTGATGAGCCCTT-3' and 5'-CCACTGTTCTTTCACTATCATTTGTCA-3' for PKC- μ (accession number NM_002742); and 5'-ACTCTCT-GCCCGACTCTCTAATGGAAG-3' and 5'-CCACAGCCTTCACAT-TTCAGTCTTTG-3' for PKC- ν (accession number NM_005813).

In situ PKC assay

PKC activity in PC-12 cells or HEK-293 cells was assayed by the modified method described previously (12). PC-12 cells or HEK-

293 cells were plated on 96-well plates (1×10^4 cells/well). Cells were treated with phorbol 12-myristate 13-acetate (PMA), DCP-LA, or linoleic acid in the presence and absence of GF109203X at 37°C for 10 min in an extracellular solution [137 mM NaCl, 5.4 mM KCl, 10 mM MgCl₂, 5 mM EGTA, 0.3 mM Na₂HPO₄, 0.4 mM K₂HPO₄, and 20 mM HEPES, pH 7.2]. Then, cells were rinsed with 100 μ l of Ca²⁺-free PBS and incubated at 30°C for 15 min in 50 μ l of the extracellular solution containing 50 μ g/ml digitonin, 25 mM glycerol 2-phosphate, 200 μ M ATP, and 100 μ M synthetic PKC substrate peptide. The supernatants were collected and boiled at 100°C for 5 min to terminate the reaction. An aliquot of the solution (20 μ l) was loaded onto a reverse-phase HPLC system (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). It was confirmed that each peak corresponds to nonphosphorylated and phosphorylated substrate peptide in an analysis by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Voyager ST-DER; PE Biosystems, Inc., Foster City, CA). Molecular weights were calibrated from the two standard spectrums, bradykinin (MW, 1,060.2) and neurotensin (MW, 1,672.9). Areas for nonphosphorylated and phosphorylated PKC substrate peptide were measured (total area corresponds to the concentration of PKC substrate peptide used here), and the amount of phosphorylated substrate peptide was calculated. Phosphorylated substrate peptide (pmol/min/cell protein weight) was used as an index of PKC activity.

For a different set of experiments, 1 μ mol of a selective PKC- ϵ inhibitor peptide (active PKC- ϵ inhibitor peptide; Glu-Ala-Val-Ser-Leu-Lys-Pro-Thr) (13) or its negative control peptide (inactive PKC- ϵ inhibitor peptide; Leu-Ser-Glu-Thr-Lys-Pro-Ala-Val) (Calbiochem) was mixed with a BioPORTER reagent (Gene Therapy Systems, San Diego, CA) that was dried for 2 h at room temperature using a vortex for 20 s. The mixture was applied to cells in serum-free DMEM and incubated at 37°C for 4 h. Then, PKC activity was assayed.

To determine the intracellular distribution of synthetic PKC substrate peptide after digitonin treatment, the peptide was labeled with fluorescein using the Fluorescein Protein Labeling Kit (Pierce, Rockford, IL). PC-12 cells were incubated at 30°C for 5 min in the extracellular solution containing 50 μ g/ml digitonin just as used for the PKC assay, except for the presence of labeled PKC substrate peptide. Then, cells were fixed with 4% (w/v) paraformaldehyde diluted with PBS at room temperature for 20 min and rinsed three times with PBS. Substrate peptide-labeled PKC was detected with an argon ion laser detector (488 nm) and visualized with a confocal laser scanning microscope (Axiovert/LSM510 META; Carl Zeiss, Oberkochen, Germany).

Small, interfering RNA and transfection

Silencing of human PKC- ϵ gene expression in HEK-293 cells was achieved by the small, interfering RNA (siRNA) technique. A duplex of 21 nucleotide siRNA with TT in the 3' overhang was a gift from Dr. N. Saito (Biosignal Research Center, Kobe University) (14). The sequences of siRNA used to silence the human PKC- ϵ gene were 5'-GCCCCUAAAGACAAUGAAGTT-3' and 5'-CUUCAUUGUCUUAGGGGCTT-3' (regions 412–430 relative to the start codon). The siRNA was transfected into HEK-293 cells using an X-tremeGENE siRNA transfection reagent (Roche Applied Science, Indianapolis, IN). Briefly, 0.1 nmol of the siRNA was incubated in serum- and antibiotics-free DMEM containing an X-tremeGENE siRNA transfection reagent for 20 min, and the solution was layered over HEK-293 cells at 37°C. Two days later, the in situ PKC assay and real-time RT-PCR were carried out.

Real-time RT-PCR

Total RNAs of HEK-293 cells untransfected and transfected with siRNA against PKC- ϵ were purified by an acid/guanidine/thiocyanate/chloroform extraction method using the Sepasol-RNA I Super kit. After purification, total RNAs were treated with RNase-free DNase I (2 units) at 37°C for 30 min to remove genomic DNAs, and 10 μ g of RNAs was resuspended in water. Then, random primers, dNTP, 10 \times RT buffer, and Multiscribe Reverse Transcriptase were added to an RNA solution and incubated at 25°C for 10 min followed by 37°C for 120 min to synthesize the first-strand cDNA. Real-time PCR was performed using a SYBR Premix Ex Taq (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 for PKC- α , - δ , - ϵ , - ι , - θ , - ζ , - η , - μ , and - ν ; or first step, 94°C for 4 min; the ensuing 40 cycles, 94°C for 1 s, 61°C for 15 s, and 72°C for 30 s for PKC- β I, - β II, and - γ . The expression level of each human PKC mRNA was normalized by that of GAPDH mRNA.

Cell-free PKC assay

PKC activity in the cell-free systems was quantified by the method described previously (15, 16). Briefly, synthetic PKC substrate peptide (10 μ M) was reacted with a variety of PKC isozymes in a medium containing 20 mM Tris-HCl (pH 7.5), 5 mM Mg-acetate, and 10 μ M ATP in the presence and absence of phosphatidylserine, diacylglycerol, DCP-LA, or linoleic acid at 30°C for 5 min. The activity of novel PKCs such as PKC- δ , - ϵ , - η , and - μ was assayed in Ca²⁺-free medium, and that of the other PKC isozymes was assayed in the medium containing CaCl₂ at concentrations ranging from 0.1 to 100 μ M. After loading on a reverse-phase HPLC system (LC-10ATvp; Shimadzu), a substrate peptide peak and a new product peak were detected at an absorbance of 214 nm. Areas for nonphosphorylated and phosphorylated PKC substrate peptide were measured (total area corresponds to the concentration of PKC substrate peptide used here), and the amount of phosphorylated substrate peptide was calculated. Phosphorylated substrate peptide (pmol/min) was used as an index of PKC activity.

Cell-free CaMKII assay

A CaMKII substrate peptide (10 μ M) was reacted with CaMKII (Calbiochem) in a reaction medium (25 μ l, pH 8.0) containing 40 mM HEPES, 5 mM Mg-acetate, 0.4 mM CaCl₂, 0.1 mM ATP, 0.1 mM EGTA, and 1 μ M calmodulin (Calbiochem) in the presence and absence of 100 μ M KN-62 at 35°C for 10 min. The reaction was terminated at 100°C for 5 min. An aliquot of the solution (10 μ l) was injected onto the column (250 mm \times 4.6 mm) (COSMOSIL 5C₁₈-AR-II; Nacalai Tesque) and loaded onto a reverse-phase HPLC system (LC-10ATvp; Shimadzu). Non-phosphorylated and phosphorylated peptide were detected at an absorbance of 214 nm (SPD-10Avp UV-VIS detector; Shimadzu). Phosphorylated substrate peptide (pmol/min) was used as an index of CaMKII activity.

Cell-free PKA assay

A PKA substrate peptide (10 μ M) was reacted with PKA (Calbiochem) in a reaction medium (25 μ l) containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 0.2 mM ATP in the presence and absence of 1 μ M H-89, a selective inhibitor of PKA, at 30°C for 10 min. The reaction was terminated at 100°C for 5 min. An aliquot of the solution (10 μ l) was injected onto the column (250 mm \times 4.6 mm) (COSMOSIL 5C₁₈-AR-II; Nacalai Tesque) and loaded onto a reverse-phase HPLC system (LC-

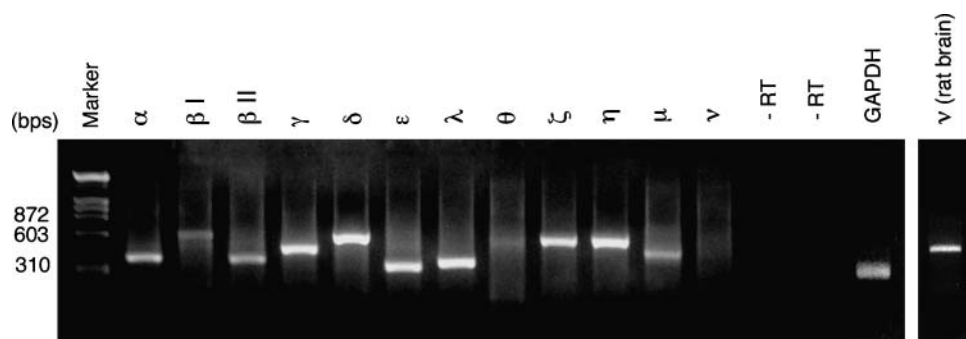


Fig. 2. RT-PCR analysis of the protein kinase C (PKC) isozymes. PCR products for the PKC isozymes as indicated and GAPDH from total RNAs of PC-12 cells are shown (reverse transcription, 40 cycles). The PCR product for the PKC- ν isozyme from total RNAs of rat brain is shown in a separate panel at right (reverse transcription, 40 cycles). -RT, PCR product without reverse transcription as a negative control.

10Atvp; Shimadzu). Nonphosphorylated and phosphorylated peptide were detected at an absorbance of 214 nm (SPD-10ATvp UV-VIS detector; Shimadzu). Phosphorylated substrate peptide (pmol/min) was used as an index of PKA activity.

Statistical analysis

Statistical analysis was carried out using ANOVA and an unpaired *t*-test.

RESULTS

DCP-LA activates PKC in PC-12 cells

In the RT-PCR analysis, PC-12 cells expressed all of the PKC isozyme mRNAs except for PKC- ν mRNA (**Fig. 2**). We

subsequently assayed PKC activity in PC-12 cells using a synthetic PKC substrate peptide that is derived from the phosphorylation site on myelin basic protein (17). It was confirmed before PKC assay that synthetic PKC substrate peptide labeled with fluorescein, which was introduced into cells using a digitonin method, was homogeneously distributed in cells (**Fig. 3**). In the reverse-phase HPLC profile, PMA, a PKC activator, produced a new peak, and that peak was abolished by GF109203X (100 nM), an inhibitor of PKC (**Fig. 4A**). In the MALDI-TOF MS analysis, the molecular weight of the product peak was 1,453.6, which corresponds to the molecular weight of phosphorylated substrate peptide (1,373.6 for nonphosphorylated substrate peptide + 80 for HPO₃) (**Fig. 4B**). In the cell-free

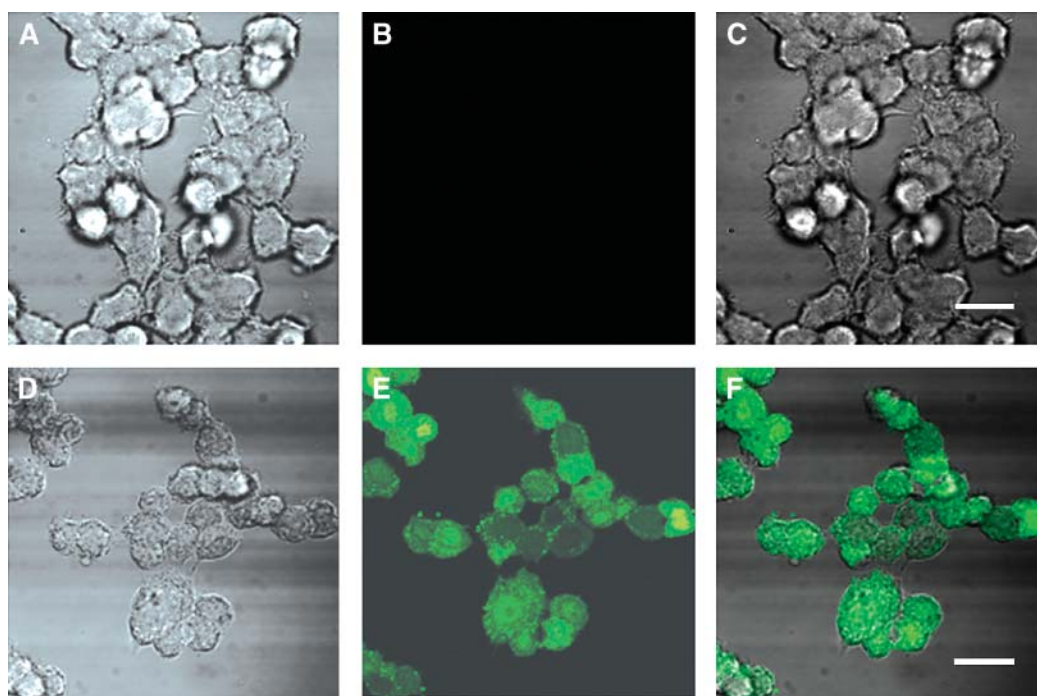


Fig. 3. Distribution of PKC substrate peptide in PC-12 cells. PKC substrate peptide was labeled with fluorescein, and the labeled peptide (green) was inserted into PC-12 cells by digitonin treatment. A–C: Nontreatment. D–F: Digitonin treatment. A, D: Phase-contrast images. B, E: Fluorescence images. C, F: Merge images. Bars = 20 μ m.

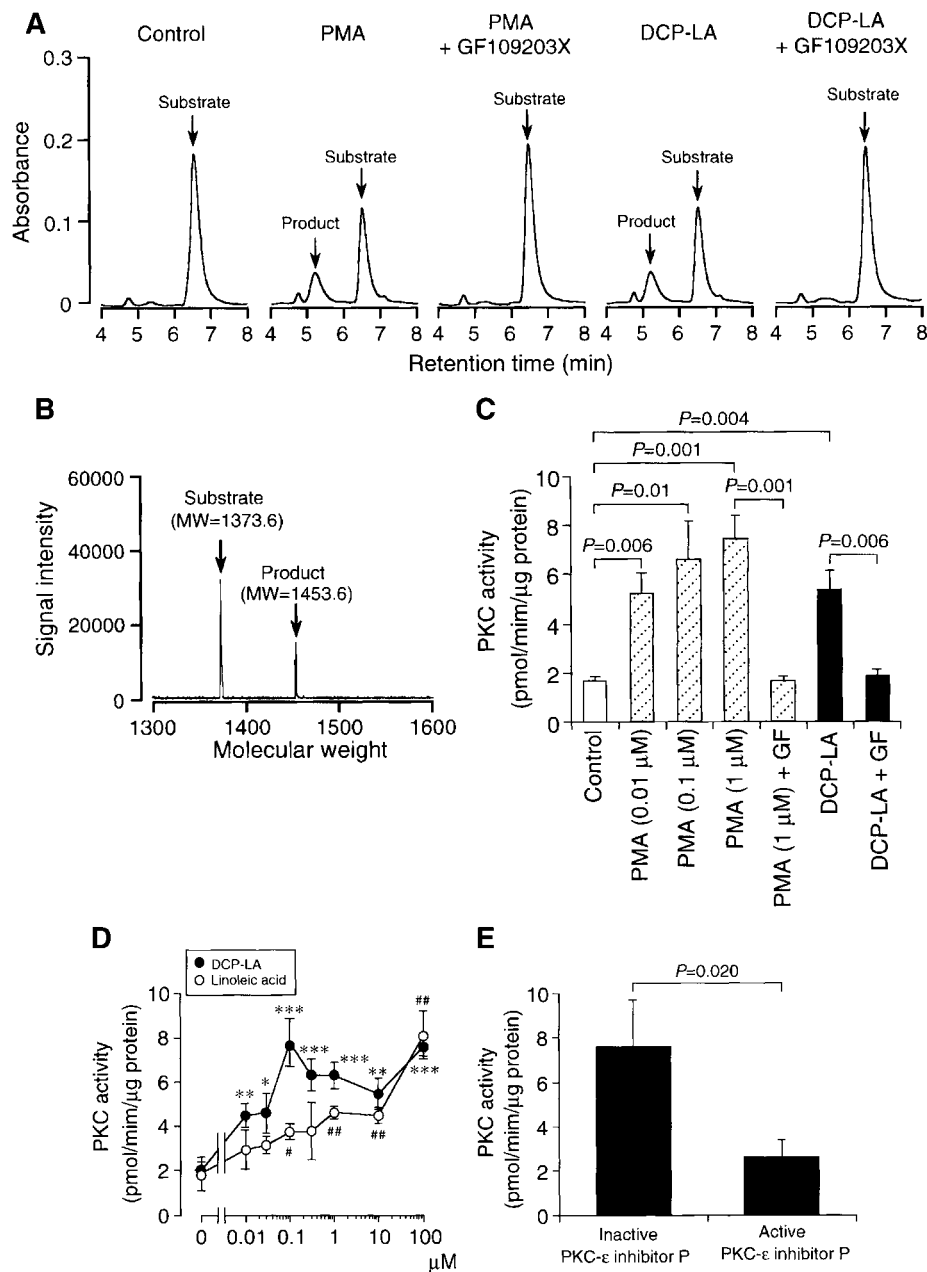


Fig. 4. DCP-LA-induced PKC activation in PC-12 cells. Phorbol 12-myristate 13-acetate (PMA; 0.01, 0.1, and 1 μ M) or DCP-LA (100 nM) was bath-applied to PC-12 cells for 10 min in the presence and absence of GF109203X (GF; 100 nM) or to cells injected with inactive PKC- ϵ inhibitor peptide (1 μ mol), and then PKC activity was quantified by reverse-phase HPLC. **A:** In the HPLC profiles, substrate peak area decreases but product peak area otherwise increases after treatment with PMA or DCP-LA, and the effect is reversed in the presence of GF109203X. **B:** The molecular weight (MW) of substrate and product was measured by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Substrate and product correspond to nonphosphorylated and phosphorylated substrate peptides, respectively. **C:** Each column represents the mean (\pm SEM) PKC activity (pmol/min/ μ g protein) ($n = 5$). P values are from unpaired t -tests. **D:** PC-12 cells were treated with linoleic acid or DCP-LA at the concentrations indicated. Each point represents the mean (\pm SEM) PKC activity (pmol/min/ μ g protein) ($n = 5$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with basal PKC activity (PKC activity in cells untreated with DCP-LA) by unpaired t -test. # $P < 0.05$, ## $P < 0.01$ compared with basal PKC activity (PKC activity in cells untreated with linoleic acid) by unpaired t -test. **E:** Each column represents the mean (\pm SEM) PKC activity (pmol/min/ μ g protein) ($n = 10$). P values are from unpaired t -tests.

CaMKII assay, CaMKII phosphorylated a CaMKII substrate peptide that was inhibited by the CaMKII inhibitor KN-62 (100 μ M), yet CaMKII never phosphorylated the PKC substrate peptide used here (Fig. 5A, B, E). PKA phos-

phorylated a PKA substrate peptide that was abolished by the PKA inhibitor H-89 (1 μ M), but no phosphorylation of the PKC substrate peptide was obtained with PKA (Fig. 5C–E). Collectively, these results indicate that the

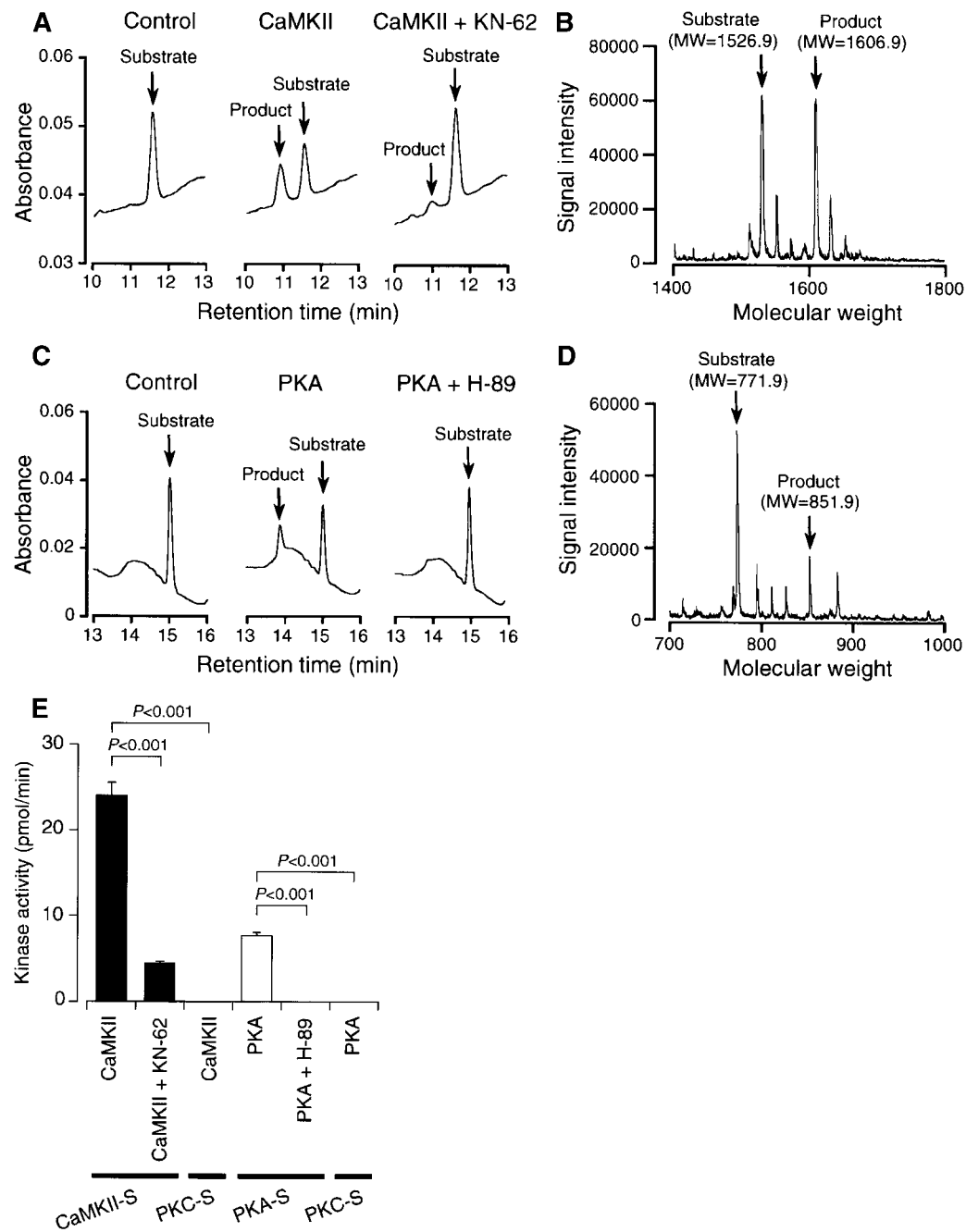


Fig. 5. High selectivity of PKC substrate peptide for PKC. Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) activity was assayed using a CaMKII substrate peptide and a PKC substrate peptide, or protein kinase A (PKA) activity was monitored using a PKA substrate peptide and a PKC substrate peptide in the cell-free systems. **A:** Reverse-phase HPLC profiles in reaction without CaMKII (Control), in reaction with CaMKII (CaMKII), and in reaction with CaMKII in the presence of KN-62 (100 μM ; CaMKII + KN-62) using a CaMKII substrate peptide. **B:** MALDI-TOF MS analysis of substrate and product in **A**. Data of **A** and **B** indicate that CaMKII phosphorylates a CaMKII substrate peptide and that the phosphorylation is blocked by KN-62. **C:** Reverse-phase HPLC profiles in reaction without PKA (Control), in reaction with PKA (PKA), and in reaction with PKA in the presence of H-89 (1 μM ; PKA + H-89) using a PKA substrate peptide. **D:** MALDI-TOF MS analysis of substrate and product in **C**. Data of **C** and **D** indicate that PKA phosphorylates a PKA substrate peptide and that the phosphorylation is blocked by H-89. **E:** Each column represents the mean (\pm SEM) activity of CaMKII or PKA (pmol/min) ($n = 4$). P values are from unpaired t -tests. CaMKII-S, CaMKII assay using a CaMKII substrate peptide; PKC-S, CaMKII assay using a PKC substrate peptide (third column) and PKA assay using a PKA substrate peptide (last column); PKA-S, PKA assay using a PKA substrate peptide.

PKC substrate peptide used here is selective for PKC and therefore that the PKC assay using the substrate peptide actually reflects PKC activity.

PMA significantly activated PKC in PC-12 cells (unpaired *t*-test), with no significant difference in PKC activation among PMA concentrations ranging from 0.01 to 1 μ M (one-way ANOVA) (Fig. 4C). PMA (1 μ M)-induced PKC activation was eliminated by GF109023X (100 nM) (Fig. 4C). DCP-LA (100 nM) also activated PKC, and this effect was blocked by GF109023X (Fig. 4A, C). The DCP-LA effect on PKC activity was concentration-dependent (10 nM–100 μ M), with the maximal effect at 100 nM ($P < 0.0001$, one-way ANOVA) (Fig. 4D). Similarly, PKC activation was also obtained with linoleic acid ($P = 0.0003$, one-way ANOVA), but linoleic acid was less potent than DCP-LA ($P = 0.001$, repeated-measures ANOVA) (Fig. 4D). These results indicate that DCP-LA is capable of activating PKC.

DCP-LA serves as a selective activator of PKC- ϵ

To identify the PKC isoforms that DCP-LA targets, PKC activity was assayed in the cell-free system. Of the nine PKC isoforms examined here (α , β I, β II, γ , δ , ϵ , μ , η , and ζ), DCP-LA (100 μ M) most strongly activated PKC- ϵ , a novel PKC (8.96 ± 0.76 pmol/min of phosphorylation), with

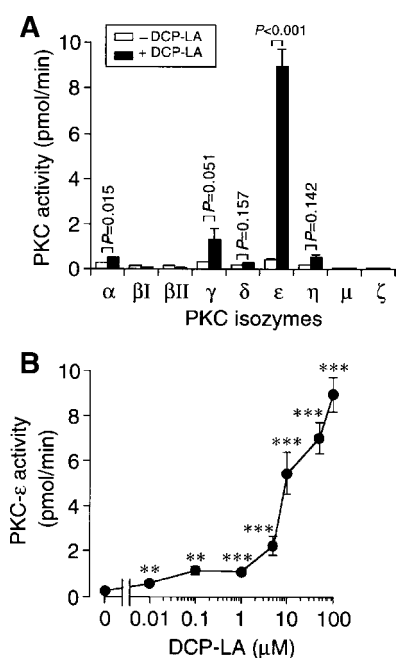


Fig. 6. Selective PKC- ϵ activation induced by DCP-LA. **A:** In the cell-free systems, the PKC isoforms as indicated were reacted in a phosphatidylserine- and diacylglycerol-free medium together with (+ DCP-LA) and without (- DCP-LA) DCP-LA (100 μ M) for 5 min, and each PKC isozyme activity was assayed by reverse-phase HPLC. Each column represents the mean (\pm SEM) PKC activity (pmol/min) ($n = 4-6$). P values are from unpaired *t*-tests. Note that the activity of novel PKCs such as PKC- δ , ϵ , η , and μ was assayed in Ca^{2+} -free medium and that other PKC assays were performed in the presence of 100 μ M CaCl_2 . **B:** Dose-dependent activation of PKC- ϵ induced by DCP-LA. Each point represents the mean (\pm SEM) PKC- ϵ activity (pmol/min) ($n = 8$). ** $P < 0.01$, *** $P < 0.001$ compared with basal PKC- ϵ activity (PKC- ϵ activity in the absence of DCP-LA) by unpaired *t*-test.

>7-fold potency over other PKC isoforms ($P < 0.0001$, one-way ANOVA), in the absence of 1,2-dioleoyl-*sn*-glycerol, a diacylglycerol, and dioleoyl-phosphatidylserine (Fig. 6A). This suggests that DCP-LA serves as a selective activator of PKC- ϵ . DCP-LA significantly activated PKC- ϵ in a concentration-dependent (10 nM–100 μ M) manner ($P < 0.0001$, one-way ANOVA), with the maximal effect at 100 μ M (Fig. 6B). For the other PKC isoforms, the maximal effect was obtained with 100 μ M DCP-LA among concentrations ranging from 10 nM to 100 μ M (data not shown).

DCP-LA-induced (100 nM) PKC activation was significantly inhibited by a selective PKC- ϵ inhibitor peptide (1 μ mol) in PC-12 cells (Fig. 4E), supporting the role for DCP-LA as a selective activator of PKC- ϵ . To obtain further evidence for this, PKC activity was assayed using siRNA against PKC- ϵ in HEK-293 cells. HEK-293 cells expressed all of the PKC isozyme mRNAs (Fig. 7A). In the real-time RT-PCR analysis, the siRNA most effectively reduced PKC- ϵ mRNA expression (0.131 of basal levels), although a reduction in the expression of the other PKC isozyme mRNAs except for PKC- β I and PKC- γ mRNAs was found to a greater or lesser extent (Fig. 7B). DCP-LA (100 nM) as well as PMA (100 nM) significantly activated PKC, which was abolished by GF109023X (100 nM), and the DCP-LA effect was significantly inhibited by siRNA against PKC- ϵ (Fig. 7C). Thus, it appears that DCP-LA preferentially activates PKC- ϵ .

DCP-LA may activate PKC- ϵ by binding to the phosphatidylserine binding site

It is believed that phosphatidylserine is required for the activation of all of the PKC isoforms (1, 2). Dioleoyl-phosphatidylserine (100 μ M) by itself activated PKC- ϵ (1.42 ± 0.05 pmol/min of phosphorylation versus 0.24 ± 0.04 pmol/min of phosphorylation for the control), but dipalmitoyl-phosphatidylserine (100 μ M) otherwise had no effect on PKC- ϵ activation (Fig. 8A). This suggests that the phosphatidylserine binding site on PKC- ϵ recognizes *cis*-unsaturated free fatty acids, but not saturated free fatty acids, at the α [1] or β [2] position on phosphatidylserine. In contrast, stearic acid (100 μ M), a saturated free fatty acid, did not activate PKC- ϵ in the absence of dioleoyl-phosphatidylserine and 1,2-dioleoyl-*sn*-glycerol (Fig. 8A). Linoleic acid activated PKC- ϵ in a concentration-dependent (5–100 μ M) manner, without dioleoyl-phosphatidylserine and 1,2-dioleoyl-*sn*-glycerol (Fig. 8B). Cotreatment with linoleic acid and DCP-LA caused no additional activation of PKC- ϵ (Fig. 8A), suggesting a common site of action on PKC- ϵ between linoleic acid and DCP-LA. Surprisingly, the DCP-LA (100 μ M) effect on PKC- ϵ activation was inhibited by dioleoyl-phosphatidylserine in a concentration-dependent (5–100 μ M) manner (Fig. 8C). Moreover, PKC- ϵ activation induced by linoleic acid or DCP-LA was abolished in the presence of dioleoyl-phosphatidylserine (100 μ M), reaching a level similar to that activated by dioleoyl-phosphatidylserine alone (Fig. 8B, D). Linoleic acid or DCP-LA, thus, may activate PKC- ϵ by binding to the phosphatidylserine binding site on PKC- ϵ , but with an affinity lower than phosphatidylserine.

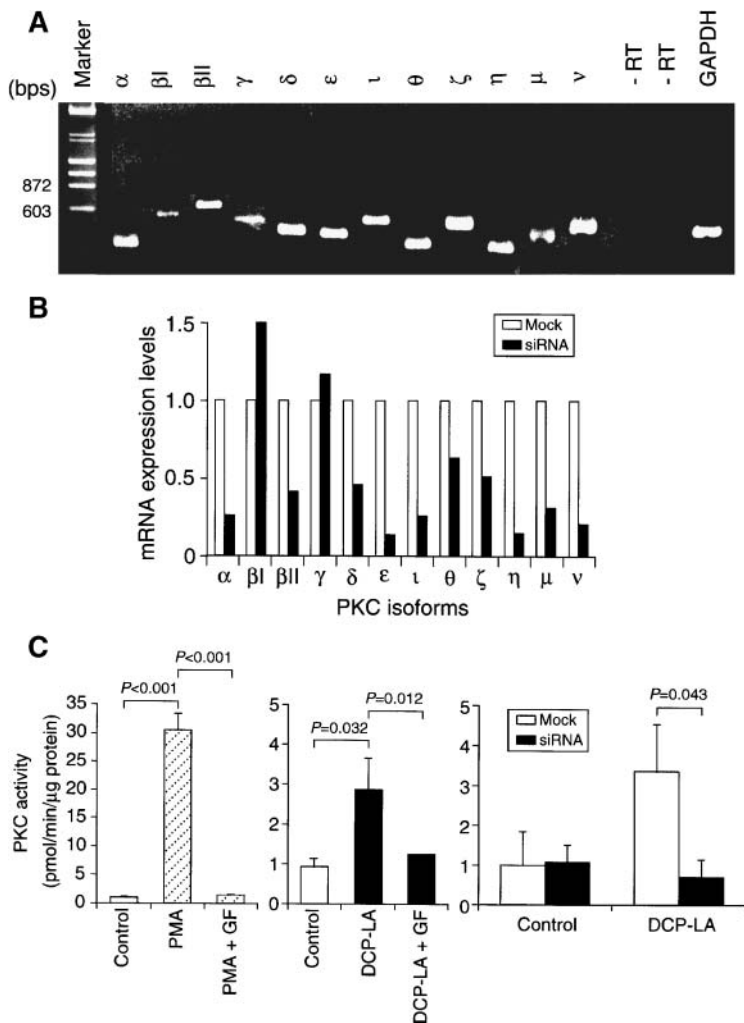


Fig. 7. Inhibition of DCP-LA-induced PKC activation by small, interfering RNA (siRNA) against PKC- ϵ in HEK-293 cells. A: RT-PCR analysis shows that HEK-293 cells express all of the PKC isozyme mRNAs (reverse transcription, 40 cycles). GAPDH, PCR product from total RNAs of HEK-293 cells; -RT, PCR product without reverse transcription as a negative control. B: HEK-293 cells were transfected without (Mock) and with siRNA against PKC- ϵ (siRNA), and RT-PCR was performed 48 h after transfection to analyze the expression level of each PKC isozyme mRNA. The expression level of each human PKC mRNA was normalized by that of GAPDH mRNA and compared by regarding the product density of each PKC mRNA from cells untransfected with the siRNA as 1.0. C: Each column represents the mean (\pm SEM) PKC activity (pmol/min/ μ g protein) ($n = 5$). P values are from unpaired t -tests. Left and middle panels, PMA (100 nM) or DCP-LA (100 nM) was applied to untransfected HEK-293 cells in the presence and absence of GF109203X (GF; 100 nM); right panel, PKC activity after nontreatment (Control) and treatment with DCP-LA (100 nM) was monitored in cells mock-treated or transfected with siRNA targeting PKC- ϵ (siRNA).

DCP-LA activates PKC- γ by a mechanism distinct from PKC- ϵ activation

PKC- γ and PKC- β II are preferentially expressed in post-synaptic cells, whereas PKC- ϵ is localized on presynaptic terminals in the brain (18, 19). Therefore, we focused upon PKC- γ , a conventional PKC, and further examined the effect of DCP-LA on its activation. Conventional PKCs are activated by diacylglycerol together with Ca^{2+} (1, 2). 1,2-Dioleoyl-*sn*-glycerol indeed activated PKC- γ in a bell-shaped concentration-dependent (5–100 μM) manner, with the peak at 10 μM , in the presence of dioleoyl-phosphatidylserine (100 μM), but no significant activation was obtained without dioleoyl-phosphatidylserine (one-way ANOVA) (Fig. 9A). Dioleoyl-phosphatidylserine (100 μM) by itself activated PKC- γ (~ 3 pmol/min of phosphorylation), with potency greater than that for PKC- ϵ (Fig. 9A–D, F). PKC- γ was not activated in the copresence of dioleoyl-phosphatidylserine (100 μM) and 1,2-dioleoyl-*sn*-glycerol (100 μM) under Ca^{2+} concentrations < 0.5 μM , although it was activated in the presence of dioleoyl-phosphatidylserine (100 μM) alone at 0.5 μM Ca^{2+} (Fig. 9C). In contrast, PKC- γ was activated in the presence of dioleoyl-phosphatidylserine (100 μM) alone or in the

copresence of dioleoyl-phosphatidylserine (100 μM) and 1,2-dioleoyl-*sn*-glycerol (100 μM) under higher concentrations of Ca^{2+} (> 1 μM), without a significant difference in PKC- γ activation between 1 and 100 μM Ca^{2+} in the reaction medium (one-way ANOVA) (Fig. 9C), supporting the notion that Ca^{2+} is required for the activation of conventional PKCs.

1-Stearoyl-2-arachidonoyl-glycerol, another diacylglycerol, never activated PKC- γ in the absence of dioleoyl-phosphatidylserine; instead, it concentration-dependently inhibited PKC- γ activation induced by dioleoyl-phosphatidylserine alone (100 μM) (Fig. 9B). Linoleic acid or DCP-LA, alternatively, activated PKC- γ without dioleoyl-phosphatidylserine and 1,2-dioleoyl-*sn*-glycerol (each ~ 1 –2 pmol/min of phosphorylation at 100 μM), but to a much lesser extent compared with PKC- ϵ activation (Fig. 9D, F). Notably, PKC- γ activation induced by linoleic acid was significantly enhanced by adding dioleoyl-phosphatidylserine (100 μM) ($P < 0.001$, repeated-measures ANOVA) (Fig. 9D). Likewise, dioleoyl-phosphatidylserine increased PKC- γ activation induced by DCP-LA (100 μM) in a bell-shaped concentration-dependent (5–100 μM) manner (Fig. 9E), and the DCP-LA concentration-responsive ef-

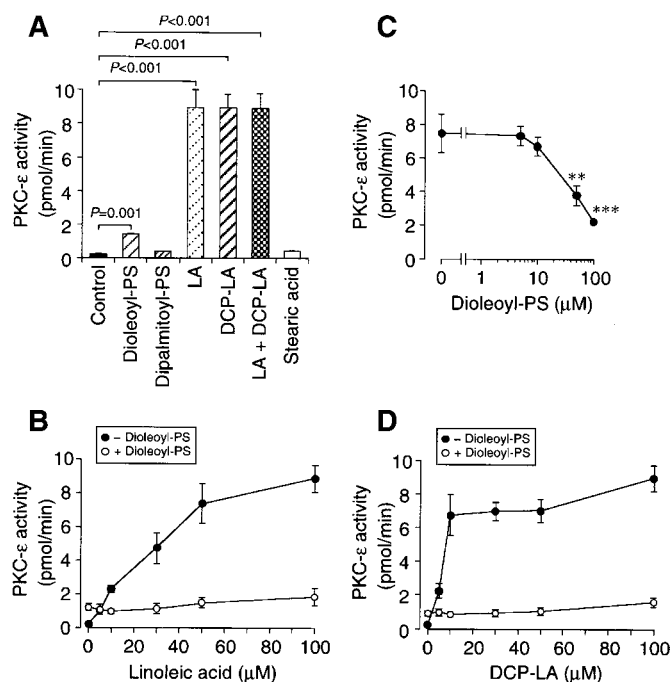


Fig. 8. Inhibition of DCP-LA-induced PKC- ϵ activation by phosphatidylserine. A: PKC- ϵ activity was quantified in the absence (Control) and presence of dioleoyl-phosphatidylserine (Dioloyl-PS; 100 μ M), dipalmitoyl-phosphatidylserine (Dipalmitoyl-PS; 100 μ M), linoleic acid (LA; 100 μ M), DCP-LA (100 μ M), linoleic acid (100 μ M) plus DCP-LA (100 μ M), or stearic acid (100 μ M). Each column represents the mean (\pm SEM) PKC- ϵ activity (pmol/min) ($n = 4-5$). P values are from unpaired t -tests. B: PKC- ϵ activation induced by linoleic acid at the concentrations indicated in the presence (+ Dioloyl-PS; 100 μ M) and absence of dioleoyl-phosphatidylserine (- Dioloyl-PS). Each point represents the mean (\pm SEM) PKC- ϵ activity (pmol/min) ($n = 5$). C: Dose-dependent effect of dioleoyl-phosphatidylserine on PKC- ϵ activation induced by DCP-LA. PKC- ϵ activation induced by DCP-LA (100 μ M) was assayed in the presence of dioleoyl-phosphatidylserine at the concentrations indicated. Each point represents the mean (\pm SEM) PKC- ϵ activity (pmol/min) ($n = 4$). ** $P < 0.01$, *** $P < 0.001$ compared with DCP-LA-induced PKC- ϵ activation in the absence of dioleoyl-phosphatidylserine by unpaired t -test. D: PKC- ϵ activation induced by DCP-LA at the concentrations indicated in the presence (+ Dioloyl-PS; 100 μ M) and absence of dioleoyl-phosphatidylserine (- Dioloyl-PS). Each point represents the mean (\pm SEM) PKC- ϵ activity (pmol/min) ($n = 5-8$).

fect on PKC- γ activation was significantly enhanced by adding dioleoyl-phosphatidylserine (100 μ M) ($P < 0.001$, repeated-measures ANOVA) (Fig. 9F).

These results indicate that the site of action of linoleic acid or DCP-LA on PKC- γ differs from that on PKC- ϵ . *Cis*-unsaturated free fatty acids are suggested to synergistically activate conventional PKCs, perhaps by binding to the C1 (cysteine-rich) domain (1, 2). Linoleic acid or DCP-LA at a concentration of 10 μ M did not affect the activity of PKC- γ induced by dioleoyl-phosphatidylserine (100 μ M) and 1,2-dioleoyl-*sn*-glycerol (Fig. 9A), but each significantly enhanced the activity induced by dioleoyl-phosphatidylserine (100 μ M) and 1-stearoyl-2-arachidonoyl-glycerol ($P = 0.0053$ for linoleic acid and $P = 0.0212$ for DCP-LA, repeated-measures ANOVA) (Fig. 9B).

Accumulating evidence has shown that PKC is activated via several pathways relevant to phospholipase C, phospholipase A₂, phospholipase D, and phosphatidylcholine-specific phospholipase C (1-3). *Cis*-unsaturated free fatty acids, which are produced via a phospholipase A₂ pathway, are involved in the activation of novel PKCs, including PKC- ϵ (1, 2). In the PKC assay using PC-12 cells, which express all of the PKC isozymes except for the ν isozyme, the *cis*-unsaturated free fatty acid linoleic acid enhanced PKC activity in a concentration-dependent (10 nM-100 μ M) manner. Likewise, the linoleic acid derivative DCP-LA enhanced PKC activity in a concentration-dependent (10 nM-100 μ M) manner, with potency greater than linoleic acid, and the effect was eliminated by GF109203X, a PKC inhibitor. This confirms that DCP-LA engages PKC activation. In an earlier study, DCP-LA was suggested to facilitate hippocampal synaptic transmission by targeting presynaptic $\alpha 7$ ACh receptors to control glutamate release via a PKC pathway (10, 11). In good agreement with the fact that DCP-LA exhibited the most beneficial effects at 100 nM, the maximal PKC activation in PC-12 cells was found at 100 nM. Collectively, DCP-LA-engaged PKC activation explains the DCP-LA action on $\alpha 7$ ACh receptor responses, glutamate release, and hippocampal synaptic transmission.

In the cell-free PKC assay with the α , β I, β II, γ , δ , ϵ , η , μ , and ζ isozymes, DCP-LA most prominently and significantly activated PKC- ϵ compared with activation of the other PKC isozymes. This provides the possibility for PKC- ϵ , but not PKC- α , - β I, - β II, - γ , - δ , - η , - μ , or - ζ , to be a target of DCP-LA. In support of this notion, the DCP-LA-induced PKC activation in PC-12 cells was prevented by a selective PKC- ϵ inhibitor peptide. Moreover, siRNA against PKC- ϵ inhibited PKC activation induced by DCP-LA in HEK-293 cells, although the siRNA reduced not only PKC- ϵ mRNA but the other PKC isozyme mRNAs except for PKC- β I and PKC- γ mRNAs. Together, the results of this study lead to the conclusion that DCP-LA serves as a selective activator of PKC- ϵ , even though the interaction with PKC- λ/ι , - θ , and - ν is not completely excluded in the siRNA experiment.

PKC- ϵ activation induced by DCP-LA occluded the linoleic acid effect, and vice versa, indicating the same site of action on PKC- ϵ between DCP-LA and linoleic acid. In the cell-free systems, DCP-LA activated PKC- ϵ in a concentration-dependent (10 nM-100 μ M) manner, with the maximal effect at 100 μ M. *Cis*-unsaturated free fatty acids are thought to activate novel PKCs in a Ca²⁺-independent and diacylglycerol-dependent manner (1, 2). Linoleic acid or DCP-LA, however, activated PKC- ϵ in the absence of 1,2-dioleoyl-*sn*-glycerol, a diacylglycerol. An established notion is that phosphatidylserine is a prerequisite for the activation of all of the PKC isozymes. PKC- ϵ was activated by dioleoyl-phosphatidylserine alone, but no activation was obtained with dipalmitoyl-phosphatidylserine, suggesting that phosphatidylserine containing *cis*-unsaturated free fatty acids, but not saturated free fatty acids, at the α [1] or β [2] position enables PKC- ϵ to activate. One of

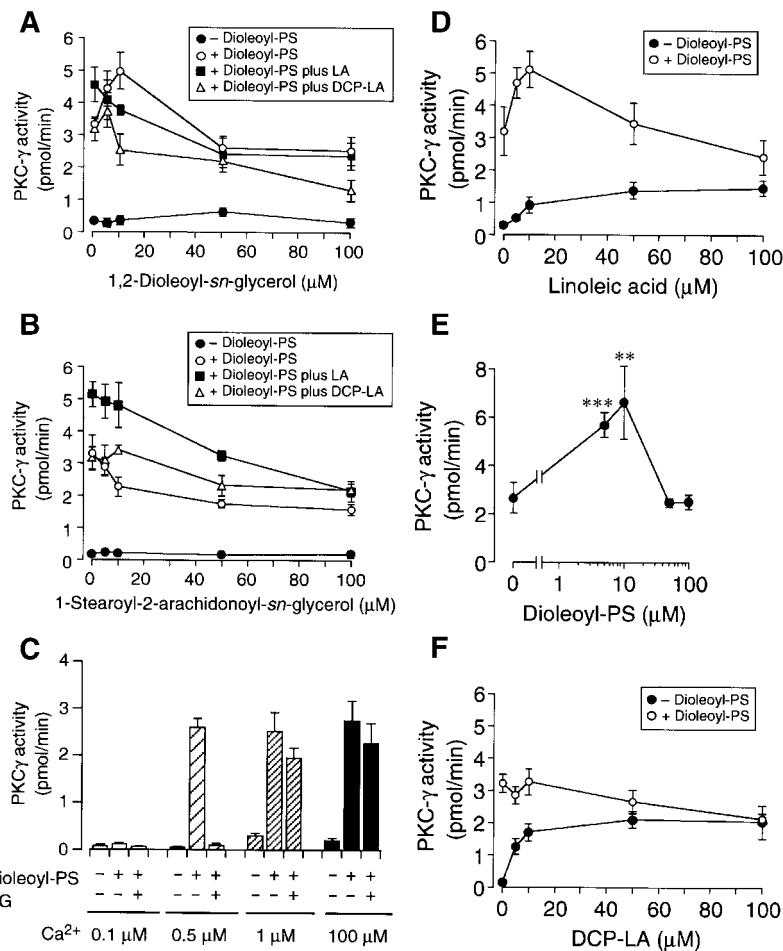


Fig. 9. PKC- γ activation induced by linoleic acid or DCP-LA. **A:** PKC- γ activity was measured in the presence and absence of 1,2-dioleoyl-*sn*-glycerol at the concentrations indicated together with and without dioleoyl-phosphatidylserine (Di-oleoyl-PS; 100 μ M), dioleoyl-phosphatidylserine (100 μ M) plus linoleic acid (LA; 10 μ M), and dioleoyl-phosphatidylserine (100 μ M) plus DCP-LA (10 μ M). Each point represents the mean (\pm SEM) PKC- γ activity (pmol/min) ($n = 4$). Note that PKC- γ activity was assayed in the presence of 100 μ M Ca^{2+} . **B:** PKC- γ activity was measured in the presence and absence of 1-stearoyl-2-arachidonoyl-glycerol at the concentrations indicated together with and without dioleoyl-phosphatidylserine (100 μ M), dioleoyl-phosphatidylserine (100 μ M) plus linoleic acid (10 μ M), and dioleoyl-phosphatidylserine (100 μ M) plus DCP-LA (10 μ M). Each point represents the mean (\pm SEM) PKC- γ activity (pmol/min) ($n = 4$). Note that PKC- γ activity was assayed in the presence of 100 μ M Ca^{2+} . **C:** Effect of Ca^{2+} concentrations on PKC- γ activation induced by dioleoyl-phosphatidylserine (100 μ M) together with and without 1,2-dioleoyl-*sn*-glycerol (DG; 100 μ M). PKC- γ activity was assayed in the presence of Ca^{2+} at concentrations ranging from 0.1 to 100 μ M. Each column represents the mean (\pm SEM) PKC- γ activity (pmol/min) ($n = 4$). **D:** PKC- γ activation induced by linoleic acid at the concentrations indicated in the presence (+ Di-oleoyl-PS; 100 μ M) and absence of dioleoyl-phosphatidylserine (- Di-oleoyl-PS). Each point represents the mean (\pm SEM) PKC- γ activity (pmol/min) ($n = 4$). **E:** Dose-dependent effect of dioleoyl-phosphatidylserine on PKC- γ activation induced by DCP-LA. PKC- γ activation induced by DCP-LA (100 μ M) was assayed in the presence of dioleoyl-phosphatidylserine at the concentrations indicated. Each point represents the mean (\pm SEM) PKC- γ activity (pmol/min) ($n = 4$). ** $P < 0.01$, *** $P < 0.001$ compared with DCP-LA-induced PKC- γ activation in the absence of dioleoyl-phosphatidylserine by unpaired *t*-test. **F:** PKC- γ activation induced by DCP-LA at the concentrations indicated in the presence (+ Di-oleoyl-PS; 100 μ M) and absence of dioleoyl-phosphatidylserine (- Di-oleoyl-PS). Each point represents the mean (\pm SEM) PKC- γ activity (pmol/min) ($n = 4$).

the striking findings in this study is that linoleic acid or DCP-LA still activated PKC- ϵ independently of dioleoyl-phosphatidylserine; conversely, dioleoyl-phosphatidylserine (100 μ M) abolished the effect of linoleic acid or DCP-LA, reaching a level similar to the PKC- ϵ activation achieved by dioleoyl-phosphatidylserine alone. Linoleic

acid or DCP-LA, accordingly, may engineer PKC- ϵ activation by binding to the phosphatidylserine binding site on PKC- ϵ , with an affinity lower than that of phosphatidylserine. Then, one would wonder why, in spite of the presence of phosphatidylserine in cells, linoleic acid or DCP-LA could activate PKC in PC-12 cells or HEK-293 cells. In

the cell-free systems, dioleoyl-phosphatidylserine at concentrations of $<10 \mu\text{M}$ had no effect on PKC- ϵ activation induced by DCP-LA. This may account for the potency of linoleic acid or DCP-LA for the in situ PKC- ϵ activation still in the presence of phosphatidylserine, although the accurate concentration of phosphatidylserine in cells is unknown.

Conventional PKCs, such as PKC- α , - β I, - β II, and - γ , on the other hand, are activated in a Ca^{2+} - and diacylglycerol-dependent manner via a G_q protein-linked receptor/phospholipase C pathway (1, 2). 1,2-Dioleoyl-*sn*-glycerol or 1-stearoyl-2-arachidonoyl-glycerol activated PKC- γ in the presence of dioleoyl-phosphatidylserine. Without dioleoyl-phosphatidylserine, however, 1,2-dioleoyl-*sn*-glycerol or 1-stearoyl-2-arachidonoyl-glycerol little/never activated PKC- γ , suggesting that phosphatidylserine, but not diacylglycerol, is required for the activation of conventional PKCs in cell-free systems. This, in light of the fact that diacylglycerol increases the ability of PKCs to associate with phosphatidylserine in the plasma membrane in situ, suggests that diacylglycerol does not directly activate PKCs. Linoleic acid or DCP-LA also activated PKC- γ in the absence of dioleoyl-phosphatidylserine and 1,2-dioleoyl-*sn*-glycerol, but to a much lesser extent compared with PKC- ϵ activation. As is not the case with PKC- ϵ , dioleoyl-phosphatidylserine enhanced PKC- γ activation induced by linoleic acid or DCP-LA, indicating that the site of action of DCP-LA or linoleic acid on PKC- γ is distinct from that on PKC- ϵ . *Cis*-unsaturated free fatty acids are suggested to synergistically enhance the activity of conventional PKCs induced by phosphatidylserine and diacylglycerol (1, 2). Such an effect was indeed obtained with linoleic acid or DCP-LA in the presence of dioleoyl-phosphatidylserine and 1-stearoyl-2-arachidonoyl-glycerol, yet linoleic acid or DCP-LA did not enhance PKC- γ activation induced by dioleoyl-phosphatidylserine and 1,2-dioleoyl-*sn*-glycerol. The reason for this discrepancy with different kinds of diacylglycerol remains an open question.

In conclusion, the newly synthesized linoleic acid derivative DCP-LA selectively activates PKC- ϵ , a novel PKC, in a phosphatidylserine- and diacylglycerol-independent manner, possibly by binding to the phosphatidylserine binding site on PKC- ϵ . DCP-LA also activates PKC- γ , but to a much lesser extent, by a mechanism distinct from PKC- ϵ activation. Thus, the results of this study may extend our knowledge of lipid signals on PKC activation. ■

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