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Synthesis and Evaluation of Dimeric Derivatives of Diacylglycerol–Lactones as Protein Kinase C Ligands

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Abstract

Protein kinase C (PKC) mediates a central cellular signal transduction pathway involved in disorders such as cancer and Alzheimer's disease. PKC is regulated by binding of the second messenger *sn*-1,2-diacylglycerol (DAG) to its tandem C1 domains, designated C1a and C1b, leading both to PKC activation and to its translocation to the plasma membrane and to internal organelles. Depending on the isoform, there may be differences in the ligand selectivity of the C1a and C1b domains, and there is different spacing between the C1 domains of the conventional and novel PKCs. Bivalent ligands have the potential to exploit these differences between isoforms, yielding isoform selectivity. In the present study, we describe the synthesis of a series of dimeric derivatives of conformationally constrained diacylglycerol (DAG) analogs (DAG–lactones). We characterize the derivatives in vitro for their binding affinities, both to a single C1 domain (the C1b domain of PKC δ) as well as to the conventional PKC α isoform and the novel PKC δ isoform, and we measure their abilities to cause translocation of PKC δ and PKC ϵ in intact cells. The dimeric compound with the 10-carbon linker was modestly more effective for the isolated PKC δ C1b domain than was the monomeric compound. For the intact PKC α and PKC δ , the shortest DAG–lactone dimer had similar affinity to the monomer and affinity decreased progressively up to the 16-carbon linker. The dimeric derivatives did not cause the Golgi accumulation of PKC δ . The present results provide important insights into the development of new chemical tools for biological studies on PKC.

Graphical Abstract

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INTRODUCTION

Protein kinase C (PKC) is a serine–threonine protein kinase that is involved in cellular signal transduction, leading to proliferation,¹ differentiation,² migration,³ and apoptosis.⁴ PKC is an important therapeutic target because of its involvement in disorders such as diabetes, cancer, heart disease, autoimmune diseases and Alzheimer's disease.^{5–8} The isozymes in the PKC family are divided into conventional PKCs (cPKC) (α , $\beta_{I/II}$, and γ), novel PKCs (nPKC) (δ , ϵ , η , and θ) and atypical PKCs (aPKC) (ζ and ι/λ).⁹ With the exception of the aPKCs, the cPKCs and nPKCs are regulated by ligand binding through their tandem C1 (C1a and C1b) domains. In the case of the cPKCs, the additional binding of Ca^{2+} to the C2 domain is required. The inactive form of PKC, which has the substrate binding domain capped by its own pseudosubstrate, is located in cytosol. Upon ligand binding, PKC shows translocation from cytosol to the plasma membrane and internal membranes. The endogenous ligand for PKC is *sn*-1,2-diacylglycerol (DAG), which is a second messenger generated downstream of receptor tyrosine kinases and G-protein coupled receptors.¹⁰ DAG is produced at the inner face of the plasma membranes, and its binding to the C1 domain causes a conformational change of PKC into the active form and translocation^{11,12} followed by signaling through multiple downstream pathways.^{13–15}

The mechanism of action of diacylglycerol and of the phorbol esters, their ultrapotent analogs, is well-understood.¹⁶ The ligand fits into a hydrophilic cleft at the top of the C1 domain, completing a hydrophobic surface and contributing additional hydrophobicity through its acyl side chains, thereby promoting membrane insertion of the C1 domain. This ternary complex of diacylglycerol–C1 domain–phospholipid bilayer is driven by hydrogen bonds between the peptide backbone of the binding cleft with the primary hydroxyl group of the diacylglycerol and one of its carbonyls, by hydrogen bonding between the second carbonyl of the diacylglycerol with the phospholipid head groups,¹⁷ and by hydrophobic interactions of the lipid acyl chains with both the acyl chains of the diacylglycerol and the face of the C1 domain.^{18,19} The influence of the side chains of diacylglycerols and diacylglycerol lactones on binding potency and biological outcome has been extensively studied.^{20,21}

Diversity among C1 domains provides multiple potential opportunities for establishing selectivity among the isoforms of PKC as well as among members of the seven families of signaling proteins with DAG-responsive C1 domains. Some, such as PKC and PKD, have two C1 domains. Others, such as RasGRP or the chimaerins, have only a single C1 domain. Among those with tandem C1 domains, the distance between them differs between the conventional PKCs, the novel PKCs, and the PKD isoforms. The separations for the conventional and novel PKCs are 14 and 21 or 22 amino acids, respectively. For PKD, the separation is considerably greater, 73 amino acids. Unfortunately, the lack of complete crystallographic or cryo-EM solution structures, even less of such structures in complexes with ligand and membranes, means that the optimal linker length for a dimeric ligand remains unknown. For those targets with two C1 domains, viz. PKC and PKD, the affinities for phorbol ester may differ between the C1a and C1b domains and the structure activity relations may be different. Furthermore, as suggested by mutational analysis, the role of the C1a and C1b domains may play different roles in the PKC translocation induced by phorbol

12-myristate 13-acetate (PMA).^{22–24} All of these differences have the potential to be exploited through appropriate bivalent ligands.

To date, several bivalent PKC ligands based on naphthylpyrrolidone,²⁵ phorbol ester,^{26,27} and isobenzofuranone²⁸ have been synthesized and evaluated for their PKC binding and membrane interaction. In no case has there been substantial capture of enhanced affinity suggestive of the simultaneous binding to both C1 domains. A complication in all of the above studies is that the linker simultaneously fulfils two functions; it provides a potential limitation on the separation of the two binding moieties, and it provides the hydrophobicity, which is well-characterized as being required for ligand activity. In the present study, a separate hydrophobic moiety is included in each of the binding moieties, although the linker also provides additional hydrophobicity. Additionally, the PKC translocation induced by bivalent ligands has not been investigated previously. In view of the functional differences between the C1a and C1b domains, monovalent and bivalent ligands might have different effects on the translocation of PKC. For a binding moiety in the present study DAG–lactones were chosen. DAG–lactones are conformationally constrained diacylglycerol analogs, which are synthetically tractable and have been optimized to provide potent PKC binding affinity as well as selectivity between classes of C1-domain-containing targets.²⁹ We describe here the design and synthesis of dimeric derivatives of DAG–lactones and characterize their PKC binding affinities and effects on PKC translocation.

RESULTS AND DISCUSSION

Design and Synthesis of Dimeric Derivatives of DAG–Lactones.

To obtain a monomeric moiety derived from DAG with sufficient affinity for PKC, an α -alkylidene group was connected to the *sn*-2 carbonyl group on the DAG–lactone template and an alkyl chain containing nine carbons was attached to the *sn*-1 carbonyl group according to the previous reports (Figure 1).^{29–32} The alkyl chain moiety is considered to be necessary for interacting with cell membranes or the C1 domain and was chosen to provide appropriate lipophilicity as determined from previous studies.²⁰ A unique feature of this series of compounds, compared to other series previously examined, is that the nine carbon alkyl chain is present both in our monomeric ligand as well as in the dimeric derivatives. In previous studies, only the linker provided substantial hydrophobicity for the dimeric derivatives. For the choice of a linker moiety, a previous report concerning dimeric benzolactams found that dimeric ligands with alkyl chains containing a linear 14 carbons as linkers increased by 200-fold their binding affinities for PKC α and for PKC δ compared to the monomeric ligands. In addition, replacement of the alkyl chain with an oligoethylene glycol chain caused a 4000- to 7000-fold decrease in affinity for PKC α .²⁵ Thus, in the present study alkyl chains were adopted as linkers for the dimeric DAG–lactones. Olefin metathesis was utilized for concise synthesis of the dimeric form of DAG–lactones, and the motion of the resulting linker moiety with olefin was restricted.

The synthesis of dimeric derivatives of DAG–lactones is described in Scheme 1. Several alkyl halides containing the terminal olefin were introduced at the α -position of *tert*-butyl decanoate **2** to yield α -alkylated compounds **3a–3g**. After the removal of the *tert*-butyl group of **3a–3g** by TFA treatment, the resulting acids were conjugated with the hydroxy group of a

DAG-lactone derivative **4**^{33,34} to yield the esters **5a–5g** by the conversion of carboxylic acids to acid chlorides using oxalyl chloride and dimethylformamide (DMF). Intermolecular olefin metathesis using the second-generation Grubbs catalyst gave dimeric derivatives of DAG-lactones **6a–6g**. Electrospray ionization time-of-flight (ESI-TOF) mass spectra of **6a–6g** were measured after flash column chromatography, indicating that the mixture of byproducts with the loss of methylene units occurred by the isomerization in the olefin metathesis process.³⁵ The products with impurities were employed for biological experiments.^{36,37}

Evaluation of the Binding Affinities of the Dimeric Derivatives of DAG-Lactones for PKC δ or for Its C1b Domain.

The binding affinities of the synthetic compounds were assessed by their ability to competitively inhibit [³H]PDBu binding to PKC and to its C1 domain. The K_i values were determined both for the PKC δ C1b (δ C1b) domain as well as for the full-length recombinant PKC δ and PKC α (Table 1 and Figure 2). While the standard practice in the field is that K_i values are reported in nM units, it is recognized that the C1 domains recognize the membrane partitioned ligand rather than that in aqueous solution.³⁸ Nonetheless, under both the usual in vitro and cellular assay conditions, the membrane concentrations of ligand are proportional to the nominal aqueous concentration; thus, this measurement is appropriate for the prediction of cellular response.

The full-length PKC δ and PKC α each contain two individual C1 domains capable of binding to DAG-lactones, but the PKCs differ in the separation between the two domains. The K_d values for the binding of [³H]PDBu to the PKC δ C1b and to the full-length PKC δ were 0.19 and 0.33 nM, respectively. All of the dimeric derivatives of DAG-lactones (**6a–g**) showed stronger binding affinity for the δ C1b domain than did the monomer **5h**. A total of three factors should contribute to this enhanced affinity. First, the dimeric constructs contain two DAG-lactone moieties per molecule, whereas the monomeric DAG-lactone contains only one. If everything else remained equal, this 2-fold increase in the relative concentration of the DAG-lactone moiety would predict a 2-fold increase in apparent potency. Indeed, at least a 2-fold enhancement was observed for linkers between 6 and 16 carbons, and even the 18-carbon linker afforded an enhancement of 1.8-fold. Second, lipophilicity has been clearly shown to influence binding potency of DAG-lactones, with reduced potency if the lipophilicity is either too low or too high.²⁰ While calculated log P (CLogP) values below 4 were clearly disadvantageous, the effect of CLogP values above 5–6 were reported to be modest.²⁰ For comparison, the CLogP of the monomer, **5h** was 4.54 and all of the dimeric compounds were above this range (e.g., **6a**, CLogP, 10.65, values calculated by ChemDraw 15.1). The dimeric DAG-lactone containing a 10 carbon atoms (10C) linker (**6c**: K_i = 0.80 nM) showed the highest binding affinity for δ C1b among all of the tested compounds. Significantly lower affinity for δ C1b was observed as the linker length was longer or shorter than 10C (K_i [nM]: **6a** (6C), 2.07; **6b** (8C), 1.17; **6d** (12C), 1.17; **6e** (14C), 1.86; **6f** (16C), 2.52; **6g** (18C), 3.00). The results suggest that the 10-carbon linker, which has a distance of approximately 14 Å (by Spartan 14; Wave Function, Irvine, CA), might be capturing simultaneously two molecules of the C1b domain. This is consistent with a hypothetical distance of 15 Å between two centers of the adjacently coherent C1a and C1b domains,

which was obtained from the model of the C1a and C1b domains that was calculated by Newton and co-workers using two C1b crystal structures without the sequence located between the C1a and C1b domains.²⁶ The decrease of the interaction with δ C1b of the dimeric DAG-lactones (**6a** and **6b**) containing shorter linkers than 10 carbons might be caused by the repulsion of two molecules of the C1b domain; thus, six and eight carbons are exceedingly short for the capture of two δ C1b domains simultaneously. However, the decrease of the interaction with δ C1b of the dimeric DAG-lactones (**6d–g**) containing longer linkers than 10 carbons might be caused by interference of the long linker with the proper spread of the binding moieties because the long, hydrophobic linker might be expected to insert into the lipid bilayer rather than to extend at the membrane surface. It is important to note that the DAG-lactones are racemic and that binding to PKC is stereospecific.³⁹ Consequently, only one-fourth of the dimeric molecules would actually have two active binding moieties capable of binding to two C1 domains. Half would have a single active moiety, and a fourth would be entirely inactive. Of course, an alternative interpretation for the optimum binding by the dimeric derivative with a linker length of 10 carbons is that the hydrophobicity is simply optimal.

In the case of full length recombinant PKC δ , the dimeric derivatives of DAG-lactones containing six-carbon (**6a**; $K_i = 2.67$ nM) and eight-carbon (**6b**; $K_i = 3.11$ nM) linkers showed K_i values similar to that for the monomer **5h** ($K_i = 2.43$ nM). As the linker length increased up to 16 carbons, the affinity for PKC δ decreased (K_i [nM]: **6c** (10C), 6.31; **6d** (12C), 13.2; **6e** (14C), 16.6; **6f** (16C), 27.1), with some increase in binding affinity then occurring for the dimeric DAG-lactone with the 18-carbon linker (**6g**, $K_i = 12.3$ nM). For PKC α , the dependence of the K_i values on the number of carbons of the linker showed a similar pattern to that for PKC δ (K_i [nM]: **6a** (6C), 8.40; **6b** (8C), 15.9; **6c** (10C), 17.4; **6d** (12C), 49.6; **6e** (14C), 48.5; **6f** (16C), 173; **6g** (18C), 30.9), indicating no evidence for isozyme selectivity (Figure 2). The clear interpretation is that there is no strong evidence for both DAG-lactone moieties in the dimeric constructs binding simultaneously to the C1a and C1b domains of either PKC isoform. One possible explanation is that, because the binding represents a ternary complex of DAG-lactone, PKC, and lipid bilayer, the constraints imposed by the lipid bilayer interfere with positioning of the second binding moiety of the dimeric DAG-lactone with the second C1 domain in the intact PKC. One possible source of this interference is that the acyl linker might be expected to insert into the lipid bilayer so as to interact with the acyl chains of the phospholipids. This interaction would draw the two DAG-lactone moieties together, preventing the desired separation predicted for an extended chain. As a further consequence, the second, now incorrectly positioned DAG-lactone moiety might then generate steric interference with the remainder of the PKC. A further factor could be that the DAG-lactone moieties showed a sufficient difference in affinity for the C1a and C1b domains so that they are only bound to the preferred domain, although their positioning would permit binding to both. The rationale is that the C1a and C1b domains display different affinities for phorbol esters and for DAG. Thus, we reported K_d values of PDBu for the isolated PKC δ C1a and C1b domains of 2.0 nM and 0.33 nM for a 6-fold difference and 130 nM versus 2.8 nM, for a 46-fold difference, in full-length PKC δ constructs possessing only a C1a domain or a C1b domain, respectively (with the other domain being deleted).⁴⁰ The difference was even greater for DAG, with values of 2400

versus 13 nM (difference of 185 fold) for the full-length PKC δ constructs possessing only a C1a domain or a C1b domain, respectively.

Observation of PKC Translocation Caused by the Dimeric Derivatives of DAG–Lactones.

PKC translocation induced by ligand binding was observed using a confocal laser scanning microscope. CHO Flp-in cells that stably express enhanced green fluorescent protein (EGFP)-tagged PKC δ were utilized. The effect on the translocation of EGFP-PKC δ by the addition of 10 μ M of the dimeric derivative of the DAG–lactone containing the six-carbon linker (**6a**), the monomer DAG–lactone (**5h**), and PDBu are shown in Figure 3a. The addition of the dimer (**6a**) caused the translocation of EGFP-PKC δ from cytosol to plasma membranes. The monomer (**5h**) also caused the translocation of PKC δ ,³² however, the translocation caused by **6a** was more clearly observed than that by **5h** at 5 min after the addition of ligands. The translocation to plasma membranes induced by PDBu was not observed more clearly than those by monomeric and dimeric DAG–lactones. To examine the effect of the longer linker length on the PKC δ translocation, experiments employing dimeric DAG–lactones, which contain the 10-carbon (**6c**), 12-carbon (**6d**), and 18-carbon (**6g**) linkers, were performed (Figure 3b). The membrane translocation induced by **6c** was observed similarly or slightly more clearly compared to that induced by **6a**. The best translocation was seen for **6c** (10 carbons) with some translocation still evident for **6d** (12 carbons) and **6g** (18 carbons). Interestingly, the potent dimeric DAG–lactones only induced plasma membrane translocation. In contrast, both the monomeric DAG–lactone and the PDBu also showed translocation adjacent to the nucleus, consistent with Golgi localization.

To check whether another PKC isoform might behave substantially differently, limited experiments were also conducted with another PKC isoform, PKC ϵ , using the human pancreatic cancer cell line LNCaP. PKC ϵ provides a contrast to PKC δ in several respects. The C1a and C1b domains of PKC ϵ have affinities within 3-fold of one another for PMA and for DAG.⁴¹ PKC ϵ has a less-complicated translocation profile than does PKC δ in that it only translocates to the plasma membrane in response to PMA (phorbol 12-myristate 13-acetate), whereas PKC δ translocates to both plasma membrane and internal membranes in response to PMA, depending on time of exposure and the extent to which PMA has equilibrated with internal membranes. Finally, the LNCaP cells possess a different phospholipid composition in their plasma membrane than do CHO cells, having greater negative charges because they are mutated in PTEN and thus have elevated levels of phosphatidylinositol-3,4,5-triphosphate. Despite these differences, the pattern of response was similar to that seen for PKC δ in the Chinese hamster ovary (CHO) cells (Figure 4). PMA (1 μ M) gave dramatic plasma membrane translocation of PKC ϵ . Modest translocation was observed for the monomer and somewhat less for **6a** (six carbons). The other compounds failed to show translocation except for faint translocation for **6g** (18 carbons).

CONCLUSIONS

The dimeric derivatives of the DAG–lactones bearing the same DAG–lactone moieties linked with variable lengths of an alkyl linker were synthesized and evaluated for their PKC binding and translocation activity. A very impressive result was that it was possible to

achieve subnanomolar potencies using a DAG–lactone moiety. This is significant because the DAG–lactone scaffold is far more synthetically accessible than that of the phorbol esters or other high-affinity PKC ligands.

As already discussed, one of the elements for the design of selective dimeric ligands would be the optimization of the two binding moieties for the C1a and C1b domains, and the DAG–lactones are well-suited for such optimization. Like the other dimeric constructs reported using other scaffolds, the current compounds failed to achieve a dramatic gain in binding affinity over the monomeric construct. A plausible explanation is that, after binding of the first DAG–lactone moiety, the linker did not favorably position the second DAG–lactone moiety to interact with the second C1 domain. Precise interpretation is of course not possible because the linker changes the lipophilicity of the compound, and it has been clearly established that lipophilicity is a critical parameter for binding affinity, with reduced affinity if a compound is either not sufficiently lipophilic or is too lipophilic. This could well explain why the dimeric compound with the 10 carbon linker was modestly more effective for the isolated PKC δ C1b domain than was the monomeric compound or the dimeric compounds with either shorter or longer linkers. Alternatively, it is possible that a 10 carbon linker or longer was sufficient to allow access of a second isolated C1b domain to the remaining free DAG–lactone moiety, accounting for the improved affinity over dimers with a shorter linker, and that the decrease with the longer linkers was related to excess lipophilicity.

For the intact PKC α and PKC δ , the behavior was distinctly different from that of the isolated PKC δ C1b domain. Here, the shortest DAG–lactone dimer had similar affinity to the monomer and affinity decreased progressively up to the 16-carbon linker. The affinity then increased slightly for the 18-carbon linker, yielding an affinity similar to that of the compound with the 14-carbon linker. This pattern of behavior suggests that the dimeric compound is only binding to the intact PKC through a single binding moiety, and the absolute difference in affinity compared to that of the isolated C1b domain is not surprising because in the intact PKC, ligand binding is coupled to conformational changes in the protein.

The translocation results are again quite promising for evaluation of the synthetic strategy. Translocation of PKC δ and of PKC ϵ by the most-active derivatives was observed, and their behavior largely mirrored that found in the binding assays. A noteworthy finding was that the dimeric derivatives did not cause Golgi accumulation of PKC δ , unlike the monomeric derivative and PDBu. This difference probably reflects the slower penetration of the dimeric derivatives into the interior of the cells over the time course examined.

Our current strategy yielded potent compounds suitable for evaluation in vitro and in intact living cells. Despite the use of linkers predicted to permit a separation that could span the distance between the tandem C1 domains in intact PKC isoforms, we saw little evidence for actual bivalent binding. An underlying concern is that aliphatic linkers, being structurally flexible, in the presence of membranes will themselves insert into the membranes, collapsing the actual separation between the two binding domains. A further problem may be that, by using a hydrophobic linker region, it complicates the optimization of lipophilicity.

Several strategies may help to address these concerns. For linkers, a polyproline structure might provide rigidity. An oligoethylene glycol linker could provide a hydrophilic linker that would sit on top of the membrane surface, with the required hydrophobicity provided separately by acyl chains such as those in the current structures. While bivalent benzolactams with an oligoethylene glycol linker showed very little activity, these compounds did not have additional elements to render the binding domains hydrophobic. Finally, heterobivalent ligands containing DAG–lactones and other C1a-selective ligands could be designed to efficiently bind to the C1a as well as to C1b domains. Our present study provides important insights into development of new chemical tools for biological studies on PKC and useful information for the discovery of therapeutic agents.

MATERIALS AND METHODS

General.

All reactions utilizing air- or moisture-sensitive reagents were performed in dried glassware under an atmosphere of nitrogen using commercially supplied solvents and reagents unless otherwise noted. CH_2Cl_2 was distilled from CaH_2 and stored over molecular sieves. For thin-layer chromatography (TLC), precoated silica gel plates (Merck 60F254) were employed. TLC plates were visualized by fluorescence quenching under UV light and by staining with phosphomolybdic acid, *p*-anisaldehyde, or ninhydrin. Flash chromatography was performed with Wakogel C-200 (Wako Pure Chemical Industries, Ltd.) and silica gel 60 N (Kanto Chemical Co., Inc.). ^1H NMR (400 or 500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on a Bruker Avance III 400 spectrometer or a Bruker AVANCE 500 spectrometer. Chemical shifts are reported in δ (ppm) relative to Me_4Si (in CDCl_3) as the internal standard. Infrared (IR) spectra were recorded on a JASCO FT/IR 4100 and are reported as wavenumber (cm^{-1}). Low- and high-resolution mass spectra were recorded on a Bruker Daltonics micrOTOF-2focus (ESI-MS) spectrometer in the positive or negative detection mode. For confocal laser scanning fluorescence images, cells were observed with a FluoView FV10i laser scanning confocal microscope (OLYMPUS, Japan) or a Zeiss LSM 510 confocal microscopy system (Carl Zeiss, Inc.).

Synthesis of DAG–Lactone Derivatives.

tert-Butyl 2-(But-3-en-1-yl)decanoate (3a; Representative Compound of 3a–3g).—Lithium diisopropylamide was prepared by the addition of *n*-BuLi in hexane (2.56 mL of 1.64 M solution, 4.2 mmol, 1.4 equiv) to a solution of diisopropylamine (0.63 mL, 4.5 mmol, 1.5 equiv) in tetrahydrofuran (THF) (1.26 mL) at 0 °C, and the mixture was allowed to sit for 10 min at room temperature. To the reaction mixture, **2** (0.685 g, 3 mmol, 1 equiv) dissolved with THF (0.34 mL) was added at room temperature, and the reaction mixture was stirred for 30 min at –78 °C. To the mixture was then added tetrabutylammonium iodide (50 mol %), hexamethylphosphoramide (0.4 mL), and 4-bromo-1-butene (0.48 mL, 4.8 mmol, 1.6 equiv) at this temperature. After stirring, the reaction mixture was warmed to ambient temperature and stirred for 40 h. The reaction was diluted with hexane and quenched with saturated aqueous NH_4Cl . The aqueous layer was extracted three times with EtOAc. The combined organic layer was dried over MgSO_4 Concentration under reduced pressure followed by flash chromatography (hexane/EtOAc = 30:1) gave compound **3a** (0.4 g, 47%).

R_f 0.5 (hexane/EtOAc = 30:1); ¹H NMR (400 MHz, CDCl₃, δ): 0.86–0.89 (m, 3H), 1.24–1.28 (m, 12H), 1.52 (s, 9H), 1.63–1.72 (m, 2H), 2.00–2.08 (m, 2H), 2.18–2.26 (m, 1H), 4.94–5.04 (m, 2H), 5.74–5.84 (m, 1H); ¹³C NMR (125 MHz, CDCl₃, δ): 14.0, 22.6, 27.2, 28.1, 29.2, 29.5, 31.6, 31.8, 32.5, 35.6, 45.8, 79.8, 114.6, 138.1, 175.6; HRMS (ESI, *m/z*): [M + Na]⁺ calcd for C₁₈H₃₄NaO₂, 305.2451; found, 305.2446.

tert-Butyl 2-(Pent-4-en-1-yl)decanoate (3b).—By the use of a procedure similar to that described for the synthesis of compound **3a**, the alkylation of **2** (0.685 g, 3 mmol) using 5-bromo-1-penten (0.57 mL, 4.8 mmol) gave compound **3b** (0.824 g, 93%). *R_f* 0.5 (hexane/EtOAc = 30:1); ¹H NMR (400 MHz, CDCl₃, δ): 0.86–0.89 (m, 3H), 1.23–1.30 (m, 12H), 1.34–1.41 (m, 4H), 1.44 (s, 9H), 1.51–1.60 (m, 2H), 2.01–2.07 (m, 2H), 2.17–2.23 (m, 1H), 4.93–5.03 (m, 2H), 5.74–5.85 (m, 1H); ¹³C NMR (125 MHz, CDCl₃, δ): 14.0, 26.6, 27.3, 28.1 (2C), 29.2, 29.4, 29.5, 31.8, 32.0, 32.6, 33.6, 46.3, 79.7, 114.4, 138.6, 175.8; HRMS (ESI, *m/z*): [M + Na]⁺ calcd for C₁₉H₃₆NaO₂, 319.2608; found, 319.2605.

tert-Butyl 2-(Hex-5-en-1-yl)decanoate (3c).—By the use of a procedure similar to that described for the synthesis of compound **3a**, the alkylation of **2** (0.685 g, 3 mmol) using 6-bromo-1-hexene (0.64 mL, 4.8 mmol) gave compound **3c** (0.618 g, 66%). *R_f* 0.5 (hexane/EtOAc = 30:1); ¹H NMR (400 MHz, CDCl₃, δ): 0.86–0.89 (m, 3H), 1.24–1.29 (m, 12H), 1.35–1.41 (m, 6H), 1.44 (s, 9H), 1.51–1.59 (m, 2H), 2.01–2.07 (m, 2H), 2.16–2.22 (m, 1H), 4.92–5.01 (m, 2H), 5.74–5.84 (m, 1H); ¹³C NMR (125 MHz, CDCl₃, δ): 14.0, 22.6, 26.8, 27.3, 28.1, 28.8, 29.2, 29.4, 29.5, 31.8, 32.4, 32.6, 33.6, 46.6, 79.7, 114.3, 138.8, 175.8; HRMS (ESI, *m/z*): [M + Na]⁺ calcd for C₂₀H₃₈NaO₂, 333.2764; found, 333.2759.

tert-Butyl 2-(Hept-6-en-1-yl)decanoate (3d).—By the use of a procedure similar to that described for the synthesis of compound **3a**, the alkylation of **2** (0.685 g, 3 mmol) using 7-bromo-1-heptene (0.73 mL, 4.8 mmol) gave compound **3d** (0.699 g, 72%). *R_f* 0.5 (hexane/EtOAc = 30:1); ¹H NMR (400 MHz, CDCl₃, δ): 0.89–0.91 (m, 3H), 1.25–1.32 (m, 16H), 1.37–1.42 (m, 4H), 1.46 (s, 9H), 1.54–1.59 (m, 2H), 2.03–2.08 (m, 2H), 2.18–2.24 (m, 1H), 4.94–5.03 (m, 2H), 5.79–5.88 (m, 1H); ¹³C NMR (125 MHz, CDCl₃, δ): 14.0, 22.6, 27.3, 28.1, 29.0, 29.2, 29.4, 29.5, 31.8, 32.5, 32.6, 33.6, 46.5, 79.7, 114.1, 139.0, 176.0; HRMS (ESI, *m/z*): [M + Na]⁺ calcd for C₂₁H₄₀NaO₂, 347.2921; found, 347.2916.

tert-Butyl 2-Octyldec-9-enoate (3e).—By the use of a procedure similar to that described for the synthesis of compound **3a**, the alkylation of **2** (0.685 g, 3 mmol) using 8-bromo-1-octene (0.80 mL, 4.8 mmol) gave compound **3e** (0.667 g, 66%). *R_f* 0.5 (hexane/EtOAc = 30:1); ¹H NMR (400 MHz, CDCl₃, δ): 0.86–0.89 (m, 3H), 1.23–1.31 (m, 18H), 1.34–1.40 (m, 4H), 1.44 (s, 9H), 1.54–1.59 (m, 2H), 2.03–2.08 (m, 2H), 2.18–2.24 (m, 1H), 4.94–5.03 (m, 2H), 5.79–5.88 (m, 1H); ¹³C NMR (125 MHz, CDCl₃, δ): 14.0, 22.6, 27.3, 28.1 (2C), 28.8, 28.9, 29.2, 29.3, 29.4, 29.5, 31.8, 32.5, 32.6, 33.7, 46.5, 79.6, 114.1, 139.1, 175.9; HRMS (ESI, *m/z*): [M + Na]⁺ calcd for C₂₂H₄₂NaO₂, 361.3077; found, 361.3077.

tert-Butyl 2-Octylundec-10-enoate (3f).—By the use of a procedure similar to that described for the synthesis of compound **3a**, the alkylation of **2** (0.685 g, 3 mmol) using 9-bromo-1-nonene (0.89 mL, 4.8 mmol) gave compound **3f** (0.401 g, 38%). *R_f* 0.5 (hexane/

EtOAc = 30:1); ^1H NMR (400 MHz, CDCl_3 , δ): 0.86–0.89 (m, 3H), 1.23–1.30 (m, 20H), 1.35–1.39 (m, 4H), 1.44 (s, 9H), 1.51–1.59 (m, 2H), 2.01–2.06 (m, 2H), 2.16–2.20 (m, 1H), 4.91–5.00 (m, 2H), 5.77–5.85 (m, 1H); ^{13}C NMR (125 MHz, CDCl_3 , δ): 14.0, 14.1, 21.0, 22.6, 27.3, 28.8, 28.9, 29.1, 29.2, 29.3, 29.4, 29.5, 31.8, 32.6, 33.7, 46.5, 60.3, 79.6, 114.0, 139.1, 176.0; HRMS (ESI, m/z): $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{23}\text{H}_{45}\text{O}_2$, 353.3414; found, 353.3414.

tert-Butyl 2-Octyldodec-11-enoate (3g).—By the use of a procedure similar to that described for the synthesis of compound **3g**, the alkylation of **2** (0.685 g, 3 mmol) using 10-bromo-1-decene (0.96 mL, 4.8 mmol) gave compound **3g** (0.649 g, 59%). R_f 0.5 (hexane/EtOAc = 30:1); ^1H NMR (400 MHz, CDCl_3 , δ): 0.86–0.89 (m, 3H), 1.23–1.29 (m, 22H), 1.33–1.42 (m, 4H), 1.45 (s, 9H), 1.51–1.60 (m, 2H), 2.00–2.07 (m, 2H), 2.16–2.22 (m, 1H), 4.01–5.02 (m, 2H), 5.76–5.86 (m, 1H); ^{13}C NMR (125 MHz, CDCl_3 , δ): 14.0, 22.6, 27.3, 28.1 (2C), 29.0, 29.2, 29.3 (2C), 29.4, 29.5 (2C), 31.8, 32.6 (2C), 33.7, 46.5, 79.6, 114.0, 139.1, 180.0; HRMS (ESI, m/z): $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{24}\text{H}_{47}\text{O}_2$, 367.3571; found, 367.3567.

(2-(Hydroxymethyl)-5-oxo-4-(propan-2-ylidene)-tetrahydrofuran-2-yl)methyl 2-Octyldodec-9-enoate (5e).—(Representative compound of **5a–5g**): To a solution of a *tert*-butyl ester derivative **3e** (0.108 g, 0.32 mmol, 1 equiv) in CH_2Cl_2 (2 mL) was added trifluoroacetic acid (TFA) (2 mL) at 0 °C, and the mixture was stirred at room temperature for 1 h followed by concentration under reduced pressure. The obtained carboxylic acid was used without further purification. To the carboxylic acid in CH_2Cl_2 (2.7 mL) was added oxalyl chloride (0.27 mL, 3.2 mmol, 10 equiv) and catalytic DMF (3 drops) at 0 °C, and the mixture was then stirred at room temperature for 2 h followed by concentration under reduced pressure. The obtained acid chloride was used without further purification. To the acid chloride in THF (6.4 mL), Et_3N (0.13 mL, 0.96 mmol, 3 equiv) and compound **4**, which was synthesized as in the previously reported procedure^{33,34} (0.12 g, 0.64 mmol, 2 equiv), were added at 0 °C, and the mixture was then stirred at room temperature for 40 h. After stirring, the solvent was removed in vacuo followed by flash chromatography ($\text{CHCl}_3/\text{MeOH}$ = 20:1) to give the title compound **5e** (77 mg, 54% yield). R_f 0.5 ($\text{CHCl}_3/\text{MeOH}$ = 20:1); ^1H NMR (400 MHz, CDCl_3 , δ): 0.86–0.89 (m, 3H), 1.23–1.33 (m, 12H), 1.39–1.65 (m, 12H), 1.87 (s, 3H), 2.01–2.06 (m, 2H), 2.27 (s, 3H), 2.34–2.39 (m, 1H), 2.63–2.84 (m, 2H), 3.60–3.69 (m, 2H), 4.13–4.29 (m, 2H), 4.93–5.01 (m, 2H), 5.76–5.85 (m, 1H); ^{13}C NMR (125 MHz, CDCl_3 , δ): 14.0, 19.9, 22.6, 24.5, 27.3, 27.4, 28.8, 28.9, 29.2, 29.3, 29.5, 31.8, 32.1, 32.2 (2C), 33.4, 45.6 (2C), 64.9, 65.1, 81.0, 114.2, 118.8, 139.0, 151.7; HRMS (ESI, m/z): $[\text{M} + \text{K}]^+$ calcd for $\text{C}_{27}\text{H}_{46}\text{KO}_5$, 489.2977; found, 489.2968.

(2-(Hydroxymethyl)-5-oxo-4-(propan-2-ylidene)-tetrahydrofuran-2-yl)methyl 2-(But-3-en-1-yl)decanoate (5a).—By the use of a procedure similar to that described for the synthesis of compound **5e**, through the removal of the *tert*-butyl group from **3a** (0.155 g, 0.68 mmol) and the subsequent formation of the acid chloride using oxalyl chloride (0.58 mL, 6.8 mmol) and a catalytic amount of DMF in DCM (5.7 mL), coupling with **4** (0.253 g, 1.36 mmol) using Et_3N (0.28 mL, 2.04 mmol) in THF (13.6 mL) gave compound **5a** (0.094 g, 35%). R_f 0.5 ($\text{CHCl}_3/\text{MeOH}$ = 20:1), ^1H NMR (400 MHz, CDCl_3 , δ): 0.86–0.89 (m, 3H), 1.25–1.29 (m, 12H), 1.44–1.77 (m, 4H), 1.87 (s, 3H), 2.00–2.14 (m, 2H), 2.26 (s, 3H), 2.43–2.63 (m, 1H), 2.73–2.94 (m, 2H), 3.60–3.74 (m, 2H), 4.15–4.29 (m, 2H), 4.97–5.06

(m, 2H), 5.70–5.83 (m, 1H); ^{13}C NMR (125 MHz, CDCl_3 , δ): 14.0, 19.9, 22.6, 24.5, 27.3, 29.2, 29.3, 29.5, 31.1, 31.2, 31.5, 31.8, 32.0, 32.1, 44.8, 44.9, 64.9, 65.2, 80.9, 115.2, 118.8, 137.6; HRMS (ESI, m/z): $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{23}\text{H}_{39}\text{O}_5$, 395.2792; found, 395.2794.

(2-(Hydroxymethyl)-5-oxo-4-(propan-2-ylidene)-tetrahydrofuran-2-yl)methyl 2-(Pent-4-en-1-yl)decanoate (5b).—By the use of a procedure similar to that described for the synthesis of compound **5e**, through the removal of the *tert*-butyl group from **3b** (0.119 g, 0.4 mmol) and the subsequent formation of the acid chloride using oxalyl chloride (0.34 mL, 4 mmol) and a catalytic amount of DMF in DCM (4 mL), coupling with **4** (0.150 g, 0.8 mmol) using Et_3N (0.09 mL, 1.2 mmol) in THF (8 mL) gave compound **5b** (0.085 g, 52%). R_f 0.5 ($\text{CHCl}_3/\text{MeOH} = 20:1$); ^1H NMR (400 MHz, CDCl_3 , δ): 0.86–0.89 (m, 3H), 1.23–1.31 (m, 12H), 1.39–1.50 (m, 6H), 1.88 (s, 3H), 2.02–2.12 (m, 2H), 2.27 (s, 3H), 2.34–2.41 (m, 1H), 2.63–2.84 (m, 2H), 3.60–3.72 (m, 2H), 4.14–4.29 (m, 2H), 4.93–5.03 (m, 2H), 5.73–5.82 (m, 1H); ^{13}C NMR (125 MHz, CDCl_3 , δ): 14.0, 20.0, 22.6, 24.6, 25.1, 26.6, 27.4, 28.1, 29.2, 29.4, 29.5, 31.8, 32.1, 33.5, 35.6, 45.4, 64.9, 65.1, 80.9, 114.8, 138.2, 151.7, 168.8; HRMS (ESI, m/z): $[\text{M} + \text{K}]^+$ calcd for $\text{C}_{24}\text{H}_{40}\text{KO}_5$, 447.2507; found, 447.2507.

(2-(Hydroxymethyl)-5-oxo-4-(propan-2-ylidene)-tetrahydrofuran-2-yl)methyl 2-(Hex-5-en-1-yl)decanoate (5c).—By the use of a procedure similar to that described for the synthesis of compound **5e**, through the removal of the *tert*-butyl group from **3c** (0.124 g, 0.4 mmol) and the subsequent formation of the acid chloride using oxalyl chloride (0.34 mL, 4 mmol) and a catalytic amount of DMF in DCM (4 mL), coupling with **4** (0.150 g, 0.8 mmol) using Et_3N (0.09 mL, 1.2 mmol) in THF (8 mL) gave compound **5c** (0.069 g, 41%). R_f 0.5 ($\text{CHCl}_3/\text{MeOH} = 20:1$); ^1H NMR (400 MHz, CDCl_3 , δ): 0.86–0.89 (m, 3H), 1.23–1.31 (m, 12H), 1.35–1.61 (m, 8H), 1.88 (s, 3H), 2.01–2.12 (m, 2H), 2.27 (s, 3H), 2.32–2.39 (m, 1H), 2.63–2.84 (m, 2H), 3.60–3.71 (m, 2H), 4.13–4.30 (m, 2H), 4.92–5.02 (m, 2H), 5.74–5.84 (m, 1H); ^{13}C NMR (125 MHz, CDCl_3 , δ): 14.0, 19.9, 22.6, 24.5, 25.1, 26.8, 27.3, 28.7, 29.2, 29.4, 29.5, 31.8, 32.1, 33.5, 45.5, 45.6, 64.8, 65.1, 81.0, 114.4, 118.8, 138.7, 151.6, 168.8; HRMS (ESI, m/z): $[\text{M} + \text{K}]^+$ calcd for $\text{C}_{25}\text{H}_{42}\text{KO}_5$, 461.2664; found, 461.2659.

(2-(Hydroxymethyl)-5-oxo-4-(propan-2-ylidene)-tetrahydrofuran-2-yl)methyl 2-(Hept-6-en-1-yl)decanoate (5d).—By the use of a procedure similar to that described for the synthesis of compound **5e**, through the removal of the *tert*-butyl group from **3d** (0.103 g, 0.32 mmol) and the subsequent formation of the acid chloride using oxalyl chloride (0.27 mL, 3.2 mmol) and a catalytic amount of DMF in DCM (2.7 mL), coupling with **4** (0.120 g, 0.64 mmol) using Et_3N (0.13 mL, 0.96 mmol) in THF (6.4 mL) gave compound **5d** (0.082 g, 58%). R_f 0.5 ($\text{CHCl}_3/\text{MeOH} = 20:1$); ^1H NMR (400 MHz, CDCl_3 , δ): 0.86–0.89 (m, 3H), 1.23–1.31 (m, 12H), 1.35–1.64 (m, 10H), 1.87 (s, 3H), 2.00–2.05 (m, 2H), 2.27 (s, 3H), 2.32–2.39 (m, 1H), 2.63–2.84 (m, 2H), 3.60–3.70 (m, 2H), 4.13–4.29 (m, 2H), 4.92–5.01 (m, 2H), 5.75–5.84 (m, 1H); ^{13}C NMR (125 MHz, CDCl_3 , δ): 14.0, 19.9, 22.6, 24.5, 27.1, 27.2, 27.3, 27.4, 28.6, 28.9, 29.1, 29.4, 29.5, 31.8, 32.0, 32.1, 32.2, 33.6, 45.0, 45.6, 64.9, 65.1, 81.0, 114.3, 138.9; HRMS (ESI, m/z): $[\text{M} + \text{Na}]^+$ calcd for $\text{C}_{26}\text{H}_{44}\text{NaO}_5$, 459.3081; found, 459.3071.

(2-(Hydroxymethyl)-5-oxo-4-(propan-2-ylidene)-tetrahydrofuran-2-yl)methyl 2-Octylundec-10-enoate (5f).—By the use of a procedure similar to that described for the synthesis of compound **5e**, through the removal of the *tert*-butyl group from **3f** (0.110 g, 0.32 mmol) and the subsequent formation of the acid chloride using oxalyl chloride (0.27 mL, 3.2 mmol) and a catalytic amount of DMF in DCM (2.7 mL), coupling with **4** (0.12 g, 0.64 mmol) using Et₃N (0.13 mL, 0.96 mmol) in THF (6.4 mL) gave compound **5f** (0.04 g, 27%). *R*_f 0.5 (CHCl₃/MeOH = 20:1); ¹H NMR (400 MHz, CDCl₃, δ): 0.86–0.89 (m, 3H), 1.23–1.33 (m, 12H), 1.39–1.65 (m, 14H), 1.87 (s, 3H), 2.01–2.06 (m, 2H), 2.27 (s, 3H), 2.34–2.39 (m, 1H), 2.63–2.84 (m, 2H), 3.60–3.69 (m, 2H), 4.13–4.29 (m, 2H), 4.92–5.01 (m, 2H), 5.76–5.85 (m, 1H); ¹³C NMR (125 MHz, CDCl₃, δ): 14.0, 14.1, 19.9, 21.0, 22.6, 24.5, 27.3, 28.8, 28.9, 29.2 (2C), 29.3, 29.4, 29.5, 31.8, 32.1, 32.2, 32.2, 33.7, 45.0, 45.6, 60.3 (2c), 64.9, 65.1, 81.0, 114.1, 139.1, 151.7; HRMS (ESI, *m/z*): [M+K]⁺ calcd for C₂₈H₄₈KO₅, 503.3133; found, 503.3126.

(2-(Hydroxymethyl)-5-oxo-4-(propan-2-ylidene)-tetrahydrofuran-2-yl)methyl 2-Octyldodec-11-enoate (5g).—By the use of a procedure similar to that described for the synthesis of compound **5g**, through the removal of the *tert*-butyl group from **3g** (0.147 g, 0.4 mmol) and the subsequent formation of the acid chloride using oxalyl chloride (0.505 mL, 4 mmol) and a catalytic amount of DMF in DCM (4 mL), coupling with **4** (0.15 g, 0.8 mmol) using Et₃N (0.12 mL, 0.86 mmol) in THF (8 mL) gave compound **5f** (0.067 g, 35%). *R*_f 0.5 (CHCl₃/MeOH = 20:1); ¹H NMR (400 MHz, CDCl₃, δ): 0.88–0.91 (m, 3H), 1.24–1.33 (m, 12H), 1.36–1.58 (m, 16H), 1.90 (s, 3H), 2.03–2.13 (m, 2H), 2.29 (s, 3H), 2.35–2.41 (m, 1H), 2.66–2.82 (m, 2H), 3.63–3.72 (m, 2H), 4.16–4.31 (m, 2H), 4.94–5.03 (m, 2H), 5.80–5.89 (m, 1H); ¹³C NMR (125 MHz, CDCl₃, δ): 14.0, 19.9, 22.6, 24.5, 27.4 (2C), 28.8, 29.0, 29.2, 29.3, 29.4 (2C), 29.5 (2C), 31.8, 32.1 (2C), 33.7 45.6, 64.9, 65.1, 81.0, 114.1, 118.8, 139.2, 151.7, 168.8, 176.4; HRMS (ESI, *m/z*): [M + Na]⁺ calcd for C₂₉H₅₀NaO₅, 501.3550; found, 501.3550.

6c (Representative Compound of 6a–6g).—To a solution of **5c** (56 mg, 0.13 mmol) in CH₂Cl₂ (3.3 mL) was added the second-generation Grubbs catalyst (0.011 g, 0.013 mmol) at room temperature, and the mixture was then stirred at that temperature for 24 h. After stirring, the solvent was removed under reduced pressure followed by flash chromatography to give the title compound **6c** (37 mg, 70%). Yields of compounds **6a–6g** are described in Table 2. Low-MS (ESI) are as follows. **6a** (*m/z*): [M + Na]⁺ calcd for C₄₄H₇₂NaO₁₀, 783.5; found, 783.5. **6b** (*m/z*): [M + Na]⁺ calcd for C₄₆H₇₆NaO₁₀, 811.5; found, 811.6. **6c** (*m/z*): [M + Na]⁺ calcd for C₄₈H₈₀NaO₁₀, 839.6; found, 839.6. **6d** (*m/z*): [M + Na]⁺ calcd for C₅₀H₈₄NaO₁₀, 867.6; found, 867.6. **6e** (*m/z*): [M + Na]⁺ calcd for C₅₂H₈₈NaO₁₀, 895.6; found, 895.6. **6f** (*m/z*): [M + Na]⁺ calcd for C₅₄H₉₂NaO₁₀, 923.7; found, 923.7. **6g** (*m/z*): [M + Na]⁺ calcd for C₅₆H₉₆NaO₁₀, 951.7; found, 951.7.

Assessment of K_i Values of the Synthesized Compounds.

Binding affinities of the synthesized compounds for the C1b domain of PKCδ, full-length PKCδ, and full-length PKCα were assayed in vitro by competition for the binding of [20-³H]phorbol 12,13-dibutyrate ([³H]PDBu)(13.5 Ci/mmol, from PerkinElmer, Boston, MA) in the presence of 100 μg/mL of phosphatidylserine (PS), as described previously.⁴²

Incubation was for 5 min at 37 °C. Values represent the mean $\pm$ SEM of triplicate binding experiments. In each experiment, competition was determined for seven concentrations of ligand, with the increasing concentrations of ligand spaced at half-log intervals, and compared to binding in the absence of competing ligand. In each experiment, triplicate measurements at each concentration of ligand were performed.

Translocation Analysis of EGFP-Tagged PKC δ in CHO Cells.

CHO cells were cultured in Ham's F12 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin, 50 units/mL and streptomycin, 0.05 mg/mL). CHO cells plated on glass bottom dishes (Matsunami Glass Ind., Ltd., Osaka, Japan) were transfected with EGFP-tagged PKC δ derived from the insertion of PKC δ into pEGFP-N1 (Clontech-Takara Bio, Kusatsu, Japan) using Lipofectamine LTX (Invitrogen, Carlsbad, CA). For confocal laser scanning fluorescence images, cells were observed with a FluoView FV10i confocal laser scanning microscope (OLYMPUS). PKC translocation caused by the addition of a DAG-lactone derivative (10 mM MeOH solution, final ligand concentration of 10 μ M) or a phorbol ester (PDBu, 10 mM MeOH solution, final ligand concentration of 10 μ M) was monitored. Images were taken before and 1, 2, 3, 4, 5, and 10 min after the addition of compounds.

Confocal Analysis of GFP-Labeled PKC ϵ Proteins.

LNCaP cells were plated at a density of 100 000 cells per plate on Ibidi microdishes (Ibidi, LLC, Verona, WI) and cultured at 37 °C in RPMI-1640 medium supplemented with 10% FBS and 2 mM L-glutamine. After 48 h in culture, cells were transfected with a GFP-tagged recombinant PKC ϵ construct using X-tremeGENE HP DNA transfection reagent (Sigma) according to the manufacturer's recommendations. After 24 h, the cells were treated as indicated with 1 μ M of PMA and 3 μ M of DAG-lactone derivatives in confocal medium (Dulbecco's modified Eagle medium without phenol red supplemented with 1% FBS), and time-lapse images were collected every 30 s using the Zeiss AIM software. Imaging was with a Zeiss LSM 510 confocal microscopy system (Carl Zeiss, Inc.) with an Axiovert 100 M inverted microscope operating with a 25 mW argon laser tuned to 488 nm. A 63 $\times$ 1.4 NA Zeiss Plan-Apochromat oil-immersion objective was used together with varying zooms (1.4 to 2 $\times$). The imaging was performed using the resources of the Confocal Microscopy Core Facility, Center for Cancer Research, National Cancer Institute.

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REFERENCES

- (1). Watanabe T, Ono Y, Taniyama Y, Hazama K, Igarashi K, Ogita K, Kikkawa U, and Nishizuka Y (1992) Cell division arrest induced by phorbol ester in CHO cells overexpressing protein kinase C-delta subspecies. *Proc. Natl. Acad. Sci. U. S. A* 89, 10159–10163. [PubMed: 1438205]

- (2). Mischak H, Pierce JH, Goodnight J, Kazanietz MG, Blumberg PM, and Mushinski JF (1993) Phorbol ester-induced myeloid differentiation is mediated by protein kinase C- α and - δ and not by protein kinase C- β II, - ϵ , - ζ , and - η . *J. Biol. Chem* 268, 20110–20115. [PubMed: 8376369]
- (3). Li C, Wernig E, Leitges M, Hu Y, and Xu Q (2003) Mechanical stress-activated PKCdelta regulates smooth muscle cell migration. *FASEB J.* 17, 2106–2108. [PubMed: 12958154]
- (4). Brodie C, and Blumberg PM (2003) Regulation of cell apoptosis by protein kinase c delta. *Apoptosis* 8, 19–27. [PubMed: 12510148]
- (5). Antal CE, Hudson AM, Kang E, Zanca C, Wirth C, Stephenson NL, Trotter EW, Gallegos LL, Miller CJ, Furnari FB, et al. (2015) Cancer-associated protein kinase C mutations reveal kinase's role as tumor suppressor. *Cell* 160, 489–502. [PubMed: 25619690]
- (6). Warwar N, Efendic S, Ostenson CG, Haber EP, Cerasi E, and Nesher R (2006) Dynamics of glucose-induced localization of PKC isoenzymes in pancreatic beta-cells: diabetes-related changes in the GK rat. *Diabetes* 55, 590–599. [PubMed: 16505220]
- (7). Bowling N, Walsh RA, Song G, Estridge T, Sandusky GE, Fouts RL, Mintze K, Pickard T, Roden R, Bristow MR, et al. (1999) Increased protein kinase C activity and expression of Ca²⁺-sensitive isoforms in the failing human heart. *Circulation* 99, 384–391. [PubMed: 9918525]
- (8). Etcheberrigaray R, Tan M, Dewachter I, Kuiperi C, Van der Auwera I, Wera S, Qiao L, Bank B, Nelson TJ, Kozikowski AP, et al. (2004) Therapeutic effects of PKC activators in Alzheimer's disease transgenic mice. *Proc. Natl. Acad. Sci. U. S. A* 101, 11141–11146. [PubMed: 15263077]
- (9). Newton AC (1995) Protein kinase C: structure, function, and regulation. *J. Biol. Chem* 270, 28495–28498. [PubMed: 7499357]
- (10). Kishimoto A, Takai Y, Mori T, Kikkawa U, and Nishizuka Y (1980) Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover. *J. Biol. Chem* 255, 2273–2276. [PubMed: 7358670]
- (11). Ogawa Y, Takai Y, Kawahara Y, Kimura S, and Nishizuka Y (1981) A new possible regulatory system for protein phosphorylation in human peripheral lymphocytes. I. Characterization of a calcium-activated, phospholipid-dependent protein kinase. *J. Immunol* 127, 1369–1374. [PubMed: 7276562]
- (12). Sakai N, Sasaki K, Ikegaki N, Shirai Y, Ono Y, and Saito N (1997) Direct visualization of the translocation of the gamma-subspecies of protein kinase C in living cells using fusion proteins with green fluorescent protein. *J. Cell Biol* 139, 1465–1476. [PubMed: 9396752]
- (13). Majumder PK, Pandey P, Sun X, Cheng K, Datta R, Saxena S, Kharbanda S, and Kufe D (2000) Mitochondrial translocation of protein kinase C delta in phorbol ester-induced cytochrome c release and apoptosis. *J. Biol. Chem* 275, 21793–21796. [PubMed: 10818086]
- (14). DeVries TA, Neville MC, and Reyland ME (2002) Nuclear import of PKCdelta is required for apoptosis: identification of a novel nuclear import sequence. *EMBO J.* 21, 6050–6060. [PubMed: 12426377]
- (15). Wang QJ (2006) PKD at the crossroads of DAG and PKC signalling. *Trends Pharmacol. Sci* 27, 317–323. [PubMed: 16678913]
- (16). Zhang G, Kazanietz MG, Blumberg PM, and Hurley JH (1995) Crystal structure of the cys2 activator-binding domain of protein kinase C delta in complex with phorbol ester. *Cell* 81, 917–924. [PubMed: 7781068]
- (17). Marquez VE, and Blumberg PM (2003) Synthetic diacylglycerols (DAG) and DAG-lactones as activators of protein kinase C (PK-C). *Acc. Chem. Res* 36, 434–443. [PubMed: 12809530]
- (18). Li J, Ziemba BP, Falke JJ, and Voth GA (2014) Interactions of protein kinase C- α C1a and C1b domains with membranes: a combined computational and experimental study. *J. Am. Chem. Soc* 136, 11757–11766. [PubMed: 25075641]
- (19). Stewart MD, Cole TR, and Igumenova TI (2014) Interfacial partitioning of a loop hinge residue contributes to diacylglycerol affinity of conserved region 1 domains. *J. Biol. Chem* 289, 27653–27664. [PubMed: 25124034]
- (20). Nacro K, Bienfait B, Lee J, Han KC, Kang JH, Benzaria S, Lewin NE, Bhattacharyya DK, Blumberg PM, and Marquez VE (2000) Conformationally constrained analogues of diacylglycerol (DAG). 16. How much structural complexity is necessary for recognition and high binding affinity to protein kinase C? *J. Med. Chem* 43, 921–944. [PubMed: 10715158]

- (21). Duan D, Sigano DM, Kelley JA, Lai CC, Lewin NE, Kedei N, Peach ML, Lee J, Abeyweera TP, Rotenberg, et al. (2008) Conformationally constrained analogues of diacylglycerol. 29. Cells sort diacylglycerol-lactone chemical zip codes to produce diverse and selective biological activities. *J. Med. Chem* 51, 5198–5220. [PubMed: 18698758]
- (22). Shindo M, Irie K, Nakahara A, Ohigashi H, Konishi H, Kikkawa U, Fukuda H, and Wender PA (2001) Toward the identification of selective modulators of protein kinase C (PKC) isozymes: establishment of a binding assay for PKC isozymes using synthetic C1 peptide receptors and identification of the critical residues involved in the phorbol ester binding. *Bioorg. Med. Chem* 9, 2073–2081. [PubMed: 11504643]
- (23). Irie K, Oie K, Nakahara A, Yanai Y, Ohigashi H, Wender PA, Fukuda H, Konishi H, and Kikkawa U (1998) Molecular basis for protein kinase C isozyme-selective binding: The synthesis, folding, and phorbol ester binding of the cysteine-rich domains of all protein kinase C isozymes. *J. Am. Chem. Soc* 120, 9159–9167.
- (24). Szallasi Z, Bogi K, Gohari S, Biro T, Acs P, and Blumberg PM (1996) Non-equivalent roles for the first and second zinc fingers of protein kinase Cdelta. Effect of their mutation on phorbol ester-induced translocation in NIH 3T3 cells. *J. Biol. Chem* 271, 18299–18301. [PubMed: 8702464]
- (25). Sridhar J, Wei ZL, Nowak I, Lewin NE, Ayres JA, Pearce LV, Blumberg PM, and Kozikowski AP (2003) New bivalent PKC ligands linked by a carbon spacer: enhancement in binding affinity. *J. Med. Chem* 46, 4196–4204. [PubMed: 12954072]
- (26). Giorgione J, Hysell M, Harvey DF, and Newton AC (2003) Contribution of the C1A and C1B domains to the membrane interaction of protein kinase C. *Biochemistry* 42, 11194–11202. [PubMed: 14503869]
- (27). Wender PA (1999) Mapping phorbol ester binding domains of protein kinase C (PKC): the design, synthesis and biological activity of novel phorbol ester dimers. *Synthesis* S1, 1401–1406.
- (28). Baba Y, Mayumi S, Hirai G, Kawasaki H, Ogoshi Y, Yanagisawa T, Hashimoto Y, and Sodeoka M (2004) Evaluation of series of isobenzofuranone dimers as PKC α ligands: implication for the distance between the two ligand binding sites. *Bioorg. Med. Chem. Lett* 14, 2969–2972. [PubMed: 15125970]
- (29). Tamamura H, Bienfait B, Nacro K, Lewin NE, Blumberg PM, and Marquez VE (2000) Conformationally constrained analogues of diacylglycerol (DAG). 17. Contrast between *sn*-1 and *sn*-2 DAG lactones in binding to protein kinase C. *J. Med. Chem* 43, 3210–3217.
- (30). Tamamura H, Sigano DM, Lewin NE, Blumberg PM, and Marquez VE (2004) Conformationally Constrained Analogues of Diacylglycerol. 20. The Search for an Elusive Binding Site on Protein Kinase C through Relocation of the Carbonyl Pharmacophore Along the *sn*-1 Side Chain of 1,2-Diacylglycerol Lactones. *J. Med. Chem* 47, 644–655. [PubMed: 14736244]
- (31). Tamamura H, Sigano DM, Lewin NE, Peach ML, Nicklaus MC, Blumberg PM, and Marquez VE (2004) Conformationally constrained analogues of diacylglycerol (DAG). 23. Hydrophobic ligand-protein interactions versus ligand-lipid interactions of DAG-lactones with protein kinase C (PK-C). *J. Med. Chem* 47, 4858–4864. [PubMed: 15369389]
- (32). Nomura W, Narumi T, Ohashi N, Serizawa Y, Lewin NE, Blumberg PM, Furuta T, and Tamamura H (2011) Synthetic caged DAG-lactones for photochemically controlled activation of protein kinase C. *ChemBioChem* 12, 535–539. [PubMed: 22238145]
- (33). Sharma R, Lee J, Wang S, Milne GW, Lewin NE, Blumberg PM, and Marquez VE (1996) Conformationally constrained analogues of diacylglycerol. 10. Ultrapotent protein kinase C ligands based on a racemic 5-disubstituted tetrahydro-2-furanone template. *J. Med. Chem* 39, 19–28. [PubMed: 8568806]
- (34). Sigano DM, Peach ML, Nacro K, Choi Y, Lewin NE, Nicklaus MC, Blumberg PM, and Marquez VE (2003) Differential binding modes of diacylglycerol (DAG) and DAG lactones to protein kinase C (PK-C). *J. Med. Chem* 46, 1571–1579. [PubMed: 12699375]
- (35). Lehman SE, Schwendeman JE, O'Donnell PM, and Wagener KB (2003) Olefin isomerization promoted by olefin metathesis catalysts. *Inorg. Chim. Acta* 345, 190–198.
- (36). Haque TS, Skillman AG, Lee CE, Habashita H, Gluzman IY, Ewing TJ, Goldberg DE, Kuntz ID, and Ellman JA (1999) Potent, low-molecular-weight non-peptide inhibitors of malarial aspartyl protease plasmepsin II. *J. Med. Chem* 42, 1428–1440. [PubMed: 10212129]

- (37). Poulsen SA, and Bornaghi LF (2006) Fragment-based drug discovery of carbonic anhydrase II inhibitors by dynamic combinatorial chemistry utilizing alkene cross metathesis. *Bioorg. Med. Chem* 14, 3275–3284. [PubMed: 16431113]
- (38). Sharkey NA, Leach KL, and Blumberg PM (1984) Competitive inhibition by diacylglycerol of specific phorbol ester binding. *Proc. Natl. Acad. Sci. U. S. A* 81, 607–610. [PubMed: 6320198]
- (39). Lee J, Sharma R, Wang S, Milne GWA, Lewin NE, Szallasi Z, Blumberg PM, George C, and Marquez VE (1996) Conformationally constrained analogues of diacylglycerol. 12. Ultrapotent protein kinase C ligands based on a chiral 4,4-disubstituted heptono-1,4-lactone template. *J. Med. Chem* 39, 36–45. [PubMed: 8568825]
- (40). Pu Y, Garfield SH, Kedei N, and Blumberg PM (2009) Characterization of the differential roles of the twin C1a and C1b domains of protein kinase C δ . *J. Biol. Chem* 284, 1302–1312. [PubMed: 19001377]
- (41). Stahelin RV, Digman MA, Medkova M, Ananthanarayanan B, Melowic HR, Rafter JD, and Cho W (2005) Diacylglycerol-induced membrane targeting and activation of protein kinase C ϵ . *J. Biol. Chem* 280, 19784–19793. [PubMed: 15769752]
- (42). Lewin NE, and Blumberg PM (2003) [^3H]Phorbol 12,13-dibutyrate binding assay for protein kinase C and related proteins. *Methods Mol. Biol* 233, 129–156. [PubMed: 12840504]

C1 domain binding module
(DAG-lactone)

Figure 1. Design of the dimeric derivatives of DAG-lactones (**6a-g**) and the structure of the monomer (**5h**). DAG-lactones, which are C1 (a and b) domain binding moieties, are shown inside pink ellipses.

Figure 2. Relation between the binding affinity of the dimeric derivatives of DAG-lactones and the carbon chain length of linkers. K_i values were determined against [^3H]PDBu binding to full length PKC or the C1b domain. Bars indicate the value from triplicate independent experiments of the $K_i \pm \text{SEM}$ for the C1b domain of PKC δ (black), full-length PKC δ (dark gray), or full-length PKC α (light gray).

Figure 3.

Translocation of EGFP-tagged PKC δ by the addition of compounds in transiently transfected CHO cells. (a) EGFP-PKC δ -expressing CHO cells were treated with 10 μ M compound (**6a**: the dimeric derivative of DAG-lactone containing the 6-carbon linker; **5h**: the monomer of DAG-lactone; PDBu: phorbol 12,13-dibutyrate). Fluorescent images were observed with confocal fluorescence microscopy at 0, 5, and 10 min after the addition of compounds. (b) EGFP-PKC δ -expressing CHO cells were treated with dimeric derivatives of

DAG–lactones containing longer linkers (10 μ M) (**6c**: 10-carbon linker; **6d**: 12-carbon linker; **6g**: 18-carbon linker). DIC: differential interference contrast.

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Figure 4.

Translocation of EGFP-tagged PKC ϵ by the addition of compounds in transiently transfected LNCaP cells. Cells were treated with PMA (phorbol 12-myristate 13-acetate, 1 μ M), with the monomeric DAG–lactone or with the dimeric DAG–lactones **6a** and **6g** (3 μ M) and imaged by confocal microscopy at the indicated times. Images are from a single experiment.

Scheme 1. Synthesis of Dimeric Derivatives of DAG–Lactones^a

^aReagents and conditions: (a) *tert*-butyl 2,2,2-trichloroacetimidate, BF₃·OEt₂, CH₂Cl₂, 45%.

(b) Lithium diisopropylamide, tetrabutylammonium iodide, tetrahydrofuran (THF)–hexamethylphosphoramide, 4-bromo-1-butene for **3a**: 47%; 5-bromo-1-pentene for **3b**: 93%; 6-bromo-1-hexene for **3c**: 66%; 7-bromo-1-heptene for **3d**: 72%; 8-bromo-1-octene for **3e**: 66%; 9-bromo-1-nonene for **3f**: 38%; 10-bromo-1-decene for **3g**: 59%. (c)

Trifluoroacetic acid, CH_2Cl_2 . (d) $(\text{COCl})_2$, catalog dimethylformamide, CH_2Cl_2 . (e) **4**, Et_3N , THF, **5a**: 35%; **5b**: 52%; **5c**: 41%; **5d**: 58%; **5e**: 54%; **5f**: 27%; **5g**: 35% (three steps).

(f) Second-generation Grubbs catalyst, CH₂Cl₂. Optical purities of products were not determined. Yields are described in the Materials and Methods section.

Table 1.
 K_i and Calculated Log P values of the Monomeric DAG–Lactone and Its Dimeric Derivatives

compound	number of carbon atoms in the linker	purity (%) ^a	PKC δ IC $_{50}$ K_i (nM) ^b	PKC δ K_i (nM) ^b	PKC α K_i (nM) ^b	CLoP ^c
5h	monomer		5.47 $\pm$ 0.81	2.43 $\pm$ 0.10	10.4 $\pm$ 0.31	4.54
6a	6	73	2.07 $\pm$ 0.08	2.67 $\pm$ 0.59	8.4 $\pm$ 1.4	10.65
6b	8	43	1.17 $\pm$ 0.05	3.11 $\pm$ 0.22	15.9 $\pm$ 1.4	11.71
6c	10	46	0.80 $\pm$ 0.05	6.31 $\pm$ 1.30	17.4 $\pm$ 5.2	12.77
6d	12	47	1.17 $\pm$ 0.34	13.2 $\pm$ 1.9	50 $\pm$ 13	13.82
6e	14	40	1.86 $\pm$ 0.41	16.6 $\pm$ 2.1	48.5 $\pm$ 6.3	14.88
6f	16	32	2.52 $\pm$ 0.46	27.2 $\pm$ 2.0	173 $\pm$ 17	15.94
6g	18	39	3.00 $\pm$ 0.76	12.3 $\pm$ 2.1	30.9 $\pm$ 3.3	16.70

^aPurities were analyzed by ESI mass spectra.

^b K_i values were measured by a competitive binding assay against [³H]PDBu binding and are the mean $\pm$ standard error of the mean (SEM) of three independent experiments.

^cCalculated log P .

Table 2.

Yields of Compounds 6a–6g

starting materials	products (yields)
5a (8 mg, 0.02 mmol)	6a (4.9 mg, 65%)
5b (51 mg, 0.12 mmol)	6b (30 mg, 64%)
5c (56 mg, 0.13 mmol)	6c (37 mg, 70%)
5d (76 mg, 0.17 mmol)	6d (9.8 mg, 14%)
5e (76 mg, 0.17 mmol)	6e (23 mg, 31%)
5f (35 mg, 0.076 mmol)	6f (15 mg, 45%)
5g (54 mg, 0.11 mmol)	6g (33 mg, 65%)