Diacylglycerol (DAG)-lactones, a New Class of Protein Kinase C (PKC) Agonists, Induce Apoptosis in LNCaP Prostate Cancer Cells by Selective Activation of PKCα*

Phorbol esters, the archetypical (PKC) activators, induce apoptosis in androgen-sensitive LNCaP prostate cancer cells. In this study we evaluate the effect of a novel class of PKC ligands, the diacylglycerol (DAG)-lactones, as inducers of apoptosis in LNCaP cells. These unique ligands were designed using novel pharmacophore- and receptor-guided approaches to achieve highly potent DAG surrogates. Two of these compounds, HK484 and HK654, induced apoptosis in LNCaP cells with much higher potency than oleoyl-acetyl-glycerol or phorbol 12,13-dibutyrate. Moreover, different PKC isoforms were found to mediate the apoptotic effect of phorbol 12-myristate 13-acetate (PMA) and HK654 in LNCaP cells. Using PKC inhibitors and dominant negative PKC isoforms, we found that both PKCα and PKCβ mediated the apoptotic effect of PMA, whereas only PKCα was involved in the effect of the DAG-lactone. The PKCα selectivity of HK654 in LNCaP cells contrasts with similar potencies in vitro for binding and activation of PKCα and PKCδ. Consistent with the differences in isoform dependence in intact cells, PMA and HK654 show marked differences in their abilities to translocate PKC isoforms. Both PMA and HK654 induce a marked redistribution of PKCα to the plasma membrane. On the other hand, unlike PMA, HK654 translocates PKCδ predominantly to the nuclear membrane. Thus, DAG-lactones have a unique profile of activation of PKC isoforms for inducing apoptosis in LNCaP cells and represent the first example of a selective activator of a classical PKC in cellular models. An attractive hypothesis is that selective activation of PKC isoforms by pharmacological agents in cells can be achieved by differential intracellular targeting of each PKC.

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The phorbol esters and related diterpenes are natural compounds that have been for many years the preferred pharmacological tools for studying protein kinase C (PKC),1 a key family of kinases implicated in growth factor- and G-protein-coupled receptor signaling. These compounds mimic the action of the lipid second messenger diacylglycerol (DAG), a relatively simple and highly flexible molecule generated by cellular phospholipases. The higher potency of phorbol esters and their greater stability compared with the second messenger DAG makes these agents the preferred activators of PKC (1, 2). Phorbol esters regulate a variety of cellular functions, including cell cycle progression, differentiation, cytoskeleton remodeling, and malignant transformation. Although phorbol esters promote mitogenesis in several cell types, accumulating data indicate that activation of PKC leads to inhibition of cell growth in many cells (3–6). Interestingly, phorbol esters induce apoptosis in several cell lines, including thymocytes, breast cancer cells, and prostate cancer cells (7–12).

The heterogeneity of effects of the phorbol esters is related to the presence of multiple phorbol ester/DAG receptors, including PKC isoforms and “nonkinase” PKC receptors. The PKC family comprises at least 10 related kinases with differential expression, subcellular distribution and biochemical regulation. PKC isoforms have been classified into three subclasses according to their structure and regulation: “classical” or calcium-dependent (“cPKCs”) α, β, βII, and γ, “novel” or calcium-independent (“nPKCs”) δ, ε, η, and θ, and “atypical” (“aPKCs”) ζ and η. A related enzyme, PKCμ or protein kinase D, is a distant relative of the PKC isoforms. Although aPKCs are insensitive to phorbol esters and DAG, cPKCs and nPKCs bind phorbol esters with high affinity in the presence of phospholipids as cofactors. PKC isoforms are subject to exquisite regulatory mechanisms and can have either overlapping or opposite biological functions (13–16).

It is well established that activation of PKC by phorbol esters triggers an apoptotic response in androgen-dependent prostate

1 The abbreviations used are: PKC, protein kinase C; DAG, diacylglycerol; PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-dibutyrate; OAG, 1-octadecyl-2-acetyl-sn-glycerol; DAF, 4,6-diamino-2-phenylindole; cPKC, classical PKC; nPKC, novel PKC; aPKC, atypical PKC; ADV, adenovirus; mo.i., multiplicity of infection; p53, plaque-forming unit(s); DN, dominant negative; FBS, fetal bovine serum; PBS, phosphate-buffered saline; GFP, green fluorescent protein; CHO, Chinese hamster ovary.
cancer cells, such as LNCaP cells (10–12, 17, 18). Elegant work from Powell and co-workers (11, 18) revealed that phorbol ester-induced apoptosis in LNCaP prostate cancer cells correlates with a persistent translocation of PKCa to membranes. We have recently demonstrated that overexpression of PKCβ in LNCaP cells markedly potentiates phorbol 12-myristate 13-acetate (PMA)-induced apoptosis, suggesting a role for PKCβ in the phorbol ester effect. Likewise, expression of a dominant negative (kinase-inactive) form of PKCβ partially inhibits PMA-induced apoptosis (17). Therefore, multiple PKC isoforms may contribute to the apoptotic effect of phorbol esters in LNCaP cells.

An interesting emerging concept is that PKC activators have varied, unique pharmacological profiles and exert in many cases discrete cellular responses. Differential responses to PKC activators have been observed for analogs such as bryostatin 1, mezerein, and 12-deoxyphorbol esters (1, 2, 5). In this paper we focus on a novel class of synthetic analogs, the DAG-lactones, which bind with high potency to the phorbol ester/DAG binding site in PKC (the C1 domain). These novel C1 domain ligands were designed through a pharmacophore-guided approach based on the crystal coordinates of the C1b domain of PKCβ in complex with phorbol-13-acetate. Remarkably, the DAG-lactones show 3–4 orders of magnitude higher affinity for PKC isoforms than natural DAGs. To generate such potent DAGs, the glycerol backbone was constrained into a rigid structure (a lactone ring), resulting in a reduced entropic penalty associated with DAG binding to the receptor (19–23). Like phorbol esters and the natural DAGs in an “open conformation,” the DAG-lactones bind to PKC and induce its activation in vitro. Moreover, these simple DAG analogs have phorbol ester-like effects in cells, such as inhibition of epidermal growth factor binding, with potencies similar to that of PDBu. Some of these compounds have displayed important antitumor activity in the in vitro cell line screen of the NCI (National Institutes of Health), but their mechanism of action is largely unknown (22). No information is available on the specificity of the DAG-lactones for individual PKC isoforms.

In this paper we show that DAG-lactones, like phorbol esters, induce apoptosis in LNCaP prostate cancer cells. A remarkable finding is that DAG-lactones and PMA exert their apoptotic effect through the activation of a different subset of PKC isoforms, as revealed by a series of pharmacological and molecular approaches. Thus, DAG-lactones represent a novel template for the design of potent selective PKC agonists, which may be useful tools to dissect PKC isozyme-specific functions in cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—PMA was obtained from LC Laboratories (Woburn, MA). GF109203X, G66976, and rottlerin were purchased from Alexis Corp. (San Diego, CA). The pan-caspase inhibitor z-VAD was obtained from Calbiochem (San Diego, CA). 4,6-Diamino-2-phenylindole (DAPI) was purchased from Sigma. Cell culture reagents and media were purchased from Life Technologies, Inc.

**Cell Culture**—The human prostate cancer cell line LNCaP was obtained from the American Type Culture Collection (Rockville, MD). LNCaP cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO2 atmosphere. LNCaP cells overexpressing Bel-2 (LNCaP-Neo/Bel-2) and the corresponding control cells transfected with empty vector (LNCaP-Neo) (24) were a gift of Dr. L. Lothstein (University of Tennessee, Memphis, TN).

**Infection of LNCaP Cells with PKC Adenovirus**—In this study we used replication-deficient adenoviruses (AdV) for overexpression of individual PKC isoforms. Generation of the PKCaAdV and PKCβAdV has been described elsewhere (17, 25–27). Kinase-inactive PKCs were generated by an Arg to Lys substitution at the ATP-binding site of the catalytic domain, and the corresponding AdVs were then generated as described in Ref. 26. AdVs were amplified in HEK 293 cells using standard techniques (28). Titers of viral stocks were normally greater than 1 × 109 pfu/cell. The absence of wild type AdV was confirmed by PCR using primers for the E1 region. An AdV for the LacZ gene (LacZAdV) was used as a control (17).

Subconfluent LNCaP cells in 6- or 12-well plates were infected with AdV for 14 h at multiplicities of infection (m.o.i.) ranging from 1 to 30 pfu/cell (in RPMI 1640 medium supplemented with 2% FBS). Following infection, the medium was replaced with fresh RPMI 1640 medium supplemented with 10% FBS and the cells were grown for an additional 24 h. Maximum expression of PKC isoforms after AdV infection was achieved with this protocol. Expression of recombinant protein remained stable for several days (17).

**Western Blot Analysis**—Cells were harvested and lysed in a buffer containing 10 mM Tris-HCl, 150 mM NaCl, 10% glycerol, 2% SDS, and 5% β-mercaptoethanol. Equal amounts of protein (10 μg) were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk and 0.1% Tween 20, PBS and incubated with anti-mouse or anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:10,000, Bio-Rad). Bands were visualized by the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Pharmacia Biotech).

**Visualization by Confocal Microscopy of GFP-PKC Translocation**—LNCaP cells were transfected with vectors encoding GFP fusion proteins for PKCa and PKCβ (29) using Lipofectamine Plus (Life Technologies, Inc.) according to the manufacturer’s instructions. Experiments were performed 3 days after transfection. Prior to observation, transiently transfected LNCaP cells were washed twice with standard medium (Dulbecco’s modified Eagle’s medium without phenol red supplemented with 1% FBS) prewarmed to 37 °C. All PKC activators were diluted to specified concentrations in the same medium, and the final concentration of solvent (ethanol) was always less than 0.01%.

**Apoptosis Assays**—To assess morphological changes in chromatin structure, cells were stained with DAPI. Cells were then trypsinized, mounted on glass slides, fixed in 70% ethanol, and stained for 20 min with 1 μg/ml DAPI. Apoptosis was characterized by chromatin condensation and fragmentation when examined by fluorescence microscopy. The incidence of apoptosis in each preparation was analyzed by counting 500 cells and determining the percentage of apoptotic cells (17).

DNA laddering was measured using the Apoptotic DNA-Ladder kit from Roche Molecular Biochemicals. For flow cytometric analysis, cells were fixed in 70% ethanol and resuspended in PBS containing propidium iodide (1 mg/ml) and RNase (40 μg/ml). Cell cycle progression and apoptosis were analyzed in an Epics XL flow cytometer (Coulter Corp., Hialeah, FL). For each treatment, 7,500 events were recorded.

**[3H]PDBu Binding**—[3H]PDBu binding to PKC isoforms was measured using the polyethylene glycol precipitation assay. A detailed description of the methodology is presented in Ref. 30. A competition of [3H]PDBu binding by different analogs, we used a fixed concentration of [3H]PDBu (3 nM) and increasing concentrations (in triplicate) of the competing nonradioactive ligand. ID50 values were determined from the competition curve, and the Kd for the competing ligand was calculated from the ID50 by using the relationship Kd = ID50/(1 + L/Kd), where Kd is the dissociation constant for [3H]PDBu and L is the concentration of free [3H]PDBu at the ID50.
used in these assays were generated by baculovirus infection of Sf9 insect cells and subsequent purification, as described previously (30).

**PKC Activity**—PKC activity was assayed by measuring the incorporation of \(^{32}\)P from \(^{32}\)P/ATP into a specific PKC substrate (a pseudosubstrate peptide), as described previously (30), using 100 \(\mu\)g/ml phospholipid vesicles (20% phosphatidylserine, 80% phosphatidylcholine) prepared by sonication. The reaction was carried out at 30 °C for 10 min. Kinase activity was linear with time over this incubation period.

**Protein Determination**—Protein determinations were made with the Micro BCA Protein Assay from Pierce, using bovine serum albumin as a standard.

## RESULTS

**Induction of Apoptosis by Synthetic DAG-lactones**—DAGs possess substantially lower potency for binding to PKCs and reduced metabolic stability compared with the corresponding phorbol ester analogs. An approach that was used to success fully generate potent DAG analogs involved constraining the glycerol backbone into a five-member ring (DAG-lactone). With the combined use of pharmacophore- and receptor-guided approaches facilitated by the crystal structure of the PKC \(\alpha\) C1b domain, it was possible to rationally design structurally simple DAG-lactone ligands that displayed binding affinities for PKC\(\alpha\) in the low nanomolar range (19–23). However, very limited information on the biological activity of these DAG-lactones is available other than their in vitro binding affinity for PKC\(\alpha\) as a receptor.

A group of 29 DAG-lactones that exhibited high binding affinities for PKC\(\alpha\) (in the low nanomolar range) were selected to generate a structurally diverse set of compounds displaying various combinations of \(R_1\) and \(R_2\) groups (general structure, Fig. 1). Their affinities for PKC\(\alpha\) expressed in terms of \(K_i\) values covered a wide range: 2–6 \(\mu\)M (10 compounds), 6–9 \(\mu\)M (3 compounds), 9–13 \(\mu\)M (6 compounds), 12–20 \(\mu\)M (4 compounds), 20–40 \(\mu\)M (2 compounds), and >40 \(\mu\)M (4 compounds). The lipophilicity range (log \(P\)) spanned between 3.5 and 6.5. Because phorbol esters exhibit pro-apoptotic activity in LNCaP prostate cancer cells, we decided to evaluate these 29 compounds for their apoptotic effect in these cells. From this group, four compounds were selected for their unique structure-activity relationship in terms of their apoptotic inducing potency. The structures of these compounds are shown together with the code names used in the text (Fig. 1). The complete set of 29 compounds, as well as the description of their syntheses and characterization, including the selected compounds for this work, will be described elsewhere.\(^2\)

LNCaP cells were treated with different DAG-lactones at a single concentration (10 \(\mu\)M) for 1 h, and apoptosis was assessed 24 h later by counting the number of apoptotic cells after DAPI staining. After such time, a maximum apoptotic response is normally observed following PKC activation (17). Both HK434 and HK654 induced –30% of apoptosis, which equals the maximum response observed with PMA under the experimental conditions used in our studies. On the other hand, DAG-lactones HK204 and HK602 showed only a modest response, as was also observed with 1-oleoyl-2-acetylglycerol (OAG), a DAG that is commonly used to activate PKC in cellular models (Fig. 2A). A representative field with apoptotic cells under the fluorescent microscope is shown in Fig. 2B. As shown in the figure, a large number of cells with morphological changes distinctive of apoptosis, including nuclear fragmentation and cell shrinkage, were observed after treatment with PMA or DAG-lactones. HK434, HK654, and PMA were able to induce a characteristic pattern of DNA fragmentation, visualized as DNA laddering in agarose gels (Fig. 2C). A concentration-dependence analysis showed that HK434 and HK654 were more potent than OAG or PDBu, a phorbol ester commonly used to activate PKC. As expected, the inactive phorbol ester 4α-PMA was totally ineffective in inducing apoptosis in LNCaP cells. The presence of apoptotic cells after treatment with DAG-lactones was also detected by flow cytometry analysis (see Fig. 4).

**Inhibition of Apoptosis by a Pan-caspase Inhibitor and Bcl-2 Overexpression**—Caspases are essential molecules for the execution of apoptosis (31). This family of Asp-directed cysteine proteases can be specifically inhibited by cell-permeable peptides. We treated LNCaP cells with the pan-caspase peptide z-VAD (50 \(\mu\)M) and evaluated the apoptotic effect of the DAG-lactone HK654 and PMA. As shown in Fig. 3A, z-VAD markedly reduced the number of apoptotic cells after HK654 or PMA treatment.

The role of Bcl-2 as a key anti-apoptotic molecule is well established in many cell types, including prostate cancer cells (31, 32). We investigated the effect of DAG-lactones in LNCaP cells transfected with a mammalian expression vector that encodes for Bcl-2. These cells (LNCaP-Neo/Bcl-2) expressed high levels of Bcl-2 compared with vector-transfected cells (LNCaP-Neo) (24). Cells overexpressing Bcl-2 showed a marked resistance to apoptosis when stimulated with either HK654 or PMA. The apoptotic responses to PMA and HK654 in Bcl-2-overexpressing cells are only 41 and 32%, respectively, of those observed in vector-transfected cells (Fig. 3B).

**Effect of PKC Inhibitors**—To confirm that the apoptotic effect of DAG-lactones in LNCaP cells is mediated by activation of PKC, we assessed the effect of these compounds in the presence of PKC inhibitors. This is important because phorbol esters and DAG have other targets in addition to PKC isozymes (2, 5, 16, 33). We first used the PKC inhibitor GF 109203X, an inhibitor of cPKCs and nPKCs capable of blocking the apoptotic effect of PMA in LNCaP cells (17). GF 109203X (5 \(\mu\)M) also blocked the apoptotic effect of HK434 and HK654 almost completely, as judged by counting of apoptotic cells after DAPI staining (Fig. 4A). The incidence of apoptosis after treatment of LNCaP cells with GF 109203X in the absence of PKC activators was less than 2% (see Ref. 17). The inhibitory effect of GF 109203X could also be ascertained by flow cytometry analysis. The DNA histograms shown in Fig. 4B revealed a large number of cells with sub-G0/G1 DNA content after HK434, HK654, or PMA.

\(^2\)J. Lee, K.-C. Han, J.-H. Kang, N. E. Lewin, S. Yan, S. Benzarria, M. C. Nicklaus, P. M. Blumberg, and V. E. Marquez, submitted for publication.
treatment, which is consistent with the presence of apoptotic cells. In all cases the effect was abolished by treatment with GF109203X. Treatment with GF109203X alone did not produce any noticeable increase in the population of cells with sub-G0/G1 DNA content.

LNCaP cells express the classical PKCα, the novel PKCδ, and the phorbol ester/DAG-unresponsive PKCζ and PKCα. Very low levels of PKCε and PKCη were also detected (17). These results agree with those reported previously by Powell and co-workers (11, 18). Previous work from the Powell laboratory has established that PKCα is a mediator of PMA-induced apoptosis in LNCaP cells (11, 18). Our recent work established that PKCδ is also a pro-apoptotic isozyme in LNCaP cells (17), suggesting the existence of overlapping roles for both PKCα and PKCδ in this cellular model. To evaluate the involvement of PKCα and PKCδ as mediators of the pro-apoptotic effect of DAG-lactones, we first used a pharmacological approach. Two PKC inhibitors known to have selectivity for PKC isozymes were used: Go6976, an inhibitor of the cPKCs (34), and rottlerin, which preferentially inhibits PKCδ (35).

FIG. 2. DAG-lactones induce apoptosis in LNCaP cells. LNCaP cells were treated with different compounds for 1 h. Apoptosis was determined 24 h later. Panel A, apoptosis induced by 100 nM PMA, 10 μM OAG, or 10 μM DAG-lactones. Panel B, LNCaP cells were stained with DAPI and nuclear morphology was assessed by fluorescence microscopy. Apoptotic cells are indicated by arrows. Cc, vehicle (control). Panel C, DNA fragmentation visualized in 2% agarose gels after staining with ethidium bromide. Lane 1, untreated cells; lane 2, HK434 (10 μM); lane 3, 10 μM HK654; lane 4, 100 nM PMA. Ms, molecular size standards. Panel D, concentration-dependence analysis of apoptosis induced by phorbol esters (PMA, 4α-PMA, and PDBu), OAG, and DAG-lactones. In panels A and D, apoptosis was determined after DAPI staining. The incidence of apoptosis in each preparation was analyzed by counting 500 cells and determining the percentage of apoptotic cells. Results are the mean ± S.E. of three independent experiments.

Effects of Overexpression of PKCα or PKCδ on HK654- and PMA-induced Apoptosis—The differential sensitivity of PMA and DAG-lactones to PKC inhibitors prompted us to explore the issue of isozyme selectivity in further detail. An approach that has been extensively used to assess PKC isoform selectivity is the overexpression of individual PKCs. We have successfully used adenoviral delivery of PKCδ to LNCaP cells as a
strategy to demonstrate the involvement of this nPKC in PMA-induced apoptosis (17). In agreement with our previous studies (17), PKC\textsubscript{H9254} overexpression markedly potentiated the apoptotic effect of PMA (Fig. 6A). The effect was proportional to the level of expression of PKC\textsubscript{H9254}, as judged by Western blot (Fig. 6C) or in vitro kinase assays (Ref. 17, and data not shown). Not surprisingly, a similar potentiation of the PMA effect was observed after infection of LNCaP cells with PKC\textsubscript{H9251}AdV (m.o.i. 1–30 pfu/cell). In this later case, the potentiation of the PMA effect also correlates with the expression levels of PKC\textsubscript{H9251}. On the other hand, a control LacZAdV was totally ineffective (Fig. 6A). Interestingly, a different pattern was observed for the DAG-lactone HK654. As in the case of PMA, overexpression of PKC\textsubscript{H9251} using the PKC\textsubscript{H9251}AdV markedly potentiated the apoptotic effect of HK654. However, infection of LNCaP cells with PKC\textsubscript{H9254}AdV did not produce any significant effect on HK654-induced apoptosis. As expected, infection with the LacZAdV was totally ineffective (Fig. 6A).

Effect of Dominant Negative PKCa (DN-PKCa) and Dominant Negative PKC\textgreek{b} (DN-PKC\textgreek{b}) Mutants on HK654- and PMA-induced Apoptosis—To further explore the role of PKCa and PKC\textgreek{b} as mediators of apoptosis in LNCaP cells, we decided to evaluate the effect of the expression of kinase-deficient mutants, which were shown to act as dominant negatives (DN) that interfere with PKC function. We used AdVs for PKCa and PKC\textgreek{b} mutants in which an Arg to Lys mutation was introduced in the ATP-binding site (DN-PKCaAdV and DN-PKC\textgreek{b}AdV) (26, 27). These mutants are kinase-inactive after expression in
LNCaP cells (17). Expression of DN-PKCs after infection with either DN-PKCaAdv or DN-PKCβAdv can be readily detected by Western blot using specific anti-PKCα or anti-PKCβ antibodies, respectively (Fig. 7C). The data in Fig. 7A show that infection of LNCaP cells with increasing m.o.i. values of DN-PKCaAdv inhibits the apoptotic effect of both PMA and HK654. On the other hand, whereas expression of DN-PKCS inhibits the apoptotic effect of PMA by ~30%, it does not affect the apoptotic effect of HK654. Taken together, these findings strongly suggest that PKCa, but not PKCβ, mediates the apoptotic effect of HK654 in LNCaP cells.

**Effect of HK654 on [3H]PDBu Binding and PKC Activation**—We next evaluated the interaction of HK654 with PKC isozymes using binding assays. The issue we wished to test was whether the differences in isozyme specificity observed in LNCaP cells could be explained by a differential pattern of recognition of the ligand. To test this hypothesis, we determined the binding affinity of HK654 for PKCα expressed in Sf9 insect cells (30). Binding was determined using a fixed concentration of the radioligand [3H]PDBu and increasing concentrations of HK654. Fig. 8 shows that HK654 very efficiently competes for [3H]PDBu binding to PKCα. The \( K_i \) for inhibition of binding to PKCα, determined from the ID\(_{50}\), was 1.6 ± 0.1 nM \( (n = 5) \). Under the same experimental conditions, the \( K_i \) for inhibition of binding to recombinant PKCβ was 5.4 ± 0.3 nM \( (n = 3) \). Therefore, HK654 binds with high affinity to both PKCα and PKCβ.

We then compared the ability of HK654 to activate kinase activity in vitro using a pseudosubstrate peptide, a substrate that can be efficiently phosphorylated both by PKCα and PKCβ (30). Fig. 9 shows that HK654 activates PKCα and PKCβ with similar potency. The maximum activation observed with HK654 was similar to that observed with 1 \( \mu \)M PMA (a concentration that induces maximum activation in our experimental conditions).
conditions), suggesting that HK654 fully activates PKCα and PKCδ. Therefore, the isoform-specific effects of HK654 in LNCaP cells cannot be explained by a differential intrinsic ability to bind and/or activate discrete PKC isoforms.

Subcellular Distribution of PKCα and PKCδ in LNCaP Cells—Experiments using subcellular fractionation by ultracentrifugation show that HK654, like PMA, translocates PKCδ from soluble (cytosolic) to particulate (membrane) fraction in LNCaP cells (data not shown). An attractive hypothesis is that the differential activation of PKC isoforms by HK654 in LNCaP cells is the consequence of a differential pattern of translocation of PKCs. To explore this issue, we used GFP-tagged PKCs. Plasmids encoding for GFP-PKCα and GFP-PKCδ were transfected into LNCaP cells and the pattern of subcellular redistribution in response to PMA and HK654 was evaluated by confocal microscopy. Fig. 10A shows that both PMA and HK654 redistribute GFP-PKCα from the cytoplasm to the plasma membrane. A quantitative analysis of translocation revealed that translocation to the plasma membrane peaked at 10 min for 1 μM PMA and at 7 min for 100 μM HK654 (Fig. 10, B and C). No translocation to the nuclear membrane was detected. However, a different pattern of translocation was observed for GFP-PKCδ in LNCaP cells. In agreement with a previous report in Chinese hamster ovary (CHO) K1 cells (29), PMA induced a rapid redistribution of GFP-PKCδ to the plasma membrane, followed by a slower redistribution to the nuclear membrane (Fig. 10, D and E). On the other hand, HK654 induced a pronounced and rapid translocation of GFP-PKCδ to the nuclear membrane. A patchy pattern of fluorescence appearing throughout the cytoplasm was observed in some cases. Although slight plasma membrane staining was observed in some GFP-PKCδ-transfected cells after HK654 treatment, a
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**A**

GFP-PKCα

0 min 5 min 10 min 20 min

PMA

HK654

**B**

1 μM PMA
Plasma Membrane

100 nM PMA
Nuclear Membrane

1 μM PMA
Nuclear Membrane

**C**

100 μM HK654
Plasma Membrane

10 μM HK654
Nuclear Membrane

100 μM HK654
Nuclear Membrane

**D**

GFP-PKCδ

0 min 5 min 10 min 20 min

PMA

HK654

**E**

1 μM PMA
Nuclear Membrane

100 nM PMA
Plasma Membrane

100 μM HK654
Nuclear Membrane

**F**

10 μM HK654
Nuclear Membrane

100 μM HK654
Plasma Membrane
DAG-lactones Induce Apoptosis in LNCaP Cells

DISCUSSION

In the present study we demonstrated that DAG-lactones, a novel class of synthetic DAG analogs, induce apoptosis in LNCaP prostate cancer cells. Unlike PMA, which mediates apoptosis through the activation of PKCa and PKCδ, HK654 activates only PKCα in LNCaP cells. The design of the γ-lactone template represents an important step in the rational synthesis of novel PKC activators with biological activity. These molecules were produced by constraining the glycerol backbone of DAG into a 13-acetate led to the generation of a series of DAG mimetics with in vitro affinities for PKC in the micromolar and nanomolar range (19-21). An important step to achieve low nanomolar binding affinities was the use of branched acyl or α-alkylidene chains in these minimal structures, a strategy that optimized the interactions of these compounds through van der Waals contacts with highly conserved amino acids of the C1 domain and with the membrane (22, 23). Our results show that, in LNCaP prostate cancer cells, the DAG-lactones HK434 and HK654 were considerably more potent than the phorbol ester PDBu and the widely used DAG analog OAG.

The heterogeneity in the cellular responses of PKC agonists highlights the complexity in the regulation of PKC isoforms. It appears that exquisite regulatory mechanisms and discrete activation of signaling pathways by individual members of the PKC family may take place in different cell types, leading to either overlapping or opposite biological functions. The data presented in this paper reveal that both PKCa and PKCδ, the most prominent cPKC and nPKC isoforms in LNCaP cells, have overlapping pro-apoptotic functions in this cell line. We have shown previously that PKCδ promotes apoptosis in androgen-sensitive prostate cancer cells (17), an effect also described in hemopoietic cells, keratinocytes, and salivary gland acinar cells (40-43). A growth-inhibitory role for PKCδ was also reported in numerous cell types. Early work from Mischak et al. (36) showed that overexpression of PKCδ in NIH 3T3 fibroblasts inhibits cell growth, in contrast to the effect observed with PKCε, which promotes cell growth and transformation in this cell type. In other cell types, such as CHO or HL-60 cells, ectopic expression of PKCδ leads G0/M arrest (37, 38). On the other hand, PKCε enhances anchorage-independent growth and metastatic potential in mammary adenocarcinoma cells (39). Different roles for PKCζ in proliferation and apoptosis have also been described in several cell types. Early work using overexpression strategies suggested that PKCζ promotes cell growth in fibroblasts (44). PKCζ also has a protective effect against apoptosis after interleukin-3 withdrawal in 32D myeloid progenitor cells (45). Furthermore, inhibition of PKCζ function is sufficient to trigger cell death in Ramos-BL B cells (46), glioma cells (47), and COS cells (48). Anti-apoptotic effects have also been ascribed to other PKC isoforms, including PKCθ and the atypical PKCs (49, 50). Despite the growth-promoting effect described for PKCζ, this cPKC inhibits proliferation or has pro-apoptotic properties in many cellular models (3, 6, 51). Given the growth inhibitory properties of PKC isoforms, an emerging theme is that PKC activation rather than PKC inhibition may have therapeutic value in appropriate systems, and indeed several PKC agonists are under evaluation as anti-cancer agents in clinical studies (52-54).

The mechanisms underlying the pro-apoptotic effects of PKC isoforms are still poorly understood. Apoptosis upon UV radiation of hemopoietic cells and keratinocytes involves the generation of an active 40-kDa fragment corresponding to the PKCδ C-terminal catalytic domain. A caspase-3 cleavage site is present in the hinge region (V3 domain) of PKCδ. Moreover, a caspase-3 inhibitor prevents the generation of the 40-kDa catalytic fragment, which suggests an important role for caspase-3 in the activation of PKCδ during apoptosis induced by UV radiation (40, 42). In LNCaP cells, however, we have shown that phorbol ester-induced apoptosis is caspase-3-independent, as judged by the lack of effect of the caspase-3 inhibitor DEVD and lack of caspase-3 cleavage (17). We hypothesize that, in LNCaP cells, phorbol esters and DAG-lactones promote the allosteric activation of PKC isoforms, and that PKCs are primary effectors or participate in a pathway that signals to apoptosis. Therefore, distinct mechanisms, namely proteolytic cleavage and allosteric activation, may be involved in apoptosis triggered by different stimuli. Whether each of these mechanisms involves the activation of different signaling pathways or occurs at different phases of the apoptotic process is still unknown. Allosteric activation of PKC, rather than proteolytic cleavage, is probably the primary mechanism of activation by phorbol esters and DAG-lactones in LNCaP cells, a hypothesis that is also supported by the fact that PKCα does not possess a caspase cleavage site in its structure, and that proteolytic cleavage of PKCζ or PKCδ was not observed upon activation with phorbol esters or HK654 in LNCaP cells (Ref. 17, and data not shown).

The subcellular redistribution or translocation of cPKCs and nPKCs from cytosol to plasma membrane is a hallmark of their activation. A striking observation in this paper is the contrasting pattern of translocation of PKC isoforms in LNCaP cells. Both PKCα and PKCζ are translocated to the plasma membrane by PMA. PKCζ is translocated to the plasma membrane after HK654 treatment, but PKCζ is primarily redistributed to the nuclear membrane by the DAG-lactone. Although we have observed some PKCζ localization at the plasma membrane after HK654 treatment, our quantitative analysis revealed that it was minimal when compared with nuclear translocation. Because a substantial proportion of PKCζ is normally associated to membranes in unstimulated cells (data not shown), we cannot rule out that this small pool of PKCζ is already present in the plasma membrane before stimulation. It may be possible that the apoptotic effect of PKC isoforms requires the phosphorylation of a PKC substrate in the plasma membrane. It has been demonstrated that PMA-induced apoptosis in LNCaP cells involves the persistent membrane translocation of PKCζ and the activation of the Raf-mitogen-activated protein kinase pathway.

Fig. 10. Translocation of PKC isoforms by DAG-lactones. Representative fluorescent images of LNCaP cells expressing GFP–PKCζ (panel A) and GFP–PKCδ (panel D) treated with 100 nM PMA or 10 μM HK654 for the times indicated in the figure. Quantitative changes in the fluorescent distribution of GFP–PKCζ and GFP–PKCδ at the plasma membrane (open symbols) and nuclear membrane (closed symbols) in response to different doses of PMA or HK654 are shown in panels B and C (for GFP–PKCζ) and in panels E and F (for GFP–PKCδ). Results are expressed as changes in the ratios of plasma membrane and nuclear membrane translocation as a function of time. Ratio of membrane translocations was calculated as the ratio of (Imem - Icyto)Icyto, where Imem represents the mean fluorescent intensity on the plasma or nuclear membrane in a given area and Icyto is the mean fluorescent intensity in a comparable area of the cytoplasm or the nucleoplasm, respectively. The quantitative values represent the average of at least three experiments with 2–3 cells evaluated in each experiment.
DAG-lactones Induce Apoptosis in LNCaP Cells

(18). Recent findings from our laboratory suggest that PKC also regulates Akt phosphorylation in LNCaP cells. Li et al. (25) have reported that translocation of PKCβ to mitochondria is an essential step for apoptosis in keratinocytes. However, we did not observe any co-localization of PKC isoforms with MitoTracker (a mitochondrial marker) in LNCaP cells upon PMA or HK654 treatment (data not shown), suggesting divergent mechanisms involved in translocation in different cell types.

An important lesson from these studies is that marked discrepancies exist between in vitro and cellular effects of PKC ligands. It is remarkable that HK654 does not discriminate weakly to phospholipids than does PKC. We know for PKC similar to the results reported here for the LNCaP cells and membrane.

In that study, as here, the DAG-lactones failed to induce translocation of PKCβ to the plasma membrane. In contrast, PKCα in CHO-K1 cells translocated to the plasma membrane in response to phosphoesters, brystostatin 1 or DAG-lactones, again similar to the results reported here for the LNCaP cells and distinct DAG-lactones. We know for PKCα that the pattern of translocation depends, among other variables, on the lipophilicity of the ligand (58). Under conditions of reduced lipophilicity, translocation to the nuclear membrane is favored. Why may that be? From other studies, we know that PKCα differs from PKCβ in its interaction with phospholipid membranes in vitro. Under conditions of sufficient calcium, PKCβ binds more weakly to phospholipids than does PKCα. Under limiting conditions, PKCα would thus require a greater hydrophobic contribution from the ligand to achieve insertion to the plasma membrane.

Although the full understanding of the structure-activity relations for PKCα localization remains to be determined, an unambiguous finding from our studies is that PKCα can be targeted pharmacologically to different intracellular compartments and that this differential targeting may be consequential. An attractive hypothesis is that “mislocalization” of a PKC isozyme may also lead to functional antagonism. Indeed, brystostatin 1 and 12-deoxyphorbol 13-phenylacetate, which translocate PKCα predominantly to nuclear membrane rather than the plasma membrane (29), antagonize PMA-mediated responses. This hypothesis still needs to be tested with DAG-lactones.

In summary, our results provide strong evidence that DAG-lactones induce apoptosis in LNCaP prostate cancer cells by selective activation of PKCs, the only cPKC present in this model. It would be important to evaluate whether this isoyme selectivity occurs in cell types other than LNCaP cells. Likewise, although PKCα is the only cPKC expressed in numerous cell types, it will be necessary to evaluate whether the DAG-lactones retain selectivity for PKCα in cells expressing other cPKCs. Because of the simplicity of their structures, DAG-lactones represent novel templates for the rational synthesis of potent selective agonists through pharmacophore and receptor-guided approaches. DAG-lactones are useful tools for studying PKC isoyme selectivity and therefore help to overcome the limitations that exist in the study of isoyme-specific functions in cellular models.

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REFERENCES


3 Y. Tanaka, T. Fujii, and M. G. Kazanietz, manuscript in preparation.
4 Q. J. Wang and P. M. Blumberg, unpublished observations.
Additions and Corrections


Identification of cathepsin B as a mediator of neuronal death induced by Aβ-activated microglial cells using a functional genomics approach.

Li Gan, Shiming Ye, Alan Chu, Kristin Anton, Saili Yi, Valerie A. Vincent, David von Schack, Daniel Chin, Joseph Murray, Scott Lohr, Laszlo Patthy, Mirella Gonzalez-Zulueta, Karoly Nikolich, and Roman Urfer

Page 5571, Fig. 4C: The first value shown at bottom of Fig. 4C should be 0.5 µM. A corrected figure is shown at right.

Also, in the Fig. 4 legend, second line from the last, within the parentheses "(em)," should be deleted. It should read "(p < 0.05) . . . ."
Diacylglycerol (DAG)-lactones, a new class of protein kinase C (PKC) agonists, induce apoptosis in LNCaP prostate cancer cells by selective activation of PKCα.

Maria Laura Garcia-Bermejo, Federico Coluccio Leskow, Teruhiko Fujii, Qiming Wang, Peter M. Blumberg, Motoi Ohba, Toshio Kuroki, Kee-Chung Han, Jeewoo Lee, Victor E. Marquez, and Marcelo G. Kazanietz

The structures of compounds HK654 and HK602 studied in this paper were incorrectly reported as N-hydroxylamides in Lee et al. (Lee, J., Han, K.-C., Kang, J.-H., Pearce, L. L., Lewin, N. E., Yan, S., Benzaria, S., Nicklaus, M. C., Blumberg, P. M., and Marquez, V. E. (2001) J. Med. Chem. 44, 4309–4312; Correction (2003) J. Med. Chem. 46, 2794). The compounds correspond instead to esters HK434 and HK204. The reader should be aware that the biological properties described for HK654 correspond instead to HK434. When taking into consideration the small difference in molecular weight (HK654, \( M_r = 397.55 \) and HK434, \( M_r = 382.54 \)), the values reported remain virtually unchanged. The slightly greater potency for the alleged HK654 in Fig. 2 is due to this difference, which resulted in testing a slightly more concentrated solution of HK434. Since discovering the problem, authentic samples of HK654 and HK602 have been synthesized and tested. They showed a nearly 1000-fold reduction in binding affinity towards PKCα. The correct structures appeared in Choi et al. (Choi, Y., Kang, J. H., Lewin, N. E., Blumberg, P. M., Lee, J., and Marquez, V. E. (2003) J. Med. Chem. 46, 2790–2793). HK434 has the attributes of isozyme specificity and apoptotic-inducing activity originally associated with the N-hydroxylamide, and therefore the main conclusions of our paper remain unchanged.

Diacylglycerols containing omega 3 and omega 6 fatty acids bind to RasGRP and modulate MAP kinase activation.

Sihem Madani, Aziz Hichami, Mustapha Cherkaoui-Malki, and Naim A. Khan

Page 1176: Dr. Cherkaoui-Malki’s name was misspelled in this article. The correct spelling is shown above.
Diacylglycerol (DAG)-lactones, a New Class of Protein Kinase C (PKC) Agonists, Induce Apoptosis in LNCaP Prostate Cancer Cells by Selective Activation of PKC α

Maria Laura Garcia-Bermejo, Federico Coluccio Leskow, Teruhiko Fujii, Qiming Wang, Peter M. Blumberg, Motoi Ohba, Toshio Kuroki, Kee-Chung Han, Jeewoo Lee, Victor E. Marquez and Marcelo G. Kazanietz

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