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, HK434 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 PKCβ. Consistent with the differences in isoform dependence in intact cells, PMA and HK654 showed 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 PKCβ 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.

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), 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 isozymes 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” (“αPKCs” ζ and δ). A related enzyme, PKCμ or protein kinase D, is a distant relative of the PKC isoforms. Although αPKCs 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 cancer cells

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The abbreviations used are: PKC, protein kinase C; DAG, diacylglycerol; PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-dibutyrate; OAG, 1-oleoyl-2-acetyl-sn-glycerol; DAFI*, 4',6-diamidino-2-phenylindole; cPKC, classical PKC; nPKC, novel PKC; αPKC, atypical PKC; AdV, adenovirus; m.o.i., multiplicity of infection; pfu, plaque-forming unit(s); DN, dominant negative; PBS, 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 PKCδ in LNCaP cells markedly potentiates phorbol 12-myristate 13-acetate (PMA)-induced apoptosis, suggesting a role for PKCδ in the phorbol ester effect. Likewise, expression of a dominant negative (kinase-inactive) form of PKCδ 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). 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 PKCδ in complex with phorbol-13-acetate. Remarkably, the DAG-lactones show 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 epithelial 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, Go6976, 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-Diamidino-2-phenylindole (DAPI) was purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA). The pan-caspase inhibitor z-VAD from BD Biosciences (San Diego, CA) was purchased from Sigma. Cell culture reagents and media were purchased from Life Technologies, Inc. (Carlsbad, CA). streptomycin at 37 °C.

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 PKCαAdV and PKCδAdV 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 higher than 1 × 1010 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 AdVs 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).

DAG-lactones, PMA, or vehicle (ethanol) were added for 1 h at different concentrations to either noninfected cells or to cells infected with different AdVs. After treatment, cells were grown in RPMI 1640 supplemented with 10% FBS for 24 h. PKC inhibitors (GF 109203X, Go6976, or rottlerin) or the pan-caspase inhibitor z-VAD were added 1 h before and during DAG-lactone or PMA treatment.

Western Blot Analysis—Cells were harvested and lysed in a buffer containing aprotinin, leupeptin, and PMSF. For live cell imaging, the Biopsite Focal Chamber System (FCS2) was inverted and attached to the microscope stage with a custom stage adapter. The cells cultured on a 40-mm round coverslip were introduced into the chamber system, which was connected to a temperature controller set at 37 °C, and medium was perfused through the chamber with a model P720 microperfusion pump (Instech, Plymouth Meeting, PA). As indicated, the perfusate to the chamber was changed to that containing the specified ligand for PKC and the same cell were then collected at 1-min intervals using LaserSharp software through a Bio-Rad MRC 1024 confocal scan head mounted on a Nikon Optiphot microscope with a 60× planapochromat lens. Excitation at 488 nm was provided by a krypton-argon gas laser with a 522/32 emission filter for green fluorescence.

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 mg/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. 27. 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 Ki for the competing ligand was calculated from the ID50 by using the relationship Ki = ID50/1 + L/Ki, where Ki is the dissociation constant for [3H]PDBu and L is the concentration of free [3H]PDBu at the ID50. PKCα and PKCδ...
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 $[gamma]^{32}$P]ATP into a specific PKC substrate (a pseudosubstrate peptide), as described previously (30), using 100 μg/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 successfully 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 PKC6 C1β domain, it was possible to rationally design structurally simple DAG-lactone ligands that displayed binding affinities for PKCα 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 PKCα as a receptor.

A group of 29 DAG-lactones that exhibited high binding affinities for PKCα (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 PKCα expressed in terms of $K_i$ values covered a wide range: 2–6 nM (10 compounds), 6–9 nM (9 compounds), 9–13 nM (6 compounds), 12–20 nM (4 compounds), 20–40 nM (2 compounds), and $>40$ nM (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 μM) 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 μM) 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 DAG-lactones in LNCaP prostate cancer cells. GF 109203X (5 μM) also blocked the apopotic 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-G$_0$/G$_1$ DNA content after HK434, HK654, or PMA treatment.

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 GF 109203X. Treatment with GF 109203X 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 PKCι. Very low levels of PKCε and PKCθ 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). Because the only cPKC present in LNCaP cells is PKCα, Go6976 represents a PKCα-selective inhibitor in our model. Fig. 5A shows that Go6976 inhibits the apoptotic effect of HK654 or PMA in a dose-dependent fashion. Go6976 did not affect the basal levels of apoptosis in LNCaP cells. Interestingly, Go6976 did not fully inhibit the apoptotic effect of PMA. In fact, the inhibitory effect of Go6976 on PMA-induced apoptosis seems to plateau at ~50% at 10–30 μM, whereas further inhibition by Go6976 was observed for HK654. This partial inhibition of PMA-induced apoptosis suggests that PKCα is not the only PKC isozyme that mediates the phorbol ester effect. Remarkably, the PKCδ inhibitor rottlerin partially blocked PMA-induced apoptosis but did not affect the apoptotic effect of HK654 (Fig. 5B). Treatment of rottlerin alone induces ~5% of apoptosis at the highest concentration tested. These experiments using PKC inhibitors suggest that, although PMA induces apoptosis through activation of PKCα and PKCδ, only PKCα is involved in the pro-apoptotic effect of the DAG-lactones.

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 isozyme 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/H9254 overexpression markedly potentiated the apoptotic effect of PMA (Fig. 6A). The effect was proportional to the level of expression of PKC/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/H9251AdV (m.o.i./11005 – 30 pfu/cell). In this later case, the potentiation of the PMA effect also correlates with the expression levels of PKC/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/H9251 using the PKC/H9251AdV markedly potentiated the apoptotic effect of HK654. However, infection of LNCaP cells with PKCAdV did not produce any significant effect on HK654-induced apoptosis. As expected, infection with the LacZAdV was also without effect (Fig. 6B). As shown previously (17), infection with PKCAdV, PKCAdV or LacZAdV (m.o.i. = 1–30 pfu/cell) induces only <2% of apoptosis in the absence of a PKC activator (data not shown). In agreement with the data using specific PKC inhibitors, these results suggest that PMA and DAG-lactones activate a different subset of PKC isozymes to promote apoptosis in LNCaP cells.

Effect of Dominant Negative PKCa (DN-PKCa) and Dominant Negative PKC8 (DN-PKC8) Mutants on HK654- and PMA-induced Apoptosis—To further explore the role of PKCa and PKC8 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 PKC8 mutants in which an Arg to Lys mutation was introduced in the ATP-binding site (DN-PKCaAdV and DN-PKC8AdV) (26, 27). These mutants are kinase-inactive after expression in

FIG. 3. Inhibition of DAG-lactone-induced apoptosis by a caspase inhibitor and Bcl-2 overexpression. Panel A, LNCaP cells were treated with 100 nM PMA (open bars), 10 μM HK654 (solid bars), or vehicle (hatched bars) in the absence or presence of the pan-caspase inhibitor z-VAD (50 μM) added 1 h before and during treatment. Panel B, LNCaP cells overexpressing Bcl-2 (LNCaP-Neo/Bcl-2) or mock-transfected cells (LNCaP-Neo) were treated with 100 nM PMA (open bars), 10 μM HK654 (solid bars), or vehicle (hatched bars). Cells were collected 24 h later and stained with DAPI. 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.

FIG. 4. Effect of the PKC inhibitor GF 109203X. LNCaP cells were treated with vehicle, 100 nM PMA, 10 μM HK654, or 10 μM HK434, either in the absence or presence of 5 μM GF 109203X, which was present 1 h before and during the treatment with DAG-lactones or PMA. Panel A, cells were collected 24 h after treatment and stained with DAPI. 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. Panel B, after staining with propidium iodide, DNA content was analyzed by flow cytometry. A representative experiment is shown. Similar results were observed in two additional experiments. Cc, control (vehicle); GF, GF 109203X.
LNCaP cells (17). Expression of DN-PKCs after infection with either DN-PKCαAdV or DN-PKCδAdV can be readily detected by Western blot using specific anti-PKCα or anti-PKCδ antibodies, respectively (Fig. 7C). The data in Fig. 7A show that infection of LNCaP cells with increasing m.o.i. values of DN-PKCαAdV inhibits the apoptotic effect of both PMA and HK654. On the other hand, whereas expression of DN-PKCδ inhibits the apoptotic effect of PMA by ~30%, it does not affect the apoptotic effect of HK654. Taken together, these findings strongly suggest that PKCα, but not PKCδ, 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 ID₅₀, 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 PKCδ (30). Fig. 9 shows that HK654 activates PKCα and PKCδ with similar potency. The maximum activation observed with HK654 was similar to that observed with 1 μM PMA (a concentration that induces maximum activation in our experimental
conditions), suggesting that HK654 fully activates PKCα and PKCδ. Therefore, the isozyme-specific effects of HK654 in LNCaP cells cannot be explained by a differential intrinsic ability to bind and/or activate discrete PKC isozymes.

Subcellular Distribution of PKCα and PKCδ in LNCaP Cells—Experiments using subcellular fractionation by ultracentrifugation show that HK654, like PMA, translocates PKCδ from the cytosol (cytosolic) to particulate (membrane) fraction in LNCaP cells (data not shown). An attractive hypothesis is that the differential activation of PKC isozymes 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 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 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
DAG-lactones Induce Apoptosis in LNCaP Cells

A

GFP-PKCα

PMA

HK654

B

C

D

GFP-PKCδ

PMA

HK654

E

F
careful quantitative analysis revealed that this effect was negligible if compared with the intensity of nuclear membrane translocation (Fig. 10F). Therefore, significant differences in the pattern of translocation of PKCδ in LNCaP cells were observed for HK654 and PMA.

**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 a-alkylidene chains in these minimal structures, a strategy that optimized the interactions of these compounds with 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 PKCδ, 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 G1/M arrest (37, 38). On the other hand, PKCδ enhances anchorage-independent growth and metastatic potential in mammalian adenocarcinoma cells (39). Different roles for PKCδ in proliferation and apoptosis have also been described in several cell types. Early work using overexpression strategies suggested that PKCδ promotes cell growth in fibroblasts (44). PKCδ also has a protective effect against apoptosis after interleukin-3 withdrawal in 32D myeloid progenitor cells (45). Furthermore, inhibition of PKCδ function is sufficient to trigger cell death in Ramos-3BL B cells (46), glioma cells (47), and COS cells (48). Anti-apoptotic effects have also been ascribed to other PKC isoforms, including PKCε and the atypical PKCs (49, 50). Despite the growth-promoting effect described for PKCα, 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 PKCα 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 PKCa and PKCδ are translocated to the plasma membrane by PMA. PKCα is translocated to the plasma membrane after HK654 treatment, but PKCδ is primarily redistributed to the nuclear membrane by the DAG-lactone. Although we have observed some PKCδ 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 PKCδ is normally associated to membranes in unstimulated cells (data not shown), we cannot rule out that this small pool of PKCδ 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 PKCs and the activation of the Raf/mitogen-activated protein kinase pathway.

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**FIG. 10. Translocation of PKC isoforms by DAG-lactones.** Representative fluorescent images of LNCaP cells expressing GFP-PKCα (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-PKCα 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-PKCα) 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 (Im − Icyt)∕Inuc, where Im represents the mean fluorescent intensity on the plasma or nuclear membrane in a given area and Icyt 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.
(18). Recent findings from our laboratory suggest that PKC also regulates Akt phosphorylation in LNCaP cells.2 Li et al. (25) have reported that translocation of PKCδ 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 between PKC isoforms in ligand binding and kinase assays (Figs. 8 and 9) but shows PKCα selectivity in translocation and apoptosis in LNCaP cells. Therefore, in vitro assays may underestimate the potential selectivity of PKC ligands in cells. Differences in in vitro binding affinities of PKC ligands for PKC isoforms and other phorbol ester receptors are normally very small (30, 33). Although in some cases this may reflect the high degree of homology between the C1 domains of individual PKCs, one cannot rule out that this may be because of the saturated amount of phospholipid (phosphatidylserine) cofactor used in in vitro binding and kinase assays, a condition that does not necessarily reflect the physiological interactions that occur in a membrane environment. In support of this concept, 12-deoxyphorbol esters, a family of analogs with anti-tumor promoter activity in the mouse skin, showed marked differences in the pattern of translocation of cPKCs and nPKCs in mouse keratinocytes (55), despite the similar potency for binding to PKC isoforms in in vitro assays (30). Likewise, we have illustrated in several systems that the structure-activity relations for ligand binding to PKC (and related receptors) is dependent upon the lipid environment. As expected from the principles of pharmacology, under phospholipid conditions differentially limiting for one receptor, apparent ligand affinity to this receptor is differentially impaired (56, 57).

The studies of Wang et al. (29, 58) both provide a marked parallel with the current findings and suggest a mechanistic basis for hybrid agonists. These authors showed that the pattern of translocation of PKCδ in CHO-K1 cells differed markedly with the specific ligand: phorbol esters, brystostatin 1, or DAG-lactones. In that study, as here, the DAG-lactones failed to induce translocation of PKCδ to the plasma membrane. In contrast, PKCα in CHO-K1 cells translocated to the plasma membrane in response to phorbol esters, brystostatin 1 or DAG-lactones,2 again similar to the results reported here for the LNCaP cells and distinct DAG-lactones. We know for PKCδ 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 PKCδ differs from PKCα in its interaction with phospholipid membranes in vitro.4 Under conditions of sufficient calcium, PKCδ binds more weakly to phospholipids than does PKCα. Under limiting conditions, PKCδ 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 PKCδ localization remains to be determined, an unambiguous finding from our studies is that PKCδ 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 isoform may also lead to functional antagonism. Indeed, brystostatin 1 and 12-deoxyphorbol 13-phenylacetate, which translocate PKCδ 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 PKCδ, the only cPKC present in this model. It would be important to evaluate whether this isoform selectivity occurs in cell types other than LNCaP cells. Likewise, although PKCδ is the only cPKC expressed in numerous cell types, it will be necessary to evaluate whether the DAG-lactones retain selectivity for PKCδ in cells expressing other PKCs. 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 isoform selectivity and therefore help to overcome the limitations that exist in the study of isoform-specific functions in cellular models.

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4 Q. J. Wang and P. M. Blumberg, unpublished observations.

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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 García-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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