Involvement of Protein Kinase C δ (PKCδ) in Phorbol Ester-induced Apoptosis in LNCaP Prostate Cancer Cells

LACK OF PROTEOLYTIC CLEAVAGE OF PKCδ*

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Phorbol esters, the activators of protein kinase C (PKC), induce apoptosis in androgen-sensitive LNCaP prostate cancer cells. The role of individual PKC isoforms as mediators of this effect has not been thoroughly examined to date. To study the involvement of the novel isozyme PKCδ, we used a replication-deficient adenovirus (PKCδAdV), which allowed for a tightly controlled expression of PKCδ in LNCaP cells. A significant reduction in cell number was observed after infection of LNCaP cells with PKCδAdV. Overexpression of PKCδ markedly enhanced the apoptotic effect of phorbol 12-myristate 13-acetate in LNCaP cells. PKCδ-mediated apoptosis was substantially reduced by the pan-caspase inhibitor z-VAD and by Bel-2 overexpression. Importantly, and contrary to other cell types, PKCδ-mediated apoptosis does not involve its proteolytic cleavage by caspase-3, suggesting that allosteric activation of PKCδ is sufficient to trigger apoptosis in LNCaP cells. In addition, phorbol ester-induced apoptosis was blocked by a kinase-deficient mutant of PKCδ, supporting the concept that PKCδ plays an important role in the regulation of apoptotic cell death in LNCaP prostate cancer cells.

Phorbol esters exert a variety of effects in cellular systems that include proliferation, malignant transformation, differentiation, and cell death (1–5). The multiplicity of effects of phorbol esters on biological systems is associated with the existence of various phorbol ester receptors, which include several PKC isoforms and novel non-kinase receptors (α- and β-chimaerins, unc-13, and Ras-GRP). The PKC family comprises at least 10 serine-threonine-kinases subject to different biochemical regulation (5, 6). PKC isoforms can be classified into three groups: calcium-dependent or "classical" (PKCs α, β1, β2, and γ), calcium-independent or "novel" (PKCs δ, ε, η, and θ), and "atypical" (PKCs ζ and ι/λ). Only the classical and novel PKC isoforms are receptors for phorbol esters and the lipid second messenger diacylglycerol.

The existence of several PKC isoforms with unique cofactor requirements, intracellular localization, and cellular/tissue distribution suggests specialized roles for each isozyme in the control of cellular functions. An issue of relevance is that individual PKC isoforms may exhibit either similar or opposite biological effects. In NIH 3T3 fibroblasts, for example, PKCδ and PKCε promote opposite effects on proliferation: whereas PKCε induces cell proliferation and malignant transformation, PKCδ inhibits cell growth (7). A second level of complexity involves the host cell: overexpression of PKCδ induces cell growth arrest in G0/G1 in Chinese hamster ovary cells and HL60 cells (8–10), but it markedly enhances anchorage-independent growth and metastatic potential in mammary adenocarcinoma cells (11). Interestingly, and of particular relevance to the present study, PKCδ induces apoptosis after DNA damage in hemopoietic cells and keratinocytes, a process that involves its proteolytic cleavage and generation of an active catalytic fragment (12, 13).

The aim of this study was to explore the role of PKCδ as a mediator of phorbol ester-mediated responses in LNCaP cells, a widely used model of androgen-dependent prostate cancer (14). Notably, phorbol esters induce apoptosis in LNCaP cells (15, 16), an effect that is also observed in thymocytes and breast cancer cells (17–19). Phorbol ester-induced apoptosis in LNCaP cells may involve the lipid second messenger ceramide (20). Powell et al. (16) reported a persistent translocation of PKCα in phorbol ester-induced apoptosis. The contribution of other PKC isoforms present in LNCaP cells as mediators of phorbol ester-mediated apoptosis, however, has not been examined to date.

To evaluate the involvement of PKCδ in phorbol ester-induced apoptosis, we used a replication-deficient adenovirus for this novel PKC isozyme (PKCδAdV). The evidence presented in this study implicates PKCδ as a mediator of phorbol ester-induced apoptosis in LNCaP cells. Interestingly, and contrary to observations in other cell types, PKCδ-mediated apoptosis in LNCaP cells does not involve its proteolytic activation by caspase-3, suggesting that allosteric activation of the enzyme is sufficient to trigger an apoptotic response in these cells.

EXPERIMENTAL PROCEDURES

Cell Culture—The human prostate cancer cell line LNCaP and U-937 human promonocytic leukemia cells were obtained from the American Type Culture Collection (Manassas, VA). LNCaP and U-937 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin (100 units/ml)-streptomycin (100 μg/ml) at 37 °C.
in a humidified 5% CO₂ atmosphere. LNCaP cells stably overexpressing Bel-2 and the corresponding vector-transfected cells (21) were a kind gift of Dr. L. Lothstein (University of Tennessee, Memphis, TN).

**PKCδ and Dominant Negative PKCδ Adenoviruses—**A XhoI-MluI fragment comprising the full-length PKCδ DNA (7) was inserted into pCA4-FLAG, a modified version of the vector pCA4 (Microbix Systems Inc., Toronto, Ontario, Canada). pCA4-FLAG includes XhoI-MluI sites for subcloning, followed by a C-terminal FLAG tag and a stop codon in frame (22). A replication-deficient adenovirus for PKCδ (PKCδAdV) was generated by standard techniques using 293 packaging cells (22, 23). Recombinant adenoviruses were isolated from single plaques and amplified in 293 cells. Titer of viral stocks were normally higher than 1 × 10⁹ pfu/cell. The absence of wild type adenovirus was confirmed by polymerase chain reaction using primers for the E1 region. An adenovirus for the LacZ gene was used as a control (22, 24).

Generation of a dominant negative PKCδ adenovirus (DN-PKCδAdV) is described elsewhere (25).

**Infection of LNCaP Cells with PKCδAdV—**Subconfluent LNCaP cells in six-well plates were infected with PKCδAdV for 14 h at different multiplicities of infection (MOIs) (1–300 pfu/cell) in RPMI 1640 medium supplemented with 2% fetal bovine serum. After removal of the virus, cells were incubated for an additional 24 h in RPMI 1640 medium supplemented with 10% fetal bovine serum. In several experiments, PMA at different concentrations (0.3–100 nM) or vehicle (ethanol) was added for 1 h following the adenoviral infection, and cells were then grown in RPMI 1640 medium supplemented with 10% fetal bovine serum for different times. For determination of cell number, cells were harvested by trypsinization (0.25% trypsin and 1 mM EDTA in Hanks’ balanced salt solution) and counted in a hemocytometer.

**PKC Activity—**PKC activity in total cellular lysates was determined by phosphorylation of the α-pseudosubstrate peptide, as described previously by Kazanietz et al. (26). Briefly, cells in six-well plates were lysed in 150 μL of lysis buffer (50 mM Tris-HCl, pH 7.4, and Complete™ protease inhibitor mixture tablets; Roche Molecular Biochemicals). The reaction was carried out in a total volume of 50 μL containing 50 μM Tris-HCl, pH 7.4, 250 μg/mL bovine serum albumin, 1 mM EGTA, 100 μg/mL phosphatidylserine, 100 mM PMA, 10 mM α-pseudosubstrate peptide, 25 mM ATP, and 7.5 mM magnesium acetate. After incubation at 30 °C for 5 min, 25 μL of each reaction was spotted onto Whatman PE-81 paper. The paper was washed three times with 0.1 M phosphoric acid and once with acetone and air-dried, and radioactivity was counted in a scintillation counter.

**[3H]PDBu Binding—**[3H]PDBu binding in cellular lysates was measured using the polyethylene glycol precipitation assay described by Sharkey and Blumberg (27). Results in A and C are expressed as mean ± S.E. of three independent experiments.

![Fig. 1](https://example.com/figure1.png)

**Fig. 1. Expression of PKCδ in PKCδAdV-infected LNCaP cells.** Cells were infected with PKCδAdV for 14 h at different MOIs, as indicated in the figure. A, cells lysates were analyzed by Western blot using anti-FLAG or anti-PKCδ antibody. Similar results were obtained in two additional experiments. B, PKC activity was measured in lysates of LNCaP cells, 24 h after infection with PKCδAdV or LacZAdV. Kinase activity was measured by phosphorylation of α-pseudosubstrate peptide as described under *Experimental Procedures.* Almost identical results were observed in two additional experiments. C, [3H]PDBu binding was determined in lysates of LNCaP cells infected with PKCδAdV or LacZAdV for 24 h, using the MOIs indicated in the figure. [3H]PDBu binding was measured by the polyethylene glycol precipitation assay described by Sharkey and Blumberg (27). Results in B and C are expressed as mean ± S.E. of three independent experiments.
microscopy. Apoptosis was characterized by chromat condensation and fragmentation. The incidence of apoptosis in each preparation was analyzed by counting 500 cells and determining the percentage of apoptotic cells.

For flow cytometry analysis, cells were fixed in 70% ethanol and stained with propidium iodide (1 mg/ml). Cell cycle progression and apoptosis was analyzed in an EPICS XL flow cytometer (Coulter Corp., Hialeah, FL). For each treatment 7500 events were recorded.

Caspase-3 activity was measured with the colorimetric assay kit from BioVision (Palo Alto, CA) that uses as a substrate the chromophore p-nitroanilide-DEVD.

RESULTS

Overexpression of PKCδ in LNCaP Cells after Infection with PKCδAdV—A recombinant adenovirus for PKCδ, PKCδAdV, was generated as described under "Experimental Procedures." The recombinant PKCδ was engineered to have a FLAG epitope-tag in frame at the C-terminal end. Infection of LNCaP cells with PKCδAdV at different MOIs resulted in a concentration-dependent expression of PKCδ, as determined by Western blotting with either anti-FLAG (Fig. 1A, top panel) or anti-PKCδ antibodies (Fig. 1A, bottom panel). Immunofluorescence using the anti-FLAG antibody reveals an efficiency of infection higher than 90% at a MOI of 10 pfu/cell (data not shown). Expression of FLAG-tagged PKCδ was detected 6 h after infection. Maximum levels of expression were obtained after 24 h and lasted for at least 7 days (data not shown).

Infection of LNCaP cells with PKCδAdV (MOI = 1–100 pfu/cell) resulted in a concentration-dependent increase in PKC activity in cell extracts (Fig. 1B), as determined by phosphorylation of α-peptide, a specific PKC substrate. The minimum MOI necessary to achieve a significant increase in total PKC activity in these cells was 10 pfu/cell. No significant changes in PKC activity were observed when LNCaP cells were infected with a LacZAdV (MOI = 100 pfu/cell). Next, we measured whether infection of LNCaP cells with PKCδAdV increases the level of phorbol ester binding, using [3H]PDBu as a radioligand. Elevated levels of phorbol ester binding were observed in cells infected with PKCδAdV but not in those infected with LacZAdV (Fig. 1C). LNCaP cells infected with LacZAdV did not show any changes in endogenous PKCδ immunoreactivity but did show high levels of LacZ expression as detected with X-Gal staining (data not shown). For most of the experiments presented here, we have used MOI of the range of 10–30 pfu/cell to avoid any potential nonspecific effects that might occur when PKCδ is overexpressed at high levels. At MOI = 10–30 pfu/cell, total cellular [3H]PDBu binding activity was elevated 3–8-fold over basal levels, respectively (see Fig. 1C).

It is well established that phorbol esters induce the redistribution of PKC isozymes from cytosolic to particulate fractions, a process known as PKC translocation. To confirm that the FLAG-tagged PKCδ was responsive to phorbol esters, we infected LNCaP cells with PKCδAdV (MOI = 10 pfu/cell) and subsequently incubated the cells with PMA for different times (Fig. 2). After extensive washing to remove the phorbol esters, cells were cultured for 24–72 h and then counted. Results are the mean ± S.E. of three independent experiments.

As expected, the phorbol ester-unresponsive PKCz is not translocated by PMA in LNCaP cells. From these data, we conclude that infection of LNCaP prostate cancer cells with PKCδAdV results in an efficient expression of a catalytically active, phorbol ester-responsive PKCδ.
Activation of PKCδ Induces Apoptosis in LNCaP Cells—Incubation of LNCaP cells with PMA (0.3–100 nM, 1 h) results in a concentration-dependent decrease in cell number compared with control (vehicle-treated) cells, as judged by cell counting at 24, 48, and 72 h after PMA treatment. The corresponding inactive isomer, 4α-PMA (100 nM), was completely ineffective (Fig. 3A). We then infected LNCaP cells with PKCδAdV at MOIs ranging from 0.3 to 100 pfu/cell and determined the cell number every 24 h for a total period of 72 h. A concentration-dependent reduction in cell number was observed after infection with PKCδAdV but not with LacZAdV (MOI = 30 pfu/cell) (Fig. 3B). The inhibitory effect of PMA was markedly enhanced in cells infected with PKCδAdV, suggesting that activation of PKCδ results in a decrease in cell count (Fig. 3C).

Morphological examination of LNCaP cells infected with PKCδAdV after PMA treatment revealed a large number of cells with characteristics distinctive of apoptosis, including cellular shrinkage and nuclear fragmentation. No signs of apoptosis were observed in either PKCδAdV- or LacZAdV-infected cells in the absence of PMA treatment (Fig. 4A). Treatment of LNCaP cells with PMA induces a pattern of DNA fragmentation characteristic of apoptosis, which can be visualized as a DNA ladder. DNA fragmentation was significantly higher in cells infected with PKCδAdV (MOI = 30 pfu/cell) after PMA treatment (Fig. 4B). PMA treatment of PKCδAdV-infected cells also results in a large number of TUNEL-positive cells (data not shown). Flow cytometry analysis of PKCδAdV-infected cells after PMA treatment revealed a large number of cells with sub-G0/G1 DNA content, consistent with the presence of apoptotic cells. The PMA effect was lower in LNCaP cells infected with LacZAdV compared with PKCδAdV-infected cells (Fig. 4C) or noninfected cells (data not shown). A dose-response

**Fig. 4.** Overexpression of PKCδ potentiates PMA-induced apoptosis. LNCaP cells were infected with either PKCδAdV or LacZAdV (MOI = 30 pfu/cell) for 14 h. Twenty-four hours later, cells were treated with either PMA (10 nM) or vehicle (ethanol) for 1 h. Apoptosis was evaluated 48 h later. A, LNCaP cells were stained with DAPI and assessed for nuclear morphology by fluorescence microscope. Apoptotic cells are indicated with arrows. Similar results were observed in three experiments. B, DNA fragmentation was monitored by electrophoresis in 2% agarose gels after staining with ethidium bromide. Lane 1, LacZAdV; lane 2, PKCδAdV; lane 3, LacZAdV + PMA; lane 4, PKCδAdV + PMA. Ms, molecular size. C, after staining with propidium iodide, DNA content was analyzed by flow cytometry. The DNA histograms show a marked accumulation of PKCδAdV-infected cells in sub-G0/G1, after PMA treatment. A representative experiment is shown. Similar results were observed in two additional experiments.

**Fig. 5.** Concentration dependence analysis of PMA-induced apoptosis in LNCaP cells. Cells were infected with PKCδAdV or LacZAdV for 14 h (MOI = 30 pfu/cell), and 24 h later, PMA was added for 1 h at different concentrations. Cells were collected 48 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.
analysis for the apoptotic effect of PMA is shown in Fig. 5. Cells were infected with either PKCδAdV or LacZAdV (MOI = 30 pfu/cell) and treated with increasing concentrations of PMA (0.1–10 nM, 1 h). The results shown in Fig. 5 revealed a markedly higher apoptotic effect of PMA in LNCaP cells infected with PKCδAdV than in noninfected cells or cells infected with LacZAdV. These findings collectively suggest that activation of PKCδ induces apoptosis in LNCaP cells.

Expression of PKC Isozymes in LNCaP Cells infected with PKCδAdV—LNCaP cells express the classical PKCα, the novel PKCδ and PKCe, the atypical PKCζ and PKCl, and PKCμ (Fig. 6). Low levels of PKCη were also observed (data not shown). PKCβ, PKCy, and PKCθ were not detected in LNCaP cells. These results agree with those previously reported by Powell et al. (16). Infection of LNCaP cells with PKCδAdV did not result in any significant change in the levels of PKCa, PKCe, PKCζ, PKCl, or PKCμ. Only a reduction in the levels of PKCε was observed after PMA treatment, both in LacZAdV and PKCδAdV-infected cells.

Effect of PKC Inhibitors, z-VAD, and Bcl-2 Overexpression—Pretreatment of LNCaP cells with the PKC inhibitor GF 109203X (bisindolylmaleimide I, 5 μM) completely blocked PMA-induced apoptosis in both noninfected and PKCδAdV-infected cells. We also used an unrelated antagonist of PKC, bryostatin I. Bryostatin I is an atypical inhibitor of PKC function: although it activates PKC isozymes in vitro and promotes few PKC-dependent effects, it fails to activate PKC-mediated responses in most cases. In those instances in which bryostatin 1 is unable to activate PKC, it blocks those responses mediated by PMA (1, 29). In LNCaP cells, bryostatin 1 (10 nM) was ineffective in inducing apoptosis. However, bryostatin 1 inhibited PMA-induced apoptosis in both noninfected and in PKCδAdV-infected cells (Fig. 7A).

It is well established that caspases, a family of Asp-directed cysteine-proteases, play a pivotal role in transducing the apoptotic signal. Caspases can be specifically inhibited in cells by cell-permeable peptides (30). Treatment of LNCaP cells with the pan-caspase inhibitory peptide z-VAD markedly reduced PMA-induced apoptosis in PKCδAdV-infected cells (Fig. 7B).

Bcl-2 plays a central role in cell death signaling by forming heterodimers with pro-apoptotic proteins Bax and Bad and inhibiting their function (31). In LNCaP cells, the apoptotic effect mediated by activation of PKCδ did not result in any noticeable changes in Bcl-2 levels (Fig. 7C). The relationship
PKCδ-mediated apoptosis in LNCaP cells does not involve the generation of a catalytic fragment. Cells were infected with PKCδAdV (MOI = 30 pfu/cell) for 14 h; 24 h later, they were treated with vehicle or 100 nM PMA (1 h), and samples were collected at the times indicated in the figure. Mw, molecular weight. A, cell lysates were subjected to Western blot analysis using an anti-FLAG or an anti-PKCδ antibody. B, incidence of apoptosis in cells infected with PKCδAdV and treated with PMA or vehicle. C, caspase-3 activity in cell lysates, evaluated with a colorimetric assay that uses as a substrate the chromophore p-nitroanilide-DEVD (see under “Experimental Procedures”). D, cell lysates were subjected to Western blot analysis with an anti-caspase-3 antibody. In C and D, U-937 cells treated with camptothecin (Cm) were used as positive controls.

PKCδ-mediated apoptosis and Bcl-2 was further explored in LNCaP cells stably transfected with a Bcl-2 mammalian expression vector (LNCaP-Bcl-2), which express higher levels of Bcl-2 compared with control (vector-transfected) cells (21). Cells overexpressing Bcl-2 showed a marked resistance to PMA induced apoptosis when infected with PKCδAdV compared with control cells (Fig. 7D). To rule out the possibility that this effect is a consequence of differential sensitivity to PKCδAdV infection, we determined PKC activity in control and Bcl-2-overexpressing cells. Similar levels of Bcl-2 were observed in both cases after infection with PKCδAdV or PMA, suggesting a similar sensitivity to adenoviral infection in both cell lines.

PKCδ-mediated Apoptosis Does Not Involve Its Proteolytic Cleavage—An emerging model postulates that activation of caspases leads to the proteolytic cleavage of kinases, which are either activated or inactivated during the apoptotic process. Recent studies have shown that PKCδ is proteolytically activated at the onset of apoptosis induced by DNA-damaging agents in hematopoietic cells and keratinocytes (12, 13). Proteolytic cleavage of PKCδ by caspase-3 at the V3 (hinge) domain of the enzyme releases a catalytically active fragment of approximately 40 kDa. In order to evaluate whether such cleavage occurs in LNCaP cells, cells were infected with PKCδAdV and then treated with PMA or vehicle. The presence of a catalytic fragment was evaluated by Western blot using either an anti-FLAG or an anti-PKCδ antibody in samples collected at different times, ranging from 15 min to 24 h. Either of these antibodies recognizes the C-terminal kinase domain of FLAG-tagged PKCδ. Interestingly, we could not detect the presence of
PKCδ catalytic fragment either at short times or after several hours of PMA treatment (Fig. 8A). Very low levels of cleavage were detected 24 h after PMA treatment. Fig. 8B revealed, however, a significant apoptotic response after 6 and 12 h of PMA treatment that reached a maximum at 24 h. Thus, PKCδ-mediated apoptosis after PMA treatment does not correlate with the generation of a catalytically active fragment of the enzyme. These results prompt us to evaluate whether caspase-3 is activated during PMA-induced apoptosis in PKCδAdV-infected cells. Fig. 8C shows that no significant changes in caspase-3 activity were detected after PMA treatment. Likewise, we did not detect any cleavage of caspase-3 under similar experimental conditions (Fig. 8D). The unexpected lack of involvement of caspase-3 was confirmed by the inefficacy of a specific caspase-3 inhibitor, DEVD, to block PMA-induced apoptosis in LNCaP cells infected with PKCδAdV (Table I). U-937 cells treated with the DNA-damaging agent camptothecin (4 μM) were used as positive controls for these experiments. In this case, a marked increase in caspase-3 activity (Fig. 8C) and caspase-3 cleavage (Fig. 8D) was observed, and DEVD markedly inhibited apoptosis (Table I).

**Inhibition of PMA-induced Apoptosis by a PKCδ Inhibitor and a Dominant Negative PKCδ Mutant—**The data presented above strongly implicate PKCδ as a mediator of PMA-induced apoptosis in LNCaP cells. To further explore the role of PKCδ, we decided to evaluate the effect of rottlerin, a selective PKCδ inhibitor, on PMA-induced apoptosis. Rottlerin preferentially inhibits PKCδ over PKCa or PKCε (32). Pretreatment of LNCaP cells with rottlerin partially inhibited PMA-induced apoptosis in noninfected cells LNCaP cells (Table I).

The involvement of PKCδ was further confirmed by introducing a kinase-deficient mutant of PKCδ in LNCaP cells. Kinase-deficient mutants of PKCδ were shown to act as dominant negatives that interfere with PKCδ function. We used an adenovirus for a PKCδ mutant in which an Arg to Lys mutation was introduced in the ATP-binding site (DN-PKCδAdV). Infection of LNCaP cells with DN-PKCδAdV results in the expression of a catalytically inactive PKCδ (Fig. 9A). Interestingly, expression of the kinase-deficient PKCδ blocks the apoptotic response of PMA (100 nM) in LNCaP cells. Infection with DN-PKCδAdV at MOIs of 30 and 100 pfu/cell resulted in 50 and 61% inhibition, respectively, of PMA-induced apoptosis (Fig. 9B).

**TABLE I**

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</tr>
<tr>
<td>No inhibitor</td>
<td>30.0 ± 1.8 (100)</td>
</tr>
<tr>
<td>+ DEVD</td>
<td>28.9 ± 0.5 (96)</td>
</tr>
<tr>
<td>+ z-VAD</td>
<td>4.5 ± 0.3 (15)</td>
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<td>%</td>
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**TABLE II**

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<th>Apoptotic cells</th>
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<tbody>
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**DISCUSSION**

To study the role of PKCδ as mediator of apoptotic responses in LNCaP cells, we overexpressed this novel PKC isoform by means of an adenoviral expression system. Adenovirus-mediated delivery of proteins has been successfully used in LNCaP cells (33, 34) and has proven to be an efficient approach at introducing PKC isoforms into cells (25). Infection of LNCaP cells with PKCδAdV allowed for a controlled expression of a catalytically active, phorbol ester-responsive PKCδ. Our results provide strong evidence that activation of PKCδ induces apoptosis in LNCaP cells.

The complexity in the regulation of PKCδ activity, which includes not only regulation by diacylglycerol but also by tyrosine phosphorylation and association to tyrosine kinases, suggests an important role for this PKC isoform in proliferation and cell death (35–38). Recent reports have implicated PKCδ as a pro-apoptotic kinase. Emoto et al. (12) have shown that exposure of U-937 cells to DNA-damaging agents results in proteolytic cleavage of PKCδ with the release of an active 40-kDa fragment corresponding to its C-terminal kinase domain. Denning et al. (13) have recently reported that in keratinocytes exposed to UV radiation, the generation of an active catalytic fragment correlates with the apoptotic event. A similar effect has been recently observed in etoposide-treated salivary gland acinar cells (39). A proteolytic cleavage site for caspase-3 has been identified at the V3 (hinge) region of PKCδ (12). Inhibition of caspases using peptide inhibitors blocks the proteolytic cleavage of PKCδ and apoptosis in U-937 cells and keratinocytes after DNA damage (12, 13). To our surprise, we found no evidence for PKCδ proteolytic cleavage after PMA activation in LNCaP cells. The unexpected lack of effect of a caspase-3 inhibitor (DEVD) to block PKCδ-mediated apoptosis and the absence of caspase-3 activation explains the lack of proteolytic cleavage for this PKC isoform. In agreement with our results, caspase-3-independent apoptosis has also been described in other systems, including human ovarian cancer cell lines treated with cisplatin (40), leukemic cells treated with arsenic trioxide (41) or tumor necrosis factor α (42), MCF-7 mammary carcinoma cells treated with tributyrin (43), and macrophages exposed to NO donors (44).

Caspase-3 is important for the typical morphologies associated with apoptosis and for the formation of apoptotic DNA ladders. In LNCaP cells, caspase-3 is a critical mediator of apoptosis induced by sodium phenylacetate (45). The present experiments have not addressed the mechanisms by which PKCδ-induced apoptosis bypasses caspase-3 activation. However, the inhibitory effect of a pan-caspase inhibitor strongly suggests the involvement of other caspases. Other alternative mechanisms, such as the recently reported association and phosphorylation of DNA-dependent kinases by PKCδ, may be implicated in PKCδ-dependent apoptosis (46). It was also shown that PKCδ-mediated apoptosis in keratinocytes involves the alteration of mitochondria function (22). Although studies in hematopoietic cells and keratinocytes have clearly shown that PKCδ is proteolytically activated at...
the onset of (12, 13), the issue of whether cleavage is required or not for apoptosis was still unanswered. Our results in LNCaP cells unquestionably show that cleavage of PKCθ is not required for apoptosis.

Activation of PKC by phorbol esters and diacylglycerol is associated with the translocation of the enzyme from the cytosolic to particulate fractions and stimulation of activity through an allosteric mechanism (6). As expected, in LNCaP cells PKCδ is redistributed to the particulate fraction after PMA treatment. Although prolonged translocation of PKC isoforms can be associated with their proteolytic cleavage by calpains and other proteases, removal of PMA in our experimental conditions results in a dissociation of PKCδ from the particulate fraction and return of the enzyme to the cytosol (data not shown), as previously shown by Blumberg and co-workers (47). The fact that apoptosis in LNCaP cells infected with PKCδAdV was only observed after phorbol ester treatment strongly suggests that allosteric activation of PKCδ is sufficient to induce apoptosis. Our results are consistent with recent studies presented by Chen et al. (48) that show that UVB radiation-induced apoptosis in JB6 epidermal cells requires translocation of PKCδ to the membrane. Interestingly, overexpression of a PKCδ catalytic fragment in HeLa and NIH 3T3 cells induced apoptosis, whereas overexpression of full-length PKCδ was ineffective (49). In the latter study, however, cells were not challenged with phorbol esters or any other stimuli. Therefore, it is likely that PKCδ-mediated apoptosis may proceed through two distinct mechanisms, namely proteolytic cleavage (after DNA damage) and allosteric activation (after direct activation). Each mechanism may involve different signaling pathways. Our results suggest that PKCδ may act as a primary effector or is involved in a pathway that signals for apoptosis.

Expression of a dominant negative PKCδ mutant inhibits PMA-induced apoptosis in LNCaP cells. It is tempting to speculate that the partial inhibition observed by expression of this dominant negative PKCδ mutant, as well as the partial inhibition observed by the PKCδ inhibitor rottlerin, reflects the involvement of other phorbol ester-responsive PKC isoforms in addition to PKCδ, namely PKCα and PKCε. In preliminary experiments using a PKCα adenovirus, we have found that this PKC isoform also mediates apoptosis in LNCaP cells. Therefore, more than one PKC isoform may signal to apoptosis in LNCaP cells. It will be important to use dominant negative forms of each PKC isoform to address this issue. Whether individual PKC isoforms promote apoptosis in LNCaP cells by similar or different mechanisms is not known. Powell et al. (16) observed a persistent membrane translocation of PKCα during PMA-induced apoptosis in LNCaP cells. Notably, a caspase cleavage site is not present in PKCα (12), which also supports a model of allosteric activation of PKC in phorbol ester-induced apoptosis. Evidence obtained from several cellular models suggests that PKC-mediated apoptosis is not restricted to PKCθ. PKCθ, an isoform highly homologous to PKCδ, mediates programmed cell death in U-937 myeloid leukemia cells in response to DNA damaging agents, an effect that involves its proteolytic cleavage (50). PKCβ is also proteolytically activated after treatment of HL60 human promyelocytic leukemia cells with the anticancer agents UCN-01, camptothecin, and etoposide. Interestingly, similar treatment results in activation of PKCα without proteolytic cleavage (51).

PKC isoforms may also signal to inhibit apoptosis (4, 53, 54). It is conceivable that the balance between expression and/or activation of different isoforms may result in either pro-apoptotic or anti-apoptotic signaling. The anti-apoptotic effect of PKC isoforms may involve the up-regulation of Bcl-2 levels (52, 55). However, we did not observe any significant changes in Bcl-2 levels after PKCδ overexpression in LNCaP cells. Our observations that overexpression of Bcl-2 in LNCaP cells prevents PKCδ-mediated apoptosis in LNCaP cells emphasizes a role for Bcl-2 as a pro-survival signal in prostate cancer cells, as described by Marcelli et al. (45). In summary, our results provide the first evidence that PKCδ is a mediator of phorbol ester-induced apoptosis in LNCaP cells. Clarifying specific biological functions of individual PKC isoforms in prostate cancer cells may underscore signaling pathways controlling cell growth or death and provide us with novel therapeutic targets for studying the progression of the disease.

REFERENCES
Involvement of Protein Kinase C δ (PKCδ) in Phorbol Ester-induced Apoptosis in LNCaP Prostate Cancer Cells: LACK OF PROTEOLYTIC CLEAVAGE OF PKC δ

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