Protein Kinase C Promotes Apoptosis in LNCaP Prostate Cancer Cells through Activation of p38 MAPK and Inhibition of the Akt Survival Pathway*

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Activation of protein kinase C (PKC) by phorbol esters or diacylglycerol mimetics induces apoptosis in androgen-dependent prostate cancer cells, an effect that involves both the activation of the classic PKCa and the novel PKCd isoforms (Fujii, T., Garcia-Bermejo, M. L., Bernabé, J. L., Caamaño, J., Obha, M., Kuroki, T., Li, L., Yuspa, S. H., and Kazanietz, M. G. (2000) J. Biol. Chem. 275, 7574–7582 and Garcia-Bermejo, M. L., Leskow, F. C., Fujii, T., Wang, Q., Blumberg, P. M., Obha, M., Kuroki, T., Han, K. C., Lee, J., Marquez, V. E., and Kazanietz, M. G. (2002) J. Biol. Chem. 277, 645–655). In the present study we explored the signaling events involved in this PKC-mediated effect, using the androgen-dependent LNCaP cell line as a model. Stimulation of PKC by phorbol 12-myristate 13-acetate (PMA) leads to the activation of ERK1/2, p38 MAPK, and JNK in LNCaP cells. Here we present evidence that p38 MAPK, but not JNK, mediates PKC-induced apoptosis. Because LNCaP cells have hyperactivated Akt function due to PTEN inactivation, we examined whether this survival pathway could be affected by PKC activation. Interestingly, activation of PKC leads to a rapid and reversible dephosphorylation of Akt, an effect that was prevented by the pan-PKC inhibitor GF109302X and the cPKC inhibitor Go6976. Inactivation of Akt function by PKC does not involve the inhibition of PI3K, and it is prevented by okadaic acid, suggesting the involvement of a phosphatase 2A in PMA-induced Akt dephosphorylation. Finally, we show that, when an activated form of Akt is delivered into LNCaP cells by either transient transfection or adenoviral infection, the apoptotic effect of PMA is significantly reduced. Our results highlight a complex array of signaling pathways regulated by PKC isoforms in LNCaP prostate cancer cells and suggest that both p38 MAPK and Akt play critical roles as downstream effectors of PKC isoforms in this cellular model.

Activation of protein kinase C (PKC) isoforms by phorbol esters and related agents induces a plethora of cellular responses, including changes in cell cycle progression, differentiation, survival, and transformation. PKC isoforms comprise a family of related serine-threonine kinases grouped on the basis of their structural and biochemical properties: “classical” calcium-dependent PKCs (“cPKCs” α, β, βI, and γ), “novel” or calcium-independent PKCs (“nPKCs” δ, ε, η, and θ) and “atypical” PKCs (“aPKCs” ζ and η/λ). Only the first two groups and the related PKC/α-protein kinase D are responsive to phorbol esters and to the second messenger diacylglycerol (DAG), the endogenous ligand for these PKCs (1–3). Phorbol ester treatment can either promote mitogenesis or inhibit cell proliferation depending on the cell type. Such heterogeneity is probably related to the multiplicity of cellular targets that mediate their responses, which include not only the PKC isoforms but also novel “non-kinase” phorbol ester receptors such as chimaerin, RasGRP isoforms, and Munc13s (4). Studies on the roles of individual phorbol ester receptors as mediators of mitogenic and survival responses have revealed a high degree of complexity in their downstream effectors. Indeed, within the PKC family some members are capable of stimulating mitogenesis, such as PKCε, whereas others such as PKCd are preferentially growth inhibitory in most cell types. An emerging theme is that this heterogeneity involves a delicate regulation of signaling pathways by individual PKC isoforms, which is probably related to a distinctive pattern of intracellular compartmentalization and access to targets (1, 2, 5–8).

Unlike most cell types, androgen-dependent prostate cancer cells undergo apoptosis in response to phorbol esters (9–11). The mechanisms underlying this atypical response are still poorly understood. Using multiple pharmacological and molecular approaches, we have previously demonstrated that both the classic PKCa and the novel PKCd mediate the apoptotic response of phorbol esters in LNCaP androgen-dependent prostate cancer cells. Although in some cell types the pro-apoptotic effect of PKCd involves its proteolytic cleavage and subsequent release of an active catalytic fragment, in LNCaP cells it de-

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† The abbreviations used are: PKC, protein kinase C; DAG, diacylglycerol; PMA, phorbol 12-myristate 13-acetate; DAPI, 4′,6-diamidino-2-phenylindole; cPKC, classic PKC; nPKC, novel PKC; aPKC, atypical PKC; AdV, adenovirus; m.o.i., multiplicity of infection; pfu, plaque-forming unit(s); FBS, fetal bovine serum; GFP, green fluorescent protein; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; PP2A, phosphatase 2a; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; MEK1, MAPK/ERK kinase 1; dsRNA, double-stranded RNA; PTEN, phosphatase and tensin homologue deleted on chromosome 10.
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PENDS, rather, on allosteric activation of the enzyme upon translocation to the plasma membrane (12, 13).

The functional dissection of mitogenic and survival signaling cascades regulated by PKCs has proved elusive due to the diversity of PKC targets and cell type differences. To date, very little information is available regarding the pathways that mediate the apoptotic effect of phorbol esters in prostate cancer cells. Among the potential effectors downstream of PKC in prostate cancer cells are the MAPK cascades. It is well established that PKC isoforms activate the ERK MAPK cascade, and evidence has pointed to a potential role for JNK and p38 MAPK cascades as PKC effectors in some cell lines (14–17). A critical role for the p38 MAPK cascade in apoptosis has been described in many cell types (18–20). The significance of each individual cascade and their relationship to PKC activation still remain to be elucidated in prostate cancer cells.

PI3K-Akt has emerged as a critical pathway for cell survival in prostate cancer cells. The PTEN tumor suppressor, a phosphatase for the lipid products of PI3K, is frequently mutated in prostate cancer, and this loss in PTEN function is associated with suppression of the PI3K-Akt pathway has surfaced as an important feature of prostate cancer cell survival, its involvement in PKC-mediated apoptosis remains to be established.

In this report we sought to investigate the signaling events regulated by PKC isoforms in LNCaP prostate cancer cells and their relationship to the apoptotic response of phorbol esters and DAG mimetics. Using a series of pharmacological and molecular approaches, we determined a critical role for p38 MAPK and Akt in PKC-mediated apoptosis. A relevant finding was that activation of PKC α leads to the dephosphorylation and inactivation of Akt, raising the attractive possibility that inhibition of Akt function upon PKC activation is a crucial event in triggering the apoptotic response of phorbol esters in LNCaP cells.

EXPERIMENTAL PROCEDURES

Materials—Phorbol 12-myristate 13-acetate (PMA), PKC inhibitors (GF109203X, Go6976, rottlerin), p38 MAPK inhibitors (SB 202190, SB 203580, and PD 169316), and the MEK1 inhibitor PD 980591 were purchased from LC Laboratories (Woburn, MA). The JNK inhibitor SP 600125 was obtained from Alexis Biochemicals. HK644 was synthesized in the laboratory of Dr. Victor Marquez (NCl, National Institutes of Health) (13). DAPI (4′,6-diamidino-2-phenylindole) was purchased from Sigma (St. Louis, MO). Cell culture reagents and media were purchased from the ATCC (Rockville, MD).

Cell Culture—LNCaP human prostate cancer cells were purchased from the ATCC. LNCaP cells (passages 2–10) were cultured in RPMI 1640 medium supplemented with 10% FBS and penicillin (100 units/ml)-streptomycin (100 μg/ml) at 37 °C in a humidified 5% CO₂ atmosphere.

Transfections—Cells were seeded in 6-well plates at ~70% confluence and transfected with different mammalian expression vectors (1–2 μg) using FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol.

Identification of LNCaP Cells with PKC and Akt Adenoviruses—Generation of AdVs for PKCα and PKCδ was described elsewhere (12, 13). AdVs for wild-type Akt1, as well as for constitutively active (Myr-Akt1) and kinase-inactive (T308A,S473A) Akt1 mutants were previously described (25). AdVs were amplified in HEK293 cells using standard techniques (26). Titers of viral stocks were normally higher than 1 × 10⁹ pfu/ml. Subconfluent LNCaP cells in 6- or 12-well plates were infected with AdVs for 14 h at multiplicity of infection (m.o.i.) values ranging from 1 to 30 pfu/cell in RPMI 1640 medium supplemented with 2% FBS. When removal of the virus, cells were incubated for an additional 24 h in RPMI 1640 medium supplemented with 10% FBS. Maximum expression after adenoviral infection was achieved using this protocol. Expression of the recombinant protein remained stable throughout the duration of the experiment. A LacZ AdV was used as a control (12, 13).

Western Blot Analysis—Cells were harvested into lysis buffer containing 50 mM Tris·HCl, pH 6.8, 1% glycerol, 2% SDS, and 50 mM β-mercaptoethanol and then lysed by sonication. Equal amounts of protein (10 μg/lane) were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk or 5% bovine serum albumin in 0.05% Tween 20 for 0.5 h and then incubated with the respective primary antibody for 1 h. Membranes were then washed three times with 0.05% Tween 20 in phosphate-buffered saline and incubated with the secondary antibody conjugated to anti-mouse or anti-rabbit horseradish peroxidase (1:3000, Bio-Rad, Hercules, CA). Bands were visualized by using the enhanced chemiluminescence (ECL) Western blotting detection system. Densitometric analysis was performed under conditions that yielded a linear response.

The following first antibodies were used: anti-PKCα (Upstate Biotechnology, Inc., Lake Placid, NY); anti-PKCδ and anti-total-JNK (Transduction Laboratories, Lexington, KY); anti-total-ATF2 and anti-phospho-ATF2 (New England BioLabs, Inc., Beverly, MA); anti-phospho-PKCα, anti-total-Akt, anti-phospho-Akt, anti-phospho-JNK, anti-phospho-PDK1, anti-phospho-p44/42 MAPK (ERK1/2), anti-phospho-p44/42 MAPK, anti-total-p38 MAPK, anti-phospho-p38 MAPK, anti-total-GSK-3, and anti-phospho-GSK-3β (Cell Signaling Technology, Beverly, MA). All of the antibodies were used at a 1:1000 dilution except for anti-PKCα, which was used at a 1:300 dilution.

Apoptosis Assays—To assess morphological changes in chromatin structure of LNCaP cells undergoing apoptosis, cells were stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma). Cells were trypsinized, mounted on glass slides, and fixed in 70% ethanol or 4% paraformaldehyde. Cells were then stained for 20 min with 1 mg/ml DAPI. Apoptosis was determined by using the fluorescein isothiocyanate (FITC)-labeled Annexin V antibody kit from Pharmingen, San Diego, CA. The percentage of apoptotic cells was determined by counting 500 cells and determining the percentage of apoptotic cells. We have previously determined that results observed by these methods essentially matched those observed by flow cytometry and correlate with DNA laddering analysis (12, 13).

Akt Kinase Assay—The Akt kinase assay was performed using the non-radioactive Akt kinase assay kit from Cell Signaling Technology, Inc., following the instructions from the manufacturer. Akt kinase activity was assayed by phosphorylation of GSK-3 fusion protein, which was analyzed by Western blot using a phospho-GSK-3 antibody.

PKC Assay—Cells were harvested into Nonidet P-40 lysis buffer (50 mM Tris·HCl, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA, 1 mM NaVO₄, 25 mM β-glycerophosphate, 50 mM NaF, and 10 mM sodium pyrophosphate) containing a protease inhibitor mixture (Sigma). Lysates were incubated with 2 μg of anti-PI3K p85α antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for 1 h and then with 30 μl of Protein A-agarose (Invitrogen, Gaithersburg, MD) at 4 °C for 1 h. Agarose beads were washed extensively and then resuspended in 30 μl of a substrate solution (100 μg of phosphatidylinositol in 50 μl of 30 mM HEPES, pH 7.3). The agarose beads were then sonicated and 200 μl of kinase buffer (15 mM β-glycerophosphate, 15 mM sodium pyrophosphate, 150 mM NaCl, 1.5 mM dithiothreitol, 20 mM HEPES), and 50 μl of 60 mM MgCl₂ was added. The kinase reaction was carried out for 20 min at 30 °C and stopped by boiling. The kinase reaction mixtures were then clarified by centrifugation at 10,000 × g for 5 min, and 5 μl of each reaction was separated by 10% SDS-PAGE. Gels were stained with Coomassie Blue and subjected to autoradiography.
out at 30 °C for 10 min in the presence of 5 μM [γ-32P]ATP. The reaction was stopped by the addition of 100 μl of 1 M HCl, and then 200 μl of chloroform/methanol (1:1) was added to the samples. Five microliters of bottom phase were spotted onto a TLC plate and run in methanol/chloroform/water/ammonium hydroxide (8:6:2:1). The plates were dried overnight and then subjected to autoradiography.

**RNA Interference**—A 21-bp dsRNA for PKCγ (CCAU-GAGUUUAUCGCCACCTT) and purchased from Dharmacon Research, Inc. The dsRNA was transfected into LNCaP cells using Oligofectamine (Invitrogen) following the instructions provided by the manufacturer. Forty-eight hours after transfection, cells were treated with 100 nM PMA for 15 min, and the cell extracts were analyzed by Western blot.

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

**RESULTS**

**Effect of p38 MAPK, JNK, and MEK1 inhibitors on PMA-induced apoptosis in LNCaP cells**—It is well established that androgen-dependent LNCaP prostate cancer cells undergo apoptosis when treated with phorbol esters (9–11). We have previously reported that short term treatment of LNCaP cells with either PMA or DAG analogs results in a large number of apoptotic cells, as determined by nuclear fragmentation assays, flow cytometry, and DNA laddering (12, 13). To begin elucidating the signaling events that mediate this effect, we first analyzed the activation of different MAPKs in LNCaP cells using phospho-specific antibodies. A rapid increase in phospho-ERK1/2 MAPK, phospho-p38 MAPK, and phospho-JNK was observed upon PMA treatment (Fig. 1). To determine whether the different cascades play any role as mediators of PMA-induced apoptosis in LNCaP cells, we used pharmacological inhibitors of the different pathways. Treatment of cells with SB 203580, a well-established inhibitor of p38 MAPK (27), inhibited the apoptotic effect of PMA in a dose-dependent fashion (Fig. 2A), suggesting a role for p38 MAPK in PKC-mediated apoptosis. To further confirm the involvement of p38 MAPK, we tested two additional p38 MAPK inhibitors, SB 202190 and PD 169316 (28). These two p38 MAPK inhibitors also reduced dose-dependently PMA-induced apoptosis (Fig. 2B and C). At a concentration of 10 μM, SB 203580, SB 202190, and PD 169316 inhibited apoptosis by 63, 62, and 69%, respectively. On the other hand, the JNK inhibitor SP 600125 (29) did not inhibit the apoptotic effect of PMA, even at very high concentrations (50 μM) (Fig. 2D). The efficacy of SP 600125 was confirmed by its ability to block c-Jun phosphorylation upon PMA treatment (Fig. 2E). We then analyzed the effect of PD 98059, an inhibitor of MEK1, the kinase responsible for the activation of ERK1/2 MAPKs. Contrary to the effect observed with the p38 MAPK inhibitors, PD 98059 (10 μM) significantly potentiated apoptosis by 50% (for 30 nM PMA) or 41% (for 100 nM PMA), as shown in Fig. 2F.

Using both pharmacological inhibitors and adenoviral delivery of wild-type PKCs and dominant-negative PKC mutants, we have previously established that both the classic PKCs and the novel PKCδ isozymes mediate the apoptotic effect of PMA in LNCaP prostate cancer cells (12, 13). An approach to achieve isozyme-specific responses consists in overexpressing individual PKC isozymes using adenoviruses (AdVs), which could be then selectively activated by a low concentration of PMA that would normally be insufficient to trigger a response (12).
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**Fig. 3. Involvement of p38 and ERK1/2 MAPKs on PKCα- and PKCδ-induced apoptosis.** A, LNCaP cells were infected with PKCα AdV, PKCδ AdV, or LacZ AdV at different m.o.i. values (3–30 pfu/cell) for 14 h. 24 h later cells were treated with 3 nM PMA or vehicle for 1 h. Apoptosis was assessed 24 h later by DAPI staining. Inset, expression of PKCα and PKCδ in cells infected with the different AdVs. B, cells were infected with PKCα AdV, PKCδ AdV, or LacZ AdV at 30 m.o.i. for 14 h. 24 h later cells were treated with 3 nM PMA for 1 h, in the presence or absence of the p38 MAPK inhibitor SB 203580 (10 μM) added 40 min before and during PMA treatment. Apoptosis is expressed as a percentage relative to control cells infected with LacZ AdV (dashed line). C, cells were infected with PKCα AdV or PKCδ AdV at 30 m.o.i. for 14 h, and 24 h later cells were treated with different concentrations of PMA (1–30 nM) for 1 h. The MEK1 inhibitor PD 98059 (30 μM) added 40 min before and during PMA treatment. The percentage of apoptotic cells was assessed 24 h later by DAPI staining. D, cells were infected with PKCα AdV or PKCδ AdV at 30 m.o.i. for 14 h. 24 h later cells were treated with different concentrations of PMA (1–30 nM) for 15 min. The MEK inhibitor PD 98059 (30 μM) was added 40 min before and during PMA treatment. Phospho-ERK1/2 levels were analyzed by Western blot using specific antibodies. No changes were observed in total ERK1/2 MAPK (data not shown). Results in A–C represent the mean ± S.E. of three independent experiments.

LNCaP cells were infected with either PKCα AdV or PKCδ AdV, which resulted in the overexpression of the corresponding PKC, as judged by Western blot analysis, [3H]phorbol 12,13-dibutyrate binding, and PKC kinase activity (see Refs. 12 and 13). PMA (3 nM), which produces only a marginal apoptotic effect in PKCδ-driven apoptosis could be specifically inhibited by the cPKC inhibitor Gö6976 and the PKCδ inhibitor rottlerin, respectively (data not shown and Refs. 12 and 13). In LNCaP cells overexpressing either PKCα or PKCδ, pretreatment with the p38 MAPK inhibitor SB 203580 significantly reduced the apoptotic effect of PMA (Fig. 3B). On the other hand, the MEK1 inhibitor PD 98059, which markedly inhibited ERK1/2 MAPK activation in LNCaP cells (Fig. 3D), potentiated PMA-induced apoptosis in cells overexpressing either PKCα or PKCδ (after infection with the corresponding AdVs at an m.o.i. of 30 pfu/cell). The effect was more pronounced in PKCα-overexpressing cells, in which the number of apoptotic cells observed after 10 nM PMA treatment was nearly doubled in the presence of the MEK1 inhibitor (Fig. 3C). Thus, a differential involvement of MAPK cascades is observed upon PKC activation in LNCaP cells.

**PMA Induces the Dephosphorylation of Akt in LNCaP Cells**—The central role of the PI3K-Akt pathway in LNCaP prostate cancer cell survival prompted us to investigate whether PKC-induced apoptosis involves any changes in this signaling pathway. It is well established that Akt is hyperactivated in LNCaP cells (21, 23). Fig. 4A shows that treatment of LNCaP cells with PMA (100 nM) leads to a rapid dephosphorylation of Akt, as revealed by Western blot using a phospho-specific anti-Akt antibody (phospho-Ser473). The maximum dephosphorylation is observed at 15–20 min, and it returns to basal levels at ~60–90 min. Similar effects were observed with an antibody against phospho-Thr308 in Akt (Fig. 4A). Akt dephosphorylation correlated with changes in activity, as determined in Akt kinase assays in immunoprecipitates using GSK-3 as a substrate. As shown in Fig. 4B, a significant reduction in Akt activity was observed in LNCaP cells upon PMA treatment. As expected, a control using the PI3K inhibitor wortmannin also resulted in lower Akt activity. The decreased Akt activity could be either related to inhibition of upstream inputs (PI3K) or effects on downstream phosphatases. Evaluation of the phosphorylation status of phosphoinositide-dependent kinase-1...
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Fig. 4. Inhibition of Akt by PMA treatment. A, LNCaP cells were treated with 100 nM PMA for different times, as indicated in the figure. Samples were collected and analyzed by Western blot using antibodies for phospho-Akt, total-Akt, and phospho-PDK1. B, LNCaP cells were treated with 100 nM PMA for 15 min or 10 μM wortmannin for 1 h, and Akt kinase activity was assayed by phosphorylation of GSK-3 fusion protein using a kit from Cell Signaling, Inc. Similar results were observed in two additional experiments. C, cells were treated with 100 nM PMA for 15 min, in the presence or absence of 100 nM wortmannin, and then PI3K activity was determined as described under “Experimental Procedures.” Two additional experiments gave similar results.

(PDK1), a direct effector of PI3K, revealed no changes in phospho-PDK1 levels upon PMA treatment (Fig. 4A). To further rule out an effect on PI3K, we next determined PI3K activity. Fig. 4C shows that PI3K activity remained basically unchanged in LNCaP cells treated with PMA. As a control, we used the PI3K inhibitor wortmannin, which totally inhibits PI3K in our assays conditions. Thus, in LNCaP prostate cancer cells PKC-induced inactivation of Akt is not a consequence of PI3K inactivation or inhibition of mechanisms that result in the activation of PI3K.

Effect of PKC Inhibitors and the DAG Analog HK654 on Akt Activity—To confirm that the PMA effect is PKC-mediated and to determine the isoforms involved, we assessed the effect of PKC inhibitors on Akt dephosphorylation. We have previously shown that the “pan” PKC inhibitor G109203X blocked PMA-induced apoptosis in LNCaP cells (12, 13). Pretreatment of LNCaP cells with G109203X dose-dependently inhibited PMA-induced dephosphorylation of Akt. A representative Western blot and the corresponding densitometric analysis are shown in Fig. 5 (A and B), respectively. We then evaluated the effect of cPKC inhibitor G66976. Because PKCo is the only cPKC present in LNCaP cells (9, 12), G66976 represents a PKCo-selective antagonist in our model. We have previously observed that G66976 significantly reduced PMA-induced apoptosis in LNCaP cells (13). Here we show that the cPKC inhibitor blocked PMA-induced Akt dephosphorylation (Fig. 5, C and D). In contrast, we did not observe any significant effect with the PKCδ inhibitor rottlerin (Fig. 5, E and F), which was previously found to partially block the apoptotic effect of PMA in our experimental model (12, 13). To further explore the involvement of PKCo, we used the DAG-mimetic HK654, an analog that we have recently described as PKCo-specific in LNCaP cells due to the specific translocation of PKCo to the plasma membrane (13). As described above with PMA, HK654 also induced a rapid dephosphorylation of Akt, without producing any noticeable changes in the level of PDK1 phosphorylation (Fig. 5, G and H). Thus, activation of PKCo results in Akt dephosphorylation in LNCaP cells. The lack of involvement of PKCo in PMA-induced Akt dephosphorylation was confirmed using small interference RNA for PKCo. Upon transfection of a dsRNA for PKCo into LNCaP cells, the expression of PKCo was substantially reduced. Nevertheless, Akt dephosphorylation observed upon PMA treatment could not be rescued under conditions in which PKCo was knocked down (Fig. 6A). The corresponding densitometric analysis of multiple experiments is shown in Fig. 6B. PKCo dsRNA was able to block PMA-induced phosphorylation of p38 MAPK and apoptosis (data not shown). These results suggest that PKCo but not PKCδ is preferentially involved in the dephosphorylation of Akt upon PMA treatment.

Effect of Okadaic Acid on Akt Dephosphorylation—To explore the potential involvement of PP2A phosphatases in the PMA-induced dephosphorylation of Akt, LNCaP cells were pre-treated with the PP2A inhibitor okadaic acid. As shown in Fig. 7A, okadaic acid prevented Akt dephosphorylation induced by PMA in LNCaP cells. Moreover, okadaic acid enhanced Akt phosphorylation by ~50% in the absence of PMA treatment. On the other hand, pretreatment of cells with orthovanadate, a broad inhibitor of tyrosine phosphatases, did not affect the basal Akt phosphorylation levels or prevent the effect of PMA on Akt dephosphorylation (Fig. 7, C and D).

Activated Akt Rescues PMA-induced Apoptosis—To further explore the relationship between PKC activation and Akt function, we expressed wild-type Akt, a constitutively active form of Akt (Myr-Akt), and a kinase-inactive form of Akt (T308A, S473A) mutant in LNCaP cells using adenoviral delivery (25). Upon infection with increasing m.o.i. values of the Akt AdVs, a more locally active Akt AdV. Upon infection with wild-type Akt AdV, high levels of phospho-Akt were also observed, which reflects the excessive PI3K input present in LNCaP cells. Interestingly, expression of either Myr-Akt or wild-type Akt significantly reduced the apoptotic effect of PMA (Fig. 8A). The effect was proportional to the expression levels of Myr-Akt or wild-type Akt. At an m.o.i. of 10 pfu/cell, the number of apoptotic cells after PMA treatment was reduced by ~50% in either case. On
the other hand, infection with a control (LacZ) AdV or the kinase-inactive Akt AdV had no effect.

In the next set of experiments, we transfected a GFP-fused Myr-Akt vector (pEGFP-Myr-Akt) into LNCaP cells. 24 h after transfection, cells were treated with PMA and the incidence of apoptosis was determined by nuclear fragmentation in transfected (green fluorescent) versus non-transfected cells. As shown in Fig. 8C, the number of apoptotic cells observed upon PMA treatment was significantly reduced in the population of cells expressing GFP-Myr-Akt compared with non-transfected cells. Importantly, when we compared apoptosis in LNCaP cells transfected with an empty vector (pEGFP), which results in the

Fig. 5. PKCa is involved in PMA-induced Akt dephosphorylation. A, C, and E, LNCaP cells were treated with 100 nM PMA or vehicle for 15 min. The pan-PKC inhibitor GF109203X, the cPKC inhibitor Go6976, or the PKCa inhibitor rottlerin were added 40 min before and during PMA treatment at the concentrations indicated in the figure. The levels of phospho- and total-Akt were determined by Western blot. G, cells were treated with the DAG mimetic HK654 (10 µM) and the samples were collected at the times indicated in the figure and analyzed by Western blot using specific antibodies against total Akt, phospho-Akt, or phospho-PDK1. B, D, F, and H show a densitometric analysis of phospho-Akt levels, normalized to the total Akt levels in each case. The dashed line (100%) indicates the expression levels in the absence of PMA treatment. Results are the mean ± S.E. of three independent experiments.

Fig. 6. Down-regulation of PKCa expression by small interference RNA does not affect PMA-induced Akt dephosphorylation. LNCaP cells were transfected with a dsRNA oligonucleotide for PKCa and 48 h later treated with PMA (100 nM, 15 min). A, phospho-Akt, total-Akt, and PKCa levels were analyzed by Western blot. B, densitometric analysis of phospho-Akt levels, normalized to the total levels. The dashed line (100%) indicates the expression levels in the absence of PMA treatment. Results are the mean ± S.E. of three independent experiments.
expression of GFP alone, no differences were observed between transfected and non-transfected cells. Taken together, these data suggest that deregulation of Akt is critical for PKC-mediated apoptosis in LNCaP prostate cancer cells.

Lack of Reciprocal Modulation of p38 MAPK and Akt upon PKC Stimulation—To determine the potential cross-talk between the p38 MAPK and Akt pathways, we first assessed whether the activation of p38 MAPK by PMA could influence the Akt phosphorylation status. As shown in Fig. 9A, none of the p38 MAPK inhibitors (SB 203580, SB 202190, and PD 169316) used at concentrations that block PMA-induced apoptosis was capable of preventing PMA-induced dephosphorylation of Akt. Furthermore, inhibition of p38 MAPK did not affect basal phospho-Akt levels (Fig. 9B). Akt phosphorylation levels were not changed by treatment with the MEK1 inhibitor PD 98059 (data not shown). Lastly, to evaluate whether activated Akt could influence the activation of p38 MAPK, we determined phospho-p38 MAPK levels in LNCaP cells infected with Myr-Akt AdV, and compared it to control (LacZ AdV-infected) cells. As shown in Fig. 9C, stimulation of p38 MAPK by PMA was not influenced by the expression of Myr-Akt. None of the AdVs per se were capable of activating p38 MAPK. These findings suggest that the p38 MAPK and Akt pathways are independently regulated by PKC in LNCaP cells.

DISCUSSION

In the present study we characterized the signaling mechanisms involved in phorbol ester-induced apoptosis in LNCaP prostate cancer cells. We have previously demonstrated that both the classic PKCα and the novel PKC8 mediate the apoptotic responses of PKC activators in LNCaP cells (12, 13). On the other hand, a different member of the nPKC subfamily, PKCe, is capable of inducing proliferation in LNCaP cells, and even promote resistance to apoptosis (29, 30). Opposite roles for the novel PKC8 and PKCe in proliferation have been reported in other models, such as NIH 3T3 fibroblasts (5). PKC8 was also shown to have a pro-apoptotic function in other cell types, including keratinocytes, salivary gland acinar cells, vascular smooth muscle cells, colon cancer cells, and hemopoietic cells (31–36). It is remarkable, however, that the mechanisms involved in each cell type seem to differ drastically from one another. Indeed, significant differences in the involvement of mitochondria and caspases in PKC8-mediated apoptosis have been observed in keratinocytes, myeloid leukemia cells, and prostate cancer cells (12, 31, 37). Activation of PKC8 in LNCaP cells by cytotoxic drugs such as etoposide and paclitaxel stimulates caspases and ceramide generation (38). Moreover, whereas in hemopoietic cells PKC8-triggered apoptosis is mediated by a proteolytically generated kinase fragment (31), in vascular smooth muscle cells the apoptotic effect seems to be totally independent of the kinase activity of the enzyme (36). In LNCaP cells, however, the results presented in this and our previous reports show that PKC inhibitors acting at the ATP-binding site of the catalytic region can efficiently block PMA-induced apoptosis. Moreover, apoptosis elicited by PKC activation in LNCaP prostate cancer cells is independent of the proteolytic cleavage of PKC8 but, rather, involves the intracellular redistribution (translocation) and allosteric activation of the enzyme (12, 13). Although all these differences may seem at first difficult to reconcile, we hypothesize that they not only reveal important cell type differences but also highlight the multiplicity of regulatory mechanisms capable of activating PKC isozymes. Heterogeneity in PKCα-mediated responses has also been described, because this cPKC was reported to mediate either mitogenic or apoptotic responses in different cell types (13, 39–41).

A key question is how each individual member of the PKC family regulates signaling cascades, and our current understanding reveals that exquisite regulatory mechanisms take place in different cellular models. One of the emerging themes in the last years has been the involvement of the individual

FIG. 7. Effect of phosphatase inhibitors on PMA-induced Akt dephosphorylation. LNCaP cells were treated for 15 min with 100 nM PMA in the presence or absence of okadaic acid (A) or orthovanadate (C), which were added 40 min before and during PMA treatment. Samples were collected and analyzed by Western blot using antibodies for phospho-Akt and total Akt. The corresponding densitometric analysis has been normalized to the total Akt levels in each case, and it is expressed as the mean ± S.E. of three independent experiments (B and D). The dashed line (100%) indicates the levels in the absence of PMA treatment.
MAPK cascades on proliferative responses of PKC isozymes. We found that in LNCaP cells PKC activation results in the stimulation of ERK1/2, p38 MAPK, and JNK. Pharmacological inhibition of p38 MAPK results in a marked reduction in the apoptotic effect of PMA, suggesting a key role for p38 MAPK in PKC-induced apoptosis. Other reports have shown that inhibition of PKC function impairs the activation of p38 MAPK upon stimulation of thrombin, vasopressin, or VEGF receptors (42–44), suggesting that PKC isozymes could indeed mediate receptor-induced stimulation of p38 MAPK. Although we have not yet explored the role of p38 MAPK isoforms as mediators of apoptosis in LNCaP cells, recent reports have shown that PKC activators increase p38 activity in HeLa cells and that p38α, but not p38γ or p38β, is activated by PKCδ in keratinocytes (45, 46). Although the mechanisms by which PKC isozymes activate p38 MAPK have not yet been defined, recent results from our laboratory reveal that in LNCaP cells PMA treatment leads to the activation of MKK3/6 (the kinase responsible for p38 MAPK activation) and its upstream kinase Ask1,2 suggesting that PKC targets signaling events further upstream of p38 MAPK. These issues are currently under investigation in our laboratory.

Inhibition of the ERK pathway with the MEK1 inhibitor PD 98059 potentiated PKC-induced apoptosis in LNCaP cells. This is consistent with the notion that the ERK cascade provides survival signals that counterbalance the apoptotic response induced by PKC activation. Thus, the balance in the activation of the different MAPK cascades is critical for determining cell fate in these cells. Interestingly, an imbalance between ERK1/2 and p38 MAPK signaling in prostate cancer cells upon inhibition of the HER-2/neu receptor was found to lead to apoptosis (20). Using a different experimental paradigm Powell and co-workers (47) have shown that persistent translocation of PKCα to the plasma membrane was sufficient to induce apoptosis in LNCaP cells, and pointed to the potential involvement of the ERK MAPK cascade in this effect. Although the precise role of the ERK1/2 MAPK cascade in prostate carcinogenesis still needs to be elucidated, it is well known that growth factor receptor signaling plays a crucial role in the control of prostate cancer cell growth, particularly in advanced stages of the disease. Moreover, histopathological studies revealed high levels of activated ERK1/2 MAPK in high grade and advanced-stage tumors, suggesting a potential contribution of this cascade in prostate cancer progression (48).

LNCaP cells express high levels of phosphorylated (active) Akt due to the lack of a functionally active tumor suppressor PTEN, a lipid phosphatase that acts as a negative regulator of the PI3K/Akt pathway. Carson et al. (21) have shown that the
PI3K/Akt pathway has a dominant effect in the survival of LNCaP cells and that inhibition of Akt function leads to apoptosis in these cells. The PI3K/Akt pathway has a prominent role in protecting prostate cancer cells from TNF- and TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis, and suppression of Akt function markedly enhanced cytokine-induced apoptotic responses (23). In this report we have determined that PMA treatment induces a rapid dephosphorylation of Akt. Thus, in LNCaP cells activation of PKC results in the inhibition of survival signaling. Importantly, we were able to rescue the apoptotic effect of PMA with an activated form of Akt, an indication that inactivation of the Akt pathway is crucial for the PKC effect. With the use of a pharmacological inhibitor of cPKCs we determined that PKC was preferentially responsible for triggering Akt dephosphorylation. The involvement of PKC was confirmed in experiments using HK654, a DAG mimetic that we have recently characterized as a selective PKC activator in LNCaP cells due to selective translocation of this PKC isozyme to the plasma membrane. Interestingly, a recent report has shown that PKCα, but not PKCδ, was able to inhibit lyso phosphatidylcholine-induced dephosphorylation of Akt in vascular smooth muscle cells (49). Although we did not yet fully characterize the mechanisms responsible for Akt dephosphorylation in LNCaP cells, our results strongly suggest the involvement of a PP2A phosphatase, as revealed by the inhibitory effect of okadaic acid on PMA-induced Akt dephosphorylation. Indeed, it has been recently demonstrated that a PP2A activity is capable of dephosphorylating Akt and disrupting insulin signaling in skeletal muscle myotubes (50). Negative regulation of Akt function by PKC does not occur via inhibition of PI3K, as revealed by the lack of changes in PDK1 phosphorylation and in PI3K activity in LNCaP cells upon PMA treatment. In agreement with our results, a recent report has shown that inhibition of Akt activity by ceramide in adipocytes involves the activation of a PP2A phosphatase and is independent of PI3K (51). Thus, we propose a novel signaling mechanism by which PKCα activates a phosphatase responsible for the dephosphorylation of Akt in prostate cancer cells. Although the identity of this PP2A phosphatase still remains to be determined, and taken into consideration the potential involvement of ceramide in PKC-mediated apoptosis in LNCaP cells (11, 38), it is tempting to speculate that both ceramide and PKC activation may regulate Akt dephosphorylation through the same PP2A phosphatase.

An important issue that emerges from this study is the requirement of multiple pathways for the apoptotic response induced by PKC in LNCaP cells. It is possible that inhibition of Akt signaling is not sufficient to trigger the apoptotic response...
in LNCaP cells growing under normal serum conditions. Indeed, expression of the kinase-inactive mutant of Akt, which acts as a dominant-negative, was not sufficient to trigger an apoptotic response in LNCaP cells. Likewise, we observed that the PI3K inhibitors wortmannin and LY294002 did not induce apoptosis in cells grown in the presence of serum (data not shown), in agreement with the report by Carson et al. (22). An attractive hypothesis is that PKC-mediated apoptosis requires the activation of caspase for inhibition of multiple pathways. Our results strongly suggest that in LNCaP prostate cancer cells both the Akt and p38 MAPK pathways are differentially activated by PKC isozymes, and the evidence presented here strongly suggests that both pathways are not interdependent in LNCaP cells. A model summarizing the signaling events regulated by PKC isozymes in these cells is depicted in Fig. 10.

In conclusion, our results provide strong evidence that both the Akt and p38 MAPK pathways are involved in PKC-mediated apoptosis in LNCaP prostate cancer cells. Collectively, these observations give novel insights into the complexity of the signaling pathways controlled by PKC isozymes and the mechanisms regulating cell survival in prostate cancer cells. Deciphering these intricate mechanisms could help to identify targets for the treatment of the disease.

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