Phorbol Ester-induced Apoptosis in Prostate Cancer Cells via Autocrine Activation of the Extrinsic Apoptotic Cascade

A KEY ROLE FOR PROTEIN KINASE Cα

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It is well established that activation of protein kinase C (PKC) by phorbol esters promotes apoptosis in androgen-dependent prostate cancer cells. However, there is limited information regarding the cellular mechanisms involved in this effect. In this report we identified a novel autocrine pro-apoptotic loop triggered by PKCα activation in prostate cancer cells that is mediated by death receptor ligands. The apoptotic effect of phorbol 12-myristate 13-acetate in LNCaP cells was impaired by inhibition or depletion of tumor necrosis factor alpha-converting enzyme, the enzyme responsible for tumor necrosis factor α (TNFα) shedding. Moreover, the apoptotic effect of conditioned medium collected after phorbol 12-myristate 13-acetate treatment could be inhibited by blocking antibodies against TNFα and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), but not FasL, as well as by RNA interference depletion of TNFα and TRAIL receptors. Moreover, depletion or inhibition of death receptor downstream effectors, including caspase-8, FADD, p38 MAPK, and JNK, significantly reduced the apoptotic effect of the conditioned medium. PKCα played a major role in this autocrine loop, both in the secretion of autocrine factors as well as a downstream effector. Taken together, our results demonstrate that activation of PKCα in prostate cancer cells causes apoptosis via the release of death receptor ligands and the activation of the extrinsic apoptotic cascade.

Protein kinase C (PKC)8 isozymes, a family of at least 10 related serine-threonine kinases, play important roles in the regulation of various cellular processes, including differentiation, proliferation, and malignant transformation, and have been widely implicated in the progression of cancer. This family of signaling kinases comprises the classical (α, β, βI, and γ), novel (δ, ε, η, and θ), and atypical (ζ and ι/ι) PKCs, which have differential patterns of cell and tissue distribution and unique modes of regulation (1). Phorbol esters, natural compounds that potently activate the classical and novel PKCs, trigger a plethora of cellular responses that vary depending on the cell type and the relative expression of individual PKC isozymes. Whereas phorbol esters are capable of promoting mitogenic or survival responses, many cell types undergo growth arrest or apoptosis in response to PKC activation. A major reason for such heterogeneity is the diversity of pathways activated by each PKC isozyme, their distinct relocation, and their differential access to substrates upon activation (2). Such functional diversity is exemplified by the novel PKCs: whereas in most cases PKCε acts as a mitogenic or anti-apoptotic kinase, PKCδ generally inhibits proliferation, or in some cell types it triggers an apoptotic response and is required for drug-induced apoptosis (3). Dissecting the signaling events regulated by individual PKCs still represents a major challenge and will certainly help to understand the functional roles of PKC isozymes in normal and cancer cells.

Androgen-dependent prostate cancer cells, such as LNCaP cells, represent one of the most studied models for phorbol ester-induced apoptosis via PKC activation (4, 5). Phorbol 12-myristate 13-acetate (PMA) stimulates apoptosis in LNCaP cells and xenografts, and it sensitizes LNCaP tumors in mice to the apoptotic effects of ionizing radiation (6, 7). We and others have assigned a key role to PKCα as a mediator of phorbol ester-induced apoptosis in LNCaP cells (8–10). Unlike observed in other cell types, activation of PKCα in prostate cancer cells is independent of its cleavage to a catalytically active form, but it rather depends on allosteric mechanisms upon translocation to membranes (8). PKCε, on the other hand, was shown to stimulate proliferation in LNCaP cells and cause progression to an androgen-independent state (11). Signaling studies have determined an essential role for p38 MAPK and ceramide as mediators of phorbol ester-induced apoptosis in LNCaP cells (6, 12), and in addition, a modulatory role for the JNK interacting protein 1 (JIP-1, an inhibitor of JNK) has been recently postulated (13), suggesting complex modes of regulation downstream of PKC in prostate cancer cells. The relative contribution of the intrinsic and extrinsic apoptotic cascades in this context remains to be determined. However, as many of the aforementioned pathways are known effectors of death receptor ligands, such as TNFα, TRAIL, or FasL, it is reasonable to speculate that phorbol ester-induced apoptosis in LNCaP cells might involve the activation of the extrinsic apoptotic pathway.

It has been known for years that phorbol esters are capable of stimulating the release of autocrine or paracrine factors that modulate PKC cellular responses. For example, conditioned medium (CM) collected from PKCe-overexpressing R6 fibroblasts stimulates DNA synthesis and causes morphologic transformation. Transforming growth factor-β release has been associated, at least in part, to the growth abnormalities caused by PKCe overexpression (14). Limited information, however, is available on the potential contribution of autocrine factors to apoptotic responses caused by PKC activation. One attractive, yet unexplored hypothesis, is that prostate cancer cell death upon phorbol ester stimulation involves the activation of an apoptotic autocrine loop that stimulates death receptors and the extrinsic apoptotic pathway.

In the present study we demonstrate that the induction of apoptosis in LNCaP prostate cancer cells by PKC involves the secretion of death

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2 The abbreviations used are: PKC, protein kinase C; FADD, Fas-associated protein with a death domain; TACE, TNFα-converting enzyme; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH2-terminal kinase; PMA, phorbol 12-myristate 13-acetate; TNFα, tumor necrosis factor α; CM, conditioned medium; DAPI, 4’,6-diamidino-2-phenylindole; RNAI, RNA interference; dS, double-stranded; ELISA, enzyme-linked immunosorbent assay; TNFR, tumor necrosis factor receptor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.
ligands. Moreover, by means of a series of pharmacological and molecular approaches, we have established that the novel PKCδ is crucial in this autocrine regulation, playing roles both in autocrine factor release as well as downstream of death receptor activation. The identification of this novel autocrine mechanism highlights the complexities of PKC signaling and may have great implications in the identification of novel targets for prostate cancer therapy.

**EXPERIMENTAL PROCEDURES**

**Materials**—PMA was purchased from LC Laboratories (Woburn, MA). DAPI (4′,6-diamidino-2-phenylindole) was obtained from Sigma. The p38 MAPK inhibitor SB203580 was purchased from LC Laboratories. The INK inhibitor S600125 was obtained from Alexis Biochemicals (San Diego, CA). Cell culture reagents and media were purchased from ATCC (Rockville, MD). Recombinant human TRAIL/Apo2L and sFlt ligand were from PetroTech, Inc. (Rocky Hill, NJ). Recombinant human TNFα was from R&D Systems, Inc. 2′,3′-Azido-bis(3-ethylbenzyazoline)-6-sulfonic acid was from Roche Diagnostics.

The following antibodies were used: mouse monoclonal anti-human TNFα (clone RDI-2C8, Research Diagnostics, Inc., Flanders, NJ); biotinylated anti-human TNFα (Research Diagnostics Inc.); monoclonal anti-human FasL (clone 2C101, Alexis, San Diego, CA); mouse monoclonal anti-human TRAIL, TNFR1, and TNFR2 (R&D Systems), anti-PKCα (Upstate Biotechnology, Inc., Lake Placid, NY); anti-PKCδ and anti-JNK (Transduction Laboratories, Lexington, KY); anti-phospho-JNK (Cell Signaling Technology, Beverly, MA); anti-caspase-8, anti-IκB, and anti-phospho-IκB (Santa Cruz Biotechnologies, Santa Cruz, CA); anti-Fas-associated protein with a death domain (FADD), anti-D4, and anti-D5 (kind gifts from Dr. Wafik el-Deiry, University of Pennsylvania), and anti-TACE (a kind gift from Dr. Marcos Milla, University of Pennsylvania).

**Cell Culture**—LNCaP human prostate cancer cells were purchased from ATCC. Cells (passages 2–10) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin (100 units/ml) from ATCC. Cells (passages 2–10) were cultured in RPMI 1640 medium and anti-TACE (a kind gift from Dr. Marcos Milla, University of Pennsylvania), anti-D5 (kind gifts from Dr. Wafik el-Deiry, University of Pennsylvania), anti-Fas-associated protein with a death domain (FADD), anti-D4, and anti-D5 (kind gifts from Dr. Wafik el-Deiry, University of Pennsylvania), and anti-TACE (a kind gift from Dr. Marcos Milla, University of Pennsylvania).

**Collection of CM**—Cells (~70% confluence) were treated with PMA (100 nM) or vehicle (ethanol) for 1 h, and then washed twice with medium to remove the phorbol ester or vehicle. After incubation for different times, CM was collected, filtered, and added to fresh LNCaP cells (~70% confluence). When indicated, CM was dialyzed using 12–14-kDa cut-off membranes for 36 h at 4 °C against RPMI medium, which was changed each 12 h.

**Western Blot Analysis**—Cells were harvested into lysis buffer containing 50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and 5% β-mercaptoethanol, and then lysed by sonication. Equal amounts of protein (20 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/phosphate-buffered saline and then incubated with the primary antibody for 1 h. After washing three times with 0.05% Tween 20 in phosphate-buffered saline, membranes were incubated for 1 h with either anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase (1:3000, Bio-Rad). Bands were visualized with the enhanced chemiluminescence (ECL) Western blotting detection system. Densitometric analysis was performed using Image software (National Institutes of Health) under conditions that yielded a linear response.

**Apoptosis Assays**—Cells were trypsinized, mounted on glass slides, and then fixed in 70% ethanol. Morphological changes in chromatin structure were assessed after staining with DAPI, as described previ-ousuly (8, 9). Apoptosis was characterized by chromatin condensation and fragmentation when examined by fluorescence microscopy. The incidence of apoptosis was analyzed by counting 500 cells followed by the determination of apoptotic cells in each preparation. We have previously determined that results observed by these methods essentially matched those observed by flow cytometry and correlate with DNA laddering analysis (8).

**RNA Interference (RNAi)**—Twenty-one bp dsRNAs were purchased from Dharmacon, Inc. (Lafayette, CO). The following target sequences were used: AAGUGCCCAAAAGAAACCUAC (TNFR1), AACAGACGUGACCCUGUCCU (TNFR2), AGCCAGAGAUGAGGACCAAC (DR5), AAGUGCAUGCAAGGGUGUGU (DR4); AAGUGCCAAAGGAACCUAC (Fas), CCAGAGUUAUCCCGACC (PKCδ), and AAACUGCAGUUGCAUCGU (control RNAi). TACE RNAi (AAACGAAACGCTGATACUGC) was purchased from Qiagen-Xeragon (Germantown, MD). dsRNAs (100 nM) were transfected into LNCaP cells using Oligofectamine (Invitrogen) following the instructions provided by the manufacturer. In each case, the optimal time for protein/RNA depletion after transfection was determined. Unless when indicated, experiments were carried out 48 h after transfection.

**Retroviral Infection of LNCaP Cells**—Retroviral vectors encoding for short hairpin RNA sequences to knock-down FADD or caspase-8, or encoding a mutated sequence for caspase-8 were kind gifts from Dr. Wafik el-Deiry (University of Pennsylvania). Empty vector p-SUPER Retro was used as a control. Retroviruses were obtained after transfection of Phoenix-Ampho packaging cells with the corresponding retroviral constructs by using Lipofectamine (Invitrogen) and collection of the supernatant. Subconfluent LNCaP cells in 6- or 12-well plates were infected with the different retroviruses for 14 h in RPMI 1640 medium supplemented with 10% fetal bovine serum and Polybrene. After removal of the retrovirus by extensive washing, cells were incubated for 24 h in RPMI 1640 medium supplemented with 10% fetal bovine serum. Selection was carried out with 1 mg/ml puromycin.

**RNA Isolation and cDNA Synthesis**—LNCaP cells were treated for 1 h with either PMA or vehicle. At different time points, cells were lysed and RNA was extracted using TRIzol (Invitrogen). Five micrograms of RNA per sample were reverse transcribed using the First-Strand cDNA Synthesis Kit (Amersham Biosciences). Each reverse transcription reaction was performed in a total volume of 50 μl.

**Real-time PCR**—PCR primers and fluorogenic probes for human TNFα were purchased from Applied Biosystems. The probes were 5′ end-labeled with 6-carboxyfluorescein (FAM). Each PCR amplification was performed in a total volume of 12.5 μl, containing 6.25 μl of 2x TaqMan Universal PCR Master Mix (Applied Biosystems), commercial target primers (300 nM), the fluorescent probe (200 nM), and 1 μl of cDNA. PCR were performed with an ABI PRISM 7700 Detection System (TaqMan; Applied Biosystems) using the following conditions: 2 min at 50 °C and 10 min at 94 °C, followed by a total of 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The PCR product formation was continuously monitored during the PCR using Sequence Detection System software version 1.7 (Applied Biosystems). The FAM signal was normalized to the endogenous glyceraldehyde-3-phosphate dehydrogenase. Results were expressed as fold increase relative to those in untreated cells.

**Enzyme-linked Immunosorbent Assay (ELISA)**—TNFα levels were determined by ELISA using 2 μg/well of coating anti-TNFα antibody. Nonspecific binding sites were blocked with 10% fetal bovine serum in phosphate-buffered saline. CM (50 μl) was added in each well and incubated overnight at 4 °C. Subsequently, 100 μl of a biotin-labeled anti-TNFα antibody (0.3 μg/ml) was added for 2 h at room temperature.
Bound antibody was detected by incubation with peroxidase-labeled streptavidine and 2,2'-azino-bis(3-ethylbenzamidine)-6-sulfonic acid, and absorbance was measured at 405 nm. TRAIL levels were determined using a Quantikine M kit from R&D Systems.

**RESULTS**

**PKC Translocation**—Translocation was determined by Western blot using a subcellular fractionation technique, as previously reported (8).

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

**RESULTS**

**PMA Stimulates the Release of Apoptogenic Factors from Prostate Cancer Cells**—LNCaP prostate cancer cells undergo apoptosis upon PMA treatment (4, 5, 8–10). To determine whether autocrine mechanisms could be involved in this effect, we first compared the activity of conditioned CM collected from PMA-treated cells (CM-PMA) and vehicle-treated cells (CM-Veh). LNCaP cells were treated with PMA (100 nM, 1 h), and then washed extensively to remove the phorbol ester. When added to fresh LNCaP cells, CM-PMA caused a significant apoptotic response, as determined by apoptotic cell counting after DAPI staining. On the other hand, CM-Veh did not cause cell death (Fig. 1, A and B). Flow cytometry analysis revealed a significant increase in the population of cells in sub-G0/G1 24 h after treatment either with PMA or CM-PMA (Fig. 1C). To determine that the effect was not because of PMA remaining in the CM despite the extensive washings, CM-PMA was dialyzed using 12–14-kDa cut-off dialysis membranes. The apoptogenic activity of the CM-PMA was indeed retained after dialysis (Fig. 1D). CM-PMA collected from androgen-independent DU-145 and PC3 cells, as well as from Tsu-Pr1 cells (originally classified as a prostate cancer cell line but later re-classified as a bladder cancer cell line) also triggered an apoptotic response when added to fresh LNCaP cells. On the other hand, CM collected from NIH-3T3 cells treated with PMA in a manner similar to the prostate cancer cells was unable to cause apoptosis when added to fresh LNCaP cells (Fig. 1E). This experiment suggests that the effect is cell type-specific, and it also serves as a control for the absence of PMA in the CM after the washings. To determine whether the apoptotic effect of PMA was entirely dependent on the release of apoptotic factors, we per-
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FIGURE 2. PKCδ is required both for the release of apoptogenic factors to the CM and as an effector of the CM. Panel A, LNCaP cells were transfected with a PKCδ RNAi duplex (48 h), or pre-incubated with either GF109203X (GF, 10 μM) or rottlerin (Rott, 10 μM) for 40 min before and during PMA treatment. Cells were then treated with 100 nM PMA (1 h), and CM-PMA was collected 24 h later and added to fresh LNCaP cells. The percentage of apoptotic cells was determined 24 h later. Data are presented as mean ± S.E. (n = 3). Panel B, CM from PMA-treated cells was added to LNCaP cells that have been previously transfected with a PKCδ RNAi duplex (48 h) or pretreated with either GF109203X (GF, 10 μM) or rottlerin (Rott, 10 μM) for 40 min before and during CM-PMA treatment. Cells were collected 24 h later and the incidence of apoptosis was determined. Data are presented as mean ± S.E. (n = 3). Panel C, representative Western blot showing the expression of PKC isozymes 48 h after transfection with a specific PKCδ RNAi duplex. Panel D, representative Western blot showing the translocation of PKCδ by the CM-PMA (n = 3).

formed consecutive washings every 15 min during the first 6 h after PMA treatment. We reasoned that this would prevent the accumulation of released autocrine factors in the CM. Remarkably, cells did not undergo apoptosis under this experimental condition (Fig. 1F), suggesting that the apoptotic effect of the phorbol ester was entirely dependent on the autocrine loop. Apoptotic activity was detected with CM from LNCaP cells collected shortly (1 h) after PMA treatment (data not shown), suggesting that the effect was probably independent of the synthesis of apoptotic factors. To further explore this issue, we collected CM from LNCaP cells treated with the protein synthesis inhibitor cycloheximide (50 μm). A significant fraction of the apoptogenic effect of CM-PMA (∼70%) was retained after “de novo” protein synthesis inhibition (Fig. 1G), suggesting that the effect was largely caused by the release of pre-formed factors.

PKCδ Has a Dual Role Both in Apoptotic Factor Release and as an Effector of CM-induced Apoptosis—PKCδ has been established as a key mediator of PMA-induced apoptosis in LNCaP cells (8, 10). To determine whether PKCδ is involved in the release of apoptotic factors, we collected CM from LNCaP cells treated with either the pan-PKC inhibitor GF109203X or the PKCδ inhibitor rottlerin (Fig. 2A). We found that CM collected after pharmacological inhibition of PKCδ had a significantly lower apoptotic effect when added to fresh LNCaP cells. GF109203X totally blocked the PMA effect. Because rottlerin is known to cause nonspecific effects unrelated to PKCδ inhibition (15), we used a RNAi approach, as we have previously described (9). PKCδ levels were reduced by ∼90% in LNCaP cells upon delivery of a specific dsRNA. This dsRNA specifically reduces the levels of PKCδ without affecting the expression of other PMA-responsive PKC isozymes present in LNCaP cells (Fig. 2C). Remarkably, CM-PMA collected from PKCδ-depleted cells has remarkably less apoptotic activity (Fig. 2A), arguing that PKCδ is required for the release of apoptogenic factors in response to PMA. PKCδ also plays a role as an effector of the apoptotic effect of CM-PMA. Indeed, when CM-PMA was added to PKCδ-depleted LNCaP cells, apoptosis was basically undetected. GF109203X and rottlerin also impaired the apoptotic effect of the CM-PMA. The inhibitory effect of rottlerin was not complete, probably because of its ability to cause apoptosis through nonspecific mechanisms (9, 15). Interestingly, the CM-PMA caused translocation of PKCδ in LNCaP cells (Fig. 2D). Thus, PKCδ appears to have a dual role, both in the release of apoptogenic factors as well as an effector.

A Role for TNFα in the PKC-induced Autocrine Loop—As TNFα is known to cause cell death in LNCaP cells (12, 16), we sought to investigate whether this cytokine was involved in the PKC-mediated autocrine effect. As a first approach we used TAPI-2, an inhibitor of TACE/ADAM17 (TNFα-converting enzyme), a metalloprotease responsible for TNFα shedding (17). CM was collected from LNCaP cells treated with PMA in the presence of increasing concentrations of TAPI-2, and then added to fresh LNCaP cells. As shown in Fig. 3A, pretreatment with the TACE inhibitor significantly impaired the apoptotic activity of the CM-PMA. The involvement of TACE was further confirmed using RNAi. We could achieve a 63 ± 8% reduction (n = 3) in TACE expression in LNCaP cells upon delivery of a specific TACE dsRNA (Fig. 3B). CM-PMA collected from TACE-depleted cells has indeed significantly lower apoptogenic activity (∼60% inhibition) when added to fresh LNCaP cells (Fig. 3C). To assess the involvement of TNFα we used a specific anti-TNFα neu-
tralizing antibody, which dose-dependently impaired the ability of the CM-PMA to cause apoptosis. At a concentration of 10 mg/ml, a 48% inhibition ($n = 3$) was observed (Fig. 4A). Real-time PCR assays showed that PMA treatment caused a marked increase in TNF-$\alpha$ mRNA levels (357 ± 92-fold after 3 h, and 1992 ± 146-fold after 6 h). Because our results using cycloheximide (Fig. 1G) suggest that the effect is largely independent of protein synthesis, we believe that the contribution of newly generated TNF-$\alpha$ is probably less important than the released of a pre-formed pool of the cytokine.

**PKC$\delta$ is Required for PMA-induced Secretion of TNF-$\alpha$**—In the next series of experiments we explored whether PKC activation could promote the secretion of TNF-$\alpha$ from LNCaP cells. TNF-$\alpha$ levels in CM-PMA (as determined by ELISA) were remarkably higher compared with those in CM-vehicle (Fig. 4B). As expected, TNF-$\alpha$ levels were significantly reduced by preincubation with the TACE inhibitor TAPI-2 as well as by TACE RNAi. The PKC inhibitor GF109703X completely blocked the secretion of TNF-$\alpha$ caused by PMA. In LNCaP cells subject to PKC$\delta$ RNAi, there was also a marked reduction in TNF-$\alpha$ levels in the CM upon PMA treatment. Taken together, these results support a critical role for PKC$\delta$ in phorbol ester-induced secretion of TNF-$\alpha$.

**TRAIL Is Also Involved in the PKC-mediated Autocrine Effect**—Other cytokines in addition to TNF-$\alpha$ are pro-apoptogenic in prostate cancer cells, including TRAIL and FasL (18–21). When a neutralizing TRAIL antibody was added to the CM-PMA, its apoptogenic activity was significantly reduced. Maximum inhibition (~30%) was achieved at an antibody concentration of 1 $\mu$g/ml (Fig. 5A). On the other hand, a blocking anti-FasL antibody was not effective, even at concentrations of 10 $\mu$g/ml (Fig. 5B). When neutralizing antibodies for TNF-$\alpha$ (1 $\mu$g/ml) and TRAIL (0.1 $\mu$g/ml) were added together to the CM-PMA, the inhibitory effect was higher than with each antibody alone, as both antibodies...
Involvement of TRAIL in PMA-induced apoptosis. Panels A and B, LNCaP cells were treated with CM-PMA for 24 h, either in the presence or absence of TRAIL, TNFα, or Fas-L blocking antibodies, or normal mouse IgG (control). Antibodies were added to the CM-PMA 30 min before and during the incubation. The incidence of apoptosis was determined 24 h later. Data are expressed as percentage of apoptosis caused by CM-PMA in the absence of antibodies. Results are presented as mean ± S.E. (n = 3). Panel C, determination of TRAIL levels by ELISA in CM-vehicle or CM-PMA collected at 8 or 24 h.

Involvement of the Extrinsic Apoptotic Pathway in the PKC-mediated Autocrine Loop—Stimulation of death receptors activates the extrinsic apoptotic pathway. TNFα and TRAIL receptors activate various signaling cascades, including the JNK, p38 MAPK, and NF-κB pathways. Whereas NF-κB mediates mainly anti-apoptotic signals in prostate cancer cells, including LNCaP cells (23, 24), emerging evidence suggests a prominent pro-apoptotic role for p38 MAPK and JNK in prostate cancer cells (9, 13, 25, 26). We found that CM-PMA significantly activates JNK and p38 MAPK in LNCaP cells, as revealed by Western blot using phospho-specific JNK and p38 MAPK antibodies. Other well known downstream effectors of death receptors, such as caspase-8 and NF-κB, also become activated, as determined by antibodies against cleaved caspase-8 and phospho-IκB, respectively. In agreement with previous studies (19, 27, 28), TNFα activates these pathways in LNCaP cells (Fig. 7, A–C). Neutralizing antibodies for TRAIL and TNFα blocked the activation of caspase-8 by CM-PMA (Fig. 7D). To determine the relative contribution of the p38 MAPK and JNK pathways in the context of the apoptotic autocrine loop, we used pharmacological inhibitors of p38 MAPK (SB203580) and JNK (SP600125). Pretreatment of LNCaP cells with either inhibitor significantly blocked the apoptotic effect of CM-PMA; inhibition was almost complete by pretreating cells with both inhibitors together (Fig. 8A). On the other hand, neither of these inhibitors affected the release of the apoptotic factors by PMA (Fig. 8B), arguing for a role for p38 MAPK and JNK as effectors of the apoptotic response.

Together suppressed apoptosis by ~65% (Fig. 5A). Determination of TRAIL levels by ELISA revealed a 2–3-fold increase in CM-PMA relative to CM-vehicle (Fig. 5C). Thus, these results suggest that TNFα and TRAIL, but not Fas-L, are involved in the PKC-mediated autocrine loop in LNCaP cells. When LNCaP cells were treated with TNFα and TRAIL at concentrations similar to those found in the CM-PMA (1 ng/ml TNFα and 0.1 ng/ml TRAIL), the percentage of apoptotic cells was only 7 ± 1%. Moreover, LNCaP cells treated with medium containing concentrations of TNFα and TRAIL 100-fold higher than those in CM-PMA led to 13 ± 2% of apoptosis. Thus, TNFα and TRAIL are required but not sufficient to cause the full apoptotic response.

Involvement of the Extrinsic Apoptotic Pathway in the PKC-mediated Autocrine Loop—Death receptors mediate the actions of TNFα and TRAIL, leading to the activation of the extrinsic apoptotic cascade. TNFR1 and TNFR2 receptors mediate the effects of TNFα, and TRAIL acts via the activation of D4 and D5 receptors (22). The relative contribution of each of these receptors was assessed using RNAi. In the case of the D4, D5, and Fas receptors, reductions in expression of 75 ± 5% (n = 3), 71 ± 2% (n = 3), and 80 ± 10% (n = 3), respectively, were achieved (Fig. 6A). TNFR1 and TNFR2 mRNA levels were also significantly reduced (in this later case reverse transcriptase-PCR was used because of the low sensitivity of available TNFR antibodies in our experimental model). We found that the apoptogenic effect of CM-PMA was significantly reduced when added to LNCaP cells in which TNFR1, TNFR2, D4, or D5 were individually depleted. On the other hand, Fas receptor RNAi did not affect the apoptotic effect of CM-PMA (Fig. 6B).

The involvement of TNFα receptors was further confirmed using blocking antibodies for TNFα receptors. As shown in Fig. 6C, incubation of LNCaP cells with either anti-TNFR1 or anti-TNFR2 specific blocking antibodies (10 μg/ml) significantly inhibited the apoptotic effect of CM-PMA. The response was somehow higher when both antibodies were used together.

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Caspase-8 and FADD RNAi Impair the Apoptotic Effect of the CM-PMA—Ligand binding to death receptors recruits FADD and procaspase-8 to the death-inducing signaling complex (DISC). FADD is a key adaptor for transmitting death receptor signals upon receptor activation, and activated caspase-8 is critical for initiating the apoptosis-executing caspase cascade (22). To further assess the contribution of the extrinsic cascade in PMA-induced apoptosis, LNCaP cells were infected with retroviruses encoding short hairpin RNAs specifically designed to knock-down caspase-8 or FADD. Using this approach we achieved a reduction of 54 ± 10 (n = 3) and 73 ± 6% (n = 3) in caspase-8 and FADD expression, respectively (Fig. 9, A and C). We observed that FADD-depleted cells were markedly resistant to apoptosis caused by the CM-PMA (Fig. 9B). Similar results were observed in caspase-8-depleted LNCaP cells. On the other hand, LNCaP cells transduced with a mismatched short hairpin RNA sequence for caspase-8 retained their sensitivity to the apoptogenic effect of CM-PMA (Fig. 9D). Taken together, these results suggest a critical role for the extrinsic apoptotic pathway in the autocrine effect triggered by PKC activation in LNCaP cells.
DISCUSSION

Several laboratories, including ours, have shown that phorbol esters trigger an apoptotic response in androgen-dependent prostate cancer cells via PKC activation (4–10), but the mechanisms involved have been partially understood. A distinctive aspect of our studies is that they underscore the existence of a PKC-activated autocrine loop that mediates phorbol ester-induced apoptosis that involves the autocrine production of death factors, including TNFα and TRAIL. Neutralizing antibodies for these cytokines, as well as inhibition or depletion of their receptors in LNCaP cells, impairs the apoptotic effect of CM collected upon PMA stimulation. The involvement of the extrinsic apoptotic cascade further supports the contribution of death receptors to the apoptotic effect. An important observation was that PKCδ plays a dual role both as a key mediator of apoptogenic factor release and as an effector.

FIGURE 8. Effect of p38 MAPK and JNK inhibitors. LNCaP cells were treated with 10 μM SB203580 (SB, p38 inhibitor), 10 μM SP600125 (SP, JNK inhibitor), or vehicle (C). Panel A, cells pre-treated with the inhibitors were treated with CM-PMA. Panel B, CM-PMA was collected from LNCaP cells pretreated with the different inhibitors and then added to fresh (untreated) LNCaP cells. The incidence of apoptosis was determined 24 h later. Results are presented as mean ± S.E. (n = 3).

FIGURE 9. CM-PMA-induced apoptosis is dependent on FADD and caspase-8. LNCaP cells were infected with retroviruses encoding short hairpin RNAs for FADD, caspase-8 (C8), or mutated (mut-C8), or infected with control retrovirus (C). Panels A and C, expression of FADD or caspase-8 in LNCaP cells. Densitometric analysis of three independent experiments (mean ± S.E.) is presented. Data are expressed as percentage of the expression in control cells. Insets, representative Western blots. Panels B and D, LNCaP infected with the different retroviruses were treated with CM-PMA. The incidence of apoptosis was determined 24 h later. Results are presented as mean ± S.E. (n = 3).
Essential Role for PKCδ in Phorbol Ester-induced Apoptosis in LNCaP Prostate Cancer Cells—The identification of the roles of individual PKC isoforms and their downstream effectors in apoptotic responses has been a subject of intense investigation. Considerable evidence supports the involvement of PKCδ as mediator of the apoptotic effect of phorbol esters and other agents, including in prostate cancer models (8–10). The role of PKCe, the other novel PKC present in LNCaP cells, still remains controversial, as studies have shown that PKCe renders cells resistant to apoptosis by PMA, whereas others have suggested a pro-apoptotic role for this PKC isozyme (10, 11). In some cell types PKCδ-mediated apoptosis is independent of its phosphorylating activity and probably involves a scaffolding function (29), however, its kinase activity is required for apoptosis in LNCaP cells (8). Whereas PKCδ-mediated apoptosis involves its proteolytic cleavage and the generation of a constitutively active catalytic fragment in several cell types, such as hematopoietic cells, keratinocytes, and salivary epithelial cells (30–32), it strictly depends on allostERIC activation and is cleavage-independent in prostate cancer cells (8). Such heterogeneity in the molecular mechanisms of activation of PKCδ emphasizes its complex regulation and cell-context dependence (33). Our results showing that PKCδ was required for the secretion of TNFα in LNCaP cells underscores a previously unknown role for this novel PKC in the context of the apoptotic response. As cells depleted from PKCδ have also an impaired response to death factors secreted to the CM, it appears that the involvement of this PKC in response to PMA occurs at multiple levels (see below).

TNFα Is Required for PMA-induced Apoptosis in LNCaP Prostate Cancer Cells—Studies from the laboratory of Weinstein (14) have initially established the potential contribution of PKC isoforms to autocrine factor release, although in those studies PKCs were shown to stimulate pro-mitogenic and transforming events. CM collected from PKCe-overexpressing R6 fibroblasts causes abnormal proliferation and malignant transformation, effects that are mediated, at least in part, by TGF-β (14). Also in the context of transformation, recent studies have shown that TNFα levels are elevated in the skin of PKCε transgenic mice and contribute to the development of metastatic squamous cell carcinomas (34). We have determined that factors known to cause apoptosis in LNCaP cells, which include TNFα and TRAIL, are released as a consequence of PKC activation. TACE/ADAM-17, a metalloprotease that promotes the shedding of the pro-form of TNFα into its mature soluble form, can be activated by PKC (35). Our results showing that TACE depletion (using RNAi) or inhibition (using TAPI-2) impaired PMA-induced apoptosis in LNCaP cells further support this hypothesis. Emerging evidence in hematopoietic cells suggests that PKCs could regulate pro-apoptotic autocrine responses (36), and a role for PKCe in LPS-induced apoptosis and TNFα secretion has been reported (37). Enhanced TNFα release in response to the PKC activator bryostatin I (in conjunction with the cdk inhibitor flavopiridol) has also been observed in human myeloid leukemia cells, an effect that is attenuated by the PKC inhibitor GF109203X. In LNCaP prostate cancer cells PKCδ seems to be critical for TNFα release in response to PMA, as determined by pharmacological inhibition of PKCδ and PKCδ RNAi. Whereas the detailed mechanisms by which PKCδ regulates TACE activity/function in prostate cancer cells are yet unknown, studies in keratinocytes have suggested that TACE activation by PKC could be mediated by reactive oxygen species (34).

Other Factors Contributing to PMA-induced Apoptosis in LNCaP Prostate Cancer Cells—Whereas our results clearly show that TNFα is a mediator of the PKC-activated autocrine loop in prostate cancer cells, it is likely that this cytokine alone is not sufficient to cause the apoptotic response. This could be inferred from the partial inhibitory effects of TAPI-2, TNFα neutralizing antibody, TNFRs blocking antibodies, and TNFRs RNAi (although in this later case complete depletion of the receptors could not be achieved). Moreover, the concentrations of TNFα released to the CM upon PMA stimulation would be insufficient to cause apoptosis in LNCaP cells. Whereas our results do not support a role for FasL, experiments using a TRAIL blocking antibody and RNAi for TRAIL receptors would argue for a role for TRAIL in the autocrine effect. TRAIL is elevated in CM from PMA-treated LNCaP cells. Nevertheless, complete inhibition could not be achieved even when both TNFα and TRAIL blocking antibodies were used together, which might suggest the involvement of additional factors. Indeed, when LNCaP cells were treated with TNFα and TRAIL at concentrations similar to those found in the CM-PMA, the apoptotic index was very small. Preliminary studies using a cytokine array suggest that several other cytokines/chemokines are elevated in CM-PMA from LNCaP cells, although it is premature at this stage to conjecture on their relative contribution to phorbol ester-induced apoptosis.

LNCaP cells are partially sensitive to TNFα and insensitive to TRAIL-induced cell death when they act as single agents. However, chemotherapeutic drugs and irradiation sensitize LNCaP cells to TRAIL or TNFα-induced cell death (12, 19, 38, 39). This led us to speculate that the autocrine regulation should involve additional mechanisms. Yet unidentified factors released to the CM and/or alternative PKC-regulated pathways, such as phosphatidylinositol 3-kinase/Akt, may sensitize cells to the effects of TNFα/TRAIL. We support this second hypothesis. Akt plays a major role in prostate cancer cell survival. LNCaP cells have high constitutive Akt activity because of loss of phosphatase and tensin homologue deleted by chromosome ten (PTEN) function, and inhibition of this pathway has been shown to sensitize prostate cancer cells to a number of apoptotic agents (40–42). We have previously shown that in LNCaP cells PMA treatment leads to the dephosphorylation and inactivation of Akt via a PP2A phosphatase. Moreover, an activated Akt mutant (Myr-Akt) protects LNCaP cells from PMA-induced apoptosis (9). It is likely that this dephosphorylation event might sensitize LNCaP cells to the effects of autocrine factors released upon PKC activation, a hypothesis supported by numerous studies showing that suppression of Akt activity makes LNCaP cells sensitive to death factors (19, 41, 42). Interestingly, treatment of LNCaP cells with CM-PMA causes a significant Akt dephosphorylation (data not shown). Although further studies would be required to understand the mechanistic basis of this effect, it is likely to believe that this suppression of Akt activity contributes to apoptosis sensitization.

A Role for the Extrinsic Apoptotic Pathway in PMA-induced Apoptosis in LNCaP Cells—Cell surface death receptors, which belong to the TNF receptor superfamily, contain a cytoplasmic death domain that enables the receptors to engage the cell apoptotic machinery. Stimulation of TNFR1 and TNFRII receptors by TNFα, or D4 and D5 receptors by TRAIL, results in the activation of the initiator caspase-8, which can propagate the apoptotic signal by direct cleavage of downstream effector caspases (22). We determined that the apoptotic effect of the CM-PMA was impaired in caspase-8-depleted LNCaP cells. In addition, depletion of the adaptor protein FADD also inhibited the apoptotic response. The involvement of the extrinsic cascade could also be inferred from the observed induction of caspase-8 cleavage, as well as by the observed phosphorylation (and therefore activation) of p38 MAPK, JNK, and IκB, well established death receptor effectors. The differential kinetics of JNK activation by TNFα and CM-PMA might relate to the effect of still unknown factors in the CM.

3 A. M. Gonzalez-Guerrico and M. G. Kazanietz, unpublished observations.
The involvement of JNK and p38 MAPK in various forms of cell death has been documented extensively. Whereas JNK is required for apoptotic cell death elicited by stimuli such as oxidative stress and UV, recent evidence supports a role for JNK in TNFα-induced apoptosis (43, 44). Using pharmacological inhibitors of JNK and p38 MAPK we determined that these two pathways are not involved in the release of apoptotic factors but instead they are effectors of the autocrine factors in prostate cancer cells. Previous studies have determined that p38 MAPK and JNK become activated by phorbol ester treatment in LNCaP prostate cancer cells (9, 13, 26), and that p38 MAPK inhibitors interfere with PMA-induced apoptosis in these cells (9), which support our experimental conclusions.

As discussed briefly above, our experiments show that the apoptogenic effect of CM-PMA was substantially reduced when added to PKCδ-depleted cells, suggesting a role for PKCδ as a death receptor effector. This observation was at first puzzling, because death receptors do not couple directly to effectors that generate diacylglycerol and activate PKC. However, some recent reports have suggested a potential link between death receptors and PKC. A paper by Kilpatrick et al. (45) showed that in neutrophils PKCδ forms a signal complex with TNFR1 and phosphatidylinositol 3-kinase in response to TNFα that greatly influences the recruitment of receptor adaptors. TNFα activation of PKCδ has been recently reported in pancreatic acinar cells (46). In intestinal cells, TNFα could cause membrane translocation of PKC isoforms, including PKCθ, an effect that correlates with increased apoptosis (47, 48). Ongoing studies in our laboratory are aimed at understanding the mechanisms of death receptor regulation of PKCδ function in prostate cancer cells.

Final Remarks—In summary, our studies have introduced a novel paradigm in phorbol ester-induced apoptosis in LNCaP prostate cancer cells that involves the autocrine production of death factors and the activation of the extrinsic apoptotic pathway. PKCδ is required both for the release of autocrine factors and for transducing apoptotic signals downstream of death receptors. In addition to the mechanistic implications, our studies may have significant therapeutic relevance. Indeed, PKC activators (including phorbol esters) are in clinical trials for various types of cancers (49–51) and greatly enhance the effectiveness of other antitumor agents and radiation in prostate cell tumor-bearing mice (6, 7).

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