Androgens Regulate Protein Kinase Cδ Transcription and Modulate Its Apoptotic Function in Prostate Cancer Cells

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Abstract

Activation of protein kinase Cδ (PKCδ), a member of the novel PKC family, leads to apoptosis in several cell types. Although the molecular bases of PKCδ activation are being unfolded, limited information is available on the mechanisms that control its expression. Here, we report that in prostate cancer cells PKCδ is tightly regulated by androgens at the transcriptional level. Steroid depletion from the culture medium causes a pronounced down-regulation of PKCδ protein and mRNA in androgen-sensitive LNCaP prostate cancer cells, an effect that is rescued by the androgen R1881 in an androgen receptor (AR)–dependent manner. Analysis of the PKCδ promoter revealed a putative androgen responsive element (ARE) located 4.7 kb upstream from the transcription start site. Luciferase reporter assays show that this element is highly responsive to androgens, and mutations in key nucleotides in the AR-binding consensus abolish reporter activity. Furthermore, using chromatin immunoprecipitation assays, we determined that the AR binds in vivo to the PKCδ ARE in response to androgen stimulation. Functional studies revealed that, notably, androgens modulate phorbol 12-myristate 13-acetate (PMA)–induced apoptosis in LNCaP cells, an effect that is dependent on PKCδ. Indeed, androgen depletion or AR RNA interference severely impaired the apoptotic function of PKCδ or the activation of p38, a downstream effector of PKCδ in LNCaP cells—effects that can be rescued by restoring PKCδ levels using an adenoviral delivery approach. Our studies identified a novel hormonal mechanism for the control of PKCδ expression via transcriptional regulation that fine-tunes the magnitude of PKCδ apoptotic responses. (Cancer Res 2006; 66(24): 11792–801)

Introduction

Protein kinase C (PKC) isoforms, a family of at least 10 related kinases, are key regulators of cellular responses that include proliferation, differentiation, transformation, survival, and apoptosis. These kinases are classified based on their structural and biochemical properties into “classic” (calcium-sensitive) cPKCs α, β, γ, δ, ε, ζ, η, and θ; and “atypical” aPKCs ξ and η. cPKCs and nPKCs are activated by the lipid second messenger diacylglycerol (DAG) and DAG mimetics such as the phorbol esters. Despite their high homology, it is now clear that each PKC has unique modes of regulation that involve phosphorylation, protein-protein interactions, and intracellular targeting—mechanisms that confer isozyme specificity in substrate phosphorylation (1–3). Activation of cPKCs and nPKCs by phorbol esters leads to a plethora of cellular responses, which could be as divergent as proliferation or antiproliferation in some cell types, and apoptosis or survival in others. A main reason for such heterogeneity in phorbol ester responses is the differential cellular expression of PKC isozymes, leading ultimately to isozyme-specific regulation of downstream signaling networks (4). The ability of DAG mimetics to cause cell arrest, apoptosis, or differentiation has served as a rationale for their use as chemotherapeutic agents; indeed, phorbol esters and related analogues, such as the bryostatins, are being tested in clinical trials for several types of malignancies (5–8).

Among the PKC gene products, PKCδ is known to play an important role as a mediator of antiproliferative and apoptotic responses. Early studies revealed that ectopic expression of PKCδ leads to growth arrest (9), and transgenic mice overexpressing PKCδ in the skin are highly resistant to tumor promotion by phorbol 12-myristate 13-acetate (PMA; ref. 10). Studies in the last years have implicated PKCδ in apoptotic responses triggered by a number of stimuli, including DNA-damaging agents, death receptor ligands, ionizing radiation, and phorbol esters (11–15). We and others have established that PKCδ mediates the apoptotic effect of phorbol esters and related activators in prostate cancer cells. PKCδ inhibition (both pharmacologic or with a dominant-negative mutant) or depletion [using RNA interference (RNAi)] impairs the apoptotic effect of PMA in LNCaP cells (15–18). In recent studies, we have determined that the apoptotic effect of PKCδ is mediated by the p38 mitogen-activated protein kinase (MAPK) pathway (19). Subsequent mechanistic analysis revealed that the effect is mediated by the autocrine secretion of death receptor ligands, including tumor necrosis factor α (TNF-α) and TNF-related apoptosis-inducing ligand (TRAIL), which are potent activators of p38. PKCδ seems to play a dual role, both in the secretion of autocrine factors as well as an effector downstream of death receptor activation (15).

PKCδ-mediated responses, including apoptosis, are tightly regulated by epigenetic mechanisms that include posttranslational mechanisms (e.g., tyrosine phosphorylation), association with specific partners, and compartmentalization (20). Despite years of extensive investigation on the molecular basis of activation and degradation of PKCs, including PKCδ, there is surprisingly very little information on the mechanisms that control the expression of individual PKC isoforms, particularly at a transcriptional level. Notably, changes in the expression of PKC isoforms, including PKCδ, have been reported in various pathologic conditions, including cancer, as well as in response to a number of physiologic
and pharmacologic stimuli (21–23). Although PKCδ expression could be modulated by estrogens, vitamin D3, or mechanical forces, presumably through genomic mechanisms (24–27), there is surprisingly very limited knowledge regarding the functional elements in the human PKCδ gene promoter or the nature of the factors that control PKCδ gene expression.

In this article, we report the identification of a novel androgen-mediated mechanism that controls the expression of PKCδ in prostate cancer cells by positively regulating PKCδ transcript and protein levels. We identified a functional androgen responsive element (ARE) that binds androgen receptor (AR) in response to androgen stimulation in the human PKCδ gene promoter. Regulation of PKCδ expression by androgens via the AR has significant functional consequences, as we determined that androgens exert a significant control of PKCδ-mediated apoptosis and signaling in prostate cancer cells.

Materials and Methods

Materials. PMA was purchased from LC Laboratories (Woburn, MA). The synthetic androgen methyl trienolone (R1881) was obtained from Perkin-Elmer Life Sciences (Boston, MA) and dihydrotestosterone was purchased from Steraloids, Inc. (Newport, RI). Epidermal growth factor (EGF), nerve growth factor (NGF), phosphatidylserine, and 4-purchased from Steraloids, Inc. (Newport, RI). Epidermal growth factor (Perkin-Elmer Life Sciences (Boston, MA) and dihydrotestosterone was fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Other elements in the human

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Cell culture. LNCaP human prostate cancer cells (from ATCC, passages 2–8) were cultured as described in ref. 15. Isolation of the PKCδ ARE and mutagenesis. A 200-bp fragment encompassing the putative PKCδ ARE was isolated by PCR from bacterial artificial chromosome (BAC) clone RP11-82B23, using the primers 5'-CCCAACGCTTTTCCATCAAAGAATGGGCC-GAGCTCAG (ARE-F) and 5'-CCGC-GGATCCGGCTCAGAAGAACCCCTCCAGGCC (ARE-R). Primers contain HindIII and BamHI restriction sites (underlined) to facilitate subcloning into pTK-Luc. Mutagenesis of the PKCδ ARE was carried out using a site-directed mutagenesis approach based on a two-step PCR reaction. The first step enabled the mutation of three key residues in the ARE sequence and generated two fragments with overlapping regions. The primers used were 5'-CCGGAAATTAGCCGGCCGCGTTATAGCGGGCTTGCG (forward) and ARE-R for one PCR reaction, and 5'-CCGCAAAACCCCGTCTATAACCGGC-CCGGCTCATTTCCCG (reverse) and ARE-F for the other. The second step involved a PCR reaction using as template the two overlapping DNA fragments and ARE-F and ARE-R as primers. The resulting 200-bp fragment containing a mutated ARE was ligated into HindIII-BamHI in pTK-Luc to generate pTK-PKCδ-mutARE.

 Luciferase assays. Cells were cotransfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with 1 μg of reporter plasmid (pTK-PKCδ ARE, pTK-PKCδ-mutARE, pTK, or a probasin-ARE reporter; ref. 28) and 50 ng of a Renilla luciferase vector (pRL-TK) in complete medium. At 24 hours later, luciferase activity was determined 16 hours after R1881 treatment. For the determination of prostate-specific antigen (PSA) mRNA levels, the following first antibodies were used: anti-PKCδ, anti-PKCε, and anti-PKCζ (Santa Cruz Biotechnology; Santa Cruz, CA) or control rabbit IgG were added to 500 μl of the purified chromatin sample and incubated overnight at 4°C. The following PCR primer sets were designed to overlap and span the androgen response region of the PKCδ promoter: 5'-CCCAACGCTTTTCCATCAAAGAATGGGCC-GAGCTCAG (forward) and 5'-CCGGAGATCCGGCTCAGAAGAACCCCTCCAGGCC (reverse), PCR amplification was carried out for 40 cycles with denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. For input, PCR was done on unpurified chromatin diluted 1,000 times.

 Apoptosis assays. Cells were treated with PMA for 1 hour; 24 hours later, cells were trypsinized, mounted on glass slides, and fixed with 70% ethanol. Morphologic changes in chromatin structure were assessed after staining with DAPI, as previously described (16). The incidence of apoptosis was analyzed by counting 500 cells followed by the determination of apoptotic cells in each preparation. Flow cytometry analysis was carried out as previously described (15, 16).

 Cell proliferation assays. Cell proliferation was monitored using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). After 2 days of treatment with R1881, EGF, or NGF, cells were stained with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium (MTS), and the plates were incubated for 2 to 4 hours at 37°C for stain development. Absorbance at 490 nm was determined in an automated plate reader. In each experiment, samples represent plates in triplicate.

 Adenoviral infections. Generation of the PKCδ adenovirus was described elsewhere (16). Adenoviruses for LacZ (16) or green fluorescent protein (GFP; Q-BIogene, Carlsbad, CA) were used as controls. Subconfluent LNCaP cells were infected with the adenoviruses for 14 hours at multiplicities of infection (MOI) ranging from 1 to 30 plaque-forming unit per cell in RPMI 1640 supplemented with 2% FBS. After removal of the virus, cells were incubated for 24 hours in RPMI 1640 supplemented with 10% charcoal-treated FBS.

 Western blot analysis. Cell harvesting and SDS-PAGE was described elsewhere (16). The following first antibodies were used: anti-PKCα, anti-PKCδ, anti-PKCε, and anti-PKCζ (Santa Cruz Biotechnology); anti-total-p38 MAPK and anti-phospho-p38 MAPK (Cell Signaling Technology, Beverly, MA); and anti-actin (Sigma). Antibodies were used at a 1:1,000 dilution except for anti-PKCα, which was used at a 1:300 dilution. Bands were visualized with the enhanced chemiluminescence detection system.

 PKC kinase assays. Prostate cancer cells were incubated in 2% charcoal-treated FBS for 48 hours, and increasing concentrations of R1881 were added for 24 hours. Cells were lysed in 50 mmol/L Tris-HCI (pH 7.4) containing 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mmol/L PMSF. Kinase assay was carried out for 10 minutes at 30°C in a buffer containing 50 mmol/L Tris-HCI (pH 7.4), 1 mmol/L MgCl2, 25 μmol/L ATP (1 μCi/mmole), 7.5 μmol/L magnesium acetate, 10 μmol/L 4-pseudosubstrate peptide, 100 μmol/L phosphatidylserine, and 1 μmol/L PMA. Reaction was stopped by spotting onto Whatman PE-81 papers. Filters were washed thrice in 0.1 mol/L phosphoric acid rinsed with acetone and allowed to dry before scintillation counting.

Reverse transcription-PCR. Total RNA was isolated from LNCaP cells using TRIzol (Invitrogen) according to the manufacturer’s protocol. Two micrograms of total RNA were treated with RNase-free DNase I (Roche, Mannheim, Germany) before the cDNA synthesis. Oligo(dT) (New England Biolabs, Beverly, MA) was used as a primer to generate cDNA. PCR was subsequently done to analyze the expression of PKCδ using the primers 5’-CTGCAAGAAGAAACATGGCGG (forward) and 5’-ATCCAGTCTCCT-CAGGAAATCT (reverse). In some experiments, LNCaP were steroid depleted and then treated for different times with R1881 (0.01–10 nmol/L) and cycloheximide (50 μmol/L, 1 hour before and during R1881 treatment). For the determination of prostate-specific antigen (PSA) mRNA levels, the primers were 5’-CATCAGGAAACAAAGCGTGAG (forward) and 5’-TGGGTT- CAAAGACTTCC (reverse). For glyceraldehyde-3-phosphate dehydrogenase, the primers were 5’-CCCTTCTATGCCACTCAATCG (forward) and 5’-CATGGTGTTGAAAGGCCAG (reverse). PCR products were separated in 2% agarose gels and visualized by ethidium bromide staining.
RNAi. Double-stranded RNA (dsRNA) duplexes for the AR were purchased from Dharmacon Research, Inc. (Lafayette, CO). The sequence was as follows: AAGCACUGCUACUCUUCAGCA. The sequences of dsRNA duplexes for PKC\(\alpha\) and PKC\(\beta\) RNAi were described elsewhere (29), and were designed based on the sequences from human PKC\(\alpha\) and PKC\(\beta\) genes (Genbank accession nos. X52479 and D10495, respectively). As a control RNAi, we used an unrelated sequence (CAUCGCUGUAGCAUCGUCU). For dsRNA transfections, cells were seeded in 6- or 12-well plates at \(-80\%\) confluency in antibiotic-free medium, and transfected using Oligofectamine (Invitrogen) according to the manufacturer's instructions. Experiments were carried out 48 hours later.

### Results

**Androgen-dependent expression of PKC isozymes in prostate cancer cells.** Individual PKC isozymes differentially control cellular processes, such as proliferation, survival, and cell death. In androgen-dependent models of prostate cancer, phorbol esters promote apoptosis via the activation of PKC\(\alpha\) (16–19). Given the importance of androgens in modulating signaling events, we asked whether they could influence PKC-mediated responses in LNCaP prostate cancer cells. We first investigated whether steroid depletion affects the expression of PKC isozymes. Cell extracts from LNCaP cells growing either in normal medium or in steroid-free medium (in which FBS was charcoal-treated) were subjected to Western blot using specific anti-PKC isozyme antibodies. A significant reduction in the levels of novel, calcium-independent PKC\(\beta\) was observed when LNCaP cells were grown in steroid-depleted medium (Fig. 1A, left). Densitometric analysis revealed that PKC\(\beta\) expression was 40 \pm 15\% \((n = 5)\) relative to that in normal medium. No significant changes were found in the levels of PKC\(\alpha\), PKC\(\epsilon\), PKC\(\zeta\), and PKC\(\xi\).

![Figure 1. Regulation of PKC\(\beta\) expression by androgens. A, left, LNCaP cells were grown for 48 hours either in normal medium or in medium in which FBS has been charcoal treated. Cell extracts were prepared and subjected to Western blot using isozyme-specific PKC antibodies. Right, LNCaP cells were grown in charcoal-treated medium either in the absence or presence of the androgen R1881; after 48 hours, cell extracts were subject to Western blot using specific anti-PKC antibodies. B, LNCaP (left), and PC3 and DU145 cells (right) were grown in charcoal-treated medium either in the absence or presence of the androgen R1881; after 48 hours, cell extracts were used to determine calcium-independent PKC kinase activity. Data are expressed as cpm of \(^{32}\)P incorporated into the substrate per minute of reaction and milligram of protein in the lysate. Columns, mean of triplicate samples; bars, SE. C, LNCaP or the LNCaP variants C4 and C4-2 were grown for 48 hours in normal medium, steroid-depleted medium, or steroid-depleted medium supplemented with 1 nmol/L R1881. PKC\(\beta\) expression was determined by Western blot (left), and PSA mRNA levels were determined by reverse transcription-PCR (RT-PCR; right). For all panels experiments were done at least thrice, which gave similar results. D, similar experiments as those described in (C) were carried out in PC3 cells. E, LNCaP cells growing in steroid-depleted medium for 48 hours were treated with R1881 for different times and concentrations. Total RNA was extracted and PKC\(\alpha\) mRNA was determined by RT-PCR. Left, time course analysis using 1 nmol/L R1881. Center, concentration-response analysis at 8 hours. Right, RT-PCR analysis of LNCaP cells growing in steroid-depleted medium supplemented with 1 nmol/L R1881 for 8 hours, either in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX; 50 \(\mu\)mol/L, 1 hour before and during R1881 treatment). Two additional experiments gave similar results.](https://cancerres.aacrjournals.org/article-pdf/66/24/11794/11794f1.pdf)
the other PKCs expressed in LNCaP cells, namely the cPKC\(\alpha\), nPKC\(\varepsilon\), and aPKC\(\zeta\) (118 ± 25%, 101 ± 5%, and 100 ± 2% relative to normal medium, as determined by densitometry, \(n = 5\) in all cases). To determine whether the effect could be ascribed to androgen depletion, LNCaP cells growing in charcoal-treated medium were supplemented with increasing concentrations of the specific androgen R1881. The androgen caused a remarkable elevation in PKC\(\delta\) levels that becomes evident at 10 to 100 pmol/L, but it did not affect the levels of PKC\(\alpha\) and PKC\(\zeta\) (Fig. 1A, right). Interestingly, PKC\(\alpha\) was also up-regulated by R1881, an effect that was evident at high concentrations of the androgen. R1881 treatment led to a dose-dependent increase in total cellular calcium-independent PKC activity, with the maximum effect observed at 1 nmol/L R1881. This effect was not seen in androgen-independent cell lines PC3 and DU145 (Fig. 1B). Elevations in PKC\(\delta\) levels in response to R1881 were also observed in the LNCaP-derivative C4 cell line that retains androgen sensitivity (30), but not in the androgen-independent variant C4-2 cells (Fig. 1C, left) or in androgen-insensitive PC3 cells (Fig. 1D). PKC\(\delta\) up-regulation in response to R1881 was also observed in normal epithelial prostate cells (kind gift of Dr. Donna Peehl, Stanford University, data not shown). As a control for the androgen responsiveness in LNCaP sublines, we measured PSA mRNA levels. As expected, R1881 treatment increased PSA mRNA in LNCaP and C4 cells, but not in C4-2 cells (Fig. 1C, right).

Next, PKC\(\delta\) transcript levels were determined in LNCaP cells in response to R1881. Androgen treatment led to a time- and dose-dependent increase in PKC\(\delta\) mRNA levels (Fig. 1E). To determine whether the androgen effect involves de novo protein synthesis, we used the protein synthesis inhibitor cycloheximide. A comparable induction of PKC\(\delta\) mRNA by R1881 after 8 hours was observed either in the presence or absence of cycloheximide, suggesting that the androgen effect is probably direct rather than involving a secondary response mediated by an androgen-induced intermediary. Therefore, the effect should involve the direct regulation of PKC\(\delta\) transcription by androgens.

**Identification of an ARE in the PKC\(\delta\) promoter.** The aforementioned direct induction of PKC\(\delta\) mRNA by androgen in prostate cancer cells prompted us to look for androgen-responsive sequences in the PKC\(\delta\) promoter. The human PKC\(\delta\) gene comprises 18 exons that span ~32 kb. Analysis of 30 kb upstream from the transcription start site using the program Genomatix revealed a single putative ARE located ~4.7 kb (NT_022517: 53130598–53130617). The sequence of the putative ARE was 5’-ATAGAGCCGGGTTTATTTAT (Fig. 2A). A BAC clone encoding for the promoter region (RP11-82B23) was obtained from the Children’s Hospital Oakland Research Institute\(^3\) and used as a template for PCR cloning of a 200-bp fragment comprising the putative ARE. This fragment was subcloned into the pTK-Luc reporter vector to generate pTK-Luc-ARE-PKC\(\delta\) (see Experimental Procedures). LNCaP cells were transfected with this reporter plasmid and then grown in steroid-depleted medium, either in the absence or presence of increasing concentrations of R1881. As shown in Fig. 2B (left), R1881 caused a concentration-dependent elevation in luciferase activity. A 35-fold increase in luciferase activity was observed with 1 nmol/L R1881. No changes in luciferase activity were observed in LNCaP cells transfected with pTK-Luc (empty vector). The androgen response was in the same range as that observed with a probasin reporter (Fig. 2B, right), a known androgen-responsive gene that contains a well-defined ARE (28).

Next, we generated a plasmid encoding a mutated PKC\(\delta\) ARE in which nucleotides TGTT, which are well conserved in AREs, were replaced by CGCG (pTK-PKC\(\delta\)-AREmut). Upon transfection into LNCaP cells, this mutated reporter was insensitive to R1881 (Fig. 2B, right). The PKC\(\delta\) ARE luciferase reporter vector did not cause any measurable response when transfected into androgen-independent prostate cancer cell lines DU145 and PC3 (Fig. 2C).

To assess the relevance of the PKC\(\delta\) ARE in vivo, we used a ChIP assay. DNA-binding proteins from LNCaP cells growing in steroid-depleted medium, before and after treatment with R1881, were cross-linked using formaldehyde, and an anti-AR antibody was used to immunoprecipitate the AR-DNA complexes. PCR analysis using specific primers designed to amplify the PKC\(\delta\) ARE revealed a significant increment of AR binding in response to R1881. A control immunoprecipitation carried out using normal IgG did not result in any band amplification under the same experimental conditions (Fig. 2D, bottom). To determine the specificity of the androgen effect, we used the AR antagonist Casodex (bicalutamide). As shown in Fig. 2D (bottom), Casodex prevented the effect of R1881. These results indicate that the motif located in position ~4.7 kb in the PKC\(\delta\) gene is an active AR-binding sequence in cells.

**Androgens modulate the apoptotic response of PMA in LNCaP prostate cancer cells.** To determine the functional relevance of the PKC\(\delta\) induction by androgens, we used a model of PMA-induced apoptosis in LNCaP prostate cancer cells. We have previously determined that RNAi depletion of PKC\(\delta\) in LNCaP cells impairs PMA-induced apoptosis in these cells, denoting its essential role as mediator of the phorbol ester effect (15). We speculated that the apoptotic effect of PMA would be impaired in androgen-deprived LNCaP cells as a consequence of PKC\(\delta\) down-regulation. Indeed, the apoptotic response of PMA in LNCaP cells growing in charcoal-treated medium was significantly lower compared with that in normal medium, as judged by the reduced number of cells with nuclear fragmentation. In agreement with these results, flow cytometry analysis revealed that the population of cells in sub-G0, which was significantly enhanced upon PMA treatment in LNCaP cells growing in normal medium, was barely detected when similar treatment was applied to cells in steroid-depleted medium (Fig. 3A). Quantification of apoptotic cells revealed a 6-fold reduction in the number of apoptotic cells in response to PMA in steroid-depleted medium (normal medium, 29 ± 1%; steroid-depleted medium, 6 ± 1%, \(n = 3\); Fig. 3B).

To assess the effect of androgens, the apoptotic effect of PMA was determined in LNCaP cells growing in steroid-depleted medium supplemented with R1881. Interestingly, the apoptotic effect of PMA was fully restored in the presence of R1881 (Fig. 3A and B). The effect of R1881 was dose-dependent, with maximum rescue observed at 1 nmol/L. A similar effect was observed with the androgen dihydrotestosterone (Fig. 3B). On the other hand, other steroids such as progesterone and dexamethasone, or T3, were ineffective in rescuing PMA-induced apoptosis (Fig. 3B). The lack of a PMA apoptotic response in steroid-depleted medium could not be attributed to apparent changes in the apoptotic machinery, because the genotoxic agent camptothecin caused similar apoptotic responses both in normal and charcoal-treated medium (Fig. 3C).

As androgens are mitogenic in LNCaP cells, it is not unreasonable to believe that the reduced apoptotic response of PMA in

\(^3\) http://bacpac.chori.org.
androgen-free medium may be due to changes in cell proliferation rate. To assess this issue, we used EGF or NGF, well-known prostatic mitogenic factors. At a concentration that induced a mitogenic response comparable with that of R1881, EGF was unable to restore the apoptotic effect of PMA. Although somehow less mitogenic than EGF, NGF was also incapable to restore the apoptotic response (Fig. 3D). These results suggest that the rescue of the apoptotic effect of PMA by androgen was not linked to changes in proliferation.

AR mediates androgen effects in LNCaP cells. To confirm the specificity of the androgen effect, we used the AR antagonist Casodex. Figure 4A shows that Casodex blocked the induction of PKCα by R1881 in LNCaP cells growing in steroid-depleted medium. Moreover, in the presence of Casodex, R1881 was unable to restore the apoptotic effect of PMA (Fig. 4B, left). Casodex also has a significant inhibitory effect on PMA-induced apoptosis in LNCaP cells growing in normal medium (Fig. 4B, right).

To further ascertain the role of androgens in PKCα regulation, we reduced AR expression in LNCaP cells using RNAi. As shown in Fig. 4C (left), expression of the AR was significantly reduced upon delivery of a specific AR dsRNA (AR levels were 26 ± 22% relative to control, n = 4). Notably, AR depletion prevented the up-regulation of PKCα by R1881 (Fig. 4C, right). Moreover, the rescue of the PMA apoptotic response by R1881 was significantly impaired in AR-depleted LNCaP cells (Fig. 4D). A control RNAi duplex did not have any effect (data not shown).

We have previously determined that PMA-induced apoptosis in LNCaP cells is mediated by the p38 MAPK pathway, as p38 inhibitors significantly impaired the apoptotic effect of PMA (19). We reasoned that under androgen deprivation conditions, PMA-induced activation of p38 should be impaired due to the reduced levels of PKCα. Indeed, although PMA caused a strong activation of p38 in LNCaP cells growing in normal medium (see our previous article, ref. 19), p38 activation in response to PMA was barely detected in cells growing in charcoal-treated medium, as determined with a phospho-p38 antibody. Notably, p38-induced activation by PMA was restored by the addition of R1881 to the steroid-depleted medium. The effect of R1881 could be detected at a concentration of 10 pmol/L and reached the maximum at 0.1 to 1 nmol/L (Fig. 5A, top), and it was fully inhibited by Casodex (Fig. 5B, bottom). To confirm the dependence of PMA-induced p38 activation on PKCα expression, we used a RNAi approach. Transfection of a PKCα dsRNA into LNCaP cells depleted PKCα without affecting the levels of other PKC isozymes present in these cells (see our previous article, ref. 15), and it impaired the up-regulation of PKCα by R1881 (Fig. 5B). In PKCα-depleted cells, R1881 was unable to restore PMA-induced p38 activation (Fig. 5C) or PMA-induced apoptosis (Fig. 5D). On the other hand,
transfection of a PKCo dsRNA into LNCaP cells, which specifically depleted PKCo, was unable to affect PKCo induction by R1881, and did not rescue PMA induction of p38 activation or apoptosis. These results not only confirm the causal link between PKCo, p38 activation, and apoptosis in LNCaP cells, but in addition they strongly support the concept that androgens, via the AR, regulate PKCo expression and consequently modulate PKCo-mediated signaling and apoptosis.

Rescue of PMA-induced apoptosis by adenoviral delivery of PKCo. Our results strongly suggest that the apoptotic effect of PMA in LNCaP cells is dependent on the expression levels of PKCo. We reasoned that reinstating PKCo levels in LNCaP cells growing in androgen-deprived medium should restore the apoptotic effect of PMA. We used a recombinant adenovirus to deliver PKCo into LNCaP cells, an approach that we have previously described (16, 17). As a negative control, we used either a GFP adenovirus (Fig. 6A) or LacZ adenovirus (data not shown), which did not cause any significant increase in PKCo levels. Notably, reinstating PKCo into LNCaP cells growing in steroid-depleted medium significantly restored PMA responsiveness. The apoptotic effect of PMA was proportional to the levels of PKCo achieved by using different MOIs (Fig. 6A). As predicted, in this context, overexpression of PKCo also restored p38 activation in response to PMA (Fig. 6A). These results not only emphasize the importance of PKCo as a proapoptotic kinase in prostate cancer cells and its modulation by androgens, but also strongly support the concept that modulation of PKCo intracellular levels is crucial for determining the magnitude of the PMA apoptotic response.

Discussion

PKCo has been implicated as an effector of signals that promote apoptosis or inhibit cell proliferation. Strong evidence supports its involvement in the control of signaling events and the expression of genes that control cell fate (13, 15, 18–20, 31). Activation of PKCo occurs in response to DAG elevations, an effect mimicked by phorbol esters, and in response to death signals (15–20, 32, 33). It is well established that PKCo activity is tightly regulated by a number of posttranslational events, including proteolytic cleavage and phosphorylation by serine/threonine and tyrosine kinases (20, 31); however, limited information is available on the mechanisms that control PKCo expression at the transcriptional level. The results

Figure 3. Androgens modulate PMA-induced apoptosis in LNCaP prostate cancer cells. LNCaP cells were grown in normal medium, steroid-depleted medium, or steroid-depleted medium supplemented with R1881 or other steroids. After 48 hours, cells were treated with PMA (100 nmol/L, 1 hour; A and B) or camptothecin (5 μmol/L, 40 hours; C). After extensive washings, cells were grown in normal medium for 24 hours and stained with DAPI for determination of apoptosis, or subjected to flow cytometry. A, left, representative micrographs of LNCaP cells stained with DAPI. Nuclear morphology is shown 24 hours after treatment with PMA (+PMA) or vehicle (-PMA). Arrows, apoptotic cells. A, right, flow cytometry analysis, 24 hours after PMA treatment. B, effect of androgens [R1881 and dihydrotestosterone (DHT), progesterone (Pr), T3, and dexamethasone (Dex)]. C, apoptotic effect of camptothecin in normal and steroid-depleted medium. Columns, mean of triplicate determinations; bars, SE (B and C). Similar results were observed in three independent experiments. D, R1881 rescue of PMA-induced apoptosis in LNCaP cells is independent of the proliferation rate. LNCaP cells were grown in steroid-depleted medium for 24 hours, and then 1 nmol/L R1881, 50 ng/mL EGF, or 20 ng/mL NGF were added to the medium for 48 hours. Left, cells were stained with MTS and the absorbance was measured at 490 nm. Right, cells were treated with PMA (100 nmol/L, 1 hour), and apoptosis was determined 24 hours later. Columns, mean of triplicate determinations; bars, SE. Similar results were observed in two additional independent experiments.
presented in this article reveal a previously unappreciated regulation of PKC\(\delta\) expression by androgens, thus underscoring a novel hormonal control of PKC function. Such regulation has considerable effect on PKC\(\delta\)-mediated apoptosis in prostate cancer cells.

Androgens were found to markedly stimulate the expression of PKC\(\delta\) protein and mRNA. Two likely scenarios were that PKC\(\delta\) induction by androgens was either mediated by a direct effect on the PKC\(\delta\) promoter or dependent on the induction of androgen-dependent genes that ultimately affect the PKC\(\delta\) promoter. Given the inability of a cycloheximide to influence androgen-mediated induction of PKC\(\delta\) mRNA, we concluded that the effect was direct rather than involving an intermediary, thus suggesting the presence of androgen-responsive sequences in the PKC\(\delta\) gene. Nuclear receptors modulate transcription by binding to DNA-response elements containing conserved hexameric sequences arranged as either monomeric sites, direct repeats, or palindromic sequences. Androgen, glucocorticoid, mineralocorticoid, and progesterone receptors bind with high affinity to the inverted repeat consensus sequence 5'-AGAACAnnnTGTTCT. Many AREs with androgen specificity resemble more direct repeats of the 5'-TGTTCT-like sequences than inverted repeats of this DNA sequence (34). Our in silico analysis of a 30-kb region upstream of the PKC\(\delta\) transcription start site revealed an ARE-like sequence located at -4.7 kb, which resembles the palindromic consensus sequence. Luciferase reporter assays showed that this sequence is responsive to androgens, and that the magnitude of the induction was similar to that observed with a probasin reporter that contains a well-defined ARE. Moreover, androgen responsiveness is lost when mutations are introduced in key residues of the ARE sequence. It is known that steroid receptor binding sites can deviate considerably from the consensus high-affinity motif and, in addition, steroid receptor recognition by low-affinity sites can be significantly enhanced by flanking sequences and coregulators (35, 36). Thus, at the present time, we cannot rule out the contribution of additional sites to the androgen response. Importantly, the -4.7-kb PKC\(\delta\) ARE is capable of binding AR in response to R1881 in LNCaP cells, as determined by ChIP, suggesting that it is functionally relevant in vivo.

The overall genomic structure of the PKC\(\delta\) gene is highly conserved from Caenorhabditis elegans to higher vertebrate organisms, with a remarkable conservation in the lengths of exons and introns in mammals (37). PKC\(\delta\) is developmentally regulated, and its expression is altered in a number of pathologic conditions or in response to various stimuli, including death factors such as TNF-\(\alpha\) and cytotoxic agents (22, 38). It has been recently found that endogenous testosterone increases coronary PKC\(\delta\) protein and activity in porcine males (39). Recent studies on the mouse PKC\(\delta\)
promoter attest to its complexity, as a range of putative transcription factor binding sites involved in processes such as development, immune response, oxidative stress, and oncogenesis, have been identified (37), which are highly conserved in humans (our own analysis; data not shown). To our knowledge, the only functional elements characterized in the PKC<sub>y</sub> promoter are nuclear factor-κB sites located immediately upstream of the transcription start of the murine gene (37). It is unknown whether these sites play a role in the context of the PKC<sub>y</sub>-mediated autocrine regulation via cytokines and death receptor activation in human prostate cancer cells (15). Analysis of the promoters of PKC<sub>d</sub> genes from multiple species revealed the presence of putative AREs (data not shown), suggesting that androgen induction of PKC<sub>y</sub> may also take place in species other than humans and swine. Curiously, studies in rats have shown that castration leads to a significant decrease in total PKC activity in the prostate (40). Although PKC<sub>y</sub> levels were not examined in those studies, PKC<sub>a</sub> expression was found to be very sensitive to circulating levels of testosterone. This is in agreement with our results showing that androgens up-regulate PKC<sub>a</sub> in LNCaP cells. PKC<sub>a</sub>, unlike PKC<sub>y</sub>, is not down-regulated in LNCaP cells growing in charcoal-treated medium, suggesting compensatory mechanisms by serum components. Although the role of PKC<sub>a</sub> in apoptosis is less clear, we have reported that this cPKC modulates the phosphorylation and activation status of the survival kinase Akt via protein phosphatase 2A in LNCaP cells (19). A preliminary analysis of the putative PKC<sub>a</sub> promoter reveals the presence of three putative AREs. The characterization of AREs in the PKC<sub>a</sub> promoter are beyond the scope of these studies, but one may envision that complex regulatory mechanisms and cross-talks may take place in the control of the expression of proapoptotic PKCs.

The mechanisms that control PKC-mediated apoptotic responses have been extensively studied in the last years, particularly in models of androgen-dependent prostate cancer. Phorbol esters cause apoptosis in LNCaP cells and xenografts; they also sensitize LNCaP tumors in mice to ionizing radiation (41). Given the emerging potential of PKC activators as anticancer agents, as reported in numerous clinical trials (5–8), there is great interest in defining the signaling events involved in their apoptotic

Figure 5. Effect of androgens on p38 activation by PMA in LNCaP cells. A, top, LNCaP cells growing in steroid-depleted medium were incubated with different concentrations of R1881 for 48 hours, and then treated with PMA (100 nmol/L, 20 minutes). Cell extracts were prepared and assessed for phospho-p38 and total p38 levels by using Western blot. Bottom, experiments were done using a fixed concentration of R1881 (1 nmol/L), either in the presence or absence of Casodex (3–10 μmol/L). B to D, LNCaP cells were transfected with dsRNA duplexes for either PKC<sub>a</sub> or PKC<sub>c</sub>. After 24 hours, cells were switched to steroid-depleted medium and incubated with R1881 for 48 hours. Cell extracts were prepared and subjected to Western blot analysis for either PKC<sub>a</sub> or PKC<sub>c</sub> (B). In (C), phospho-p38 and total p38 levels were determined in cell extracts in response to PMA (100 nmol/L, 20 minutes). In another set of experiments, the apoptotic response of PMA (100 nmol/L, 1 hour) was determined (D). Columns, mean of triplicate determinations; bars, SE. Similar results were observed in three to five independent experiments.

Figure 6. Elevation in PKC<sub>c</sub> levels in androgen-deprived LNCaP cells by adenoviral delivery restores the apoptotic effect of PMA. A, LNCaP cells growing in steroid-depleted medium were infected for 14 hours with either a PKC<sub>c</sub> or a control (GFP) adenovirus (AdV), at different MOIs. After 24 hours, cells were switched to steroid-depleted medium and incubated with either PKC<sub>c</sub> or control (LacZ) adenovirus. Two additional experiments gave similar results.
effect. Although individual PKC isozymes exert differential roles in the control of cell death and survival through the activation of distinct pathways, studies from several laboratories unambiguously point to PKCδ as a crucial mediator of phorbol ester–induced apoptosis in prostate cancer cells (16–18). The p38 MAPK was identified as a downstream effector of PKCδ-induced apoptosis in LNCaP cells (19), and here we show that p38 activation by PMA is impaired by RNAi depletion of PKCδ. When androgen-sensitive LNCaP cells are deprived of androgen, PKCδ is down-regulated, and both activation of p38 and apoptosis induced by PMA are abrogated, thus supporting the causality of the PKCδ-p38-apoptosis link. Furthermore, p38 activation and apoptosis in response to PMA, as well as the expression levels of PKCδ in LNCaP cells, are restored by androgens in an AR-dependent manner. In addition, the apoptotic effect of PMA is recovered by ectopic expression of PKCδ in the absence of androgens, a clear indication that PKCδ-mediated responses are highly sensitive to the expression levels of the kinase. As indicated above, PKCδ exerts its effects via stimulation of the autocrine secretion of death factors, including TNF-α and TRAIL, ultimately leading to the activation of p38 and the extrinsic apoptotic cascade (15). Interestingly, androgen-depletion results in a marked reduction in the release of death factors from LNCaP cells due to PKCδ down-regulation. Therefore, the multiple mechanisms controlled by PKCδ, namely the release of death receptor factors and the regulation of signaling events, are highly sensitive to transcriptional control by androgens.

In summary, our studies established that androgens are regulators of PKCδ expression in prostate cancer cells. Our findings have potential important implications in prostate cancer therapy. Recent work has shown that androgens can suppress the growth of androgen-independent tumors and revert androgen-independent prostate cancer xenografts to an androgen-stimulated phenotype in vivo (42). In addition, AR has been recently reported to be essential for UV-mediated apoptosis in prostate cancer cells (43). Our results suggest that transcriptional control of PKCδ by androgens may serve as a mechanism that fine-tunes responses in androgen-sensitive prostate cancer cells, and significantly modulates apoptosis via activation of this kinase. Because androgen deprivation and AR inhibition represent standard approaches in prostate cancer treatment, one may hypothesize that this could alter PKCδ expression and modify the sensitivity to treatments aimed at killing cancer cells. It is therefore likely that down-regulation of PKCδ may occur upon androgen withdrawal and may contribute to androgen resistance in patients, an issue that warrants investigation. An attractive possibility is that androgen-resistant cells gain sensitivity to androgens upon activation of PKCδ or its downstream effectors. An apparent contradiction, however, is that androgen-independent cellular models, such as PC3 and DU145, are not deficient in PKCδ, and therefore one cannot rule out that downstream effectors of PKCδ are either not expressed or are dysfunctional in these cells, or that PKCδ activation is insufficient to overcome survival signals in these cells. We believe that the scenario is even more complex and that the balance in the expression/functionality of PKC isozymes is more relevant. Indeed, RNAi depletion of novel PKCε sensitizes androgen-resistant cells to PMA-induced apoptosis, which is in agreement with the prosurvival role for this kinase. This supports the concept that alterations in the balance of PKC isoform expression and/or activation could dictate whether prostate cancer cells become sensitive or resistant to therapy. The identification of this novel regulatory mechanism of PKCδ function may have significant implications in normal prostate physiology and in the context of prostate cancer therapy.

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References
4. Yang C, Kazanietz MG. Divergence and complexities in the control of cell death and survival through the activation of PKC and -epsilon in NIH 3T3 cells induces opposite effects on growth, morphol-
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Androgens Regulate Protein Kinase Cδ Transcription and Modulate Its Apoptotic Function in Prostate Cancer Cells
