Phorbol 12-myristate 13-acetate (PMA) potently induces apoptosis of LNCaP human prostate cancer cells. Here, we show that C4-2 cells, androgen-hypersensitive derivatives of LNCaP cells, also are sensitive to PMA-induced apoptosis. Previous reports have implicated activation of protein kinase C (PKC) isozymes α and δ in PMA-induced LNCaP apoptosis using overexpression, pharmacological inhibitors, and dominant-negative constructs, but have left unresolved if other isozymes are involved, if there are separate requirements for individual PKC isozymes, or if there is redundancy. We have resolved these questions in C4-2 cells using stable expression of short hairpin RNAs to knock down expression of specific PKC isozymes individually and in pairs. Partial knockdown of PKCδ inhibited PMA-induced C4-2 cell death almost completely, whereas near-complete knockdown of PKCα had no effect. Knockdown of PKCε alone had no effect, but simultaneous knockdown of both PKCα and PKCε in C4-2 cells that continued to express normal levels of PKCδ inhibited PMA-induced apoptosis. Thus, our data indicate that there is an absolute requirement for PKCδ in PMA-induced C4-2 apoptosis but that the functions of PKCα and PKCε in apoptosis induction are redundant, such that either one (but not both) is required. Investigation of PMA-induced events required for LNCaP and C4-2 apoptosis revealed that p38 activation is dependent on PKCδ, whereas induction of retinoblastoma protein hypophosphorylation requires both PKCα signaling pathways and is downstream of p38 activation in the PKCδ pathway.

The phorbol ester phorbol 12-myristate 13-acetate (PMA) induces extensive apoptotic death of LNCaP androgen-sensitive human prostate cancer cells (1) in a process reported to involve protein kinase C (PKC) isozymes α and δ (2–4). C4-2 is an androgen-hypersensitive cell line derived from LNCaP cells by Chung and co-workers (5, 6) in a two-step process.
kinase (JNK)-1 and JNK2 (12, 13), and by induction of p21WAF1/CIP1 and hypophosphorylation of the retinoblastoma protein (Rb) (14). Deyrophosphorylation of Akt (12), truncation of E-cadherin (15), and induction of ceramide synthase activity (16) by PMA have been described in LNCaP cells. All of these events, except activation of ERKs and possibly JNKs, appear to be necessary for PMA-induced apoptosis to occur (12–15). Examination of some of these events during PMA treatment of our PKC knockdown clones revealed that p38 activation requires PKCδ, whereas Rb hypophosphorylation requires both PKCδ and either PKCα or PKCε. Furthermore, p38 activation is required for PMA-induced Rb hypophosphorylation, indicating that PKCδ acts through a separate signaling pathway than PKCα and PKCε to activate Rb. Our results show that PMA-induced apoptosis of C4-2 cells requires a more complex combination of PKC isozymes than recognized previously and that different PKC signaling pathways control different events required for apoptosis.

**EXPERIMENTAL PROCEDURES**

**shRNA Expression Constructs**—The shRNA expression vector pSHAG-1 (17), a derivative of pENTR/D-TOPO (Invitrogen) containing a human U6 small nuclear RNA promoter upstream of BseRI and BamHI cloning sites, was a gift from Dr. G. J. Hannon (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). For selection of stably transfected cells, we inserted a neomycin or blasticidin resistance cassette under the control of the herpes simplex virus thymidine kinase promoter and polyadenylation signals into the EcoRV site of pSHAG-1 to yield pSHAG-1-neo and pSHAG-1-bla. Double-stranded oligodeoxynucleotides encoding shRNAs targeting human PKCα, PKCδ, and PKCε were designed using the online program shRNA Retriever, developed by R. Sachidanandam and J. Faith (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), and following suggestions of the Tuschl laboratory (29). All had the following structure: mRNA target = N1–27C28; oligonucleotide A = (reverse complement of N1–27)-GAAGCTTGN1–27C28, with every third A or C of the sense N1–27 converted to G or T, respectively, up to a maximum of four base changes; and oligonucleotide B = exact reverse complement of the entire oligonucleotide A, bounded by GATC (BamHI overhang) at its 5′-end and CG (BseRI overhang) at its 3′-end. Each target sequence was compared with all other sequences in the complete coding sequence of human PKCα, PKCδ, and PKCε and either PKCα or PKCε-induced apoptosis of C4-2 cells requires a more complex combination of PKC isozymes than recognized previously and that different PKC signaling pathways control different events required for apoptosis.

**Fig. 1.** Modified pSHAG-1 (17) containing two shRNA expression cassettes. +1 indicates the start of transcription from the U6 promoters. U6 promoter, human U6 small nuclear RNA promoter; Neo and Bla, neomycin and blasticidin resistance cassettes, respectively.

The coding region of that cDNA was reverted to the wild type G, using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

**Cell Culture and Transfections**—LNCaP and C4-2 cells were obtained from American Type Culture Collection and UroCor Labs (Oklahoma City, OK), respectively. Cells were maintained as monolayer cultures in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM glutamine (complete RPMI 1640 medium) in a humidified atmosphere of 5% CO2 at 37 °C. shRNA expression constructs were transfected into C4-2 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were incubated with plasmid DNA and Lipofectamine 2000 in complete RPMI 1640 medium for 18 h; the medium was replaced with fresh complete RPMI 1640 medium; and 12–18 h later, cells were replated in 100-mm dishes. 24–36 h after replating, selection was initiated by adding 500 μg/ml Geneticin or 10 μg/ml blasticidin. After ~2 weeks of selection, individual colonies were isolated and expanded; PKC isoforms were determined by immunoblot analyses; and aliquots of selected clones were promptly frozen in liquid nitrogen. Human wild-type PKCε in pIREShyg or the pIREShyg vector alone was transfected into C4-2 clone α4+1, with PKCα and PKCε knocked down. Selection of these transfectants in increasing concentrations of hygromycin (100–600 units/ml) yielded pools of cells used in the experiments of Fig. 6. In all cases, cells subjected to multiple transfections and drug selections were kept in culture for the minimal time to obtain stable clones for analysis and were otherwise kept frozen in liquid nitrogen.

**Immunoblot Analyses**—All cell lysis buffers contained a protease inhibitor mixture (Roche Applied Science) and 10 mM HEPES, 2 mM sodium phosphate inhibitors sodium fluoride (50 mM), active site orthovandate (0.2 mM), and p-nitrophenyl phosphate (10 mM). For immunoblotting of PKC isoforms, poly(ADP-ribose) polymerase, and phosphorylated and total c-Jun and Rb, total cell lysates were prepared in radioimmune precipitation assay buffer (18). For analyses of MAPKs, cells were lysed by using phosphatase-free lysis buffer containing β-glycerophosphate (10 mM), 1 mM EDTA, and inhibitors and centrifuged at 20,000 × g for 10 min, and the supernatants were analyzed. Cytoplasmic and membrane fractions were prepared by Dounce homogenizing cells in buffer without detergent (10), pelleting nuclei and unbroken cells by centrifugation at 800 × g, and then centrifuging post-nuclear supernatants at 100,000 × g for 1 h. The supernatants (cytoplasm) were recovered, and the pellets were Dounce-homogenized in buffer containing 1% Triton X-100 and centrifuged at 20,000 × g for 15 min to separate non-nuclear membranes (supernatants) from Triton-insoluble particles (pellets).

Samples in NuPAGE lithium dodecyl sulfate sample buffer and antioxidant (In Vitrogen) were electrophoresed on precast SDS-polyacrylamide gels minigels (Bio-Rad) and electrophoretically transferred to polyvinylidene difluoride membranes. Immunoblot analyses were performed using rabbit polyclonal or mouse monocular primary antibodies and horseradish peroxidase-linked donkey anti-rabbit Ig or sheep anti-mouse Ig secondary antibodies (1:5000 dilution; Amersham Biosciences) with enhanced chemiluminescence detection (ECL Plus, Amersham Biosciences). Primary antibodies for PKCα, PKCδ, and PKCε were from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody that detects the native 115-kDa and 85-kDa cleavage products of poly- (ADP-ribose) polymerase was obtained from Cell Signaling Technology (Beverly, MA). Antibodies for phospho-Ser-637/638, phospho-Ser-602/603, and phospho-Thr-386, phospho-Thr-388, and phospho-Thr-622 were from Cell Signaling Technology.

**Cell Viability Assay**—Viable cells were quantified by incubating the cells with the tetrazolium compound 5-(4,5-dimethylthiazol-2-yl)-3-(4,5-
ment of C4-2 cells with several concentrations of PMA yielded induced apoptosis to induction of apoptosis, similar to our observations on PKC isoforms in PMA-induced apoptosis. We also detected very low amounts of calcium- and diacylglycerol-activated guanine nucleotide exchange factor-II (MEK) inhibitor U0126 was from Promega. LNCaP and C4-2 cells also express mRNAs for PKCδ, PKCε, and PKCγ. LNCaP and C4-2 cells express the same subset of PMA-activated PKC isoforms as LNCaP cells. As shown by RNase protection and immunoblot analyses, C4-2 cells express PMA-activated PKCδ, PKCε, and PKCγ, but not PKCθ, PKCβII, PKCν, or PKCθ. LNCaP and C4-2 cells also express mRNAs for the three known PKD isozymes: PKD/PKC, PKCγ, and PKD2. Regarding other PMA-activated proteins, both cell lines express Munc13-2 and MRCKβ, but not Munc13-1, Munc13-3, or MRCKα. We also detected very low amounts of calcium- and diacylglycerol-activated guanine nucleotide exchange factor-II by RNase protection, but were unable to detect this protein with available antibodies (data not shown).

Because previous data implicated PKCs and PKCδ in PMA-induced LNCaP apoptosis, we initially targeted these isoforms for shRNA-directed knockdown in C4-2 cells. We also targeted PKCe because we found that overexpression of PKCe in LNCaP cells changed the effect of bryostatin-1 from inhibition of PMA-induced apoptosis to induction of apoptosis, similar to our observations on PKCa-overexpressing LNCaP cells (3). Several oligodeoxynucleotide pairs encoding shRNAs targeting multi-

![Figure 2](http://www.jbc.org/)

**Fig. 2. Effect of PMA on growth of C4-2 cells.** Cells were plated with complete RPMI 1640 medium in 96-well plates (8 wells/condition). PMA or Me2SO vehicle was added 24 h later, and the numbers of viable cells, including adherent and detached cells, were counted at the indicated times by MTS assay as described under "Experimental Procedures." A, 4500 C4-2 cells were plated per well and counted 72 h after addition of the indicated concentrations of PMA or 0.006% Me2SO vehicle (O). B, 5000 LNCaP and 4500 C4-2 cells were plated per well. Cells were counted at the indicated times after addition of 10 nM PMA or 0.006% Me2SO vehicle. The actual numbers of LNCaP and C4-2 cells at the time of PMA addition (t = 0, 24 h after plating) were 8473 ± 284 and 8556 ± 243, respectively; the data were normalized to yield 8500 cells at t = 0. ▲, LNCaP cells + vehicle; ○, C4-2 cells + vehicle; ▲, C4-2 cells + PMA; ○, C4-2 cells + PMA + C4-2 cells were plated per well, and 10 nM PMA was added 24 h later. At the indicated times after PMA addition, the culture medium was replaced with fresh medium without PMA, and the cells were counted 72 h after PMA was added.
that PMA induces higher expression of PKCα with pSHAG-1bla containing shRNA expression cassettes for PKCα containing shRNA expression cassettes for PKCα with pSHAG-1neo containing shRNA expression cassettes for PKCα.

The same strategy was used to generate C4-2 clones stably low in C4-2 cells, stably transfected clones were selected. Because sequential transfections yielded by competitive reverse transcription-PCR (data not shown). The construct for expressing these two shRNAs was transfected into C4-2 cells. Because sequential transfections yielded significant toxicity and because transfection efficiency is usually low in C4-2 cells, stably transfected clones were selected. The same strategy was used to generate C4-2 clones stably transfected with pSHAG-1bla encoding two shRNAs targeting PKCβ or PKCε. The levels of PKCα, PKCβ, and PKCε in the knockdown clones are shown in Fig. 3A. PKCo was knocked down to barely detectable levels (clones α1 and α2), whereas knockdowns of PKCβ and PKCε were less complete, but >60% (clones β1, β2, ε1, and ε2). Because we had observed previously that PMA induces higher expression of PKCα, but not of PKCβ or PKCε, in LNCaP cells (10), we also measured PMA-induced membrane translocation of PKCα in α1 cells. As shown in Fig. 3B, minimal PKCo was translocated to non-nuclear membranes by PMA in α1 cells relative to parental C4-2 cells. PKCo knockdown clone α1 was subsequently transfected with the PKCo shRNA expression construct and selected with blasticidin to yield clones with PKCo and PKCo knocked down (clone α1+δ) or with PKCo and PKCo knocked down (clones α+ε1 and α+ε2) (Fig. 3A). Also, clone δ2 was subsequently transfected with the PKCo shRNA expression construct and selected with Geneticin to yield clone δ2+α, with PKCoα and PKCoΔ knocked down (Fig. 3A).

Recent reports have shown that even 21-nucleotide double-stranded RNAs can activate the interferon system and the double-stranded RNA-activated protein kinase PKR in certain cells, including up-regulation of numerous interferon-stimu-
A fragment was performed after an additional 24 h. Vehicle (0.006%) was added 24 h later; and an enzyme-linked immunosorbent assay for detection of cytoplasmic histone-associated DNA fragments was performed after an additional 24 h. Enrichment = $A_{405 \text{ nm}}$ of PMA-treated cells divided by $A_{405 \text{ nm}}$ of vehicle-treated cells. B. Cells were plated in 6-well plates with complete RPMI 1640 medium; PMA (10 nm) or MeSO vehicle (0.006%) was added 24 h later; and total cell lysates were prepared after an additional 48 h. Immunoblot analyses were performed with an antibody that recognizes the intact 115-kDa and 85-kDa cleavage products of poly(ADP-ribose) polymerase. See the Fig. 3 legend for descriptions of clones.

Effects of PKC Isozyme Knockdowns on PMA-induced DNA Fragmentation and Cleavage of poly(ADP-ribose) Polymerase. A. Protein level in total cell lysates of untreated C4-2 clones. C2 indicates untransfected C4-2 cells. Clone α+1 has both PKCα and PKCε knocked down (see the Fig. 3 legend for further description); α+ε1pIRES is clone α+1 transfected further with the empty expression vector pRREShyg; and clone α+ε1wt is clone α+1 transfected further with wild-type PKCε in pRREShyg. B. Viability of PMA-treated cells. Cells were plated and treated with 10 nm PMA or MeSO vehicle, and MTS assays were performed as in the Fig. 4 legend. ○, C4-2 + vehicle; □, C4-2 + PMA; △, α+ε1pIRES + PMA; ○, α+ε1wt + PMA.

Fig. 5. Effects of shRNA-directed knockdown of PKC isozymes on PMA-induced DNA fragmentation and cleavage of poly(ADP-ribose) polymerase. A, cells (2.0 × 10^4/well) were plated in 96-well plates with complete RPMI 1640 medium; PMA (10 nm) or MeSO vehicle (0.006%) was added 24 h later; and an enzyme-linked immunosorbent assay for detection of cytoplasmic histone-associated DNA fragments was performed after an additional 24 h. Enrichment = $A_{405 \text{ nm}}$ of PMA-treated cells divided by $A_{405 \text{ nm}}$ of vehicle-treated cells. B, cells were plated in 6-well plates with complete RPMI 1640 medium; PMA (10 nm) or MeSO vehicle (0.006%) was added 24 h later; and total cell lysates were prepared after an additional 48 h. Immuno blot analyses were performed with an antibody that recognizes the intact 115-kDa and 85-kDa cleavage products of poly(ADP-ribose) polymerase. See the Fig. 3 legend for descriptions of clones.

Effects of PKC Isozyme Knockdowns on PMA-induced DNA Fragmentation and Cleavage of poly(ADP-ribose) Polymerase. A. Protein level in total cell lysates of untreated C4-2 clones. C2 indicates untransfected C4-2 cells. Clone α+1 has both PKCα and PKCε knocked down (see the Fig. 3 legend for further description); α+ε1pIRES is clone α+1 transfected further with the empty expression vector pRREShyg; and clone α+ε1wt is clone α+1 transfected further with wild-type PKCε in pRREShyg. B. Viability of PMA-treated cells. Cells were plated and treated with 10 nm PMA or MeSO vehicle, and MTS assays were performed as in the Fig. 4 legend. ○, C4-2 + vehicle; □, C4-2 + PMA; △, α+ε1pIRES + PMA; ○, α+ε1wt + PMA.
cated in PMA-induced apoptosis and because its overexpression has been reported to render LNCaP cells resistant to PMA (24), in contrast to our own observations, it is important to determine whether exogenous expression of PKCα could overcome the effects of the shRNAs targeting PKCa and PKCe. Clone α+ε1, with PKCa and PKCe knocked down, was transfected further with human wild-type PKCe in pIREShyg or with the pIREShyg vector alone, and pools of hygromycin-resistant subclones were selected and labeled α+ε1wt and α+ε1pIRES, respectively. We chose to express human PKCe mRNA rather than another mammalian PKCe mRNA that has mismatches relative to the PKCe shRNAs to achieve replacement of PKCe levels without excessive overexpression. As shown in Fig. 6, exogenous expression of PKCe reverted the partial PMA resistance of clone α+ε1 back to PMA sensitivity similar to that of parental C4-2 cells. Northern blot analyses revealed that expression of the two PKCe shRNAs was markedly lower in the cells overexpressing PKCe than in clones α+ε1 and α+ε1pIRES, whereas expression of the two PKCa shRNAs remained high in all of these clones (data not shown). Possibly, the overexpressed PKCe overtaxed and led to degradation of the shRNAs targeting it. Thus, although the reversion of α+ε1wt cells to PMA sensitivity ruled out the possibility that clone α+ε1 developed resistance to PMA spontaneously, the possibility that an effect of the PKCe shRNAs unrelated to knockdown of PKCe contributed to the PMA resistance of clone α+ε1 could not be ruled out completely. However, the latter possibility is unlikely since the α+ε1wt cells continued to express the two PKCa shRNAs, and expression of the PKCe shRNAs alone in C4-2 cells had no effect on PMA sensitivity.

Effects of MAPK Inhibitors on PMA-induced Death—As in LNCaP cells (3, 12, 13), PMA activated MAPKs of the p38 (presumably the α isozyme), JNK (JNK1 and JNK2), and ERK (ERK1 and ERK2) families in C4-2 cells as assessed by induction of dual Thr/Tyr phosphorylation (Fig. 7). We measured the effects of pharmacological inhibitors of p38, JNK, and ERK on PMA-induced death (Fig. 8A), in agreement with the findings of Tanaka et al. (12) in LNCaP cells. Inhibition of PMA activation of JNK1 and JNK2 by SP600125, measured by inhibition of PMA-induced phosphorylation of c-Jun, revealed that expression of the two PKCa shRNAs was markedly lower in the cells overexpressing PKCe than in clones α+ε1 and α+ε1pIRES, whereas expression of the two PKCa shRNAs remained high in all of these clones (data not shown). Possibly, the overexpressed PKCe overtaxed and led to degradation of the shRNAs targeting it. Thus, although the reversion of α+ε1wt cells to PMA sensitivity ruled out the possibility that clone α+ε1 developed resistance to PMA spontaneously, the possibility that an effect of the PKCe shRNAs unrelated to knockdown of PKCe contributed to the PMA resistance of clone α+ε1 could not be ruled out completely. However, the latter possibility is unlikely since the α+ε1wt cells continued to express the two PKCa shRNAs, and expression of the PKCe shRNAs alone in C4-2 cells had no effect on PMA sensitivity.

Effects of MAPK Inhibitors on PMA-induced C4-2 Cell Death—As in LNCaP cells (3, 12, 13), PMA activated MAPKs of the p38 (presumably the α isozyme), JNK (JNK1 and JNK2), and ERK (ERK1 and ERK2) families in C4-2 cells as assessed by induction of dual Thr/Tyr phosphorylation (Fig. 7). We measured the effects of pharmacological inhibitors of p38, JNK, and ERK on PMA-induced C4-2 cell death. Under our conditions of pretreatment with inhibitor for 1 h, followed by incubation of C4-2 cells with inhibitor + 10 nM PMA for 4 h, we found that p38 was required for PMA-induced cell death (Fig. 8A), in agreement with the findings of Tanaka et al. (12) in LNCaP cells. Inhibition of PMA activation of JNK1 and JNK2 by SP600125, measured by inhibition of PMA-induced phosphorylation of c-Jun, had a small but significant inhibitory effect on PMA-induced C4-2 cell death (Fig. 8B). (PMA-induced

FIG. 7. PMA-induced activation of MAPKs. Cells were plated with complete RPMI 1640 medium in 6-well plates; PMA was added 24 h later; and lysates were prepared as described under “Experimental Procedures” at the indicated times after PMA addition or 1 h after addition of 0.006% Me2SO vehicle (0). Immunoblot analyses were performed as described under “Experimental Procedures.” Phospho p38, Phospho JNK, and Phospho ERK indicate detection with antibodies against dual phosphorylated (Thr/Tyr) p38, JNKs, and ERKs, respectively.

FIG. 8. Effects of MAPK inhibitors on PMA-induced death. Cells were plated with complete RPMI 1640 medium in 96-well plates (for MTS assays) or 6-well plates (for immunoblot analyses). MAPK inhibitors were added 24 h later, 30 min before addition of PMA (10 nM) or Me2SO vehicle (0.006%), and then both drugs were removed 4 h after addition of PMA. The numbers of viable cells, including adherent and detached cells, were counted 72 h after addition of PMA by MTS assay. For immunoblot analyses of phosphorylated and total c-Jun and ERKs, lysates were prepared as described under “Experimental Procedures” at the indicated times after PMA addition. A, p38 inhibitor SB203580. B, JNK inhibitor SP600125. p values were determined using an unpaired t test with Welch’s correction. C, MEK inhibitor U0126.
expression of c-Jun was not inhibited by SP600125. A larger effect may have been masked by the inhibitory effect of SP600125 alone on C4-2 cell growth. Inhibition of PMA activation of ERK1 and ERK2 by the MEK inhibitor U0126 had no effect on PMA-induced C4-2 cell death (Fig. 8C).

PKC Isozyme Dependence of PMA-induced p38 Activation and Rb Hypophosphorylation—PMA-induced dual phosphorylation of p38 was inhibited markedly by knockdown of PKCδ, but was unaffected by simultaneous knockdown of PKCα and PKCe or by knockdown of PKCδ alone (Fig. 9A). Fig. 9B shows that PMA-induced hypophosphorylation of Rb was inhibited by knockdown of PKCδ and partially by simultaneous knockdown of PKCα and PKCe, but was unaffected by knockdown of either PKCα or PKCe alone. Thus, PMA-induced Rb activation requires both the PKCδ and PKCα/e signaling pathways. Inhibition by SB203580 of PMA-induced p38 activation also prevented Rb hypophosphorylation (Fig. 9C), indicating that the latter requires p38 activation. Thus, PKCδ signals through p38, whereas PKCα and PKCe must signal through a separate pathway to activate Rb. These conclusions are summarized in Fig. 10.

Further evidence for separate PKCδ and PKCα/e signaling pathways has been obtained from preliminary microarray analyses that revealed some PMA-induced changes in gene expression affected by knockdown of PKCδ and others affected by simultaneous knockdown of PKCα and PKCe. One such gene is CYR61 (cysteine-rich 61), which encodes a protein shown to inhibit Taxol-induced apoptosis of breast cancer cells (25). Induction of CYR61 expression by 10 nM PMA for 12 h was unaffected by knockdown of PKCδ, PKCα, or PKCe alone, but was 3.5–7-fold higher when both PKCα and PKCe were knocked down. Thus, either PKCδ or PKCe can markedly attenuate PMA-induced expression of CYR61. PMA-induced E-cadherin expression and truncation, shown previously to be required for PMA-induced LNCaP apoptosis (26), was not affected by knockdown of PKCδ, PKCα, PKCe, or PKCα + PKCe (data not shown), suggesting that these responses to PMA involve either additional redundancy among PKC isoforms or one or more non-PKC PMA receptors.

DISCUSSION

PMA-induced apoptosis of LNCaP androgen-sensitive human prostate cancer cells has been studied for several years, and different reports have implicated PKCα and/or PKCδ as a PMA receptor in death induction (2–4, 12). Although the consensus from the previous studies put forth in a recent review (8) is that activation of either PKCα or PKCδ is sufficient to trigger LNCaP apoptosis, we reasoned that ambiguities in the previous data left such a conclusion open to question. In particular, we questioned the PKC isozyme specificities of pharmacological inhibitors at high doses and kinase-negative PKC constructs that inhibit PMA-induced apoptosis. For example, we found that overexpression of one PKC isozyme markedly altered PMA-induced membrane translocation of other isoforms and that inhibition of PMA-induced LNCaP cell death by exogenous expression of the PKCα regulatory domain was accompanied by inhibition of membrane translocation of PKCα, PKCδ, and PKCe (26). In our report on bryostatin-1-induced death of LNCaP cells overexpressing PKCα, we noted that overexpression of high amounts of PKCα in membranes of these cells has no effect on cell growth in the absence of drug and that bryostatin-1 induces prolonged membrane translocation of PKCδ in both parental and PKCα-overexpressing LNCaP cells (3). This left the possibility that neither PKCα nor PKCδ alone could induce LNCaP cell death, although other possibilities were discussed. Here, we have shown that C4-2 cells, androgen-hypersensitive metastatic derivatives of LNCaP cells, are as sensitive as LNCaP cells to PMA-induced apoptosis and that apoptosis requires a more complex combination of PKC isoforms than indicated previously for LNCaP cells. Partial knockdown of PKCδ and near-complete knockdown of PKCα showed clearly that PMA-induced C4-2 cell death requires PKCδ, but not PKCα. However, a contributory role for PKCα...
and the necessity of a second PKC signaling pathway were shown by simultaneous knockdown of PKCα and PKCe.

Although PKC-induced C4-2 apoptosis requires multiple PKC signaling pathways, we cannot rule out the possibility that novel, more selective PKC activators might induce death through a single pathway. For example, Kazanietz and co-workers (4, 12) reported that the diacylglycerol lactone HK654 translocates PKCα and PKCδ to non-nuclear and nuclear membranes of LNCaP cells, respectively, while inducing apoptosis and concluded that apoptosis induction is through selective activation of PKCα. A slight possibility exists that activation of PKCδ by PMA induces both pro- and anti-apoptotic signals and that the anti-apoptotic signals must be offset by PKCα or PKCe, whereas activation of PKCα alone induces only apoptotic signals in LNCaP cells. However, given that HK654 activates PKCδ as well as PKCα in vitro (4) and that we have detected substantial amounts of PKCδ in non-nuclear membranes of LNCaP cells in the absence of drug (3), it seems more likely that HK654 acts through PKCδ as well as PKCα in inducing LNCaP apoptosis.

The observation that simultaneous knockdown of PKCα and PKCe yields only partial inhibition of PKC-induced C4-2 cell death may reflect our inability to achieve near-complete depletion of both isozymes in the same cells or may indicate that there is additional redundancy among PKC receptors in this signaling pathway. The reasons for the inability to knock down PKCα and PKCe may be technical, such as competition among shRNAs for RNA interference components, or may reflect a requirement for one of these isozymes to maintain cell growth. The latter possibility would not be incompatible with a requirement for PKCα or PKCe in PKC-induced cell death since PKA yields abnormally prolonged activation of these isozymes, whereas growth factors in serum yield only transient activation via tightly regulated generation of diacylglycerol. We have also found that, whereas PKCe is eventually down-regulated by PMA in LNCaP cells, especially in the minority of cells refractory to PKC-induced apoptosis, PKA-resistant LNCaP cells developed in our laboratory recover normal PKCε expression (10).

Our discovery of a contributory role for PKCe in PKC-induced C4-2 cell death is the first indication of a pro-apoptotic role for this oncogenic isozyme in prostate cancer cells and is in marked contrast with results of another group who reported that overexpression of high amounts of PKCe in LNCaP cells renders the cells androgen-insensitive and resistant to apoptosis induction by PMA (24). We have stably overexpressed several different amounts of human wild-type PKCe in LNCaP cells, and all of our clones remained as sensitive to PKA-induced apoptosis as the parental LNCaP cells, with the highest overexpressors killed by brayostatin-1,3 similar to our observations of PKCα-overexpressing LNCaP cells (3). We can only speculate how the other group obtained such markedly different results. One possibility is that their PKCε-overexpressing LNCaP cells, which were also androgen-insensitive, had been grown in medium containing insufficient androgen, resulting in co-selection of androgen- and PKA-insensitive cells. Such cross-resistance to PKA has been reported in serum starvation-resistant LNCaP cells developed by Chen et al. (27).

Previous reports on the roles of MAPKs in PKC-induced LNCaP apoptosis have indicated a requirement for one or more p38 isoforms (12), conflicting results on the importance of JNKs (12, 13, 28), and no effect or a slight inhibitory effect of ERK activation (12, 13). Our observation of partial inhibition of PKC-induced C4-2 cell death by the JNK inhibitor SP600125 (Fig. 8A) is in between the results of Tanaka et al. (12), who found that up to 50 μM SP600125 has a minimal effect on LNCaP apoptosis induced by 100 nM PMA, and Engedal et al. (13), who reported that expression of the JNK-binding domain of the JNK-interacting protein markedly inhibits PKC-induced LNCaP apoptosis. Also, Chen et al. (28) reported that overexpression of JNK1 induces apoptosis of LNCaP cells. Additional work will be required to confirm a role for JNKs in PKC-induced prostate cancer cell death. The absence of an effect on PKC-induced C4-2 cell death by doses of the MEK inhibitor U0126 that blocked activation of ERK1 and ERK2 (Fig. 8C) differs slightly from the results of Tanaka et al. (12), who reported some potentiation of PKC-induced LNCaP cell death by another MEK inhibitor, PD98059. The difference may reflect higher specificity of U0126 for ERKs or the shorter PMA treatment (1 h) used by Tanaka et al., which does not yield maximal cell death.

Our data showing the dependence of PKC-induced p38 activation on PKCe are in agreement with the results of Tanaka et al. (12), who reported that transient transfection of LNCaP cells with a small interfering RNA targeting PKCe blocks PKC-induced p38 activation, although they did not show the data. These authors also concluded that either PKCα or PKCe can act through p38 to induce apoptosis based on data that the p38 inhibitor SB203580 inhibits PKC-induced apoptosis of LNCaP cells overexpressing either PKCα or PKCe. However, PMA still induces endogenous PKC5 in the PKCε overexpression model. Our data showing inhibition of PKC-induced p38 dual phosphorylation by knockdown of PKCe, but not by knockdown of PKCα, PKCe, or PKCα + PKCe, indicate that activation of p38 by PMA is directed by PKC5, not by PKCα or PKCe. We have further shown that PKC-induced Rb hypophosphorylation requires PKC5-directed p38 activation as well as a PKCαε signaling pathway that does not involve p38 (Fig. 10). These data confirm that PKCα and PKCe act through a separate signaling pathway than PKCe to activate Rb. The attenuation of PKC-induced CYR61 expression by PKCα or PKCe, but not by PKCe, provides further evidence for distinct signaling pathways, although no evidence for an effect of CYR61 on Rb has been reported. To the best of our knowledge, this is the first demonstration of separate PKC signaling pathways, including a unique isozyme and a redundant pair of isozymes, affecting a PKC-induced cellular alteration. Further experiments will be necessary to uncover signals unique to the PKCαε pathway that are required for PKC-induced Rb hypophosphorylation.

The possibility that additional PKA receptors are required for PKC-induced C4-2 cell death remains to be determined. Also, since C4-2 cells retain a functional androgen receptor and are androgen-hypersensitive, our results showing PKC-induced C4-2 cell death in the presence of medium containing 10% fetal bovine serum do not rule out a requirement for the androgen receptor. It is possible that PKC-induced death of C4-2 cells, like that of LNCaP cells, requires the androgen receptor to limit activation of NF-κB by PMA. Still, it is significant that these cells have retained sensitivity to PKC-induced death after progressing through two rounds of selection in castrated mice to a faster growing subline with much higher metastatic ability compared with LNCaP cells.

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