Although protein kinase C (PKC) has been widely implicated in the positive and negative control of proliferation, the underlying cell cycle mechanisms regulated by individual PKC isozymes are only partially understood. In this report, we show that PKC mediates phorbol ester-induced G1 arrest in lung adenocarcinoma cells and establish an essential role for this novel PKC in controlling the expression of the cell cycle inhibitor p21. Activation of PKC with phorbol 12-myristate 13-acetate (PMA) in early G1 phase impairs progression of lung adenocarcinoma cells into S phase, an effect that was completely abolished by specific depletion of PKCδ, but not PKCα. Although the PKC effect was unrelated to the inhibition of cyclin D1 expression, PKC activation significantly up-regulated p21 and down-regulated Rb hyperphosphorylation and cyclin A expression. Elevations in p21 mRNA and protein by PMA were mediated by PKCδ but not PKCα. Studies using luciferase reporters also revealed an essential role for PKCδ in the PMA-induced inhibition of Rb-dependent cyclin A promoter activity. Finally, we showed that the cell cycle inhibitory effect of PKCδ is greatly attenuated by RNA interference-mediated knock-down of p21. Our results identify a novel link between PKCδ and G1 arrest via p21 up-regulation and highlight the complexities in the downstream effectors of PKC isozymes in the context of cell cycle progression and proliferation.

Activation of protein kinase C (PKC) is known to cause major effects on cell proliferation, survival, and differentiation. Phorbol esters, natural compounds that activate PKC and mimic the action of the lipid second messenger diacylglycerol, can impact on cell cycle progression both in positive or negative manners, resulting in either stimulation of mitogenesis or cell cycle arrest, or even in apoptosis, depending on the cell type (1–4). Such a wide range of biological responses triggered by phorbol esters relates to the multiplicity of intracellular effectors for these compounds, which include the classic PKCs (α, β1, βII, and γ), the novel PKCs (δ, ε, η, and θ), as well as PKC-related kinases and non-kinase phorbol ester receptors (5, 6). The abbreviations used are: PKC, protein kinase C; Cdk, cyclin-dependent kinase; PMA, phorbol 12-myristate 13-acetate; RNAi, RNA interference; FBS, fetal bovine serum; AdV, adenovirus; m.o.i., multiplicity of infection; psu, plaque forming unit(s); dsRNA, double-stranded RNA; Q-PCR, Quantitative Real-time RT-PCR; RT, reverse transcription; GFP, green fluorescent protein; CRE, cAMP response element; MTS, 3-(4,5-di-methylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.
the vast majority of these early studies has relied heavily on overexpression strategies or the use of pharmacological tools and dominant-negative mutants of questionable specificity. In this report, we explore the role of PKC isozymes as mediators of phorbol ester-induced control of proliferation in lung cancer cells. Thus far, little is known about the role of individual PKCs in cell cycle control in lung cancer cells, particularly those isozymes that could be potentially growth inhibitory. Despite the limited mechanistic information available, ongoing efforts aimed at targeting PKC isoforms for lung cancer therapy are being developed (8,30). PKC isozymes have also been linked to a chemo-resistant phenotype in lung cancer and have been implicated in tobacco carcinogenesis (31–33). By using RNAi to knock-down individual PKCs in a lung cancer cell model, we have determined an absolute requirement of PKCδ for phorbol ester-induced G1 arrest. This novel PKC is essential for phorbol ester-induced up-regulation of p21 and inhibition of cyclin A promoter activity.

EXPERIMENTAL PROCEDURES

Materials—Cell culture medium was purchased from Invitrogen. PMA and its inactive isomer 4α-PMA were obtained from LC Laboratories (Woburn, MA) and dissolved in ethanol. The pan-PKC inhibitor GF109203X (bisindolylmaleimide I) was from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA). Go6976 was purchased from Alexis (San Diego, CA). Propidium iodide was from Sigma.

Cell Culture—H441 and H358 lung adenocarcinoma cancer cell lines (bronchoalveolar type) were used. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 units/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM) at 37 °C in a humidified 5% CO2 atmosphere.

Cell Proliferation Assays—Cells (1 × 105) cells were seeded 6-well plates. After incubation for 24 h in normal medium, cells were treated with different concentration of PMA for 1 h. Cells were trypsinized at different times and counted in a hemocytometer. For the MTS assay, H358 cells were seeded in 96-well plates. After incubation for 24 h in normal medium, cells were treated with different concentrations of PMA for 1 h. Cells were washed with phosphate-buffered saline and incubated for an additional 24-h period. Absorbance at 490 nm was determined with the CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, WI). When PKC inhibitors were used, they were added to the culture 30 min before and during PMA treatment.
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FIGURE 2. Time-dependent inhibition of G1/S progression by PMA. H358 cells were synchronized in G0 by serum starvation. Cells were released with 10% FBS and then treated with 100 nM PMA, added for 1 h after serum release at the times indicated in the figure. 24 h after serum release cells were stained with propidium iodide and cell cycle distribution analyzed by flow cytometry. Representative panels depicting cell cycle distribution are shown. Similar results were observed in three independent experiments.

Adenoviral Infections—H358 cells were infected with a replication-deficient adenovirus (AdV) for PKCα or a control LacZ AdV (4, 17) for 14 h at different multiplicities of infection (m.o.i.) in RPMI 1640 medium without FBS. After removal of the AdV by extensive washing, cells were incubated in RPMI 1640 medium supplemented with 10% FBS. Expression of recombinant protein was readily detected after 14 h and remained stable for several days (data not shown). Amplification of AdVds was carried out in 293 packaging cells. Titers of viral stocks were normally higher than 1 × 10⁹ pfu/cell. In some experiments infections were carried out 24 h after transfection of cells with dsRNA (see below).

Cell Cycle Assays—H358 cells were seeded in 60-mm dishes (≈50% confluency) and serum-starved for 24 h. Approximately 80% of the cells were synchronized in G0 after serum starvation. Synchronized cells were incubated in RPMI 1640 medium with or without PMA for 1 h and then collected at selected times for propidium iodide staining (0.1 mg/ml) followed by flow cytometry analysis, as reported previously (4).

Western Blot Analysis—Cells were lysed in a buffer containing 50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and 5% β-mercaptoethanol. Cell extracts (20 μg of protein/lane) were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Bedford, MD). After blocking with 5% milk in 0.05% Tween 20/phosphate-buffered saline, membranes were incubated with the primary antibody for 1 h. Either anti-mouse or anti-rabbit horseradish peroxidase (1:3000, Bio-Rad) were used as secondary antibodies. Bands were visualized with a chemiluminescence detection kit (ECL, Amersham Biosciences). Densitometric analysis was performed under conditions that yielded a linear response using Image software.

The following first antibodies were used: anti-PKCα (Upstate Biotechnology Inc., Lake Placid, NY); anti-PKCβ, anti-cyclin A, anti-cyclin B1, anti-cyclin D1, anti-p21, anti-p27, and anti-cdk4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-PKCβ, anti-PKCε, anti-PKCδ, and anti-PKCζ (BD Transduction Laboratories); and anti-phospho T821 Rb (AbCam, Cambridge, MA).

RNA Interference—dsRNAs were purchased from Dharmacon Inc. (Dallas, TX). The following targeting sequences were used: PKCα (AATCCTGTGCAAGAGGCTGTC), PKCβ (AACATGAGTTATCGCCACC), and p21 (AACATCTGGCCTGGACTT). dsRNAs were transfected into H358 cells using Oligofectamine (Invitrogen) following the protocol provided by the manufacturer. Experiments were carried out 48 h after transfection.

Quantitative Real-time RT-PCR—Cells were lysed in 500 μl of TRIzol (Invitrogen), and total RNA was extracted according to the manufacturer’s protocol. Total RNA (20 ng) from each sample was used for cDNA synthesis using reverse transcription reagents from Applied Biosystems Inc. (5.5 mM MgCl₂, 2 mM dNTP, 2.5 μM oligo(dT) or 2.5 μM random hexamers, 8 units of RNase I, and 25 units of Multiscribe reverse transcriptase per 20 μl of reaction). cDNA (2.5 μl) was subjected to 40 amplification cycles of Q-PCR (Applied Biosystems Prism 7000 sequence detection system) using Taqman universal PCR master mix in a 25-μl reaction. For human p21 mRNA, each real-time PCR reaction contained 1.25 μl of 20× p21 TaqMan Gene Expression Assay (Applied Biosystems Inc.). For human cdk4 mRNA, each real-time PCR reaction contained 150 nm forward primer 5′-ACAAGTGTTGAACAGTCAAGCT, 200 nm reverse primer 5′-GCATATGGAACAGGAAGGACT, and 150 nm TAMRA probe 5′-VIC-ATTCCAGTATACAGGCCTGTTGTTTACACTCT-TAMRA. Each sample was analyzed in duplicate, and mRNA levels were quantified by reference to a standard curve using the Prism 7000 sequence detection software. p21 mRNA expression was normalized to cdk4 mRNA expression, which did not vary during serum stimulation or PMA treatment.

Cell Transfections and Promoter Analyses—H358 cells (≈5 × 10⁵, ~80% confluency) in 35-mm dishes were transiently co-transfected with +CRE and −CRE cyclin A promoter-luciferase vectors (34) using 5 μl of Lipofectamine Plus reagent, 1 μg of cyclin A promoter-luciferase plasmid, and either 1 μg of a human papilloma virus type-18 E7 expression vector or 1 μg of empty vector. A Renilla luciferase expression plasmid (0.01 μg, pRL-CMV, Promega) was co-transfected for normalization of transfection efficiency. After an overnight recovery, cells were serum-starved for 24 h, treated with either 10 nM PMA or vehicle for 1 h, and stimulated with 10% FBS for different times. Cyclin A promoter-driven luciferase activity was determined and normalized to Renilla luciferase, as previously described (34).

RESULTS

PMA Induces G1 Arrest in Bronchoalveolar Cells via PKC Activation—To determine whether phorbol esters affect proliferation of lung adenocarcinoma cells, asynchronous H358 and H441 cells were treated with different concentrations of PMA (1 nM to 1 μM, 1 h). PMA was removed by extensive washing, and cell number was determined at different times. Fig. 1A shows that PMA treatment caused a dose-dependent reduction in cell number, both in H358 and H441 cells. No evidence of apoptosis was detected upon PMA treatment, as judged by the lack of nuclear fragmentation and the absence of a sub-G0/G1 population upon flow cytometry analysis (data not shown). Assessment of cell proliferation in H358 cells with an MTS assay also revealed a significant dose-dependent inhibition by PMA. The inactive analog 4α-PMA (100 nM), on the other hand, was ineffective. The "pan" PKC inhibitor GF109203X (bisindolylmaleimide I) completely blocked the effect of PMA (Fig. 1B). Thus, the effect was mediated by PMA-responsive PKC.
isozymes and not by other endogenous phorbol ester receptors present in these cells (13).

To determine whether phorbol ester treatment influences the G1/S transition, we synchronized H358 cells in G0 by serum starvation and examined the effect of PMA upon serum release. The maximum percentage of cells in G0 (80%) was achieved upon 24-h serum starvation (data not shown). H358 cells entered S phase 12 h after serum release, with the maximum percentage of cells in S phase observed at 18 h (Fig. 1C). At 24 h, the majority of cells were in G2 (data not shown). When H358 cells were treated with PMA (100 nM, 1 h) at t = 0 h, cells were unable to progress into the S phase (Fig. 1C and D). The arrest caused by PMA was fully blocked with the GF109203X but not by Go6976, an inhibitor of the classic PKCs. Representative flow cytometry experiments are depicted in Fig. 1D. Analysis of the expression of cyclins in untreated cells revealed that cyclin D1 is significantly up-regulated 6 h after serum release, which corresponded with an increase in Rb phosphorylation (Fig. 1E). As expected, cyclins A and B become elevated at later time points. Treatment of H358 cells with PMA significantly impaired cyclin A and cyclin B up-regulation. Analysis of cdk inhibitors revealed that PMA caused a strong and sustained up-regulation of p21, without any significant up-regulation in the levels of p27. Because up-regulation of p21 prevents Rb dephosphorylation, we determined phospho-Rb levels using a phospho-specific antibody (phospho T821 Rb). Consistent with the p21 up-regulation, a marked reduction in phosphorylated Rb was detected in PMA-treated cells.

Studies in various cell models have suggested differential roles for PKC in early- and late-G1 phase. Thus, we decided to examine the consequence of adding PMA (100 nM) for 1 h at different times after serum release in H358 cells (Fig. 2). The inhibitory effect of PMA was only observed when added at early times in G1 but not at later time points. Indeed, addition of PMA at t = 0–8 h impaired cell progression into the S phase, whereas addition of PMA at t ≥ 12 h was clearly ineffective, suggesting that only PKC activation in early-mid G1 phase caused cell cycle arrest.

The Inhibitory Effect of PMA on G1/S Progression Is Mediated by PKCδ—H358 cells express three phorbol ester-responsive PKCs: one classic PKC (PKCα) and two novel PKCs (PKCδ and PKCζ). In addition, they express the phorbol ester unresponsive PKCε (Fig. 3A). Growth inhibitory roles have been ascribed to PKCα and/or PKCδ in various cell models. The lack of effect of Go6976, the inhibitor of classic PKCs (see Fig. 1D), led us to speculate that PKCδ may be a key mediator of the growth inhibitory effect of PMA in H358 cells. To address this issue we depleted individual PKCs in H358 cells using RNAi. Upon transfection of specific dsRNAs for either PKCα or PKCδ, reductions of 69 ± 19% and 81 ± 9% in PKCα and PKCδ levels were achieved, respectively (Fig. 3, B and C). Analysis of cell proliferation in knock-down cells revealed...
remarkable differences in each case. Cell cycle analysis revealed that normal progression into the S phase upon serum stimulation (in the absence of PMA treatment) was not affected when either PKC was knocked down. However, in PKCδ-depleted cells, PMA was unable to cause G1 arrest, because the cells progress normally into the S phase. On the other hand, PMA fully arrested H358 cells in which PKCα has been depleted (Fig. 3D), suggesting that this isozyme was dispensable for the PMA effect. Taken together, these results indicate that PKCδ mediates the anti-proliferative effect of PMA in H358 cells.

**p21 Is Required for PMA- and PKCδ-induced G1 Arrest in H358 Cells**—Up-regulation of p21 in response to phorbol esters has been reported in several cellular models. H358 cells are p53-null (35), thus p21 up-regulation in response to PMA in these cells (see Fig. 2E) is p53-independent. To determine the requirement of p21 in PMA-induced G1 phase arrest, a specific dsRNA was designed, which depleted this cdk inhibitor by 66% upon delivery into H358 cells (Fig. 4A). No effect was observed using an unrelated dsDNA designed to deplete GFP (data not shown). Next, we examined the effect of PMA on G1/S progression in p21-depleted cells. Indeed, upon p21 RNAi the G1 arrest induced by PMA was significantly impaired (Fig. 4B and C). Moreover, the loss of Rb phosphorylation in response to PMA was also significantly inhibited upon depletion of p21 (Fig. 4D). Thus, p21 is required for PMA-induced G1 arrest in H358 cells.

**Overexpression of PKCδ Inhibits H358 Cell Proliferation**—To further establish a role for PKCδ as inhibitor of proliferation in H358 cells, we assessed the effect of PKCδ overexpression using an adenoviral delivery approach. After infection of H358 cells with increasing multiplicities of infection (m.o.i. values) of a PKCδ AdV, elevated levels of this PKC were readily detected (Fig. 5A). PKCδ overexpression caused a significant reduction in cell number. No evidence of apoptosis was observed (data not shown). On the other hand, a control LacZ AdV, which has the same backbone as the PKCδ AdV, did not reduce H358 cell number at an m.o.i. = 300 pfu/cell (Fig. 5B). Notably, PKCδ overexpression in H358 cells led to a significant elevation in p21 levels. Similar results were observed in H441 cells (Fig. 5A).

We next assessed whether p21 depletion could affect the inhibitory effect of PKCδ. H358 cells were subjected to p21 RNAi and then infected with the PKCδ AdV (m.o.i. = 100 pfu/cell). The anti-proliferative effect observed by PKCδ overexpression (assessed 48 h after infection with the PKCδ AdV) was significantly impaired in p21-depleted cells compared with control cells (Fig. 5C). The rescue effect was partial (~50%), probably due to the strong and persistent nature of the stimulation caused by PKCδ overexpression and to the fact that p21 knock-down in H358 cells was not complete (see Fig. 4A). Nevertheless, this effect was not observed when an unrelated control dsRNA (GFP) was delivered into H358 cells. Taken together, these experiments strongly support an anti-proliferative role for PKCδ in human bronchoalveolar cells and indicate that p21 is a major effector of PKCδ in this context.

**PKCδ Mediates PMA-induced Elevations in p21**—Based on the information presented above, we hypothesized that PKCδ may be required for p21 up-regulation in response to PMA. To prove this concept we determined p21 levels in response to PMA in PKC knock-down cells. Notable differences in p21 expression were found between PKCα- and PKCδ-depleted cells. Indeed, although in PKCα knock-down H358 cells...
PMA-induced elevations in p21 protein levels were minimally affected, the PMA effect was blunted after PKC/H9254 RNAi. PKC/H9254 RNAi also impaired PMA-induced up-regulation of p21 in H441 cells (Fig. 6A). To further strengthen these results, we next assessed p21 mRNA levels by Q-PCR. A time-course analysis showed that p21 mRNA levels in H358 cells are elevated as a consequence of PMA treatment (Fig. 6B). Maximum levels were observed 12 h after incubation with PMA. In agreement with the results observed at the protein level, p21 mRNA up-regulation in response to PMA was essentially blunted in PKC/H9254 knockdown H358 cells but not affected upon depletion of PKC/H9251 (Fig. 6C). These results unambiguously define the requirement of PKC/H9254 for p21 up-regulation in response to PMA treatment in H358 cells.

**PKCδ Controls the Expression of the Cyclin A Promoter**—The inhibitory effect of PMA on the expression of cyclin A in H358 cells (see Fig. 1E) prompted us to explore whether PKCδ, and more specifically PKCδ, controls the activity of the cyclin A promoter. The cyclin A promoter contains several regulatory elements, including CRE (cAMP response element) and E2F sites clustered near the transcription start sites. We used a cyclin A promoter construct, p284/cyclinA-Luc (or +CRE), which comprises 284 bp spanning from −120 to +164 of the cyclin A promoter upstream of the luciferase reporter (34). This vector was transfected into H358 cells, and promoter activity was subsequently determined at different times after serum stimulation, either in the absence or presence of PMA (100 nM, 1 h, added at t = 0). A time-dependent increase in luciferase activity was observed in control cells. However, this activity was essentially blunted by PMA treatment (Fig. 7A). As expected, luciferase activity of the CRE-mutated promoter (p225/cyclin A, or −CRE, which comprises bp −61 to +164 of the cyclin A promoter (34)) was less than that containing the wild-type CRE (Fig. 7, compare scales in A and B). However, its activity was also impaired by PMA (Fig. 7B). This result shows that the CRE site is not required for the inhibitory effect of PMA and suggests that the E2F site may be the target of PMA action. Indeed, we found that transfection of human papillomavirus-18 E7, which inactivates RB and allows for constitutive E2F activity, largely eliminated the inhibitory effect of PMA on the cyclin A promoter. Moreover, the rescue of promoter activity by E7 was independent of the CRE site (Fig. 7C). Together, these data indicate that the inhibitory effect of PMA on cyclin A promoter activity and gene expression is mediated through the E2F site, a result that agrees well with the stimulatory effect of PMA on p21 levels.

To determine whether promoter activity was controlled in a PKC izyme-dependent manner, we performed similar experiments in PKCα- and PKCδ-depleted cells. Remarkably, the inhibitory effect of PMA on cyclin A promoter activity was completely abolished upon PKCδ RNAi. PKCα knock-down, on the other hand, was unable to rescue the inhibitory effect of PMA on the promoter activity (Fig. 7D).

**DISCUSSION**

Because phorbol esters exert profound effects on cell proliferation, it is essential to understand the mechanisms by which their cellular effec-
tors control the different constituents of the cell cycle machinery. In that regard, our studies have established that PKC\(\text{\textgreek{p}}\) is a member of the novel PKC family, mediates the anti-proliferative effect of PMA in lung adenocarcinoma cells. PKC\(\text{\textgreek{p}}\) is responsible for the elevations in the cdk inhibitor p21 in response to PMA, which consequently leads to inhibition of G1/S progression. Striking differences exist with regard to PKC specificity in this process, as PKC\(\text{\textgreek{p}}\) is the only classic PKC expressed in H358 cells, does not mediate p21 up-regulation and inhibition of cell cycle progression in response to PMA.

Evidence accumulated in the last years points to distinct roles for individual PKC isozymes in the control of cell cycle progression, both at the levels of G1/S and G2/M transitions (2, 3, 28). Early studies in vascular endothelial cells have shown that phorbol esters potentiate the mitogenic effect of growth factors when added in early G1 phase, but they inhibit DNA synthesis in late G1 phase (22). These bi-directional effects closely associate to the control of G1 cdk and result from either activation or inhibition of Rb protein phosphorylation. Hypophosphorylation of Rb and other pocket proteins (p107 and p130) upon PKC\(\text{\textgreek{p}}\) activation has been detected in several models, including intestinal epithelial cells, vascular smooth muscle cells, and fibroblasts (2, 22, 28, 36), although the underlying mechanisms are not fully understood and may vary from one cell type to another. In H358 cells, we found that the G1 arrest caused by PMA after serum release only occurs when the phorbol ester was added at the initial time points, suggesting that PKC was indeed targeting early regulatory events. Interestingly, PMA does not inhibit cyclin D1 expression in H358 cells, as we detected a substantial induction of cyclin D1 protein (Fig. 1E) and mRNA by the phorbol ester (data not shown). In other cell types, such as in microvascular endothelial cells, PKC\(\text{\textgreek{p}}\) delays the induction of cyclin D1 following serum stimulation (37). Other studies have reported no changes in cyclin D1 in response to PKC activation (36). Despite these major cell type differences in the control of cyclin D1 expression by PKC, our data are suggestive of alternative mechanism for PMA-induced G1 phase arrest that involves p21 in lung cancer cells.

PMA causes a fast and robust increase in the levels of p21 (mRNA and protein) in H358 bronchoalveolar cells. In this model the induction of p21 represents a key event in the G1 arrest by phorbol ester activation. Indeed, the effect of PMA was significantly impaired when p21 was depleted with RNAi. Our results also revealed a remarkable selectivity for PKC isozymes in the control of p21 induction, both at the level of protein and mRNA. p21 up-regulation was basically abolished in PKC\(\text{\textgreek{p}}\)-depleted cells, whereas PKC\(\alpha\) RNAi did not cause any effect. These results contrast with those reported recently in intestinal epithelial cells, where p21 up-regulation correlates with PKC\(\alpha\) activation and was not
affected by PKCδ RNAi (27). This is not surprising, because major differences in PKC isoform-specific functions exist among different cell types. Unlike PKCδ, which is generally regarded as a growth inhibitory protein in the vast majority of cell lines, PKCα promotes mitogenic signaling in various cell types (38–41), which suggests that the inhibitory role for PKCα is probably restricted to a few cell types. These cell type differences are normally related to differential intracellular localization and access to targets and may well explain the differences observed among the different studies. It has been reported that, in cells in which PKCδ activation is apoptogenic, such as in prostate cancer cells (4, 17), cell death is preceded by p21 up-regulation and Rb dephosphorylation (42). Thus, p21 is probably a key component of anti-proliferative and apoptotic responses mediated by PKCs, particularly those involving PKCδ. Although the generalization of this concept still needs to be determined, our experiments using p21 knock-down cells strongly support that this is the case in lung cancer cells.

Our studies also reveal that in H358 cells, PKCδ activation impairs the expression of cyclin A, which is normally required for the activation of cdk2 in S phase. Cell cycle–dependent expression of the cyclin A promoter is regulated by E2F-pocket protein complexes. The cyclin A promoter also contains a cAMP-responsive element (CRE) element that is probably constitutively occupied in G1-phase cells and regulated by mitogenic signaling via phospho-CREB. We found that PKCδ activation markedly inhibits the activity of a cyclin A–luciferase reporter and that this effect was independent of the CRE site. Interestingly, the PKCδ effect was overcome by the expression of human papillomavirus E7, which inactivates Rb and allows for constitutive E2F signaling. Our results therefore suggest that the inhibitory effect of PKCδ on cyclin A promoter activity is probably secondary to the pocket protein inactivation and E2F release. This effect is mediated by PKCδ and not by PKCα, as determined by RNAi. All of these results agree well with our data showing that PMA and PKCδ up-regulate p21, a cdk inhibitor that blocks pocket protein inactivation.

In summary, our studies established PKCδ as a key component of the phorbol ester response leading to p21 induction, prevention of Rb dephosphorylation, and G1 arrest in lung cancer cells. In addition to uncovering a relevant mechanism underlying PKC control of cell cycle, our studies may have important implications in carcinogenesis. For example, polycyclic aromatic hydrocarbon metabolites, which are constituents of tobacco smoke, have been recently found to inhibit the activity of PKC isoforms, including that of PKCδ (43). One may speculate that impairing the activation of growth inhibitory PKCs could greatly impact on cell cycle regulatory mechanisms and therefore represent a causative or added factor to lung carcinogenesis. Various PKC
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analog (including phorbol esters) are currently undergoing clinical trials for the treatment of various hyperproliferative and neoplastic diseases (1, 44–47); our studies may also have important implications for understanding the molecular basis of their therapeutic effects. The characterization of the novel PKCδ-p21 link highlights the complexities in the signaling events downstream of PKC activation in the context of cell proliferation and provides mechanistic rationale for the development of isozyme specific strategies for therapeutic interventions.

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