Protein Kinase C δ Inhibits the Proliferation of Vascular Smooth Muscle Cells by Suppressing G₁ Cyclin Expression*

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Shinya Fukumoto‡, Yoshiki Nishizawa, Masayuki Hosoi, Hidenori Koyama, Kenjiro Yamakawa, Shigeo Ohno§, and Hirotoshi Morii

From the Second Department of Internal Medicine, Osaka City University Medical School, Osaka 545, Japan and the §Department of Molecular Biology, Yokohama City University School of Medicine, Yokohama 236, Japan

To elucidate the physiological role of protein kinase C (PKC) δ, a ubiquitously expressed isofrom in vascular smooth muscle cells (VSMC), PKC δ was stably overexpressed in A7r5 cells, rat cloned VSMC. The [³H]thymidine incorporation in A7r5 overexpressed with PKC δ (DVs) was suppressed to 37.1 ± 13.2% (mean ± S.D.) of the level in control or A7r5 transfected with vector alone (EVs). The reduction of [³H]thymidine incorporation was strongly correlated with overexpressed PKC levels. Moreover, transient transfection of a dominant negative mutant of PKC δ restored the reduced proliferation in DVs. Flow cytometry analysis demonstrated that DVs were arrested in the G₀/G₁ phase of the cell cycle. Expression of cyclins D₁ and E and retinoblastoma protein phosphorylation were reduced, while the protein levels of p27 were elevated in DVs as compared with EVs. There were no significant differences in the expression of c-fos, c-jun, c-myc, cyclin D₂, D₃, cyclin-dependent kinase 2, cyclin-dependent kinase 4, and p21 among the clones. We conclude that PKC δ inhibits the proliferation of VSMC by arresting cells in G₁ via mainly inhibiting the expression of cyclin D₁ and cyclin E.

Proliferation of vascular smooth muscle cells (VSMC) plays a central role in the progression of atherosclerotic lesions (1, 2). In VSMC, activation of protein kinase C (PKC) has been shown to regulate cell differentiation and proliferation as well as modulate agonist-stimulated phospholipid turnover and increase the contractile force (3–5).

PKC is a complex family including three types of isozymes (6, 7). The first group is the conventional PKCs (cPKC) (α, βI, βII, γ), which are Ca²⁺-dependent and activated by phorbol esters. The second is the novel PKCs (nPKC) (δ, ε, η, θ, μ), which are also activated by phorbol esters in a Ca²⁺-independent manner. The third is the atypical PKCs (ζ, ι, λ), which are activated in a phosphatidylinositol-dependent but Ca²⁺- or phorbol ester-independent manner (4, 7, 8). The different PKC isozymes are thought to have distinct functions regarding phosphorylation of specific substrate proteins (7). Evidence accumulated to date suggests that each PKC isozyme has a distinct role in cell proliferation. For example, PKC δ, a major isozyme ubiquitously expressed in most mammalian cells, was reported to inhibit growth and promote apoptosis in A549 lung cancer cells (9, 10). PKC α was also reported to inhibit proliferation and induce differentiation in melanoma cells (11), while PKC ε was shown to induce cell growth (10, 12). However, little is known about the molecular mechanism by which individual PKC isozymes control cell proliferation.

We found that PKC δ was expressed abundantly in rat aorta and rat VSMC, but the physiological role of PKC δ in VSMC and the signaling events leading from it remains to be elucidated. In this study, we first demonstrated the role of PKC δ in VSMC growth and the cellular mechanism involved. The cell cycle was arrested in the G₁ phase in PKC δ-overexpressing VSMC. The inhibition of cell proliferation by PKC δ was associated with suppression of G₁ cyclin expression and retinoblastoma protein (pRb) phosphorylation.

EXPERIMENTAL PROCEDURES

Materials—All dishes and plates were obtained from Becton Dickinson (San Jose, CA). All reagents, unless otherwise stated, were purchased from Sigma.

Cells and Cell Culture—Rat VSMC, A7r5, obtained from the American Type Culture Collection were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS) (Life Technologies, Inc.) and used within eight passages. Before mitogenic stimulation, subconfluent cells were arrested in the quiescent state by a culture in DMEM containing 0.2% FCS for 72 h. Cell viability was greater than 95% by trypan blue exclusion for all experiments.

Generation of PKC δ Stable Overexpressing Cell Lines—Full-length PKC δ cDNA was cloned into the expression vector SRD (13). A7r5 cells were transfected with either SRD vector alone as a control (designated as EVs) or with the PKC δ expression vectors (designated as DVs) by the calcium phosphate precipitation method followed by 15% glycerol treatment. The transfected cells were subsequently grown in selection medium. SRD-based construct-transfected cells, being with the neomycin-resistant gene as a selection marker, were grown in DMEM containing 400 μg/ml G418. After 15–20 days in selection medium, single colonies were removed and subsequently examined for the expression of PKC protein by Western blot analysis.

Northern Blot Analysis—Northern blot analysis was performed as described previously (15). The probes used were as follows: EcoRI fragments of mouse PKC α cDNA (16), mouse PKC βI cDNA (16), mouse PKC ι cDNA (16), mouse PKC γ cDNA (16), mouse PKC δ cDNA (14), mouse PKC ε cDNA (13), mouse PKC η cDNA (17), mouse PKC ζ cDNA (18), cDNAs for human c-fos, c-jun, and c-myc (obtained from the Japanese Cancer Research Resources Bank), mouse cyclins D₁, D₂, D₃, cyclin-dependent kinase (cdk) 2 and cdk4 (kindly provided by Dr. H. Matsushima, University of Tokyo, Tokyo, Japan), and EcoRI fragments of rat glycyldaldehyde-3-phosphate dehydrogenase cDNA (19). Quantification of the relative signal intensities was performed with a densitometer using the software NIH Image (Bethesda, MD), and the results were expressed in arbitrary units.

Protein Preparation—The cultured VSMC were homogenized in ice-
cold lysis buffer (20 mM Tris/HCl, pH 7.4, 300 mM sucrose, 2 mM EGTA, 5 mM EDTA, 0.3% (v/v) mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 5 mM 1,4-dithiothreitol, 600 μM diisopropyl fluorophosphate, and 25 μg/ml leupeptin). The homogenate was sonicated and then spun at 100,000 × g for 30 min, and the supernatant was used as the cytosolic fraction. The pellet was resuspended in lysis buffer containing Nonidet P-40 and agitated at 4 °C for 45 min. The homogenate was then sonicated and centrifuged at 100,000 × g for 30 min. The supernatant was used as the particulate fraction. Total cellular lysates were obtained by homogenizing cells in lysis buffer with 1% Nonidet P-40 and subjecting them to agitation for 45 min, followed by spinning at 100,000 × g for 30 min.

**Western Blot Analysis**—Total cellular protein samples (10–20 μg) were fractionated in 10% SDS-polyacrylamide gels and electrophoresed onto polyvinylidene fluoride membranes (Immobilon-P, Millipore, Bedford, MA). The following highly specific polyclonal antibodies were used for the analyses: antibodies directed against peptides derived from α, β, γ, δ, ε, and ζ subtypes of PKC (Life Technologies, Inc.) and antibodies for cyclin D1, cyclin E, p21, p27, pRB, cdk2, and cdk4 (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Western blots were developed by the ECL system (Amersham). Specificities of the signals of PKC α and δ were confirmed by using other antibodies obtained from Santa Cruz Biotechnology Inc. The densitometer was used to quantify the relative signal intensities of the bands as described above.

**[3H]PDBu Binding Assay**—[3H]Phorbol 12,13-dibutyrate (PDBu) binding was measured to estimate the levels of phorbol ester binding PKC molecule as described previously (14). Cells plated in a 48-well plate were washed twice with binding solution (DMEM, 1 mg/ml bovine serum albumin, 10 mM HEPES, pH 7.0) and incubated in the presence of 10 nM [3H]PDBu (DuPont NEN) at 37 °C for 30 min. Nonspecific binding was determined by incubating cells with [3H]PDBu in the presence of 10 μM unlabeled PDBu. Incubations were terminated by rapid washes with ice-cold phosphate-buffered saline and followed by a lysis with 0.1 M NaOH. Radioactivity in the lysates was determined by liquid scintillation counting.

**Measurement of Ca2+−dependent and -independent Protein Kinase C Activities**—Cells were stimulated with 100 nM 12-O-tetradecanoylphorbol-13-acetate (TPA) for 15 min. The cytosolic fractions and Nonidet P-40-solubilized particulate fractions obtained as stated above were applied to DE52 columns equilibrated with lysis buffer. PKC were eluted with lysis buffer containing 0.1 M NaCl, and PKC activity in these fractions was measured as described previously (14). The PKC assay mixture containing 20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 20 μM ATP, and 0.5 μCi of [γ-32P]ATP (3000 Ci/mmol) (DuPont NEN) was incubated with 50 μg/ml myelin basic protein (mBP), 15 μg/ml bovine serum albumin, 25 μg/ml phosphatidylserine (10 μM), and 25 μg/ml cardiolipin for nPKC activity or in the presence of 0.5 mM Ca2+, and 25 μg/ml phosphatidylethanolamine for cPKC activity or in the presence of 0.5 mM EGTA, 0.5 mM EDTA, and 25 μg/ml cardiolipin for nPKC activity. The reaction was spotted onto DE52 paper, washed three times with 1% H3PO4, and counted in a liquid scintillation counter.

**Measurements of DNA Synthesis and Cell Proliferation**—A7r5 and transduced cells (EVs and DVs) were seeded in 24-well plates in DMEM with 10% FCS. Cell proliferation was determined by counting cell densities, cells grown in 24-well plates to confluency were kept for 4 additional days with daily medium changes and followed by cell counting. DNA synthesis was assessed by [3H]thymidine (DuPont NEN) incorporation as described previously with some modification (15). After 72 h of serum deprivation, cells in 48-well plates were stimulated with 10 ng/ml PDGF-BB (Genzyme, Cambridge, MA) for 24 h and labeled with [3H]thymidine for the last 2 h. Cells suspended by trypsinization were centrifuged at 5,000 rpm for 3 min. Sediments were washed twice with PBS, and fixed in 70% ethanol overnight at 4 °C. After washing once with PBS, fixed cells were incubated in 1 mg/ml RNase at 37 °C for 30 min and followed by staining of the DNA with propidium iodide (1 μg/ml) at 4 °C for 2 h under shaking. The cell cycle distribution was analyzed by FACScan® (Becton Dickinson) on the software “Cell FITTM DNA System” (Becton Dickinson).

**PKC δ and G1 Cyclins**

Transfection of Dominant Negative Mutants into PKC δ Stably Overexpressed VSMC—Dominant negative mutants of PKC δ (9) were expressed transiently in VSMC by transferrin/polylysine-mediated gene delivery method (20) using a “Transferrinfection Kit” (Bender Med Systems, Vienna, Austria). As a control, empty vector alone was transfected. After seeding at 4 × 104 cells/well in a 48-well plate, cells were grown for 24 h in DMEM containing 10% FCS then incubated with 50 μM desferrioxamine at 37 °C for an additional 20 h. After the medium was changed, equal volumes of incubation mixture containing 20 μM chloroquine and various concentrations of transferrin/polylysine-conjugate-DNA complex were added. The basal concentration (1 unit) of transferrin/polylysine-conjugate-DNA complex consisted of 25 μg/ml of expression or empty vector in Hepes-buffered saline (HBS; 20 mM Heps, pH 7.3, 150 mM NaCl) and an equal volume of 25 units/ml transferrin/polylysine in HBS (1 μg of DNA for 4 × 104 cells). After incubation with various concentrations of mixture at 37 °C for 4 h, the cells were washed, deprived of serum for 72 h, and analyzed for DNA synthesis.

**Statistical Analysis**—Statistical analysis was done by using analysis of variance combined with multiple comparison (Scheffe’s type). These statistical analyses were carried out using Stat View IV on a personal computer (Macintosh Centris 650). The data were expressed as the mean ± S.D.
The activity was presented as pmol/min/mg of protein. cPKC activities were determined by using Ca\(^{2+}\) and phosphatidyserine as cofactors. nPKC activities were determined by using EGTA, EDTA, and cardiolipin as cofactors. The PKC activities were measured in the absence or the presence of 100 nM TPA for 15 min. Values are expressed as mean ± S.D. (n = 3). This experiment was repeated three times with reproducible results.

| Fraction | PKC family | TPA− | TPA+ |
|----------|------------|------|------|
|          |            | EV6  | DV9  | EV6  | DV9  |
| Soluble  | cPKC       | 98.7 ± 7.08 | 102.3 ± 8.75 | 53.9 ± 5.02 | 60.4 ± 5.56 |
|          | nPKC       | 21.7 ± 2.04 | 27.0 ± 0.99 | 15.7 ± 2.37 | 19.2 ± 2.25 |
| Particle | cPKC       | 33.8 ± 1.49 | 43.3 ± 5.16 | 72.9 ± 8.64 | 84.1 ± 7.83 |
|          | nPKC       | 22.5 ± 2.22 | 48.1 ± 2.41\(^a\) | 30.3 ± 3.51 | 61.0 ± 3.32\(^a\) |

\(^a\) Significant difference from EV6 (p < 0.05, multiple comparison, Scheffe’s type).

RESULTS

Constitutive Overexpression of PKC δ in Rat VSMC, A7r5—We used A7r5, a clonal cell line of rat VSMC for the experiments. This cell line has a monochonal character and has been used as a model system to examine proliferation and signal pathways including mitogen-activated protein kinase, phospholipase D, and PKC (21, 22). We examined the expression of PKC α, βI, βII, γ, δ, η, and ζ in A7r5 by Northern blot analyses and found that PKC α and δ were abundantly expressed (data not shown). PKC β, η, and ζ were also detectable (data not shown). To understand the role of PKC δ in VSMC and to elucidate the molecular signaling event leading from PKC δ, PKC δ was stably transfected to A7r5. Incorporation of vector and exogenous PKC δ DNA into genomic DNA of A7r5 was confirmed by Southern blot analysis (data not shown). DVs showed 2.1–3.0- and 2.0–3.8-fold higher PKC δ levels than A7r5 or EVs as determined by Western blot and Northern blot analyses, respectively (Fig. 1, A and B). No differences in mRNA and protein levels of PKC α were observed among DVs, A7r5, and EVs (Fig. 1, A and B).

PDBu binding activities, a simple method used to estimate the levels of overexpressing PKC isozymes (23), in DVs were consistently 1.4–1.8-fold higher than in A7r5 or EVs (Fig. 1C). Although PDBu binding assay measured more than just PKC δ, elevated PDBu binding activities in DVs seemed to reflect the expression levels of PKC δ because no significant changes in mRNA or protein levels of PKC α, another abundantly expressed isoform, were appreciated. PKC activities were also measured in EV6 and DV9, representatives of EVs and DVs. cPKC activities in both soluble and particle fractions were comparable between DV9 and EV6 (Table I). In contrast to cPKC, more than 50% of nPKC activity existed in the particle fraction even before cells were stimulated with TPA (Table I). The level of nPKC activity in the particle fraction of DV9 was 2-fold higher than that of EV6. There was no significant difference in nPKC activity between the soluble fractions of DV9 and EV6. Expression of α smooth muscle actin, a phenotypic marker for VSMC, in DV9 was as abundant as in EV6 as determined by Western blot analysis (data not shown).

PKC δ Suppresses Proliferation of A7r5—DNA synthesis was markedly suppressed in DVs compared with EVs 24 h after PDGF stimulation (Fig. 2A). The level of \(^{3}H\)thymidine incorporation was negatively correlated with that of PDBu binding (Fig. 2B) suggesting that DNA synthesis was inversely associated with levels of PKC δ expression. The time course of thymidine incorporation showed remarkable suppression and delay of onset in DV9 compared with EV6 or A7r5 (Fig. 2C). DNA synthesis in A7r5 or EV6 started 12 h after PDGF stimulation with the maximal accumulation observed at 24 h. In contrast, it started in DV9 24 h after PDGF stimulation, and the peak was observed at 48 h.

Cell numbers in the presence of 10% FCS for A7r5, EV6, and DV9 were counted every 24 h for 8 consecutive days (Fig. 2D). The proliferation of DV9 was significantly reduced as compared with A7r5 and EV6. Calculated doubling time of A7r5, EV6, and DV9 was 37.2 ± 0.8, 36.3 ± 4.8, and 62.7 ± 7.0 h, respectively. Moreover, the maximal cell density of DV9 was much lower than that of A7r5 or EV6 suggesting that DV9 cells were more susceptible to contact inhibition of growth (A7r5, 12.9 ± 0.35 × 10\(^4\) cells/cm\(^2\); EV6, 13.0 ± 0.38 × 10\(^4\) cells/cm\(^2\); DV9, 3.16 ± 0.45 × 10\(^4\) cells/cm\(^2\)).

To further elucidate the effect of PKC δ on cell growth, cell cycle distribution was determined by flow cytometry. When EV6 cells were deprived of serum for 72 h, 75.4 ± 4.77% of cells was arrested in the G0/G1 phase of the cell cycle. When the cells were treated with PDGF for 24 h, 26.4 ± 7.32 and 15.6 ± 6.00% of EVs were distributed in the S and G2/M phase, respectively (Fig. 3A). Similar cell cycle distribution was observed in A7r5 (data not shown). In contrast, 83.2 ± 2.28% of DV9 cells were still in the G0/G1 phase 24 h after PDGF stimulation, and relatively few had progressed to the S and G2/M phase 36 and 48 h after the stimulation (Fig. 3B). Thus, PKC δ suppresses cell proliferation by inhibiting and delaying G1/S transition in the cell cycle.

To further elucidate the involvement of PKC δ in the regulation of cell proliferation, we examined the effect of expression of a dominant negative mutant of PKC δ. This mutant harbors an amino acid substitution at Lys-376 in the catalytic domain, which is crucial for ATP binding and is conserved among several protein kinases (9). Transfection of the dominant negative mutant of PKC δ increased PDGF-stimulated DNA synthesis in DV9 in a dose-dependent manner (Fig. 4B). In contrast, transfection of the mutant did not affect PDGF-stimulated DNA synthesis in EV6 (Fig. 4A).

Suppression of G1 Cyclin Expression in PKC δ-overexpressing Cells—We next examined the mechanism underlying PKC δ suppression of cell proliferation. There was no difference in the PDGF-stimulated increase in mRNA levels for c-fos, c-jun, or c-myec among A7r5, EV6, and DV9 (Fig. 5). The maximal mRNA levels for c-fos, c-jun, and c-myec after stimulation with PDGF were observed at 30–45 min, 45–60 min, and 3 h, respectively, in each of the three cell lines (data not shown).

Because PKC δ-overexpressing cells appear to be arrested in G1 or delayed in G1/S transition, we examined the expression of G1 cyclins and cdks necessary for progression to S phase (24). Three types of cyclin D (D1, D2, and D3) were detected in A7r5 by Northern blot analysis. In A7r5 or EV6, the mRNA levels of cyclin D1 peaked at 6 h after PDGF stimulation, while cyclin D2 and D3 were constitutively expressed and did not oscillate (Fig. 6A). This result was in agreement with previous reports (25, 26) showing the distinct expression of three types of cyclin D underlying different regulation. In marked contrast, a PDGF-stimulated increase in cyclin D1 mRNA levels was only observed at 12 h with a maximal level at 18 h in DV9 (Fig. 6A).
6 h after PDGF stimulation mRNA levels of cyclin D1 in DV9 were significantly lower than those in EV6 (Fig. 6B).

Induction of cyclin D1 mRNA by PDGF was at least partly dependent on de novo protein synthesis, since treatment of cells with cycloheximide abolished PDGF-induced cyclin D1 mRNA at 18 h in both EV6 and DV9 (data not shown). Furthermore, treatment of cells with 100 μg/ml actinomycin D 6 h after PDGF stimulation showed that the half-life of cyclin D1 mRNA in DV9 was similar to that in EV6 (data not shown) suggesting that the stability of the mRNA for cyclin D1 was not altered in DV9.

Cyclin E mRNA levels were also increased by PDGF stimu-

Fig. 2, A, the levels of [3H]thymidine incorporation in PKC δ-overexpressing cells. Each group of cells was made quiescent by a culture in medium containing 0.2% FCS for 72 h. Thereafter PDGF-BB (10 ng/ml) or vehicle was added for 24 h, and the cells were labeled with [3H]thymidine for the last 2 h. [3H]thymidine incorporation into the trichloroacetic acid-precipitable fraction was determined. The results were adjusted with cell numbers. EV1 and EV6 are A7r5 transfected with vector alone, and DV9, DV11, DV12, and DV13 represent A7r5 overexpressed with PKC δ. Open column, vehicle; closed column, PDGF. The values are expressed as mean ± S.D. (n = 6). This study was performed three times with reproducible results. *, significant difference from A7r5 or EVs (p < 0.05, multiple comparison, Scheffe’s type). B, negative correlation between the levels of thymidine incorporation and PDBu binding. PDGF-stimulated [3H]thymidine incorporation and [3H]PDBu binding levels were determined in A7r5, EVs, and DVs. Each plot represents a fold increase in the transfected cells as compared with A7r5 and is expressed as the mean ± S.D. C, the kinetics of DNA synthesis induced by PDGF-BB in A7r5, EV6, and DV9. Quiescent cells were treated with 10 ng/ml PDGF-BB for the indicated time. DNA synthesis was determined by [3H]thymidine incorporation as described under “Experimental Procedures.” Open circles, closed circles, and open squares represent A7r5, EV6, and DV9, respectively. Values are expressed as mean ± S.D. (n = 6). This study was reproducible in three separate experiments. D, effect of overexpression of PKC δ on cell number increase. Each group of cells was plated in 24-well plates on day 0 at a density of 4 × 10⁴ cells/well in DMEM supplemented with 10% FCS. Cell numbers were counted every 24 h. Open circles, closed circles, and open squares represent A7r5, EV6, and DV9, respectively. Values are expressed as mean ± S.D. (n = 6). The experiment was reproducible in three individual experiments. *, significant difference from day 1 (p < 0.05, multiple comparison, Scheffe’s type) in A7r5 and EV6. **, significant difference from day 1 (p < 0.05, multiple comparison, Scheffe’s type) in DV9.
**Fig. 4.** Effect of a dominant negative mutant of PKC δ on the DNA synthesis of either vector alone transfected cells (EV6 (A)) or PKC δ-overexpressing cells (DV9 (B)). A dominant negative mutant of PKC δ was transiently transfected into EV6 and DV9 by the transferrin/polylysine-mediated gene delivery system as mentioned under “Experimental Procedures.” The basal concentration (1 unit) of the transfection mixture consisted of 1 μg of DNA for 4 × 10^6 cells. After incubation in various concentrations of transfection mixture, the cells were washed, deprived of serum for 72 h, and subjected to DNA synthesis analysis. 1, incubation with transfection mixture without DNA; 2, incubation of vector alone with 10 units of DNA; 3, 4, 5, incubation of dominant negative mutant with 0.1, 1.0, and 10 units of DNA, respectively; open column, without PDGF stimulation; closed column, with PDGF stimulation. The values are expressed as mean ± S.D. (n = 6). This study was reproducible in three distinct experiments. * significant difference from the others (bars 1–4) (p < 0.05, multiple comparison, Scheffe’s type).

**DISCUSSION**

In this study, we first showed that PKC δ suppressed VSMC proliferation, which was closely associated with inhibition of G1/S transition and expression of G1 cyclins (cyclin D1 and cyclin E).

**PKC δ Suppresses Proliferation of VSMC**—We successfully established a VSMC line in which PKC δ was overexpressed and found that PKC δ had a profound inhibitory effect on VSMC proliferation. Using representative clones (DV and EV), we found that total PDWhu binding activities, which were up-regulated by PKC δ transfection, were inversely correlated to the levels of PDGF-stimulated DNA synthesis. Furthermore, dominant negative PKC δ compromised the inhibitory effect of overexpressed PKC δ on DNA synthesis. An inhibitory role of PKC δ in the cell proliferation was also reported in fibroblast (6, 12). Taken together with these observations, PKC δ appears to be an antimitogenic signaling molecule. However, the cell cycle regulators governed by PKC δ might be specific in cell species. In VSMC overexpressed with PKC δ, cell cycle was delayed or arrested in the G1 phase, while in the Chinese hamster ovary cell, Watanabe et al. (23) reported that PKC δ arrested the cell cycle at G2/M but not G1/S transition.

Expression of PKC δ is shown to be regulated by cell density in NIH 3T3 cells (29) and C6 glioma cells (30). Also in A7r5, PKC δ mRNA levels, but not PKC α, were significantly up-regulated by cell confluency (data not shown) suggesting that PKC δ is involved in signaling from cell-cell interaction. This idea may be supported by the observation that PKC δ-overexpressing cells ceased growing at much lower cell density than control cells. It will be important to elucidate the physiological stimuli, including cell-cell interaction, and their signaling cascades leading to PKC δ expression.

**Cell Cycle Regulation by PKC δ**—PKC δ has been shown to be involved in PDGF-induced expression of the c-fos and c-myc genes (31, 32). Hirai et al. (9) also demonstrated that in NIH 3T3 cells PKC δ activates AP1/Jun transcription factor suggesting that these early responsive genes may mediate the function of PKC δ. However, we observed no changes in mRNA levels of c-fos, c-jun, or c-myc following PDGF stimulation in PKC δ-overexpressing VSMC, although we did not measure the transcriptional activity of AP1/Jun or Myc/Max. Thus, PKC δ
may suppress the growth of VSMC downstream of these early responsive genes.

We clearly demonstrated that phosphorylation of pRb and PDGF-stimulated expression of cyclin D1 and cyclin E, but not cdk2, cdk4, cyclin D2, or cyclin D3, were markedly suppressed in PKCd-overexpressed VSMC. Expression of cyclin D and E and phosphorylation of the pRb family are indispensable for the progression of the G1 phase and for entry into the S phase of the cell cycle in mammalian cells (33–35). Cyclin D/cdk4 and cyclin E/cdk2 phosphorylate pRb during early-middle G1 phase and late G1 phase, respectively (24). Although we did not measure cyclin D/cdk4 and cyclin E/cdk2 kinase activities, downregulation of cyclin D1 and cyclin E proteins may result in the abrogation of their associated kinase activities and pRb phosphorylation. Cyclin/cdk activities are also inhibited by cdk inhibitors such as p21 and p27 (27, 28). The protein level of p27, but not p21, was elevated in PKCd-overexpressing VSMC. Thus, PKCd suppressed cyclin D1 and cyclin E and up-regulated p27, and this may have led to abrogation of pRb phosphorylation and cell cycle progression.

Phorbol ester has been shown to have a stimulatory (36), an inhibitory (37), or no (38) effect on cyclin D1 expression in a cell type-dependent manner. However, which PKC isozyme is involved in the regulation of cyclin D1 expression has yet to be determined. We first directly demonstrated that PKCd could suppress cyclin D1 expression in VSMC. It is of interest to

FIG. 6. Cyclin and cdk expression in PKCd-overexpressing cells. Quiescent EV6 and DV9 cells were stimulated for indicated periods with 10 ng/ml PDGF, and the cells were harvested for RNA or protein isolation. For Northern blot analysis (A and B), total RNA (10 μg/lane) was separated on 1.0% agarose gel, blotted onto a nylon membrane, and hybridized with indicated cDNA probes. For Western blot analysis (C), samples (10–20 μg) were fractionated on SDS-polyacrylamide gels, electroblotted onto polyvinylidene fluoride membranes, and incubated with indicated antibodies. A, time course of mRNA levels for cyclins D1, D2, and D3 after PDGF stimulation in EV6 and DV9. B, mRNA levels of cyclins and cdks in EV6 and DV9. Right panels show the summarized results obtained from three separate experiments. Each mRNA level was adjusted by density of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The values are expressed as mean ± S.D. (n = 3). *, significant difference from EV6 (0 h) or EV6 (18 h); ***, significant difference from EV6 (6 h); and ****, significant difference from EV6 (0 h) and DV9 (0 h), respectively; †, significant difference from EV6 at the indicated time; ††, significant difference from EV6 (0 h) or EV6 (18 h). (Significant differences mean that p values were below 0.05 by multiple comparisons (Scheffe’s type).) C, protein levels of cyclin, cdk, cdk inhibitors, and phosphorylation of pRb in EV6 and DV9. Each study was repeated three times with reproducible results.
determine how PKC δ regulates cyclin D1 expression in VSMC. In this study we showed that the regulation of the level of cyclin D1 protein by PKC δ was associated with its mRNA level (Fig. 6). In addition, the half-life of cyclin D1 mRNA was not affected by PKC δ transfection, suggesting that mRNA levels for cyclin D1 are subjected to transcriptional control by PKC δ. We also observed that the transcription of cyclin D1 was regulated through de novo protein synthesis, which was in agreement with a previous report (39). PKC δ may regulate cyclin D1 transcription via this newly synthesized protein, since treatment of EV6 and DV9 cells with cycloheximide suppressed cyclin D1 mRNA induction by PDGF to the same level (data not shown).

The mechanism of the suppression of cyclin E protein and mRNA by PKC δ is not clear. It is known that cyclin E is regulated by a transcription factor, E2F (40, 41), whose activity is controlled by phosphorylation of pRb (34). Thus, abrogation of pRb phosphorylation by PKC δ may regulate cyclin D1 expression without affecting cyclin D1 expression (38), it is also conceivable that PKC δ suppresses the cyclin E expression directly in a cell cycle-independent manner.

Recently specific PKC β inhibitor was reported to ameliorate diabetic vascular dysfunction (42). Clarifying specific biological functions of distinct PKC isozymes in VSMC may provide us with a therapeutic approach for preventing the progression of atherosclerotic disease.

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