Proliferation and migration of vascular endothelial cells are essential features of angiogenesis and wound repair (1, 2). Both of these processes, as well as formation of a vessel tube, require tightly regulated changes in the endothelial actin cytoskeleton. Upon cell contact, proliferating endothelial cells become quiescent and develop a static actin cytoskeleton, a prominent feature of which is a peripheral actin band (3, 4). When contact inhibition is released, such as occurs during vascular injury or following the matrix dissolution that accompanies angiogenesis (5), endothelial cells lose this peripheral actin band, reorganize the actin filaments, reenter the cell cycle, and proliferate. Structures that often develop in migrating fibroblasts include actin stress fibers, filopodia, and lamellipodia, or ruffling (5, 6).

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Activation of the protein kinase C (PKC) family with phorbol esters induces endothelial proliferation and angiogenesis, but which of the events that constitute angiogenesis are affected by individual members of the PKC family is unknown. In rat capillary endothelial (RCE) cells, serum stimulation increased expression of a single PKC isoenzyme, PKC\(\alpha\), and its translocation to the periphery. Conditional overexpression of a dominant-negative mutant of PKC\(\alpha\) markedly inhibited RCE proliferation, as well as closure of a “wound” by RCE migration and formation of capillary rings and tubules in vitro. PKC\(\alpha\) inhibition delayed the endothelial cell cycle at the G\(_2\)/M phase and prevented formation of actin stress fibers and filopodia but not lamellipodia. The defect in cell morphology and wound closure in PKC\(\alpha\)-kn cells was reversed by overexpressing kinase-active PKC\(\alpha\), indicating that these RCE functions depend upon PKC\(\alpha\) substrates. Thus, PKC\(\alpha\) is required for multiple processes essential for angiogenesis and wound repair, including endothelial mitosis, maintenance of a normal actin cytoskeleton, and formation of an enclosed tube.

This work was supported by National Institutes of Health Grants HL47032 and HL51043 (to J. A. W.) and a training grant from the National Institutes of Health to the Cardiovascular Division of Beth Israel Hospital (to S. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 272, No. 45, Issue of November 7, pp. 28704–28711, 1997

28704 This paper is available on line at http://www.jbc.org
PKC9 in Endothelial Proliferation and Migration

was replaced with Trp to obtain a kinase-negative (kn) PKC9 protein that competes with the native form for substrates, as shown for other PKC isoforms (26–28). Similar mutations in the kinase domain of other isoenzymes have conferred upon them the ability to bind, but not phosphorylate, its substrates and thus to act as a dominant-negative inhibitor (29). After overexpression of the immunoprecipitated construct was determined to verify the presence of the desired mutation and to check for undesired random mutations. The constructs were transfected into rat capillary endothelial (RCE) cells (a gift of Robert D. Rosenberg, MIT, Ref. 29) using the Lipofectin method (Life Technologies, Inc.). The RCE cells were cultured in M199 media with 15% fetal bovine serum (FBS), 2 mML-glutamine, 1% non-essential amino acid, 0.8 mg/ml Geneticin (Life Technologies, Inc.). Cells that stably excrete 13-acetate, 7.5 mM dithiothreitol, and 225 nmol of deoxycholate that did not affect cell morphology or function of normal RCE was added to the media to prevent concomitant proliferation. The monolayer was wounded by scraping approximately 300 μm with a 1–200-μl pipette tip (Marsh Biomedical Products, Inc.). The distance of the gap was measured under a 4× phase objective of a light microscope, monitoring with a solid state TV camera (COHU Electronics), and captured with a Sony video graphic system (monitor, PVM97; printer, UP980MD).

Western Blot Analysis—Cell lysates were prepared by addition of 1 ml of lysis buffer (phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml phenylmethylsulfonyl fluoride, 20 mM/l aprotinin) per 1 × 107 cells. Samples were run on a 10% SDS-polyacrylamide gel and electrophoretically transferred to Immobilon-P membranes (Millipore). The membranes were then hybridized with either anti-PKC9 or immunoenzyme-specific antibody (Santa Cruz) or Flag antibody (IBI) in PBS containing 5% dry milk and detected via enhanced chemiluminescence (Amersham Corp.).

Northern Blot Analysis—Subconfluent monolayers of RCE cells (in 100-mm plates) were treated with trypsin and washed with phosphate-buffered saline (PBS). The cell pellet was resuspended in lysis buffer (140 mM NaCl, 1.5 mM MgCl2, 0.5% Triton X-100, 15 mM Tris, pH 8.3) and incubated on ice for 10 min. The supernatant was mixed with an equivalent volume of proteinase buffer (25 mM EDTA, 300 mM NaCl, 2% SDS, 200 mg/ml proteinase K), and incubated at the supernatant and incubated at 65°C for 1 h. Total RNA was extracted with phenol/chloroform, precipitated with ethanol, and resuspended in diethyl pyrocarbonate-treated water. For Northern transfer analysis, 20 μg of total RNA was subjected to electrophoresis on a 1.5% formaldehyde–agarose gel and transferred to GeneScreen Plus membrane according to the manufacturer’s recommendations. The blot was UV cross-linked (Stratalinker, Stratagene, Inc.) and hybridized with random-primer cDNA probes at 65°C for 3 h in Quik-Hyb solution (Stratagene, Inc.). Blots were washed under high stringency conditions and subjected to autoradiography.

Kinase Activity Assay—Subconfluent monolayers of RCE cells (in 100-mm plates) were treated with trypsin and counted and then washed with phosphate-buffered saline (PBS). 1.0 × 107 cells from each plate were resuspended in 1 ml of ice-cold lysis buffer for 10 min and then homogenized by repeated aspiration through a 21-gauge needle. Cell debris was removed by centrifugation at 3000 rpm for 4°C for 15 min. 1 μg of anti-PKC9 polyclonal antibody or bovine serum albumin (BSA, as control) was added to the supernatant and incubated for 1 h at 4°C. Agarose-conjugated protein A (20 μl) was added and incubated at 4°C overnight with gentle rocking. The immunoprecipitates were collected by centrifugation at 2500 rpm for 5 min at 4°C and washed 4 times with PBS. The pellet was suspended in 50 μl of PBS and stored in ice before the kinase assay. The kinase activity assay was carried out using a Protein Kinase C Enzyme Assay Kit (Amersham Corp.), 25 μl of assay mixture (50 mM Tris/HCl, pH 7.5, 0.05% sodium azide, 2 mol % l-o-phosphatidylserine, and 6 μg/ml phospholipase 12-myristate 13-acetate, 7.5 mm diothiothreitol, and 225 μg/ml PKC substrate peptide, 25 μl of ATP buffer (50 mM Tris/HCl, pH 7.5, 0.05% sodium azide, 150 mM ATP, 45 mM magnesium acetate), and 0.2 μg/ml of γ-[32P]ATP was added to each immunoprecipitation sample and incubated for 20 min at room temperature. In the experiment designed to test the activity of the constitutively active mutant, no phospholipid was added to the assay mixture. The reaction was terminated with 100 μl of stop reagent. Peptide binding papers were used to separate phosphorylated peptide. The papers were washed with 5% acetic acid twice and then were transferred to scintillation vials for 3H counting. The presence of native PKC9 or kinase-negative mutants in the immunoprecipitate was confirmed by immunoblotting with either anti-PKC9 or anti-Flag antibodies.

Cell Growth Analysis—Cell growth was determined by counting the cells with a Coulter Counter. Subconfluent cells were synchronized in serum-free medium for 2 days and then seeded at 2.0 × 104 per 35-mm plate in 3 ml of complete medium. After being cultured for indicated periods, the RCE were washed with PBS, treated with trypsin, and suspended in the medium for counting.

Endothelial "Wounding" Assay—RCE cells were cultured on 22 × 22-mm glass coverslips in 6-well plates in complete medium until confluent. In some experiments, serum was withdrawn from the media to prevent concomitant proliferation. The monolayer was wounded by scraping approximately 300 μm with a 1–200-μl pipette tip (Marsh Biomedical Products, Inc.). The distance of the gap was measured under a 4× phase objective of a light microscope, monitoring with a solid state TV camera (COHU Electronics), and captured with a Sony video graphic system (monitor, PVM97; printer, UP980MD).

Capillary and Tube Assay—Collagen gel (Vitrogen 100, Celsrix) and Matrigel (MATRIGEL Basement Membrane matrix, Becton Dickinson) were used. RCE were prepared in 12-well plates following the manufacturer’s instructions. RCE cells (approximately 80% confluent) were transfected with tetracycline, and treated with trypsin, and 1 × 105 to 5 × 105 cells were seeded on the top of plates with complete M199 medium. The photographs were taken under a 20× (for capillary ring structures) or a 4× (for tubule structures) phase objective of a light microscope, monitoring with a system described above.

Immunofluorescence Analysis—For localization of PKC9, cells (approximately 70% confluent) cultured on coverslips were washed with PBS and then were fixed with 2% paraformaldehyde in PBS for 10 min. The excess fixative was quenched with 0.1 mA glycine in 1% BSA/Hanks’ (137 mM NaCl, 5.4 mM KCl, 0.9 mM dextrose, 4.2 mM NaHCO3, 0.42 mM NaHPO4, 0.44 mM KH2PO4, pH 7.4). The cells were then permeabilized with 1% Triton X-100 in PBS for 10 min, washed in 1% BSA/Hanks’, and blocked with 10% goat serum in 1% BSA/Hanks’ for 1 h. The cells were placed in 2% goat serum in 1% BSA/Hanks’ containing a 1:50 dilution of stock anti-PKC9 antibody (Santa Cruz) for 1 h, washed in 1% BSA/Hanks’ with 0.05% Triton X-100, placed in BSA/Hanks’ containing a 1:40 dilution of fluorescein isothiocyanate–dextran-labeled anti-rabbit IgG solution for 90 min, washed again to remove the excess label, and mounted on coverslips with FluoroSave (Calbiochem) before analysis.

For F-actin localization, RCE cells (approximately 70% confluent) were cultured on coverslips, washed with PBS, fixed in 3.7% formaldehyde for 5 min, rendered permeable with 0.2% Triton X-100/PBS for 5 min, and incubated with 0.1 mg/ml rhodamine-phalloidin (Molecular Probes) for 20 min. The coverslips were mounted with FluoraSave. Cells were viewed on a Nikon C-H200 microscope with a 520 nm (excitation) and 580 nm (emission) filters. The fluorescence images were photographed with Kodak T-MAX 400 film.

Cycle Cell Analysis—RCE cultured in 6-well plates (approximately 70% confluent) were maintained in serum-free M199 medium for 3 days and then were stimulated with 15% FBS (complete medium). After incubation for indicated periods, the culture medium was removed, and the cells were washed with PBS. 250 μl of hypotonic buffer (0.5 mM Tris/HCl, 4 mM sodium citrate, 0.1% Nonidet P-40, 1.5 mM spermin, pH 7.5) with 0.05% trypsin was added to the cells. After 10 min incubation at room temperature, 210 μl of hypotonic buffer with 0.05% trypsin inhibitor and 0.01% RNase A was added and incubated for 10 min at room temperature, followed by addition of 210 μl of hypotonic buffer with 0.04% propidium iodide. The stained nuclei were gently pipetted several times and transferred to 12 × 75-mm polypropylene tubes. The propidium iodide fluorescence of individual nuclei was measured using a FACScan flow cytometer (Becton Dickinson).

Statistical Analysis—Statistical evaluation of the data was performed by use of Student’s t test. A value of p < 0.05 was considered statistically significant.

RESULTS
To determine which of the known phospholipid ester-sensitive PKC isoenzymes were expressed in RCE cells, both cytosolic and membrane (particulate) fractions of both quiescent and serum-stimulated RCE cells were subjected to electrophoresis
and blotted with antibodies specific to PKC α, β, γ, δ, ε, η, and θ isoenzymes, as well as PKCζ, an isoenzyme not responsive to phorbol ester. Of these isoenzymes, only PKCβ, γ, δ, ζ, and a small amount of α were detectable in either the cytosolic or particulate fraction of RCE, similar to our previous findings with human umbilical vein endothelial cells (15); serum stimulation for 36 h did not increase expression of α, γ, or δ isoenzymes but did induce a severalfold increase in PKCζ at both the protein and the RNA levels (Fig. 1). Thus, these results suggested the hypothesis that prevention of the action of PKCζ might alter endothelial proliferation induced by serum or phorbol esters.

**The PKCζ-kn Mutant Inhibits Native PKCζ Activity and Co-localizes with Activated PKCζ in RCE Cells**—To test the above hypothesis, a constitutively active, kinase-negative mutation of PKCζ was constructed with a PKCζ cDNA (22, 23) and transfected into RCE, and stable clones of these cells (PKCζ-kn) were selected by G418 resistance and analyzed by Northern and Western blotting. In Western blotting with the PKCζ antibody, which would be expected to recognize either native or mutated PKCζ, cells transfected with PKCζ clones (PKCζ-ca or PKCζ-kn) expressed much more anti-PKCζ-reactive protein than did the control cells that expressed endogenous PKCζ only (Fig. 2A). Only single bands at approximately 85 kDa were detected with this antibody. In a parallel Western blot obtained with the anti-Flag M2 antibody, only the cells transfected with the Flag epitope-tagged PKCζ mutants (PKCζ-ca and PKCζ-kn) were reactive, thus indicating the existence of cloned PKCζ (Fig. 2B). Although there was some constitutive expression of the mutants in the clones tested, the expression significantly increased after the cells were induced with dexamethasone. Examination of PKC activity in immunoprecipitates of control RCE (expressing the vector only), PKCζ-ca cells, and constitutively active PKCζ (PKCζ-ca) revealed that the PKCζ mutant not only did not possess intrinsic PKC activity but also was able to inhibit most intrinsic PKCζ activity in a dominant-negative fashion (Fig. 3). As expected in the immu-
noprecipitant containing constitutively active PKCo, as well as native PKCo, a significant portion of the activity was independent of phospholipids (phosphatidylserine). Removal of the transfected PKCo using the Flag antibody (Fig. 3B) revealed equal precipitations of native PKCo in all samples. As was the case with protein expression, some enzymatic activity or inhibition was evident constitutively in the mutant-expressing clones and was significantly increased by dexamethasone. These experiments suggest that the PKCo-kn inhibitor was effective in preventing the action of PKCo in vitro.

Immunohistochemical examination of wild-type RCE cells revealed that, in quiescent nonconfluent cells, PKCo is largely located in the cytoplasm and nucleus; stimulation with serum or contact with other cells induces translocation from the cytoplasm to the cell surface (Fig. 4, A and C). The constitutively active mutants (either −kn or −ca) were found to translocate to the surface membrane, similar to that seen with cellular activation, even in the absence of serum or cell-to-cell contact (Fig. 4D). Thus, the intracellular location of the PKCo-kn mutant coincides with, and therefore could potentially inhibit, the activated form of PKCo in intact cells.

**Inhibition of PKCo Prevents Endothelial Proliferation, Closure of an Endothelial Wound, and Formation of Capillary Ring and Tubes**—To determine the effects of PKCo inhibition on endothelial proliferation, quiescent control and PKCo-kn RCE cells were stimulated with media containing enriched serum concentrations, and cell counts were determined every 24 h thereafter for 8 days (Fig. 5). Statistical analysis revealed that RCE proliferation was neither inhibited nor significantly enhanced by PKCo-ca. In contrast, the slope of the growth curves of the PKCo-kn cells was significantly decreased compared with that of the control cells before reaching the plateau of the growth curve. These results showed that overexpression of the PKCo-kn inhibitor markedly inhibited RCE growth, thus suggesting that PKCo is required for serum-induced mitogenesis of capillary endothelium. Of note is that only slight inhibition was seen with PKCo-kn before induction, suggesting that the inhibitor reached levels sufficiently high to alter the phenotype only after induction. To confirm that these findings resulted from the effect of the inhibitor on the substrates of the targeted protein, we transfected the PKCo-kn cells with a PKCo-ca cDNA to create the PKCo-knR (reversal) RCE cell line and examined them to see whether the inhibitory effects would be reversed. The double transfected cells grew at a slower rate than did the control cells but significantly faster than PKCo-kn cells. Since expression of PKCo-ca did not exert a significant independent effect on RCE growth, these results suggest that competition with the inhibitor for the substrates caused this reversal and that PKCo is required for normal RCE cell growth.

To determine whether inhibition of PKCo was associated with reduced cell motility, we compared the ability of control, PKCo-ca, and PKCo-kn RCE to cover a 300-μm wound placed in a field of confluent cells of each culture. Impaired coverage of the defect by the PKCo-kn RCE cells was evident by 10 h after wounding. Measurement of the time required to close the gap revealed complete obliteration of the wound in 20 h in the wild-type RCE cells and the PKCo-ca cells, which did not differ significantly. In contrast, the PKCo-kn RCE closed the wound much more slowly (Fig. 6) and did not achieve full closure until 30 h after wounding. In the experiments shown, all RCE cells were incubated in the absence of serum so that proliferation did not contribute to coverage of the wound. The experiments were repeated in the presence of serum, and qualitatively similar results were obtained (data not shown).

To confirm that this effect resulted from inhibition of PKCo,
we also examined the PKC\(\theta\)-knR (reversal) RCE cells, with the expectation that this product would compete with the PKC\(\theta\)-kn inhibitor for substrates. The double transfected cells closed the wound significantly faster than did the PKC\(\theta\)-kn cells, although not as fast as the control cells, indicating that the effect of PKC\(\theta\)-kn was only partially blocked. Thus, these results suggest that the PKC\(\theta\) participates in events necessary for wound closure, one of which is endothelial migration.

Next, we asked whether expressing PKC\(\theta\)-kn would also prevent formation of capillary structures in an in vitro model of angiogenesis in a three-dimensional gel containing either collagen or a basement membrane matrix. When cultured in a collagen gel, normal RCE and PKC\(\theta\)-ca cells formed small rings resembling early capillaries in cross-section (Fig. 7, A and C) and occasional tube-like structures. PKC\(\theta\)-kn cells, in contrast, formed very few capillary rings (Fig. 7, B and C). When cultured in a Matrigel plate, normal RCE and PKC\(\theta\)-ca cells formed capillary tube-like structures (Fig. 8A), and the number of these tubules appeared to increased after induction of PKC\(\theta\)-ca cells with dexamethasone (Fig. 8C). The number of tube structures of PKC\(\theta\)-kn cells appeared to be less than that of control RCE and PKC\(\theta\)-ca cells, especially after induction with dexamethasone (Fig. 8C). Therefore, in addition to inhibiting endothelial proliferation and migration, PKC\(\theta\)-kn may retard formation of three-dimensional capillary structures, including rings and tubes.

PKC\(\theta\)-kn Delays the RCE Cell Cycle in G\(_2\)/M and Prevents Formation of Actin Stress Fibers and Filopodia—In the next set of experiments, we tested whether the decrease in RCE proliferation by PKC\(\theta\)-kn could be attributed to a delay in progression through the RCE cell cycle. Cell cycle analysis revealed that the PKC\(\theta\)-kn RCE cells demonstrated a delay at the G\(_2\)/M phase (Fig. 9A–C), 32% of the cells accumulating in this phase, compared with 12% of control cells and 7% of the PKC\(\theta\)-ca cells (data not shown), at 24 h following addition of serum after deprivation (Fig. 9D). In contrast to these differences, the length of time required to complete G\(_1\) phase, as judged by the time of an increase in the percentage of cells in S-phase following release from quiescence, was not significantly different in control RCE cells, the PKC\(\theta\)-ca cells and PKC\(\theta\)-kn cells (mean of 12 h in each case). Thus, these results suggest that the inhibition of cell proliferation in PKC\(\theta\)-kn RCE is caused by a specific delay at the G\(_2\)/M phase of the cell cycle; PKC\(\theta\) is not
required for progression through $G_1$ and entry into S phase.

During the course of the experiments, the PKC$\varepsilon$-kn RCE cells demonstrated differences in morphology from either the wild-type RCE or PKC$\varepsilon$-ca RCE cells. Specific morphological differences were noted between subconfluent wild-type or control (vector-transfected) RCE cells and PKC$\varepsilon$-kn cells. The control cells showed a polar migratory shape, typical of capillary endothelial cells and were spread. The PKC$\varepsilon$-kn cells revealed an abnormally rounded shape and were mostly found in clusters or colonies. Because of this observation, and the role that overall PKC activation had been shown to play in maintenance of the actin cytoskeleton in endothelial and other cell types (30), we asked whether prevention of PKC$\varepsilon$ activity would affect formation of filamentous actin structures. Immunohistochemical examination revealed that wild-type RCE developed prominent stress fibers composed of filamentous actin, with limited amounts of actin in a dense peripheral band (Fig. 10, A and B). Actin containing filopodia were also seen in the wild-type cells (Fig. 6C and Fig. 10A, small arrowheads) as well as in the PKC$\varepsilon$-ca RCE cells, which also demonstrated prominent actin stress fibers. In contrast, the PKC$\varepsilon$-kn RCE demonstrated a very prominent dense peripheral band of actin but had few of these stress fibers or filopodia (Fig. 10, C–F); instead, in the cytoplasm, actin appeared largely as small globules not organized into filaments. Cell ruffling (lamellipodia) around the edges of both control RCE and the cluster of PKC$\varepsilon$-kn cells (Fig. 10C) was clearly identified, indicating that PKC$\varepsilon$ is not a critical factor in this aspect of cytoskeletal function. In the PKC$\varepsilon$-knR cells (in which PKC$\varepsilon$-kn cells were transfected with a PKC$\varepsilon$-ca cDNA), F-actin arrangement and cell morphology regained the characteristics of those in the control RCE cells, suggesting further that PKC$\varepsilon$ is involved in the formation of F-actin stress fibers (Fig. 10, G and H).

Finally, because of the critical role that adherence via integrin receptors has been shown to play in angiogenesis (31), we examined the effect of PKC$\varepsilon$-kn expression on adhesion of RCE to vitronectin. No impairment of adhesion of the endothelial cells to vitronectin was noted in the PKC$\varepsilon$-kn cells (data not shown), suggesting that this PKC isoenzyme may not participate in signaling events that are critical to the ability of integrin or other receptors to bind to an insoluble matrix.

**DISCUSSION**

This study reveals that PKC$\varepsilon$ is a necessary component of the signal transduction pathway that leads to endothelial mitosis and formation of cytoskeletal structures by filamentous actin. These observations were made using a PKC$\varepsilon$-kn inhibitor designed by methods shown by others (26–28) working on other PKC isoenzymes to be a specific and potent inhibitor. The effectiveness of this inhibitor is suggested by the inhibition of PKC$\varepsilon$ activity in the in vitro enzymatic assay. Furthermore, the fact that changes in endothelial cell function significantly increased upon expression of the mutated gene at higher levels, following induction in the same cells, indicated that the observed phenotype resulted from this inhibitor. That the PKC$\varepsilon$-kn inhibitor is specific for the substrates of PKC$\varepsilon$ is suggested by the reversal of the phenotype by overexpression of PKC$\varepsilon$ in the affected cells. Thus, although it is not ruled out that other isoenzymes or unrelated proteins can also interact with these unidentified substrates, it seems likely that the

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**FIG. 9. Cell cycle analysis.** Single cell suspensions of the control or PKC$\varepsilon$-kn RCE cells were stained with propidium iodide, and DNA content per cell was analyzed by fluorescence-activated flow cytometry, approximately 10,000 events per sample. A–C, computer-aided analysis of the fluorescence-activated flow cytometry tracings of freely growing cells (approximately 80% confluent). D, time course analysis. The values of each time point (y axis) are expressed as the percentage of the total events. The results shown are representative of three similar experiments.
periphery, is stimulated by one or more of the molecules engaged in cell-to-cell contact and then acts on cytoskeletal substrates to initiate formation of actin stress fibers and filopodia.

Overall activation of the PKC family by at least some physiologic mitogens or by phorbol esters has been shown to be both necessary and sufficient for endothelial proliferation and angiogenesis in several in vitro and in vivo models (13–15). In contrast, others who used different experimental models and circumstances have shown that addition of PKC-activating phorbol esters can arrest endothelial cells in G₂ phase by inhibiting the action of cdc² and can also either facilitate or retard DNA synthesis, depending upon the phase of G₁ in which they are added (18, 32). This complexity suggests that individual isoenzymes of PKC, separated either temporally or by intracellular localization, might mediate these disparate effects. Overexpression of the constitutively active PKCₜ mutant does not inhibit cell cycle progression or RCE proliferation, thus suggesting that the inhibitory effects observed following phorbol esters do not result from stimulation of this isoenzyme. On the other hand, the results seen with the PKCₜ-kn cells suggest that this individual PKC family member is required for mitosis and formation of actin cytoskeletal structures and thus is a plausible candidate for the mediator of the angiogenic effect exerted by phorbol esters. Since endothelial proliferation and angiogenesis resulting from basic fibroblast growth factor (15) or vascular endothelial growth factor (14) can be blocked by chemical PKC inhibitors, it is possible that PKCₜ mediates angiogenesis induced by these agents as well.

The molecular mechanism by which PKCₜ regulates angiogenesis and cell growth appears to involve several interdependent processes. PKCₜ is located in the RCE cell nucleus in both permissive and stimulated cells, suggesting that it may regulate transcription and/or progression of the cell cycle. Additionally, translocation of PKCₜ to the plasma membrane upon cell-cell contact or mitogenic stimulation allows PKCₜ to participate in both the regulation of the cytoskeleton of the individual endothelial cell as well as the signaling cascade involved by cell-cell contact. A critical process in angiogenesis. The failure to form capillary ring structures in PKCₜ-kn cells indicates that PKCₜ mediates a pathway, likely triggered by signals from neighboring cells together with the underlying matrix, that leads to the formation of a three-dimensional tube. It is also evident that PKCₜ is a critical mediator in the organization of RCE cytoskeletal structure. The decreased cell motility seen in the PKCₜ-kn cells in the wound healing assay could result from lack of either filopodia or actin stress fibers that are involved in generating of tracking force necessary for cell locomotion (33). Adhesion of endothelial cells to vitronectin and formation of lamellipodia (ruffles), while apparently critical components for cell migration and proliferation (34), are not, by themselves, sufficient for these events to occur in endothelial cells.

The underlying substrates utilized by PKCₜ to cause these effects are not known. The PKCₜ-kn phenotype in RCE has some similarities to that found to occur in fibroblasts in which the small GTP-binding protein rho was inhibited. Those studies found that rho and/or cdc42 is a central mediator of the formation of actin stress fibers and filopodia (5, 35), as well as cell cycle progression (36, 37) and transcription (38) in fibroblasts. Furthermore, recent studies reveal that at least one of rho's targets is a serine-threonine kinase protein kinase N (PKN) (also known as PRK) (39, 40), whose catalytic domain is partially homologous to that of some PKC isoenzymes (41, 42), with which it shares some substrates in vitro (43). As is the case with rho in fibroblasts, inhibition of PKCₜ in RCE does not diminish formation of ruffling edges (lamellipodia) nor the ac-

FIG. 10. Fluorescence micrographs of control and PKCₜ-kn cells stained for actin with rhodamine-conjugated phalloidin. A and B, control RCE cells. Small arrowheads indicate the filopodia structures. C and D, PKCₜ-kn cell line 1; E and F, PKCₜ-kn cell line 2. Small arrows in C indicate the lamellipodia structures (ruffles). G and H, PKCₜ-knR cells (PKCₜ-kn cells transfected with PKCₜ-ca cDNA). Prominent F-actin stress fibers appear in the control RCE cells (A and B), whereas prominent granules of F-actin are shown in the PKCₜ-kn RCE cells (C–F), as well as the dense peripheral bands of actin seen at the periphery.

The potential relevance of these observations to the understanding of the intracellular mechanisms that regulate angiogenesis and repair of a damaged vessel is shown by the reduction in endothelial proliferation, wound healing, and capillary tube and ring formation in the endothelial cells expressing the specific PKCₜ inhibitor, PKCₜ-kn. PKCₜ's location is predominantly in the nucleus and cytoplasm of nonconfluent, unstimulated RCE, indicating that matrix contact is not itself sufficient to cause translocation of the isoenzyme to the surface membrane. PKCₜ migrates to the cell periphery upon either serum stimulation or cell contact. Since endothelial cells undergo cytoskeletal reorganization and reenter the cell cycle as a prelude to migration and proliferation once contact inhibition is released (3), it seems likely that PKCₜ, when localized to the cell
cumulation of cytoplasmic vesicles. These functions appear to depend upon rac activation (6), which consequently does not appear to require PKN. Thus, our data do not permit us to rule out the possibility that rho or cdc42 share a similar pathway with PKN to effect formation of actin stress fibers and filopodia. Two observations argue against a model in which PKN and rho are in the same pathway leading to cell cycle progression and proliferation. First, a major difference can be seen in their effects on the cell cycle in that inhibition of rho prevents exit from G1, at least in fibroblasts (37), whereas the PKCθ-kn cells in these experiments exhibited normal G1/S transition but were blocked in G2/M phase and did not undergo mitosis. Second, overexpression of PKCθ, the PKC isoenzyme that is the most homologous to PKCθ (73.3% identity at the amino acid level in the catalytic domain), prevents, rather than stimulates, endothelial proliferation (44), an effect not shared by PKCθ in these studies. Thus, it appears that the substrates utilized by PKC isoenzymes in vivo are very sensitive to minor changes in sequence, as has been noted in studies comparing intracellular substrates of the catalytic domains of closely related conventional isoenzymes (45–47). If we assume that rho’s effects on cell morphology and/or cell cycle progression are mediated by PKN (which has not yet been shown), it is likely that PKN and PKCθ do not share the same substrates, since the degree of dissimilarity is much greater (57.3% identity in the catalytic domain) between PKCθ and PKN than is the case with PKCθ. Thus, it seems more likely that PKCθ is in a pathway distinct from, and parallel to, that of rho, at least in its effects on cell cycle progression and mitosis.

Acknowledgments—We thank Drs. John White and Robert Rosenberg for the pGRE vector and the rat capillary cells, respectively, and Drs. Elizabeth Harrington, Masao Yukawa, James Chang, and Laila von Andrian for advice and technical assistance.

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