Endothelial cell-pericyte cocultures induce PLA_2 protein expression through activation of PKCα and the MAPK/ERK cascade

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Abstract **Little is known about the regulatory mechanisms of endothelial cell (EC) proliferation by retinal pericytes and vice versa. In a model of coculture with bovine retinal pericytes lasting for 24 h, rat brain ECs showed an increase in arachidonic acid (AA) release, whereas Western blot and RT-PCR analyses revealed that ECs activated the protein expression of cytosolic phospholipase A_2 ($cPLA_2$) and its phosphorylated form and calcium-independent intracellular phospholipase A_2 ($iPLA_2$). No activation of the same enzymes was seen in companion pericytes. In ECs, the protein level of phosphorylated extracellular signal-regulated kinase (ERK) 1/2 was also enhanced significantly, a finding not observed in cocultured pericytes. The expression of protein kinase C-α (PKCα) and its phosphorylated form was also enhanced in ECs. Wortmannin, LY294002, and PD98059, used as inhibitors of upstream kinases (the PI3-kinase/Akt/PDK1 or MEK-1 pathway) in cultures, markedly attenuated AA release and the expression of phosphorylated forms of endothelial $cPLA_2$, PKCα, and ERK1/2. By confocal microscopy, activation of PKCα in perinuclear regions of ECs grown in coculture as well as strong activation of $cPLA_2$ in ECs taken from a model of mixed culture were clearly observed. However, no increased expression of both enzymes was found in cocultured pericytes. Our findings indicate that a sequential activation of PKCα contributes to endothelial ERK1/2 and $cPLA_2$ phosphorylation induced by either soluble factors or direct cell-to-cell contact, and that the PKCα-$cPLA_2$ pathway appears to play a key role in the early phase of EC-pericyte interactions regulating blood retina or blood-brain barrier maturation.—** Anfuso, C. D., G. Lupo, L. Romeo, G. Giurdanella, C. Motta, A. Pascale, C. Tirolo, B. Marchetti, and M. Alberghina. **Endothelial cell-pericyte cocultures induce PLA_2 protein expression through activation of PKCα and the MAPK/ERK cascade. *J. Lipid Res.* 2007.** 48: **782–793.**

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The structural basis of the blood-brain barrier (BBB) is not only a specialized brain capillary endothelial cell (EC) but encompasses a highly organized complex consisting of an endothelium, pericytes, and astrocytic foot processes. For the endothelium to form a selective barrier between the blood stream and tissues, vascular maturation requires the formation of tight EC-to-cell contacts, the downregulation of endothelial proliferation, the deposition of a basal lamina to which the endothelium tightly adheres, and the recruitment of supporting cells to the vessel walls such as pericytes or smooth muscle cells (1–3).

Pericytes have received much less attention than the other components of microvascular beds. These contractile cells of mesenchymal lineage have long processes paralleling the capillary axis and short processes encircling the capillary. The current view of the role of pericytes in early stages of angiogenesis states that the initial endothelial tubes form without pericyte contact and that subsequent acquisition of pericyte coverage leads to vessel remodeling and maturation (4). This view has been challenged because it was shown that pericytes invest in actively sprouting and remodeling vessels (4, 5). Retinal pericytes have a mitogenic effect on retinal microvascular ECs (6), which may have important implications in new vessel formation. In an in vitro model of BBB established by coculturing immortalized ECs with brain pericytes that expressed tumor growth factor (TGF)-β1, the permeability of ECs was decreased significantly (7). Furthermore, in

Abbreviations: AA, arachidonic acid; $AACOCF_3$, arachidonoyl trifluoromethyl ketone; Akt, amino kinase terminal; BBB, blood-brain barrier; BEL, bromoenol lactone; $cPLA_2$, cytosolic phospholipase A_2; EC, endothelial cell; ERK, extracellular signal-regulated kinase; $iPLA_2$, calcium-independent intracellular phospholipase A_2; MAPK, mitogen-activated protein kinase; MEK-1, MAP/ERK kinase-1; PDK1, phosphoinositide-dependent protein kinase 1; PI3 kinase, phosphatidylinositol-3-kinase; PLA_2, phospholipase A_2; PKC, protein kinase C; TGF, tumor growth factor; vWF, von Willebrandt factor; WPB, Weibel-Palade body.

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mixed cultures, pericytes have an antiproliferative effect on ECs (8–10). However, the molecular basis of the induction signal is not yet clear, whether soluble factors (pericyte-derived angiopoietin-1 or TGF-β) or cell-to-cell contact.

Neovascularization and the vascular maturation process are under the control of phosphorylated p44/p42 mitogen-activated protein kinase (MAPK) and phosphorylated protein kinase C (PKC) isoforms upstream of p44/p42 MAPK and represent the main signaling cascades that become activated in angiogenic ECs (11). The two main sources of angiogenic signals (i.e., growth factors and the extracellular matrix) are transduced through the extracellular signal-regulated kinase (ERK) PI3K/Akt/ERK signaling system. Cytosolic phospholipase A_2 ($cPLA_2$), which regulates the provision of arachidonic acid (AA), cyclooxygenase-2, and prostaglandin E_2 release, is highly upregulated in angiogenic ECs during tumor progression (12), promoting integrin αVβ3-mediated EC adhesion, spreading, and angiogenesis through prostaglandin-cAMP-PKA-dependent activation of the small GTPase Rac (13).

To study these subjects, several in vitro models of cocultures have been used. For instance, astroglial cells were cultivated with brain ECs to produce a coculture model to study the permeability of cell layers (14). In the retina, macroglia (astrocytes and Müller cells) can enhance the barrier properties of retinal blood vessels by the production of factors that contribute to tight junction formation. Although mutual structural and functional interaction between astrocytes and ECs has been documented, less is known about the interaction between pericytes and ECs in mature microvessels. The potent vasoconstrictor endothelin-1 is a putative endothelium-pericyte signal regulating multicellular functional units of microvessels by a mechanism partially sensitive to PKC activation (15). Thus, although we are beginning to understand that pericytes are important in a number of situations in vessel formation, maintenance, and dysfunction (retinal diseases), we lack knowledge about how and what they signal to the endothelium.

The aim of this study was to scrutinize the reaction conditions that enable retinal pericytes to maintain the differentiated BBB phenotype in quiescent EC cultures or to induce EC maturation during the angiogenic state (angiogenic sprouting). This last phase may activate the expression of several specific proteins less expressed under quiescent conditions (no vessel formation). Incidentally, these proteins may represent potential targets for anti-angiogenesis therapy in ocular diseases as well as cancer. We hypothesized that the activation of the PKC-ERK1/2-$cPLA_2$ pathway may be involved in several endothelial functions, including BBB promotion, as well as angiogenesis. A role for PKC and PLA_2 to mediate cell-to-cell interactions is suggested, as several agonists, including endothelin-1, which increased prostacyclin production, increased at the same time PKC, MAPK, and PLA_2 activities in both isolated ECs (16) and pericytes (15). In addition, monocyte adhesion to human umbilical vein endothelial cells activated monocytic PKCα, PKCε, and both calcium-independent intracellular phospholipase A_2 ($iPLA_2$) and $cPLA_2$ activities (17). PLA_2s are a diverse group of enzymes that catalyze the hydrolysis of the *sn*-2 substituent from glycerophospholipid substrates to yield a free fatty acid and 2-lysophospholipid acceptors (18). Among the PLA_2s is an 85 kDa $cPLA_2$ that requires Ca^{2+} for catalysis and a calcium-independent PLA_2, $iPLA_2\beta$, for which several potential functions have been proposed, including a housekeeping role in phospholipid remodeling and a signaling role in cell growth, apoptosis, secretion, inflammation, and oxidant-induced cell injury (19–21). A number of observations that $iPLA_2$ has a functional role in cellular signaling in addition to its roles in AA release and phospholipid remodeling have been reported (22). $iPLA_2$ mediates the phosphorylation of transcription factors through a PKA-dependent pathway (23).

Activation of $cPLA_2$ is associated with a redistribution of the enzyme within the cell (24–26). The hydrolysis of glycerophospholipids to liberate fatty acids is in fact regulated by $cPLA_2$ translocation to membranes and by MAPK-catalyzed phosphorylation (19, 25, 27). There are several reports indicating that $cPLA_2$ may be a substrate for PKC phosphorylating activity (17, 28). Our recent work has also provided in vitro evidence that PKC upstream activates $cPLA_2$ after stimulation of ECs by oxidized LDL or of pericytes by β-amyloid peptides (29, 30). To date, direct evidence for any of the PKC isoforms being involved in the signaling pathway during endothelial cell-pericyte coupling has not been defined. Therefore, in cocultures of immortalized rat brain GP8.39 ECs and retinal pericytes (an in vitro model of BBB), we investigated the induction of PLA_2s in both cell types and illustrated the role of some PKCs and MAPKs, comparing the data obtained with those of their respective monocultures. We were able to show that in a coculture system, with or without a direct cell-to-cell contact, endothelial PKCα/δ and $cPLA_2$ protein expression were activated compared with those that evaluated in ECs grown alone.

EXPERIMENTAL PROCEDURES

Materials

Reagent-grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) or E. Merck (Darmstadt, Germany). Collagenase-dispase, fatty acid-free BSA, and the kinase inhibitors wortmannin, LY294002, PD98059, bromoenol lactone (BEL), and arachidonoyl trifluoromethyl ketone ($AACOCF_3$) were purchased from Calbiochem (La Jolla, CA). Primary antibodies against $cPLA_2$ (mouse monoclonal), PKCα (mouse monoclonal), rabbit polyclonal antibody against ERK1 or ERK2, mouse monoclonal antibody to phospho-ERK1/2, goat polyclonal anti-phospho-PKCα, mouse monoclonal antibody against actin, and rabbit polyclonal von Willebrandt factor (vWF) antibody were purchased from Santa Cruz Biotechnology, Inc. $iPLA_2$ (rabbit polyclonal) antibody was from Cayman Chemical Co. Rabbit monoclonal anti-phospho-$cPLA_2$ was obtained from Cell Signaling Technology, Inc. (Beverly, MA). All other chemicals were reagent grade.

Cell cultures

Immortalized rat brain ECs (GP8.39) were generously provided by Dr. John Greenwood (Department of Clinical Ophthal-

mology, University College, London) (31) and fed with F10 Ham's medium supplemented with 10% fetal bovine serum, 80 μg/ml heparin, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cell line was already characterized, and our cell cultures were prepared and characterized according to previously described procedures (31, 32). Morphological changes and cell viability determined by [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide] test were assessed as reported previously (29, 33). Pure microvessel pericyte cultures were prepared from bovine retinas as described previously (34). The isolated cells were then cultured in DMEM supplemented with 2 mM glutamine, 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Cocultures without direct cell-to-cell contact

Culture plates and inserts (Transwells; Corning, Inc., Corning, NY) were coated on the upper side with 150 μl of a 2 mg/ml solution of rat tail collagen containing 10-fold concentrated DMEM plus 0.3 M NaOH. The coating was dried for 1 h at 37°C and rinsed twice with water and once with Ca^{2+}- and Mg^{2+}-free PBS before being placed in complete medium. Before performing cocultures, ECs or pericytes were placed (40,000 cells/cm^2) and seeded onto six-well plates treated with collagen or skin porcine gelatin, respectively. At the same time, the two cell types were plated on the Transwell inserts (40,000 cells/cm^2) with the same substrate coverages. At confluence, ECs or pericytes on inserts were placed onto the plates containing, respectively, pericytes or ECs on the bottom (culture medium; 1 ml within the insert and 2 ml in the outer well; pericyte-to-EC ratio, 1:1). After a coincubation for 24 h in EC/pericyte medium (50% DMEM plus F10 Ham's, each containing 10% FBS), inserts containing conditioning cells were discarded, whereas cells on the plate were washed three times with ice-cold PBS and scraped with a rubber policeman. Lysates were performed as described (29, 33). EC or pericyte (40,000 cells/cm^2) monocultures were established in parallel as controls.

Cocultures with direct cell-to-cell contact

To make an in vitro model of the BBB based on direct contact of cells, pericytes were first plated on the outside of the polycarbonate membrane (40,000 cells/cm^2) of the Transwell inserts (six-well type, 0.4 μm pore size) and directed upside down in the well culture plate. After pericytes had adhered, the Transwells were inverted and reinserted onto six-well plates, and ECs were plated on the top surface of the insert (40,000 cells/cm^2). After coincubation for 24 h, cells grown on both sides of the inserts were scraped with a rubber policeman and saved separately.

AA release

Cultures of ECs or pericytes were established on six-well plates and incubated with 0.3 μCi/dish [1-^{14}C]AA (Dupont NEN) in F10 Ham's (ECs) or DMEM (pericytes) medium containing 2 mM glutamine and 5% fetal calf serum for 24 h at 37°C. The cells were washed three times, 5 min per wash, with HBSS buffer and coincubated without direct cell-to-cell contact with pericytes or ECs in serum-free DMEM (50%)-F10 Ham's (50%) medium containing 0.5% BSA as a trap for released AA in the absence or presence of 5 mM EDTA or in the presence of inhibitors such as wortmannin (60 nM) plus LY294002 (20 μM), PD98059 (25 μM), $AACOCF_3$ (50 μM), or BEL (25 μM) dissolved in DMSO stock solution for 24 h. The effects of PI3-kinase or MEK1 inhibitors (24 h exposure) and $AACOCF_3$ or BEL or EDTA on AA release were also tested in the prelabeled cells (control cultures in solo). After incubation, the medium was removed and centrifuged for 5 min. The supernatant fraction was concentrated by lyophilization to 1 ml, and an aliquot (100 μl) was taken to determine the radioactivity in total medium by liquid scintillation spectroscopy, normalizing to protein. Then, it was acidified with 1 M HCl to bring the pH to 3.0 (medium turns yellow) and frozen for subsequent analysis. Acidified medium was extracted three times in equal volumes of ethyl acetate (33). The organic layer was evaporated under nitrogen, and the residue was dissolved in ethanol. In some analyses, AA and eicosanoid derivatives were separated on silica gel 60 TLC plates developed with the organic phase of ethyl acetate-isooctane-acetic acid-water mixture (11:5:2:10, v/v). The lipid zones were located with I_2 vapor or by preparing autoradiographs (Berthold DAR-signal analyzer) and scraped into scintillation vials, and the radioactivity was measured by liquid scintillation spectroscopy. Identification of AA (main spot) and lipids (minor bands) was based on a comparison of their TLC mobilities with those of authentic unlabeled standards.

Preparation of cells for fluorescence confocal microscopy

To detect the expression and translocation of the PKCα isoform as well as $cPLA_2$, confocal microscopy was performed on ECs or pericytes cultured on microscope cover glasses placed on a six-well plate for noncontact cocultures as described above. Mixed cultures of ECs and pericytes (1:1) were also performed; glass coverslips were placed onto six-well plates before gelatin coating. ECs and pericytes were mixed and plated in 2 ml of 50% EC/50% pericyte medium.

After 24 h of coincubation, cells were fixed by adding 4% paraformaldehyde in PBS and processed for immunocytochemistry as described previously (29) using either anti-PKCα or anti-$cPLA_2$ antibodies. Distribution of PKCα and $cPLA_2$ immunocomplexes was observed by confocal immunofluorescence microscopy using a Leica TCS NT confocal laser scanning microscope. Single lower power scans were followed by 16–22 serial optical sections of randomly chosen cells in four to five fields per coverslip. The average fluorescence (mean ± SD) intensity (pixels) in individual cell bodies was measured throughout the stack. Each condition was tested on a total of 60–80 cells, resulted from at least three coverslips obtained from at least two different cultures.

Western blotting

After 24 h of coincubation, ECs and pericytes were lysed as described previously (29). The protein content of the cell lysate was quantified by Lowry's assay, and immunoblots were assessed as described elsewhere (29). Membranes were incubated with primary antibodies against $cPLA_2$ (mouse monoclonal; 1:500 dilution), $iPLA_2$ (rabbit polyclonal; 1:1,000 dilution), PKCα (mouse monoclonal; 1:2,000 dilution), or phospho-ERK (mouse monoclonal; 1:250 dilution). The membranes were then incubated with secondary antibodies for 1 h at room temperature, and the immunocomplexes were detected by enhanced chemiluminescence reagent (ECL; Amersham).

Total RNA isolation and RT-PCR

Total cellular RNA was purified from confluent and quiescent GP8.39 cells and pericytes using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions and redissolved in 30 μl of RNase-free water. RNA concentrations and purity were estimated by optical density at 260 and 280 nm. First-strand cDNA was reverse-transcribed in a 20 μl reaction volume with 200 units of SuperScript II, 50 ng of random examers, 125 mM deoxynucleoside triphosphate, 10 mM dithiothreitol, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 3 mM $MgCl_2$ (Invitrogen Life

Technologies). The reaction, carried out at 42°C for 50 min, was stopped at 70°C for 10 min. PCR was performed in 10 μl final volumes containing 1 μl of cDNA, 8 pmol of each primer, 1.7 mM deoxynucleoside triphosphate mix, and 1 unit of TaqMan Gold DNA polymerase (Applied Biosystems) in 1× buffer II. Cycling parameters were as follows: initial denaturing, 95°C for 10 min; denaturing, 95°C for 35 s; annealing at specific temperature (55–60°C) for 35 s; extension, 72°C for 45 s; and a final extension step at 72°C for 5 min. The appropriate number of PCR cycles was established as described below. Specific primers for rat $cPLA_2$, $iPLA_2$, and GAPDH genes (synthesized by Invitrogen Life Technologies) were used in semiquantitative RT-PCR on GP8.39. A 450 bp product, using a forward primer (5′-AAGGCCAAGTGACACCAGCC-3′) and a reverse primer (5′-GAAACAGAGCAACGAGATGGG-3′), was amplified for rat $cPLA_2$. A 353 bp product, using a forward primer (5′-AAGTGAACGTGTTCGAGAGG-3′) and a reverse primer (5′-GATGATGCGGCTGTGATGG-3′), was amplified for $iPLA_2$. The primers used for GAPDH were 5′-TAGACAAGATGGTGAAGG-3′ (forward) and 5′-GCAGGGATGATGTTCTGG-3′ (reverse), and their amplification product was 650 bp. Specific primers for bovine $cPLA_2$, $iPLA_2$, and β-actin genes (synthesized by Invitrogen Life Technologies) were used in semiquantitative RT-PCR assays on pericytes. A 293 bp product, using a forward primer (5′-ATTGCCCGACTATCATTCAC-3′) and a reverse primer (5′-ATGCTGTGGGTTTGCTTAG-3′), was amplified for $cPLA_2$. A 343 bp product, using a forward primer (5′-ATCACAGCCACATCATCAGC-3′) and a reverse primer (5′-CCATGATGTTGCAGCGAGC-3′), was amplified for $iPLA_2$. For β-actin, the primers used were 5′-CCCGTGCTGCTGACCGAG-3′ (forward) and 5′-ACCGTGTTGGCGTAGAGG-3′ (reverse), and their amplification product was 590 bp.

GAPDH and β-actin genes were used as internal control templates to normalize relative changes of target mRNA in RT-PCR on GP8.39 and pericytes, respectively. For each target gene to be analyzed and for each separate experiment, the exponential phase of amplification was determined such that product formation was related to starting template. Preliminarily, PCR was performed at various cycles, with positive and negative controls, to determine the cycle at which products became visible within the exponential phase of amplification. The conditions were chosen so that none of the RNA analyzed reached a plateau at the end of the amplification protocol (i.e., they were in the exponential phase of amplification). Thirty-two, 32, and 34 cycles of amplification for $cPLA_2$, $iPLA_2$, and GAPDH, respectively, were chosen to yield the linear increase of PCR products before reaching plateaus in GP8.39. The same conditions were also ensured by 31, 32, and 30 cycles of amplification for $cPLA_2$, $iPLA_2$, and β-actin, respectively, in PCRs on pericytes. We usually performed a negative control using RT-PCR instead of cDNA to check genomic DNA contamination. Furthermore, DNA sequencing of PCR products confirmed the specificity of our amplifications. Sequences were determined by the Centro Ricerca Interdipartimentale Biotecnologie Innovative (CRIBI)sequencing service at the University of Padova (Italy).

The PCR products were separated on 1.5% agarose gels in 1× Tris-acetate-EDTA buffer and stained with ethidium bromide. The bands on the ultraviolet light-transilluminated gel were converted into images, and their amounts were quantified with Scion Images software (Scion Corp., Frederick, MD).

Statistical analysis

Data are given as means ± SEM. One-way ANOVA was performed to test for differences between groups. The Student-Newman-Keuls test was performed. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

AA release

In ECs in noncontact cocultures with pericytes for 24 h, a statistically significant increase in AA release versus controls (ECs grown alone, no additions) was observed (**Fig. 1A, B**).

Treatment of ECs in cocultures with wortmannin plus LY294002 (PI3-kinase inhibitors), PD98059 (MEK1 inhibitor), $AACOCF_3$, or BEL induced a quite similar and sharp decrease in AA liberation compared with untreated cocultures (Fig. 1A, B). Treatment with PI3-kinase or MEK1 inhibitors significantly ($P < 0.01$) suppressed pericyte-induced AA release, suggesting that PI3-protein kinase may be involved in free AA production. No significant dif-

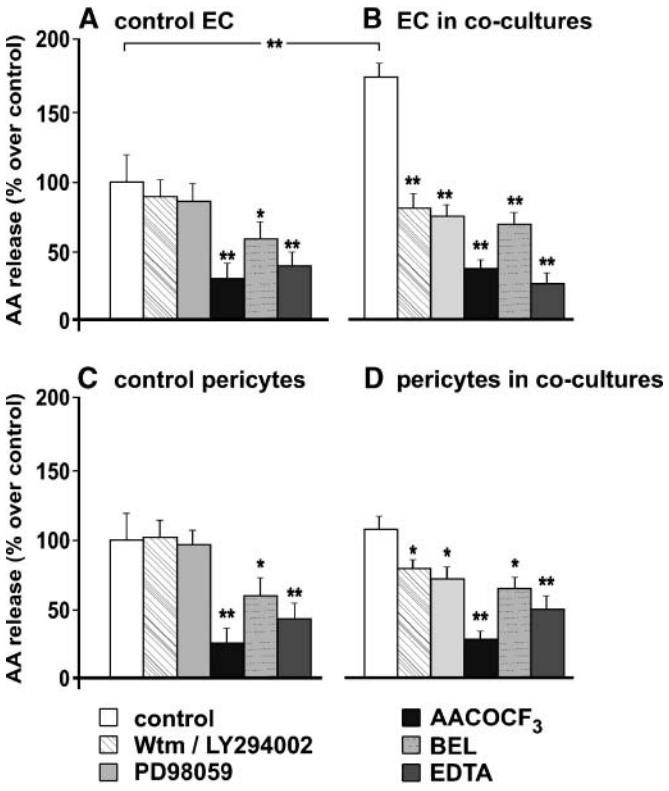

Fig. 1. Effects of inhibitors on [1-^{14}C]arachidonic acid (AA) release in GP8.39 endothelial cells (ECs) and pericytes in cocultures. ECs (A) or pericytes (C) were independently prelabeled with 0.3 μCi/dish (six-well plate) of [1-^{14}C]AA in F10 Ham's (ECs) or DMEM (pericytes) medium containing 2 mM glutamine and 5% fetal calf serum for 24 h and washed three times with HBSS medium containing 0.5% BSA before incubation with pericytes (B) or ECs (D) in noncontact cocultures alone or in combination with PI3-kinase, MEK1, or PLA_2 inhibitor. Cells were challenged with wortmannin (Wtm; 60 nM) plus LY294002 (20 μM), PD98059 (25 μM), arachidonoyl trifluoromethyl ketone ($AACOCF_3$; 50 μM), bromoenol lactone (BEL; 25 μM), or 5 mM EDTA for 24 h in serum-free DMEM (50%)-F10 Ham's (50%) medium. Endothelial or pericyte control cultures were also incubated in the presence of vehicle (DMSO) alone (i.e., no coculture conditions and without inhibitors). AA release into the culture medium was determined after extraction with ethyl acetate, as described in Experimental Procedures. Values represent means ± SEM from three separate experiments. Statistically significant differences (* $P < 0.05$, ** $P < 0.01$) are indicated by asterisks.

ferences were observed for endothelial control cultures (no additions) versus cell monocultures treated with wortmannin plus LY294002. No significant differences in AA release were observed for either pericyte control cultures (no additions) versus inhibitor-treated cell monocultures (Fig. 1C, D) or for pericytes in noncontact cocultures with ECs versus pericytes grown alone.

To clarify the role of group VI $iPLA_2$ upon pericyte stimulation in ECs, we evaluated the effect of PLA_2 inhibitors such as $AACOCF_3$, BEL, and EDTA. The enzyme activity insensitive to BEL represents the Ca^{2+}-dependent PLA_2, whereas that insensitive to EDTA represents Ca^{2+}-independent PLA_2. Our results demonstrated that treatment of unstimulated ECs or pericytes for 24 h with $AACOCF_3$, BEL, and EDTA resulted in, respectively, 71% (statistically significant at $P < 0.01$), 45% (statistically significant at $P < 0.05$), and 62% (statistically significant at $P < 0.01$) or 75% (statistically significant at $P < 0.01$), 44% (statistically significant at $P < 0.05$), and 56% (statistically significant at $P < 0.01$) decrease in AA release (Fig. 1A, C). All of these findings reflect the inhibition of basal PLA_2 activity, which is the sum of $cPLA_2$ and $iPLA_2$ activities. As shown in Fig. 1B, $AACOCF_3$, BEL, and EDTA were able to significantly inhibit stimulated AA release measured in the lysates of ECs in coculture. $AACOCF_3$, an inhibitor of both $cPLA_2$ and $iPLA_2$ (29), at 50 μM suppressed the pericyte-enhanced AA release by 77%, whereas 25 μM BEL was able to decrease the pericyte-enhanced AA release by 57%. In addition, EDTA suppressed the pericyte-enhanced AA release by 83%. These results demonstrate that pericyte-stimulated AA release (an indirect index of total PLA_2 activity) in ECs was mediated by both PLA_2 enzymes, even though $cPLA_2$ activation may be the predominant fraction.

PLA_2 expression and the effect of kinase inhibitors

The participation of calcium-dependent PLA_2, in addition to calcium-independent PLA_2, in cell-induced AA liberation remained to be elucidated in all coculture models investigated to date. To clarify the role of both phospholipases upon cell stimulation in ECs and pericytes, we evaluated PLA_2 protein expression by Western blot analyses. Our results demonstrated that, in a coculture system that prevented physical contact but allowed the diffusion of soluble factors for 24 h, ECs significantly expressed $cPLA_2$ and $iPLA_2$ protein greater than ECs in solo cultures, respectively, by 55% (statistically significant at $P < 0.05$) and 85% (statistically significant at $P < 0.01$) (**Fig. 2**). Equal loading in each lane was demonstrated by similar intensities of actin. These results demonstrated that in ECs, pericyte-stimulated total PLA_2 activity (Fig. 1A) might be mediated by both PLA_2 enzymes. In contrast, pericytes in the same coculture system showed no significant increase in either $cPLA_2$ or $iPLA_2$ protein expression. As shown in Fig. 2, the presence of pericytes in the coculture induced an increase in the endothelial phosphorylated forms of p42/p44 MAPK (ERK1/2). No changes in total ERK1/2 protein expression were observed. Blots using anti-phospho-p42/p44 MAPK antibodies for pericytes did not show any change. These data indicate that in ECs, the

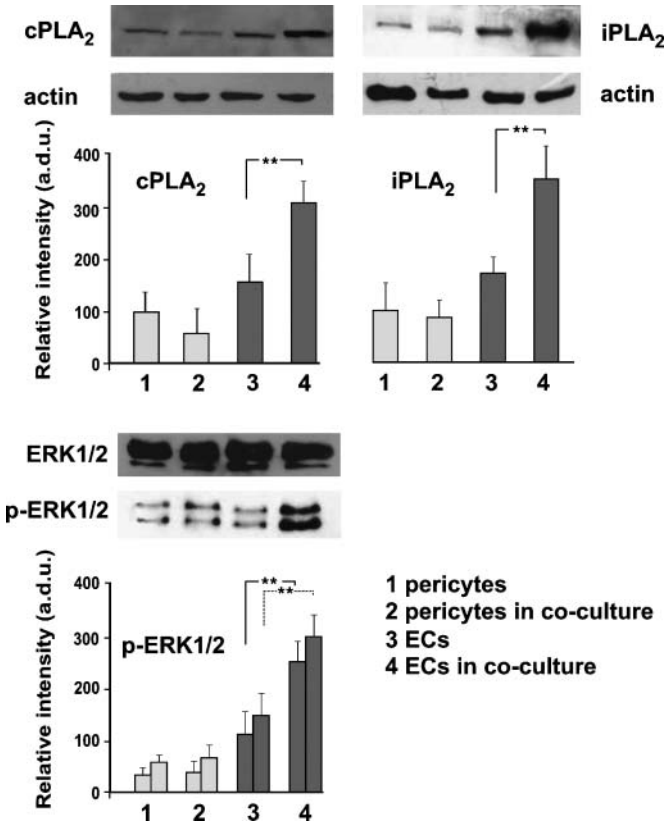

Fig. 2. Western blot analyses of cytosolic phospholipase A_2 ($cPLA_2$), calcium-independent intracellular phospholipase A_2 ($iPLA_2$), total extracellular signal-regulated kinase (ERK) 1/2, and phosphorylated forms of ERK1/2 protein expression in bovine retinal pericyte and GP8.39 EC cocultures without direct contact. ECs were incubated in DMEM (50%)-F10 Ham's (50%) medium containing 10% FBS in Transwell chamber inserts (0.4 μm), and pericytes were seeded in the chamber beneath at the bottom of the plastic wells and vice versa. Harvested cells were lysed at 24 h after coincubation, and individual cell lysates were resolved by SDS-PAGE. Expressed proteins were independently revealed with $cPLA_2$ monoclonal antibody, $iPLA_2$ polyclonal antibody, ERK1/2 polyclonal antibody, anti-phospho-ERK monoclonal antibody, or actin monoclonal antibody as described in Experimental Procedures. Immunoreactive bands were visualized by chemiluminescence (ECL system; Amersham). All blots were controlled for equal loading by actin monoclonal antibody and were scanned to calculate the fold increase in antigen level. The values, expressed as arbitrary densitometric units (a.d.u.), were obtained by the reading of blots and are means ± SEM of at least three independent experiments, each performed in triplicate; bar graphs represent net intensity of protein bands using the Scion Image program. Representative gel analyses are shown. ** $P < 0.01$, comparing cells in coculture with control cells grown alone, by pairwise Student's *t*-test.

ERK kinase phosphorylation and PLA_2 activation (Fig. 1) are coincident.

In addition, as shown in **Fig. 3**, ECs coincubated for 24 h with pericytes in Transwell chambers (in this method of coculture, again, the pericytes are not in direct contact with ECs but are separated from them by the medium and a semipermeable membrane that can be crossed by soluble mediators) showed increases in the phosphorylated forms of $cPLA_2$, PKCα, and ERK1/2 by 95, 128, and 300%,

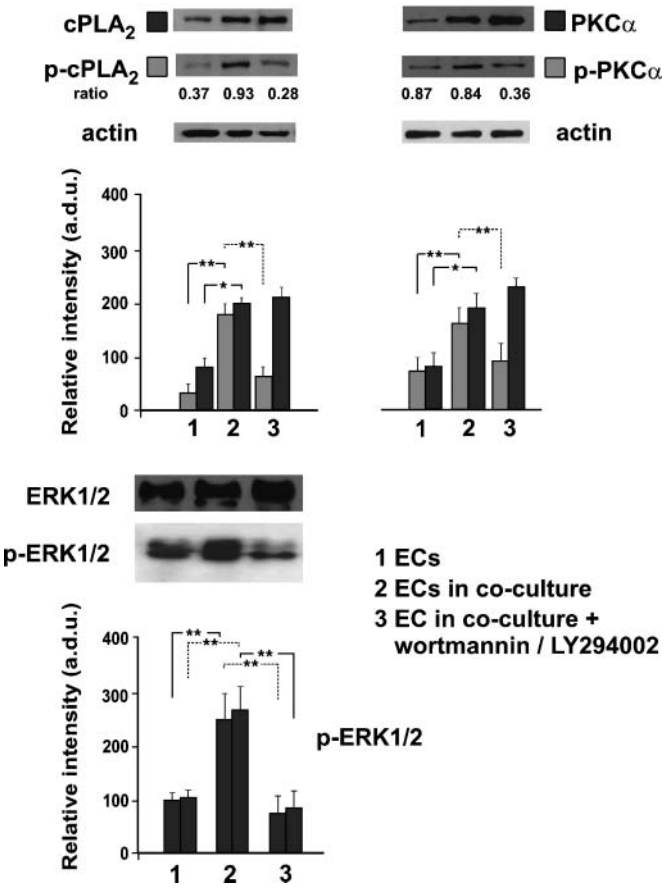

Fig. 3. Western blot analyses of the effects of the protein kinase (PI3-kinase) inhibitors wortmannin and LY294002 on the expression of total protein and phosphorylated forms of $cPLA_2$, protein kinase C (PKCα), and ERK1/2 in GP8.39 ECs cocultured with bovine retinal pericytes in a noncontact system. ECs and pericytes were incubated in DMEM (50%)-F10 Ham's (50%) medium containing 10% FBS for 24 h in the absence or presence of wortmannin (60 nM) plus LY294002 (20 μM). Harvested ECs were lysed at 24 h after treatment in coculture, lysates were resolved by SDS-PAGE, and expressed proteins were revealed with monoclonal antibody against $cPLA_2$, monoclonal antibody against PKCα, polyclonal antibody against ERK1/2, monoclonal anti-phospho-$cPLA_2$, polyclonal anti-phospho-PKCα, or monoclonal anti-phospho-ERK1/2 antibodies. All blots were controlled for equal loading by actin monoclonal antibody. The ratios of band intensity, phospho-$cPLA_2$/total $cPLA_2$ or phospho-PKCα/total PKCα are indicated. The blots were probed with the ECL Western blot detection system and exposed to film (Kodak) for the same period of time. Bar graphs represent net intensity of protein bands using the Scion Image program. Values represent means ± SEM from three independent experiments. Representative gel analyses are shown. * $P < 0.05$, ** $P < 0.01$, comparing cocultured ECs with control cells grown alone, by pairwise Student's *t*-test. a.d.u., arbitrary densitometric units.

respectively, compared with control cells grown alone. Equal loading in each lane was demonstrated by similar intensities of actin. In GP8.39 ECs, the constitutive phosphorylated form of $cPLA_2$ is seen at the ~40% level, whereas constitutive p-ERK1/2 were at 50%, as shown previously (29). Thus, the increase of $cPLA_2$ synthesis and phosphorylation (p-$cPLA_2$/$cPLA_2$ ratio from 0.37 to 0.93) may support an increase in $cPLA_2$ activity (Fig. 1).

To investigate the possible signaling mechanisms by which pericytes may mediate the phosphorylation of endothelial $cPLA_2$, we used inhibitors of PI3-kinase phosphorylation upstream of phosphorylating activities of endothelial PKCα and ERK1/2. Wortmannin (60 nM) plus LY294002 (20 μM), both inhibitors of upstream PI3-kinase, which regulates PKC phosphorylation, were able to significantly inhibit (p-$cPLA_2$/$cPLA_2$ ratio from 0.93 to 0.28) the stimulated expression of phosphorylated $cPLA_2$ measured in the lysates of cocultured ECs for 24 h in the presence of both drugs (Fig. 3). Preincubation of ECs and pericytes with wortmannin or LY294002 or a mixture of both inhibitors for 24 h did not result in significant changes in the viability of GP8.39 ECs ([3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide] test; mean of four independent experiments) compared with untreated controls (data not shown).

The inhibitor mixture also suppressed to almost the basal level the expression of the pericyte-enhanced phosphorylated form of endothelial PKCα (p-PKCα/PKCα ratio from 0.82 to 0.36), whereas the same inhibitors were able to decrease significantly pericyte-stimulated ERK1/2 phosphorylation by 3.3-fold. PKC is involved in the regulation of intracellular PLA_2 in several cell types. The strong effect of upstream phosphorylating activity inhibitors we observed in ECs may be attributable to the pivotal function of PKC isoforms in the control by basal phosphorylation of many target regulatory proteins all over the cell compartments, which also include PLA_2, in both early (contraction and metabolism, for instance) and late (differentiation and proliferation) cellular events.

Therefore, these data indicate that immortalized ECs had good levels of endogenous $cPLA_2$ significantly increased by 24 h of exposure to medium conditioned by the copresence of pericytes. Treatment of ECs with kinase inhibitors reduced the cell content of $cPLA_2$ phosphorylated protein to the control level, providing evidence that soluble pericyte factors induced $cPLA_2$ protein increase by PKCα and MAPK action. Interestingly, skin fibroblasts were used as control cells for cocultures with ECs. Factors released in cultures by them had no comparable effects to pericytes (data not shown).

Upregulation of $cPLA_2$ and $iPLA_2$ mRNA by pericytes

As the presence of pericytes in coculture increased $cPLA_2$ and $iPLA_2$ protein synthesis in ECs, we next assessed $cPLA_2$ and $iPLA_2$ mRNA levels by RT-PCR after pericyte stimulation for 24 h. As depicted in **Fig. 4A**, pericytes induced a significant increase of endothelial $cPLA_2$ mRNA (~2.1-fold; $P < 0.01$ compared with control levels). We extended our study to mRNA expression of Ca^{2+}-independent PLA_2 (type VI $iPLA_2$). Results shown in Fig. 4A indicate that, in ECs grown in serum-containing medium in the presence of retinal pericytes, the type VI $iPLA_2$ gene was constitutively expressed and that pericyte-soluble factors caused an increase (~2.4-fold compared with basal levels) in $iPLA_2$ gene expression. In contrast, parallel evaluations for $cPLA_2$ and $iPLA_2$ mRNA in cocultured pericytes (Fig. 4B) did not show any significant change compared with cell monolayers.

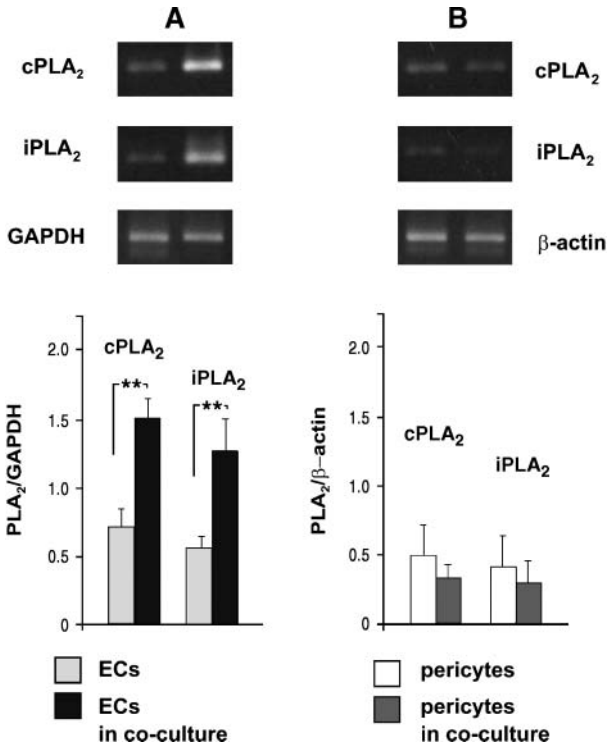

Fig. 4. Effects of coculture conditions on $cPLA_2$ and $iPLA_2$ mRNA expression in GP8.39 rat brain immortalized ECs (A) and bovine retinal pericytes (B). ECs were cocultured with retinal pericytes in DMEM (50%)-F10 Ham's (50%) medium containing 10% FBS for 24 h in a noncontact system with retinal cells seeded on the bottom side of Transwell chamber inserts and vice versa. After the incubation period, total cellular RNA was isolated from independently harvested cells using TRIzol reagent and phenol-chloroform extraction and used for RT-PCR analyses. Specific primers for control GAPDH (ECs) or β-actin (pericytes) were used. Values represent means ± SEM from three independent experiments. ** $P < 0.01$, comparing cocultured ECs with respective control cells grown alone, by pairwise Student's *t*-test.

Expression of PKCα in different coculture systems

Because endothelial PKCα is involved in stimulated p42/p44 MAPK phosphorylation by pericytes via the Raf-MEK pathway, indirectly suggesting that PLA_2 activation in ECs is mediated by PKCα, we analyzed the effect on PKCα expression of contact and noncontact conditions between cells in coculture. As shown in **Fig. 5**, in either the noncontact model (Fig. 5A) or the contact model (Fig. 5B) [i.e., ECs in contact with pericytes grown on the opposite sides of Transwell chamber inserts (pericyte processes made contact with opposing cell types through the semipermeable membrane)], the stimulation of endothelial PKCα protein expression reached almost the same values (2.0- to 2.5-fold). Equal loading in each lane was demonstrated by similar intensities of actin. On the contrary, pericyte PKCα did not show any stimulation (no statistical difference at $P < 0.05$); that is, the presence of ECs was without significant effect on pericyte protein expression.

Incidentally, in another set of experiments, mixed cultures of ECs and pericytes grown in contact on the same glass slide (Fig. 5C) showed the formation of net-like structures (phase-contrast micrograph).

Confocal microscopy

We next investigated by immunofluorescence microscopy the subcellular localization of PKCα in ECs and pericytes incubated in coculture for 24 h. To estimate PKCα fluorescence, ECs, double stained with a polyclonal vWF antibody coupled to a green fluorescent protein-labeled secondary antibody (to highlight EC architecture) and with a monoclonal PKCα antibody coupled to red Cy3 antibody, were acquired separately with the FITC and Cy3 filters, and the intensity of fluorescence was determined and corrected for background measured in areas devoid of cells.

As illustrated in **Fig. 6**, fusion of confocal images (Fig. 6A) reveals PKCα faintly accumulating in the cytoplasm of control ECs grown alone, as shown by Cy3 in red (Fig. 6C; PKCα staining). After cocultivation for 24 h with pericytes, merged confocal images (Fig. 6A′) revealed considerable overlap of FITC with vWF and Cy3 with PKCα (yellow or orange) in the perinuclear area (white arrows) and widespread throughout the cell. Strong red fluorescence (Fig. 6C′; PKCα staining) suggests increased protein expression, and a major part of the pool of the PKCα isoform seems to be translocated into the perinuclear region and nucleus of ECs. vWF secretory granules are diffuse all over the cell body, and stimulation with pericytes in cocultures results in distinct clustering of a subset of Weibel-Palade bodies (WPBs) in the perinuclear region of the cell (Fig. 6B, B′). Emission intensities of FITC (Fig. 6B, B′) and Cy3 (Fig. 6C, C′) were also evaluated with Leica confocal software, and pixel values inside the cells on a scale of 0–200 pixels (fluorescence arbitrary units) are reported in the graph for quantitative analysis.

$cPLA_2$ expression in ECs before and after cocultures is shown in **Fig. 7**. Cells were double stained with a polyclonal vWF antibody coupled to a green fluorescent protein-labeled secondary antibody (to highlight EC architecture) and with a monoclonal $cPLA_2$ antibody coupled to red Cy3 antibody. Intensity of fluorescence was determined separately with the FITC (Fig. 7B, B′) and Cy3 (Fig. 7C, C′) filters, and the merged images are shown (Fig. 7A, A′). The merged image (Fig. 7A′) shows that the immunofluorescent signal of total $cPLA_2$ (yellow by overlapping of green/FITC and red/Cy3) is significantly greater in EC cocultures with pericytes than in ECs grown alone. Quantitative analysis of $cPLA_2$ emission intensity (fluorescence arbitrary units) demonstrated enhanced expression of the enzyme in cocultured ECs.

A major expression of PKCα and $cPLA_2$ was not induced in pericytes in the presence of ECs compared with control pericytes in the absence of ECs. Quantitative analyses of PKCα and $cPLA_2$ emission intensity were very similar in controls and in pericytes in cocultures, demonstrating no activation of both pericyte enzymes by ECs (data not shown). In addition, results concerning mixed cultures of ECs and pericytes grown for 24 h on the same glass slide in the well (mixed model with cells in direct physical contact) to

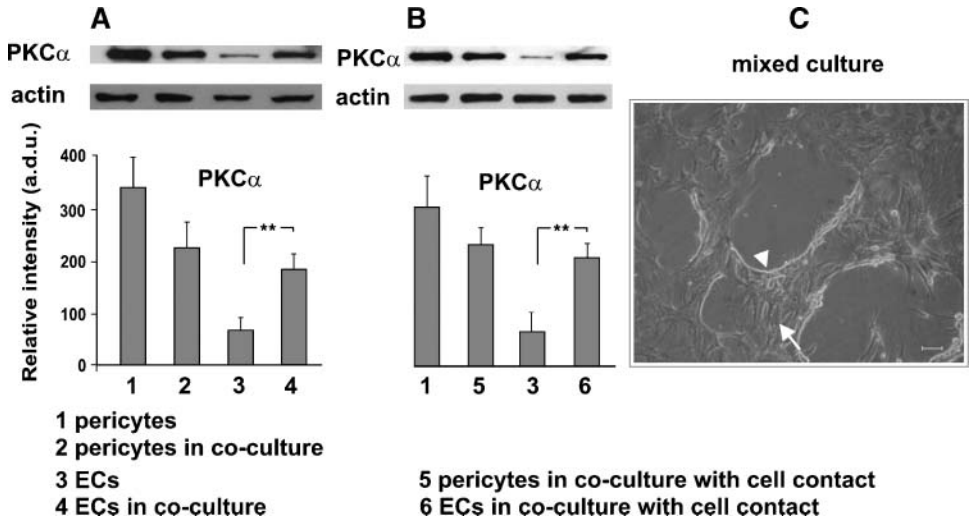

Fig. 5. Effects of the coculture conditions on PKCα protein expression (Western blots) either in retinal pericytes or GP8.39 ECs. A, B: ECs were grown on the noncontact system (A) or in contact with pericytes grown on opposite sides of Transwell chamber inserts (B). All blots were controlled for equal loading by actin monoclonal antibody. Bar graphs represent net intensity of PKCα bands using the Scion Image program. Values are means ± SEM from at least three independent experiments. Representative gel analyses are shown. ** $P < 0.01$, comparing cocultured cells with cell controls grown alone, by pairwise Student's *t*-test. a.d.u., arbitrary densitometric units. C: In another set of experiments, mixed cultures of ECs and pericytes grown in contact in the same glass slide showed the formation of net-like structures (phase-contrast micrograph; magnification, ×100). Note the EC phenotype morphology associated with the lumen of capillary-like areas (arrowhead) and the irregular shape and overlapping morphology of pericytes (arrow).

observe PKCα reciprocal modulation show an intense red signal for PKCα in ECs compared with a faint signal in large pericytes (data not shown). In a similar manner, confocal micrographs of ECs and pericytes in cocultures grown on the same glass showed that the immunofluorescent signal of total $cPLA_2$ was significantly greater in ECs than in pericytes (data not shown).

DISCUSSION

Mural cells such as smooth muscle cells and pericytes are important for stabilizing EC-to-cell contacts because they may influence vessel type-specific differences of the endothelial phenotype. Most research on the in vitro BBB has been performed with ECs with or without astrocytes (35). In the blood-nerve barrier, where cells equivalent to astrocytes are not found, endoneural pericytes support the integrity of the blood-nerve barrier determined by peripheral nerve microvascular ECs (36). In addition, pericytes induce microvascular endothelial maturation, which requires the downregulation of EC proliferation and the formation of adhering and tight junctions. All these processes could be mediated by AA derivatives that function as a phenotypic switch (exocrine and autocrine loop). This signaling may be achieved directly through physical contact (gap junctions, *N*-cadherin expression) or indirectly through an exchange of soluble mediators (4, 37, 38).

Although the in vitro growth conditions of ECs may be different from those seen in vivo, studies of their proliferation and cell interactions in vitro can be faithfully reproduced. This allows us to begin to determine the cross-talk between ECs and pericytes, which may play a role in neovascularization in vivo. To examine the role that pericytes might play in the formation of the BBB, we used immortalized rat brain EC-bovine retinal pericyte cocultures to mimic the phenotype cell-to-cell interactions that occur in microvessels in vivo. In this study, we tested the hypothesis that activation of the PKCα-ERK1/2-$cPLA_2$ pathway is involved particularly in several endothelial functions, including angiogenesis and BBB promotion.

Using the coculture system, we demonstrated that AA release, $cPLA_2$, and $iPLA_2$ protein expression were enhanced in ECs when they were cocultured with pericytes compared with those measured in EC or pericyte monolayers alone (transcriptional reprogramming of ECs when exposed to pericytes). The switch for endothelial $cPLA_2$ as well as $iPLA_2$ protein synthesis and activity, induced by neighboring pericytes that share the same culture medium and that are separated by a microporous insert (juxtacrine signaling previously unknown), might serve to produce endothelium-derived prostaglandins (PGE_2 and $PGF_2\alpha$) and prostacyclin. Our preliminary results indicated that cyclooxygenase-2, but not 15-lipoxygenase, was highly expressed in ECs cultured directly with pericytes for 24 h. Prostanoids may act as endogenous stimulators of angiogenesis (39), vascular cell adhesion molecule-1 expression, EC migration via autocrine and paracrine loop (40), and finally as a protection against early atherothrombogenesis.

It is known from previous studies that pericytes (or conditioned medium of retinal pericytes) inhibit EC migration and proliferation in cell culture (8–10). This inhibitory

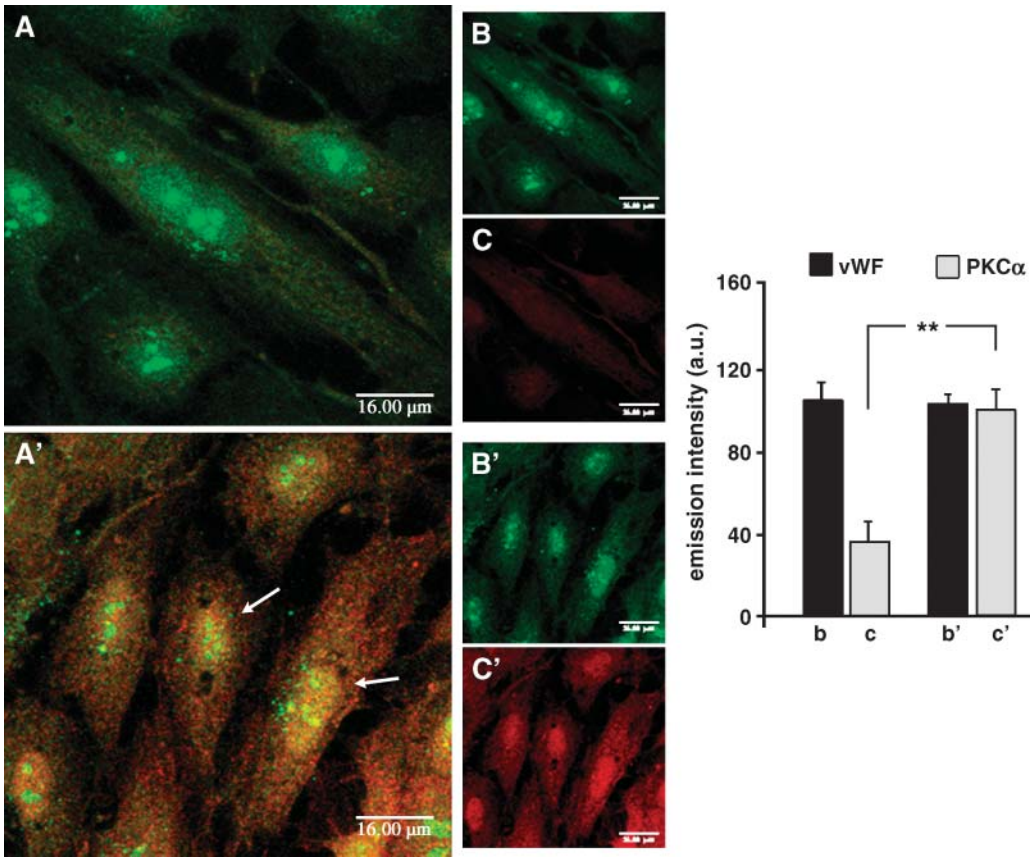

Fig. 6. Confocal microscopy of PKCα localization and expression in GP8.39 ECs. Cells were cultured in DMEM (50%)-F10 Ham's (50%) medium containing 10% FBS on glass coverslips in Transwell chambers and incubated for 24 h in the presence or absence of retinal pericytes, as described in Experimental Procedures. Cell monolayers were washed, fixed, permeabilized, and stained with a monoclonal anti-PKCα antibody coupled to a red fluorescent protein-labeled Cy3 secondary antibody and with a polyclonal anti-von Willebrandt factor (vWF) antibody coupled to a green fluorescent protein-labeled secondary antibody (to highlight EC architecture). Images of control ECs (cells grown alone) are shown in A (merge), B (vWF staining), and C (PKCα staining) (magnification, ×100). Images of ECs grown in the presence of pericytes in a noncontact model are shown in A′ (merge), B′ (vWF staining), and C′ (PKCα staining) (magnification, ×100). The images were analyzed by the determination of whole cell emission intensity (Leica TCS NT software version 2.5). Bars represent mean intensity values [pixels; in arbitrary units (a.u.)] of vWF or PKCα staining in panels B and B′ and C and C′, respectively. In control ECs, immunofluorescent signals (red Cy3) are almost detectable in either the cytoplasm or the membrane system (C). Coculture conditions strongly induced red immunofluorescence (PKCα activation) within the EC cytoplasm, particularly in the nuclear region (C′). The experiments were repeated on several occasions (three to four independent batches of cells) with nearly identical results. Values are means ± SEM. ** $P < 0.01$, comparing PKCα values in cocultured ECs with respective control cells grown alone, by pairwise Student's *t*-test.

or antiangiogenic activity is mediated by pericyte TGF-β, which becomes activated mainly when pericytes make contact with ECs. ECs in turn release platelet-derived growth factor-β, which induces pericytes to migrate toward the endothelium and to make contact (3, 41). Thus, cells of the vessel wall and ECs reciprocally activate mechanisms, mediated by extracellular factors, that induce coordinated vascular differentiation.

Investigating signaling pathways triggered intracellularly, we report here that the pericyte presence induces a sustained phosphorylation of $cPLA_2$ and ERK1/2, with a concomitant increase in PKCα protein expression, regardless of the type of cell-to-cell interactions (i.e., either in the noncontact or the contact coculture model).

Our results suggest that proteins that play a role in signaling are upregulated particularly in ECs after 24 h in coculture with pericytes. These observations are consistent with previous published data demonstrating transient activation of PKC and increased phosphorylation of 85 kDa myristoylated adenine-rich C-kinase substrate protein induced by astrocytes in proliferating cerebromicrovascular ECs (42).

In our study, signaling pathways were explored using pharmacological inhibitors of PI3-kinase (wortmannin plus LY294002) and MEK1 (PD98059). Wortmannin plus LY294002 blocked the pericyte-induced enhanced expression of phosphorylated forms of $cPLA_2$, PKCα, and ERK1/2, whereas both inhibitors had similar effects in blocking

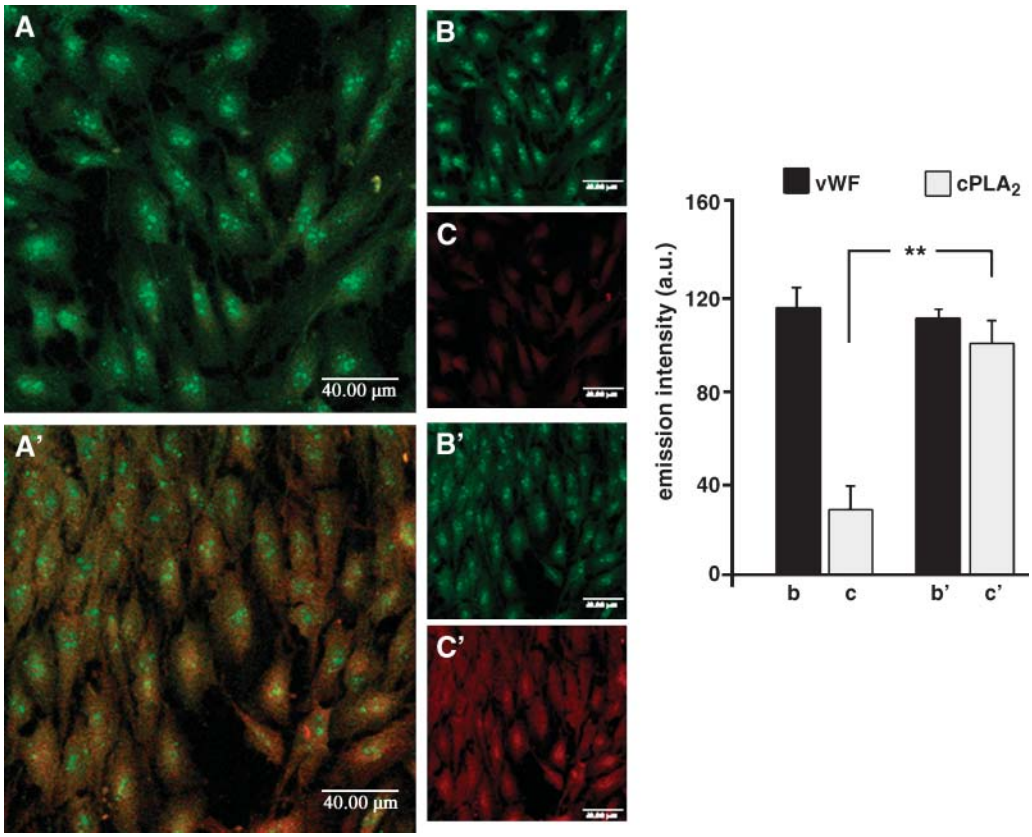

Fig. 7. Confocal microscopy of $cPLA_2$ localization and expression in GP8.39 ECs. Cells were cultured in DMEM (50%)-F10 Ham's (50%) medium containing 10% FBS on glass coverslips in Transwell chambers and incubated for 24 h in the presence or absence of retinal pericytes, as described in Experimental Procedures. Cell monolayers were washed, fixed, permeabilized, and stained with a monoclonal anti-$cPLA_2$ antibody coupled to a red fluorescent protein-labeled Cy3 secondary antibody and with a polyclonal anti-vWF antibody coupled to a green fluorescent protein-labeled secondary antibody (to highlight EC architecture). Images of control ECs (cells grown alone) are shown in A (merge), B (vWF staining), and C ($cPLA_2$ staining) (magnification, ×40). Images of ECs grown in the presence of pericytes in a noncontact model are shown in A′ (merge), B′ (vWF staining), and C′ ($cPLA_2$ staining) (magnification, ×40). The images were analyzed by the determination of whole cell emission intensity (Leica TCS NT software version 2.5). Bars represent mean intensity values [pixels; in arbitrary units (a.u.)] of vWF or $cPLA_2$ staining in panels B and B′ and C and C′, respectively. The experiments were repeated on several occasions (three to four independent batches of cells) with nearly identical results. Values are means ± SEM. ** $P < 0.01$, comparing $cPLA_2$ values in cocultured ECs with respective control cells grown alone, by pairwise Student's *t*-test.

stimulated AA release in EC cocultures. These findings suggest that activation of PKCα, ERK1/2, and PLA_2 enzymes may involve a mechanism dependent on PI3-PDK1 kinases. Extensive studies have provided evidence that 3′-phosphoinositide-dependent protein kinase and PDK1 phosphorylate and activate members of the ABC protein kinase family and play an important role in the regulation of cell survival, differentiation, and proliferation (43).

More than 12 PKC isozymes, the conventional PKCs (α, β, γ), novel PKCs (δ, ε, η), and atypical PKCs (ζ), have been found over the years. Previously, it was demonstrated that PKC isoforms (α, β, γ, δ, ε, ζ) were detected in vascular ECs (44–48). The expression pattern of PKC isozymes, including α, β, δ, ε, and the calcium- and diacylglycerol-independent ζ isoform, was also demonstrated to be present in pericytes (44, 45, 49). Ca^{2+}-dependent PKCα isoform is required for ERK signaling to trigger gene expression of $cPLA_2$. Previously, we have demonstrated that PKC activation is required for ERK-$cPLA_2$ phosphorylation after stimulation of ECs by oxidized LDL (29). Here, we have investigated how the interaction between the MAPK cascade and PKC affects ERK1/2 activation and phosphorylation upon cell-to-cell interaction in coculture. Our results suggest that activation of $cPLA_2$ through the activation of PKCα is the main mechanism by which the cross-talk between pericytes and ECs develops. In fact, total PKC expression and PKCα translocation from the cytosol to the membrane system and the nucleus was increased in cocultures compared with both cell cultures grown alone. It has been established that PKCα is a predominantly cytosolic protein but is able to translocate to the membrane fraction after stimulation. The exact subcellular location, plasma membrane or nucleus, of PKC after stimulation varies depending on the cell line and the stimulus (17, 50, 51).

vWF is an adhesive glycoprotein involved in primary hemostasis and synthesized in ECs, where it is stored in WPBs. vWF secretory granules are diffuse all over the cytoplasm, and intracellular vWF trafficking encompasses exocytosis on EC stimulation after vascular injury. In addition, stimulation with cAMP-increasing agonists (forskolin and epinephrine) results in distinct clustering of a subset of WPBs (escaping rapid secretion) in the perinuclear region of the cell (52). cAMP-dependent perinuclear recruitment of WPBs might provide a means to limit the excessive release of prothrombotic and proinflammatory mediators stored in WPBs under physiological conditions. Furthermore, cAMP-increasing agents improve barrier function in EC monolayers accompanied by a tightening of cell-to-cell contacts (53), probably by stimulating the phosphorylation of protein involved in the junctions or in cytoskeleton (54). It has also been shown that dibutyryl cAMP induced a phenotypically differentiated cell as well as the establishment of endothelial permeability barriers (tightening of EC cell-to-cell contacts) and increased the recruitment and cytoskeleton association of the tight junctional proteins (occludin, ZO-1, ZO-2) (55). Cocultures of ECs with smooth muscle-like 10T1/2 mimic the same effect. Therefore, the likeliest explanation for the nuclear localization of vWF observed in our confocal micrograph preparations of confluent ECs (Fig. 7) is that pericyte-soluble factors, favoring cell retraction, stress fiber disruption and cytoskeletal remodeling and antagonize the central granular movement from the Golgi area to the cell plasma membrane.

In agreement with our previous results in ECs (33), the intense staining of $cPLA_2$ seems to be associated with the nuclear compartment (Fig. 7). Translocation of $cPLA_2\alpha$ from the cytosol to the perinuclear membrane, where downstream eicosanoid-synthetic enzymes such as cyclooxygenase and 5-lipoxygenase are localized, has been reported by several groups (24, 25, 33). Indeed, targeting of $cPLA_2$ to the Golgi and endoplasmic reticulum membranes in CHO and MDCK cells activated by intracellular Ca^{2+} concentration increase has been demonstrated (26). More recently, in human ECs after stimulation with the calcium-mobilizing agonist A23187, relocation of $cPLA_2\alpha$ occurred to the endoplasmic reticulum, the Golgi, and primarily to both the inner and outer surfaces of the nuclear membrane (27). This process appears to be cell-specific and dependent on the proliferation state of ECs. Tashiro et al. (56) recently confirmed the presence of nuclear localization and export signals within the $cPLA_2\alpha$ protein.

In summary, our results demonstrate that the presence of pericytes activates the endothelial PKCα/ERK1/2 pathway, an essential step for augmented $cPLA_2$ expression in ECs regardless of coculture conditions. AA-derived prostanoids in endothelium might act as endogenous stabilizers of cell-to-cell contacts and stimulators of angiogenesis.

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