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Vascular Endothelial Cell Cyclic Nucleotide Phosphodiesterases and Regulated Cell Migration: Implications in Angiogenesis

Stuart J. Netherton and Donald H. Maurice

Department of Pharmacology and Toxicology, Botterell Hall, Queen's University, Kingston, Ontario, Canada

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ABSTRACT

Angiogenesis is necessary during embryonic development and wound healing but can be detrimental in pathologies, including cancer. Because initiation of angiogenesis involves migration and proliferation of vascular endothelial cells (VECs) and cAMP-elevating agents inhibit these events, such agents may represent a novel therapeutic avenue to controlling angiogenesis. Intracellular cAMP levels are regulated by their synthesis by adenylyl cyclases and hydrolysis by cyclic nucleotide phosphodiesterases (PDEs). In this report, we show that human VECs express variants of PDE2, PDE3, PDE4, and PDE5 families and demonstrate that the levels of these enzymes differ in VECs derived from aorta, umbilical vein, and microvascular structures. Selective inhibition of PDE2 did not increase cAMP in any VECs, whether in the absence or presence of forskolin, but it did inhibit migration of all VECs studied. Inhibition of PDE4 activity decreased migration, and in conjunction with forskolin, increased cAMP in all VECs studied. PDE3 inhibition potentiated forskolin-induced increases in cAMP and inhibited migration in VECs derived from aorta and umbilical vein but not in microvascular VECs. In experiments with combinations of PDE2, PDE3, and PDE4 inhibitors, a complex interaction between the abilities of these agents to limit human VEC migration was observed. Overall, our data are consistent with the hypothesis that PDE subtype inhibition allows different effects in distinct VEC populations and indicate that these agents may represent novel therapeutic agents to limit angiogenesis in complex human diseases.

Angiogenesis, the formation of new blood vessels from preexisting vascular structures, is both a necessary developmental and survival process with the potential to also be detrimental, promoting certain disease processes (D'Angelo et al., 1997; Griffioen and Molema, 2000; Davis et al., 2002; Auerbach et al., 2003; Gerritsen et al., 2003; Favot et al., 2003). During wound healing, embryonic development, and menses, angiogenesis provides developing tissues with necessary nutrients and oxygenation (D'Angelo et al., 1997; Griffioen and Molema, 2000). Indeed, stimulating angiogenesis after injury has been proposed as a means of reducing the damage that often accompanies reperfusion of ischemic tissues after injury (Griffioen and Molema, 2000). In contrast, aberrant or excessive angiogenesis allows vascularization of solid tumors and provides routes through which cancer cells may metastasize. A better understanding of the steps controlling angiogenesis should further advance our attempts to stimulate angiogenesis when warranted and inhibit it when required. Inhibition of angiogenesis would clearly be beneficial in limiting the growth of solid tumors and progression of diabetic retinopathy, and chronic inflammation associated with rheumatoid arthritis. Stimulating angiogenesis could have beneficial consequences in the treatment of coronary artery disease and in the limb ischemia associated with diabetes (Griffioen and Molema, 2000).

Angiogenesis requires the cooperation of several distinct cell types; with vascular endothelial cells (VECs) perhaps being the most important. VECs line every blood vessel in the body and constitute the majority of cells in capillaries (Auer-

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ABBREVIATIONS: VEC, vascular endothelial cell; VEGF, vascular endothelial cell growth factor; bFGF, basic fibroblast growth factor; PDE, phosphodiesterase; HAEC, human aortic endothelial cell; bAEC, bovine aortic endothelial cell; HUVEC, human umbilical vein endothelial cell; HMVEC, human microvascular endothelial cell; BAY 31-9472, 5-({9-[1-(1-hydroxyethyl)-4-phenylbutyl]-6-oxo-6, 9-dihydro-1*H*-purin-2-yl} methyl)-2-methoxy-*N*-methylbenzenesulfonamide; IBMX, 3-isobutyl-1-methylxanthine; DMEM, Dulbecco's modified Eagle's medium; TCA, trichloroacetic acid; RIMAC, radial invasion of matrix by aggregated cells; PCR, polymerase chain reaction; Ro, Ro 20-1724 [4-(3-butoxy-4-methoxy-benzyl)-2-imidazolidinone; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)-adenine]; 2-D, two-dimensional; 3-D, three-dimensional; EC, endothelial cell.

bach et al., 2003). The ability of VECs to emerge from their basement membrane and migrate toward an angiogenic stimulus is thought to initiate the neovascularization process. Such proangiogenesis stimuli can be released by tumors, activated lymphocytes, or wound-associated macrophages (Auerbach et al., 2003). Vascular endothelial growth factor (VEGF) is probably the most widely acknowledged initiator of angiogenesis (Ferrara, 1999; Griffioen and Molema, 2000). VEGF released from tumor cells, macrophages, and other immune cells in response to hypoxia (Brown et al., 1997) stimulates vascular relaxation (a prerequisite for endothelial cells to enter the angiogenic cascade) via NO production by VECs (Griffioen and Molema, 2000), increases VEC permeability (Ziche et al., 1997; Cao et al., 2001), and increases expression of VEC VEGF receptors during periods of hypoxia or ischemia (Forsythe et al., 1996). In addition, basic fibroblast growth factor (bFGF) (Griffioen and Molema, 2000; Favot et al., 2003), acidic fibroblast growth factor, and transforming growth factor-α are other potent positive regulators of angiogenesis (Ferrara, 1999).

The intracellular second messengers cAMP and cGMP are generated by adenylyl and guanylyl cyclases, respectively, and play an important role in modulating the migratory and proliferative tendencies of many cell types, including VECs (Favot et al., 2003). Most effects of cAMP or cGMP are thought to be related to activation of protein kinase A or protein kinase G, respectively, and phosphorylation of multiple selective cellular substrates (D'Angelo et al., 1997; Fleming et al., 2004). Cellular cAMP and cGMP levels are dynamically regulated by both the rate of their synthesis by cyclases and their hydrolysis by cyclic nucleotide phosphodiesterases (PDEs). Eleven distinct PDE gene families (PDE1–11) are defined based on sequence, catalytic, and regulatory considerations (Maurice et al., 2003). Although members of the PDE1, PDE2, PDE3, PDE10, and PDE11 families of enzymes can hydrolyze cAMP or cGMP, PDE4, PDE7, and PDE8 enzymes selectively hydrolyze cAMP. In contrast, PDE5, PDE6, and PDE9 family enzymes selectively hydrolyze cGMP (Maurice et al., 2003). A limited number of studies have characterized PDE activities in VECs and shown that some bovine, rat, and human VECs contain activities consistent with expression of PDE1, PDE2, PDE3, PDE4, and PDE5 family enzymes (Lugnier and Schini, 1990; Ashikaga et al., 1997; Lugnier et al., 1999; Sadhu et al., 1999; Keravis et al., 2000; Miro et al., 2000; Thompson et al., 2002; Creighton et al., 2003; Favot et al., 2003; Maurice et al., 2003).

The aims of this study were to identify the PDEs expressed in VECs from both human and bovine large conduit arteries [human aortic endothelial cells (HAECs) and bovine aortic endothelial cells (bAECs)], human veins [human umbilical vein endothelial cells (HUVECs)] as well as human microvasculature structures [human dermal microvascular endothelial cells (HMVECs)] and to begin to assess the therapeutic potential of controlling angiogenesis by inhibiting VEC migration through selective PDE inhibition. To be more specific, we aimed to determine the impact of selective PDE inhibition on both VEC cyclic nucleotide levels as well as the effects of these treatments on migration of these cells. We submit that by simultaneously analyzing responses of VECs derived from differences species and different human vascular structures our analysis should allow for our aims to be achieved.

Materials and Methods

Cell Culture of VECs. HAECs and HMVECs were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). bAECs were a generous gift from Dr. D. Lillicrap (Queen's University, Kingston, ON, Canada), and HUVECs were a generous gift from Dr. L. Bajzar (McMaster University, Hamilton, ON, Canada). HAECs, HMVECs, and HUVECs were cultured using EGM-2-MV media from Cambrex Bio Science Walkersville, Inc. Tissue culture reagents for bAEC (Dulbecco's modified Eagle's medium, calf serum, HEPES, penicillin/streptomycin, and trypsin-EDTA) were purchased from Invitrogen (Carlsbad, CA). Cells were passaged 1:3 when they became confluent, being used in experiments between passages 3 and 10.

Processing of VECs and Protein Determinations. Confluent cultures of bAECs, HAECs, HUVECs, or HMVECs were lysed and homogenized in a lysis buffer containing 52.5 mM Tris-HCl, pH 7.4, 5.25 mM $MgCl_2$, 5 mM benzamidine, 1 mM EDTA, 100 mM dithiothreitol, 200 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1% Triton X-100, and 50 mM sodium fluoride. Cellular debris was removed by centrifugation at 1000*g* (3000 rpm) for 3 min. The 1000*g* supernatants were stored at 4°C until cAMP/cGMP PDE activity assays were conducted. Protein concentration of lysates was determined using the bicinchoninic acid protein assay system from Pierce Chemical (Rockford, IL), according to the manufacturer's recommendations, using bovine serum albumin as the standard.

PDE Activity Assays. cAMP and cGMP PDE activities were assayed by a modification of Davis and Daly (1979) as described previously (Rose et al., 1997), using final concentrations of 1 μM cAMP or 1 μM cGMP. Contributions of PDE2, PDE3, PDE4, or PDE5 to total VEC cAMP or cGMP PDE activities were determined pharmacologically using maximally effective selective concentrations of inhibitors, as determined previously (Rose et al., 1997). The concentration of the PDE2 inhibitor BAY 31-9472 (100 nM) used in this study was chosen to be 20-fold greater than the IC_{50} value (3.9 nM) against PDE2 but significantly below the IC_{50} value for inhibition of other PDEs ($IC_{50} \geq 39$ μM against PDE3B, PDE4B, and PDE5A; E. Bischoff, personal communication). PDE1 activities were determined by supplementing the assay buffer with calcium (100 μM) and calmodulin (10 U/ml). Total PDE activity [3-isobutyl-1-methylxanthine (IBMX)-sensitive fraction] in VEC lysates for cAMP or cGMP was determined using the broad-spectrum, nonselective PDE inhibitor IBMX (500 μM).

Determination of Cellular Cyclic Nucleotide Levels. For these studies, confluent monolayers of VECs were incubated overnight with either [^{3}H]hypoxanthine (20 μCi/ml serum, 2 μM; bAECs) or [^{3}H]adenine (10 μCi/ml serum, 2 μM; HAECs, HUVECs, and HMVECs) to label intracellular cyclic nucleotide metabolic pools as described previously (Maurice et al., 1993). After removal of labeling media, cells were rinsed with phosphate-buffered saline, trypsinized, and resuspended in a defined volume of DMEM (8×10^5 cells/ml). Aliquots (500 μl) of cell suspension were transferred to 24-well plates coated with 0.25% gelatin and placed in an incubator (37°C, 5% CO_2) for 2 h. VECs were treated with PDE inhibitors for 1 min after which forskolin was added for an additional 5 min. Reactions were terminated by the addition of 500 μl of ice-cold 10% TCA [final 5% (v/v)]. cAMP was isolated and purified via column chromatography using neutral alumina and levels of [^{3}H]cAMP and [^{14}C]cAMP (used as internal control) were determined using liquid scintillation as described previously (Palmer et al., 1998). The amount of cAMP in each well (measured as [^{3}H]cAMP) was expressed as a percentage of the total ^{3}H in each well. [^{3}H]Hypoxanthine allows for the homogeneous labeling of both the ATP and GTP metabolic pools (Palmer et al., 1998), which could only be used with the bAECs, as the human endothelial cells did not take up the [^{3}H]hypoxanthine, and hence [^{3}H]adenine had to be used to measure cAMP levels but unfortunately not cGMP levels in the HAECs, HUVECs, and HMVECs using our methodology. [^{3}H]cGMP in bAECs was purified via sequential

column chromatography using neutral alumina and Dowex 50 resin columns, as described previously (Palmer et al., 1998).

Immunoblotting of Endothelial Cell Lysates. Confluent monolayers of VECs were lysed directly in 2× SDS-PAGE buffer. Samples resolved by SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA), and incubated with a blocking solution containing 20 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween 20, supplemented with 5% powdered nonfat milk for 1 h. Individual membranes were probed for PDE2A, PDE3A, PDE3B, PDE4B, PDE4D, and PDE5, with immunoreactive species visualized by enhanced chemiluminescence using a horseradish peroxidase-conjugated secondary antisera (Liu and Maurice, 1998). In these studies, several antisera were used. A PDE2A N-terminal antibody was purchased from FabGennix, and aliquots of the following antibodies were a gift from FabGennix Corporation (Shreveport, LA): PDE3A (1:500 dilution) and PDE3B (1:500 dilution). Two commercially available PDE3B antibodies (both 1:100 dilutions; both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA), a PDE3A/3B antibody (1:1000 dilution, generously provided by Dr. J. Beavo, University of Washington, Seattle, WA), a PDE3B antibody (1:4000 dilution; generously provided by the ICOS, Bothell, WA) along with the FabGennix PDE3B antibody were used to probe and identify PDE3B. PDE3A (1:1000 dilution), PDE4B (1:4000 dilution), and PDE4D (1:4000 dilution) were all graciously provided by the ICOS. The PDE5 specific antibody was a gift from Dr. S. S. Visweswariah (Indian Institute of Science, Bangalore, India).

Radial Invasion of Matrix by Aggregated Cells. Migration of bAEC in a three-dimensional type I collagen matrix was performed using the RIMAC assay, as described previously (Vernon and Sage, 1999; Netherton et al., 2002). In brief, cell aggregates were formed by the hanging drop method over 3 days. Aggregates were encased in 0.6 mg/ml type I collagen (rat tail), supported with a Nytex mesh disc. After collagen polymerization, discs were transferred to 96-well plates, containing 100-μl volume of DMEM supplemented with the test agent(s). Cells were allowed to migrate for 2 days, with aggregates being exposed to VEGF or bFGF and/or PDE inhibitors during the entire 2-day migration period, after which they were fixed and stained (formalin-crystal violet). Discs of fixed/stained cells were mounted on glass slides with poly(vinyl) alcohol, and digital images were taken. Scion Image software (Scion Corporation, Frederick, MD) was used to analyze the distance traveled by the VECs, by averaging the maximum distance of migration along 64 radii that was digitally superimposed over each image, as described previously (Vernon and Sage, 1999; Netherton et al., 2002).

Transwell-Based Migration Assays. Transwell cell culture chamber inserts (polycarbonate, tissue culture-treated, 6.5-mm diameter, and 8.0-μm pore size) were purchased from Corning (Acton, MA). Transwells were coated with 0.25% gelatin for 60 min before addition of cells to the upper chamber of the wells. Based on preliminary studies, 30,000 bAECs, 50,000 HAECs, 75,000 HUVECs, or 35,000 HMVECs were used. After a 2-h preincubation period in DMEM, VEC-containing Transwells were transferred to wells containing VEGF (10 nM) or VEGF (10 nM) and specific PDE inhibitors. VECs were allowed to migrate for 4 h after which the cells were fixed and stained (formalin-crystal violet). Cells remaining on the top of the filter were removed and VECs that had migrated to the lower part of the insert were counted from five random fields of view for each insert, and averaged, to determine the number of cells that had migrated for each condition.

Reverse Transcription-Polymerase Chain Reaction Amplification of PDE3B mRNA in bAECs. RNA was isolated and purified from confluent monolayers of bAECs by TRIzol (Invitrogen). bAEC mRNA was reverse transcribed and amplified by PCR with primers and reactions described previously (Tilley and Maurice, 2002).

General Reagents. Radioactive chemicals [^{3}H]cAMP, [^{3}H]cGMP, [^{14}C]cAMP, 5′-[^{14}C]AMP, 5′-[^{14}C]GMP, [^{3}H]hypoxanthine, and [^{3}H]adenine were from PerkinElmer Life and Analytical Sciences (Boston, MA). BAY 31-9472 was obtained from Bayer AG (Wuppertal, Germany), and sildenafil was purified as described previously (Francis et al., 2003). Calmodulin, Ro 20-1724 (Ro), cilostamide, forskolin, and *erythro*-9-(2-hydroxy-3-nonyl)-adenine (EHNA) were purchased from Calbiochem-Novachem (Mississauga, ON, Canada), sodium fluoride, and IBMX were from Sigma Diagnostics Canada (Mississauga, ON, Canada). Tris-HCl, benzamidine, EDTA, EGTA, dithiothreitol, phenylmethylsulfonyl fluoride, and Triton X-100 were obtained from ICN Biomedicals (Montreal, QC, Canada), whereas leupeptin was from Roche Diagnostics (Laval, QC, Canada). Reverse transcription-PCR kit (containing reverse transcriptase), agarose gel DNA purification kit, and plasmid mini-prep kit were all purchased from QIAGEN (Mississauga, ON, Canada). DNA *Taq* polymerase, 6× loading dye and GeneRuler DNA ladder are from MBI-Fermantas (Burlington, ON, Canada). EcoRI, its reaction buffer, PCR buffer, $MgCl_2$, and dNTPs were purchased from Invitrogen. pBlueScript II+KS was purchased from Stratagene (La Jolla, CA). Potassium chloride (KCl), calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$), magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$), and potassium dihydrogen orthophosphate (KH_2PO_4) were purchased from BDH (Ontario, Canada). Affi-gel 601 and the column supports were from Bio-Rad (Ontario, Canada). The bicinchoninic acid protein assay and bovine serum albumin were from Pierce Chemical (Ontario, Canada). Nytex rings (outer diameter 5.2 mm and inner diameter 2.8 mm) were manufactured by Tetko (Briarcliff Manor, NY), and type I rat tail collagen (BD Biosciences, Bedford, MA) was used in the RIMAC assay. All other chemicals were of reagent grade and purchased from Fisher Scientific (Ontario, Canada).

Statistical Analysis. Statistical analysis was performed using a paired Student's *t* test, or one-way analysis of variance test, when applicable, with 95% a confidence interval. Data are presented as either means ± S.E.M. (figures and tables) or as percentages (in text) from at least three independent experiments

Results

Characterization of bAEC, HAEC, HUVEC, and HMVEC cAMP-Hydrolyzing PDE Activities. Characterization of cAMP PDE activities expressed by the four VECs chosen for our studies was accomplished with a strategy of selective pharmacological inhibition using PDE family-selective inhibitors. Although our data identify PDE2, PDE3, and PDE4 family activities as catalyzing cAMP hydrolysis in most, the relative abundance of each in individual VEC types differed markedly (Table 1). Thus, the PDE3 family-selective inhibitor cilostamide inhibited cAMP PDE activity in bAECs and HAECs by 15 and 36%, respectively, but only minimally

TABLE 1
cAMP PDE activity in VECs

cAMP PDE activity inhibited by addition of specific PDE inhibitors or stimulated after addition of Ca^{2+}/calmodulin is shown. PDE1 activator 100 μM Ca^{2+}/10 U calmodulin, PDE2: first generation PDE2 inhibitor EHNA (10 μM), second generation PDE2 inhibitor BAY 31-9472 (100 nM), PDE3 inhibitor 1 μM cilostamide, PDE4 inhibitor 10 μM Ro 20-1724, and nonselective PDE inhibitor IBMX (500 μ M). No addition (total activity) refers to the cAMP PDE activity present in the absence of any PDE inhibitors. Data are means ± S.E.M. calculated from all experiments with each VEC type (bAEC, n = 8–16; HUVEC, n = 4–8; HMVEC, n = 4–8) where all cells were between passages 3 and 10.

Addition	bAEC	HAEC	HUVEC	HMVEC
	pmol/min/mg of protein			
None (total activity)	470 ± 35	192 ± 30	121 ± 12	152 ± 15
Ca^{2+}/CaM	3.9 ± 12	28 ± 14	8.5 ± 7	10 ± 14
Cilostamide	71 ± 12	69 ± 15	9 ± 10	10 ± 12
Ro 20-1724	212 ± 24	87 ± 19	65 ± 10	120 ± 21
BAY 31-9472	45 ± 23	5.8 ± 7	13 ± 8	26 ± 9
EHNA	50 ± 42	8 ± 2	16 ± 8	24 ± 9
IBMX	445 ± 36	223 ± 45	121 ± 17	145 ± 15

CaM, Ca^{2+}/calmodulin.

impacted cAMP PDE activities in HUVECs (7%) and HMVECs (6%). PDE4 activity was the dominant cAMP PDE activity in all four VECs studied (Table 1). Indeed, the PDE4-selective inhibitor Ro 20-1724 reduced cAMP PDE activities in bAECs, HAECs, HUVECs, and HMVECs by 45, 45, 54, and 79%, respectively (Table 1). Using either the first generation PDE2-selective inhibitor EHNA or the more selective second generation PDE2 inhibitor BAY 31-9472, PDE2 activity accounted for <17% of cAMP PDE activity in each of the four VECs studied (Table 1). Combinations of these agents (BAY 31-9472, cilostamide, and Ro 20-1724) caused an additive inhibition of cAMP PDE activity in the VECs studied, and reduced cAMP PDE activity in each to levels comparable with those seen using the broad-selectivity PDE inhibitor IBMX (Table 1). None of the VECs studied expressed any detectable PDE1 activity using either calcium/calmodulin-based activation of cAMP hydrolysis (Table 1) or inhibition with vinpocetine (data not shown). Because the impact of combined PDE2, PDE3, and PDE4 inhibition was comparable with that achieved with IBMX, it is likely that if other PDEs are expressed in these cells that they probably account for a very small percentage of total cAMP PDE activity. Although some reports have indicated that PDE expression can be altered as a consequence of cell passage, and we have reported such a phenomenon for PDE3A expression during the process of phenotypic modulation that accompanies culturing of rat or human aortic vascular myocytes (Dunkerley et al., 2002), no passage-dependent changes in any cAMP PDE activities were noted in this study.

Characterization of bAEC, HUVEC, and HMVEC cGMP-Hydrolyzing PDE Activities. Using an approach identical to that described above, the PDE enzymes catalyzing cGMP hydrolysis in these four VECs were also determined. Although cGMP hydrolytic activity in each of the VEC cultures was markedly lower than that seen for cAMP (Table 2), our data clearly identify PDE2 and PDE5 activities in each with PDE5 activity consistently representing the dominant activity in each (Table 2). As was the case when cAMP PDE activity was measured, addition of calcium/calmodulin (Table 2) or vinpocetine (not shown) had no effect, and the combined impact of PDE2 and PDE5 inhibition was comparable with that achieved using the broad-spectrum inhibitor IBMX (Table 2). Again, no passage-dependent changes in cGMP PDE activities were noted in this study.

TABLE 2

cGMP PDE activity in VECs

cGMP PDE activity inhibited by addition of specific PDE inhibitors or stimulated after addition of Ca^{2+}/calmodulin is shown. PDE1 activator 100 μM Ca^{2+}/10 U calmodulin, PDE2: first generation PDE2 inhibitor EHNA (10 μM), second generation PDE2 inhibitor BAY 31-9472 (100 nM), PDE5 inhibitor sildenafil (100 nM), and nonselective PDE inhibitor IBMX (500 μM). No addition (total activity) refers to the cGMP PDE activity present in the absence of any PDE inhibitors. Data are means ± S.E.M. calculated from all experiments with each VEC type (bAEC, n = 5–8; HUVEC, n = 4–8; HMVEC n = 4–6), where all cells were between passages 3 and 10.

Addition	bAEC	HAEC	HUVEC	HMVEC
	pmol/min/mg of protein			
None (total activity)	137 ± 25	9 ± 1.5	25 ± 4	11 ± 4
Ca^{2+}/CaM	1 ± 5	2.5 ± 1	6.7 ± 2	1 ± 0.7
BAY 31-9742	18 ± 12	1.5 ± 1	1.6 ± 3.4	5.5 ± 4.3
EHNA	33 ± 9	1.5 ± 1.2	9 ± 4	5.1 ± 2.8
Sildenafil	76 ± 21	5.5 ± 1	11.1 ± 3.8	7.3 ± 1.3
IBMX	134 ± 25	8.5 ± 1.2	23.5 ± 5	14 ± 6

CaM, Ca^{2+}/calmodulin.

Immunoblot Analysis of PDE Expression in bAECs, HAECs, HUVECs, and HMVECs. Based on our inhibitor studies, we concluded that bAECs, HAECs, HUVECs, and HMVECs all expressed variants of each PDE2, PDE3, PDE4, and PDE5 families of enzymes, albeit in different proportions. Because most PDE families encode several variants, and individual variants can have unique regulatory characteristics, we next used an immunoblot-based approach to identify the PDE2, PDE3, PDE4, and PDE5 family variants expressed in these cells. Consistent with our analysis indicating that PDE2 was expressed by all four VECs, immunoblot analysis identified a 102-kDa anti-PDE2A immunoreactive protein in lysates of each (Fig. 1A). It is interesting that marked differences were observed when we characterized the PDE3 variants expressed in the different VECs. Thus, although immunoblot analysis of the three human VECs identified multiple PDE3A variants in each cell type (Fig. 1, B and C), this analysis of bAECs identified expression of these PDE3A variants (Fig. 1B) and of a 135-kDa PDE3B (Fig. 1C). PDE3B expression was not observed in any human VECs (Fig. 1C). Consistent with this immunoblot-based analysis, a reverse transcription-PCR-based amplification and sequencing approach confirmed expression of PDE3B in bAECs and its absence from each human VECs (not shown). It is regrettable that the functional significance of expression of PDE3B in bAECs will require development of PDE3 subfamily selective inhibitors as such agents are not yet available and those that are available inhibit both PDE3A and PDE3B enzymes. Consistent with PDE4 activity representing the dominant cAMP PDE activity in each VEC studied, immunoblot analysis identified several PDE4 variants in each cell type. Indeed, 63-kDa PDE4B2 (Fig. 1D) and 95-kDa PDE4D3 and

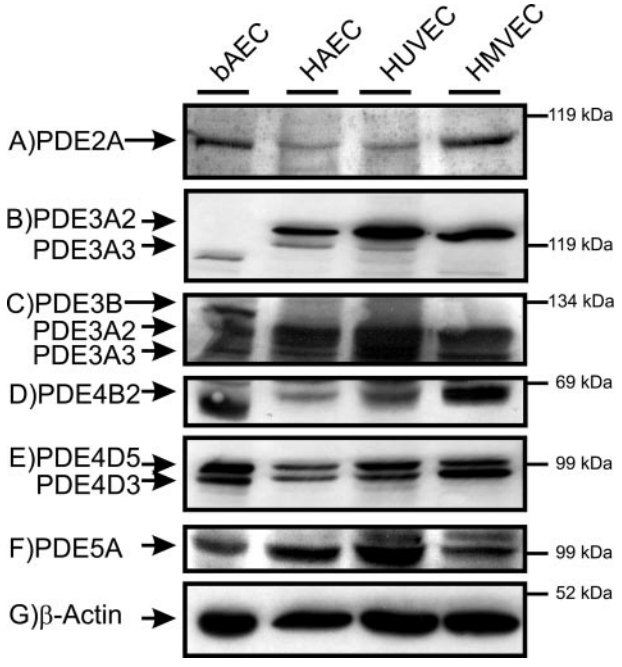

Fig. 1. Immunoblot analysis of VECs for specific PDE enzymes. Confluent monolayers of VECs were lysed directly in 2× Laemlli, separated by SDS-PAGE, and analyzed by immunoblot with PDE2A- (A), PDE3A- (B), PDE3A/B- (C), PDE4B- (D), PDE4D- (E), PDE5- (F), and β-actin (G)-specific antibodies. Note: lack of a PDE3A-immunoreactive proteins in bAECs in B, but presence in C is caused by a human-specific PDE3A antibody used in B.

105-kDa PDE4D5 (Fig. 1E) proteins were detected when lysates of each VEC were studied when probed with anti-PDE4B- or anti-PDE4D-selective antisera, respectively. Consistent with PDE5 activity being detected in each type of VEC studied, immunoblot analysis identified a 100-kDa PDE5A1 variant was detected in all four VEC studies (Fig. 1F). Immunoblot analysis with antisera directed at PDE1A, PDE1B, PDE1C, PDE4A, and PDE7 showed that none of these enzymes were significantly expressed in any of the four VECs studied. Together, these data are internally consistent with the activity measurements obtained using PDE family-selective inhibitors and demonstrate that each of the VECs chosen for study express multiple PDE family variants, albeit in different proportions.

PDE Inhibitor-Mediated Increases in VEC cAMP. Having characterized the VECs PDE expression profiles, we next ascertained the impact of inhibiting these enzymes on endothelial cell cAMP levels in the absence and in the presence of an activator of adenylyl cyclase, namely, forskolin. When used alone, forskolin (0.1–10 μM) caused a modest concentration-dependent increase in cAMP in each VEC tested, which only achieved significance at a concentration of 10 μM in HAECs and HUVECs (Fig. 2). Whereas incubation of VECs alone with selective inhibitors of either PDE2, PDE3, or PDE4 did not significantly increase their cAMP contents (Figs. 2 and 3), when used in combination with forskolin (10 μM), each the PDE3 inhibitor, cilostamide, and the PDE4 inhibitor Ro, 20-1724, significantly increased cAMP to levels significantly higher than those seen with forskolin alone (Fig. 2). Because a direct cGMP-mediated competitive inhibition of PDE3 could have complicated interpretation of the effects of the PDE2 inhibitors (Maurice and Haslam, 1990), each PDE2 inhibitor (BAY 31-9742 and EHNA) was tested in the presence or absence of the PDE3 inhibitor cilostamide. In neither situation did either BAY 31-9472 (Fig. 3) or EHNA (not shown) significantly potentiated forskolin-induced increases in cAMP in any VECs. Overall, these data indicate that certain cAMP PDEs (PDE3 and PDE4) can act in concert with activated adenylyl cyclases to regulate VEC cAMP levels in VECs, whereas others (PDE2), although present, do not. Using our methods, cGMP levels in bAECs were not altered by PDE inhibition either in the absence or presence of atrial natriuretic peptide (1–1000 nM; not shown) and were unable to be measured in the human endothelial cells examined (see *Materials and Methods*).

Effects of PDE Inhibition on Migration of VECs. The impact of PDE inhibition on migration of VECs in response to gradients of VEGF, or bFGF, was determined using either a two-dimensional (2-D) model of cell migration (Transwells; bAECs, HAECs, HUVECs, and HMVECs) or one in which cells were encased with collagen and allowed to migrate within a three-dimensional (3-D) context (RIMACs; bAECs). Our analysis of the impact of PDE inhibition on VEC migration clearly demonstrated that inhibition of cAMP hydrolysis markedly antagonized VEGF- or bFGF-induced migration of VECs. In addition, we identified potentially important differences in the relative impact of inhibitors of PDE2, PDE3, or PDE4 in these cells. Although VEGF- or bFGF-induced migration in all four VECs studied was each markedly reduced when PDE4 activity was inhibited (Figs. 4–8), PDE2 or PDE3 inhibitors were more selective in their effects. Thus, BAY 31-9472 inhibited bAEC, HAEC, HUVEC, or HMVEC VEGF-stimulated migration by 45, 52, 31, or 29%, respectively (Figs. 5–8), and PDE3 inhibition reduced bAEC, HAEC, and HUVEC migration in response to VEGF by 36, 52, and 31%, but it was completely without effect when used in HMVECs (Fig. 8). Because cAMP regulates a myriad of effects in most cells, including VECs, inhibition of two or more PDE activities could in principle have had subadditive, additive, or synergistic inhibitory effects on VEC migration. To investigate this, we tested the effects of combinations of these inhibitors. Again, results of these studies point to marked differences between responses to these treatments when different VECs were studied. When cilostamide and Ro 20-1724 were combined, the resulting inhibition of VEGF-induced migration was additive in HAECs, nonadditive in bAECs, and subadditive in either HUVECs (Fig. 7) or HMVECs (Fig. 8). Likewise, when combined, BAY 31-9742 and Ro 20-1724 inhibited 10 nM VEGF induced migration additively in bAECs (Fig. 5), but not in HAECs (Fig. 6). The broad-spectrum PDE inhibitor IBMX completely inhibited HAEC migration to a gradient of VEGF (Fig. 6). In addition, when results obtained in our comparative studies of bAEC migration in our 2-D and 3-D migration assay were compared, PDE inhibitors were shown to have context-dependent effects (Figs. 4 and 5). Indeed, whereas PDE4 inhibition had a dom-

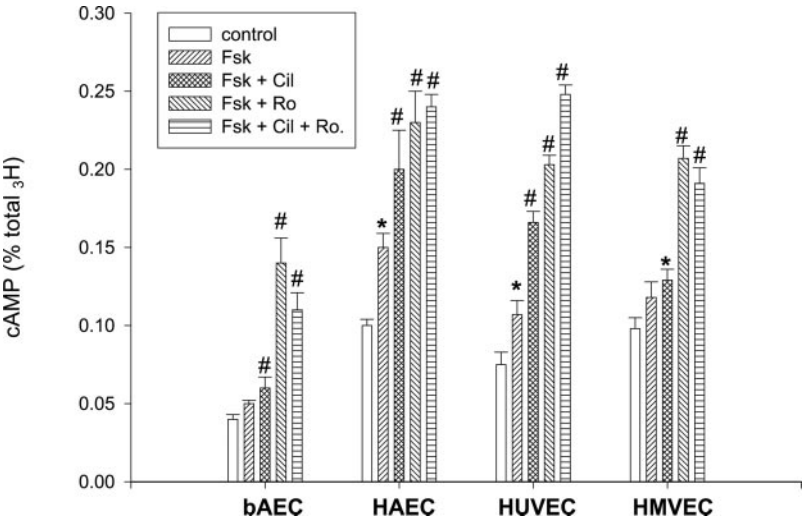

Fig. 2. Effect of PDE3 and PDE4 inhibition on VEC cAMP levels. VECs were incubated with a PDE3 inhibitor [1 μM cilostamide (Cil)] or a PDE4 inhibitor (10 μM Ro), alone or in combination, for 1 min followed by a subsequent 5-min incubation with 10 μM forskolin (Fsk). Reactions were terminated with the addition of 10% TCA [5% (w/v) final], and cAMP, measured as a percentage of total ^{3}H, was purified by chromatography as described under *Materials and Methods*. cAMP levels with inhibitor treatment alone were bAEC, Cil (0.03%), Ro (0.03%); HAEC, Cil (0.12%), Ro (0.1%); HUVEC, Cil (0.07%), Ro (0.07%); and HMVEC, Cil (0.1%), Ro (0.11%). Asterisk (*) indicates statistical difference ($P < 0.05$) against control cAMP levels; # indicates statistical difference ($P < 0.05$) against 10 μM Fsk.

inant effect in the 2-D migration assay, only PDE3 inhibition attenuated migration of these cells in this the 3-D context with PDE4 inhibition have negligible effects. Because prolonged incubation of VECs in 2-D and 3-D contexts could in principle have had disparate effects because of altered PDE expression, we measured cAMP PDE activities in HUVEC grown in 2-D and 3-D contexts. These different culturing environments had no impact whatsoever on HUVEC cAMP PDE activities (not shown).

Discussion

The hypothesis that inhibition of angiogenesis might be achieved by reducing VEC proliferation or migration is consistent with recent reports (Ilan et al., 1998; Griffioen and Molema, 2000; Favot et al., 2003; Folkman, 2003). In this context, studies using bovine (bAEC) or human (HUVEC) cells have indicated that increasing VEC cAMP levels may represent a novel therapeutic approach to achieving this goal (Favot et al., 2003; Fleming et al., 2004). Although these earlier studies showed that this approach could work in model systems, they did not assess its potential in microvascular beds, vascular structures that are more likely to be involved in supplying VECs for angiogenesis in vivo in humans. Here, we have studied these models (bAECs and HUVECs) performed cross-species comparisons within similar vascular structures (bAECs versus HAECs) and extended our analysis to include HMVECs.

Although elevation of VEC cAMP by specifically activating one of the nine families of adenylyl cyclases could form the basis of this approach, no drugs presently exists that differentiate between these enzymes. In contrast, numerous drugs exist that selectively inhibit enzymes from each of the PDE families expressed in VECs. Thus, in this study we examined the PDE expression profile of bAECs, HAECs, HUVECs, and HMVECs, determined the impact of their inhibition on cAMP handling in these cells, and determined the effect of these treatments on inhibition of angiogenic factor-induced VEC migration.

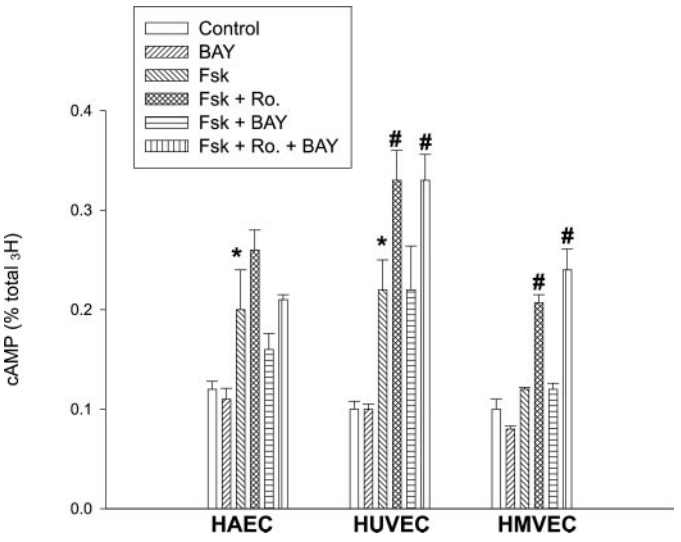

Fig. 3. Effect of PDE2 and PDE4 inhibition on VEC cAMP levels. In the constant presence of cilostamide (1 μM), VECs were further incubated with either a PDE2 inhibitor [100 nM BAY 31-9742 (BAY)] or a PDE4 inhibitor (10 μM Ro), alone or in combination for 1 min, followed by a subsequent 5-min incubation with 10 μM forskolin (Fsk). Reactions were terminated with the addition of 10% TCA [5% (w/v) final], and cAMP, measured as a percentage of total ^{3}H, was purified by chromatography as described under *Materials and Methods*. Note: BAY alone, in the absence of cilostamide, resulted in a 0.11% cAMP level. Asterisk (*) indicates statistical difference ($P < 0.05$) against control cAMP levels; # indicates statistical difference ($P < 0.05$) against 10 μM Fsk.

Our data confirm the expression of PDE2, PDE3, PDE4, and PDE5 in bAECs and HUVECs (Lugnier and Schini, 1990; Ashikaga et al., 1997; Lugnier et al., 1999; Keravis et al., 2000, Miro et al., 2000; Favot et al., 2003), unequivocally established that these enzymes are also expressed in HAECs and HMVECs, and demonstrate that PDEs were expressed to differing levels in these cells. Our data highlight potentially important differences between bovine and human aortic VECs as well as between the three human VECs studied. In particular, bAECs were shown to express significant levels of PDE3B, an enzyme not expressed by any of the human VECs studied. Expression of PDE3B in bAECs is noteworthy because we have shown previously that PDE3B is an exclusively particulate enzyme (Liu and Maurice, 1998), a fact that perhaps accounts for the observation of Keravis et al. (2000) that >60% of bAEC PDE3 activity was particulate. In addition, our data demonstrate clear vascular bed-based differences in the expression profile of PDEs in VECs. Moreover, our analysis of VEC migration inhibition with PDE inhibitors is consistent with our main hypothesis that selective effects on migration of specific VECs may be feasible using a strategy aimed at targeting individual PDEs expressed within these cells.

Although it is generally accepted that the VECs most likely to be involved in initiating angiogenesis are those present in microvascular structures (Griffioen and Molema, 2000; Auerbach et al., 2003), there is currently a paucity of information regarding the role of cAMP in regulating the functions of these cells. In addition, fewer studies have assessed the relative contributions of PDEs to cAMP-regulated cellular functions in microvascular endothelial cells than in VECs derived from larger blood vessels, especially in humans. In one report (Thompson et al., 2002), rat pulmonary microvascular endothelial cells were found to be devoid of cGMP PDE activity and to express cAMP-hydrolyzing PDE4 variants PDE4A and PDE4B. In contrast, our study demonstrates that HMVECs express significant PDE2 and PDE5 cGMP PDE activity and that PDE2, in addition to PDE4, each catalyzes cAMP hydrolysis. One commonality between our findings with HMVECs and those of Thompson et al. (2002) is the dominance of PDE4 activity in these cells, although the individual PDE4 variants that account for this activity in rat and human were clearly different. Thus, although HMVECs expressed PDE4B2, an enzyme identified in rat pulmonary artery endothelial cells, HMVECs were shown to express significant PDE4D3 and PDE4D5, enzymes not found in rat cells. A comparison of PDE2 expression between the individual human VECs studied allows a hypothesis first proposed by Sadhu et al. (1999) to be tested. Thus, in their earlier work, Sadhu proposed that PDE2 would be more abundant in microvascular endothelial cells than in those derived from larger blood vessels. A comparison of levels of PDE2 expression by the three human VECs used in our study confirms Sadhu's prediction. Recent work by several groups has demonstrated that the impact of PDE inhibition on cell function is reflective of the subcellular compartment in which the enzymes are localized, rather than its contribution to total

cAMP hydrolytic activity (Yarwood et al., 1999; Dodge et al., 2001; Brunton, 2003). Our analysis of PDE inhibitor-mediated control of VEC migration is internally consistent with a role for PDE compartmentation in VECs and further established a potential role for differential PDE effects in 2-D and 3-D models of cell migration. Thus, when the impact of PDE inhibition on bAEC migration was measured using conventional 2-D migration assays, the magnitude of the effects of these agents was, for the most part, proportional to their contribution to cAMP PDE activity in broken cell lysates. In striking contrast, however, when the impact of PDE inhibitors on VEGF-induced migration was studied in a 3-D migration assay, the PDE3 and PDE4 inhibitors had unexpected effects. Indeed, whereas PDE3 inhibition virtually abolished VEGF-induced migration of bAECs in the 3-D system, PDE4 inhibition had no effect. It is interesting that no such differences were noted when bFGF, rather than VEGF, was used to induce migration. Because our data obviate an effect attributable to differences in PDE expression in the 2-D versus 3-D assays, we propose that our data are best explained by a mechanisms related to altered coupling between distinct cAMP pools and selected PDEs in these two situations. Although substantial work remains to more firmly establish this mechanism, it is perhaps relevant that bFGF-induced migration was recently shown to be dependent on MAPK-mediated signaling, whereas VEGF-induced motility was more dependent on activation of downstream of PKCs (Cao et al., 2001) and probably involved increases in cGMP-mediated signaling. Because none of the human VECs formed the cell aggregates required for analysis in the RIMAC assay, a similar comparison with these cells was not possible.

In addition to migration assay (2-D versus 3-D) and growth factor (VEGF versus bFGF)-dependent differences in the inhibition of VEC migration by individual PDE inhibitors, our data also elucidate mechanistically important differences between the effects of inhibiting single versus multiple PDE activities in VECs. For example, when HUVEC or HMVEC migration to VEGF was studied, the impact of simultaneous PDE3 and PDE4 inhibition on migration was less than that seen with PDE4 inhibition alone. Because our analysis of cAMP dynamics after combined selective inhibition of PDE3 and PDE4 activities in HUVECs and HMVECs had shown that these treatments resulted in an approximately additive effect on cAMP levels, we had initially predicted that dual PDE3 and PDE4 inhibition would have similarly affected migration in an additive manner. Although a possible explanation for the apparent antagonistic effect of combined PDE3 and PDE4 inhibition on migration of these cells may stem from the impact of VEGF-induced increases in cGMP (Ferrara, 1999; Lang et al., 1999; Wohlfart et al., 1999; Rivero-

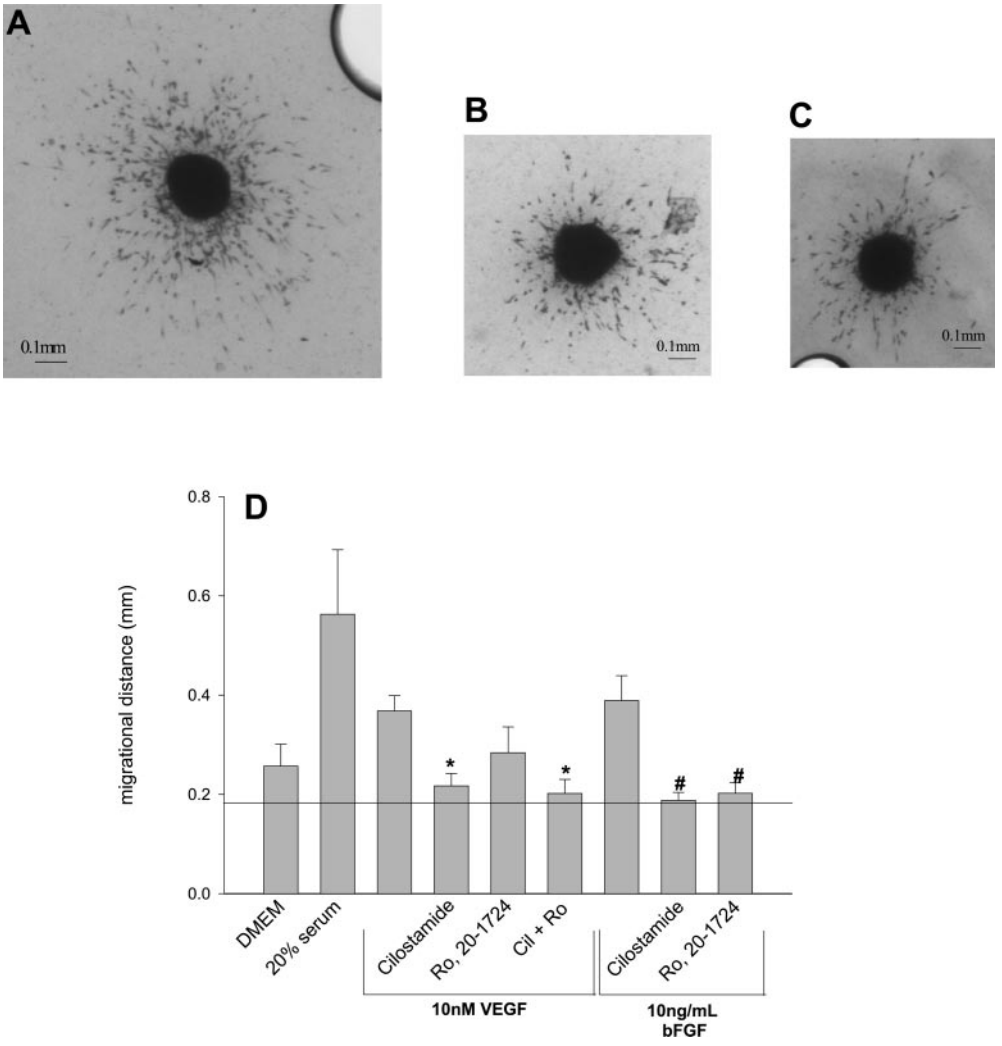

Fig. 4. Migration of bAECs in 3-D type I collagen matrix. bAEC migration in a 3-D type I collagen matrix was assessed using the RIMAC methodology (see *Materials and Methods*). Cell aggregates of 10,000 cells were allowed to migrate for 2 days under experimental conditions. Representative aggregates included 10 nM VEGF-induced migration (A), 10 nM VEGF + 1 μM cilostamide (PDE3 inhibitor) (B), 10 nM VEGF + 10 μM Ro 20-1724 (PDE4 inhibitor) (C), and pooled RIMAC data (D). Serum (20%) = 10% fetal bovine serum + 10% baby calf serum. Horizontal line indicates diameter of the aggregates, above which represented the migrational distance of bAECs from the edge of the aggregates. Asterisk (*) indicates statistical difference ($P < 0.05$) against 10 nM VEGF-induced migration; # indicates statistical difference ($P < 0.05$) against 10 ng/ml bFGF-induced migration.

Vilches et al., 2003; Kelly et al., 2004), and effects of cGMP on PDE2 (cGMP stimulated cAMP/cGMP PDE) and PDE3 (cGMP-inhibited cAMP/cGMP PDE), further efforts will be required to formally test this hypothesis. However, this proposed mechanism is consistent with previous reports that cGMP-elevating agents, although inhibitory on VSMC migration (Jacob et al., 2002; Zhang et al., 2003) can promote migration of certain VECs (Ziche and Morbidelli, 2000; Kawasaki et al., 2003; Kook et al., 2003). In addition, the absence of an antagonistic effect when bAECs were studied is consistent with this model because PDE3B, which is perhaps the dominant PDE3 in these cells, is significantly less sensitive to the inhibitory influences of cGMP (for review, see Maurice et al., 2003).

Of relevance to our studies, recent reports have begun to assess differences in signaling between macrovascular and microvascular endothelial cells (ECs). In this context, Creighton et al. (2003) recently reported that increases in macrovascular EC Ca^{2+} were associated with decreases in cAMP, whereas no impact of Ca^{2+} were noted in microvascular ECs. Likewise, differences in integrin expression

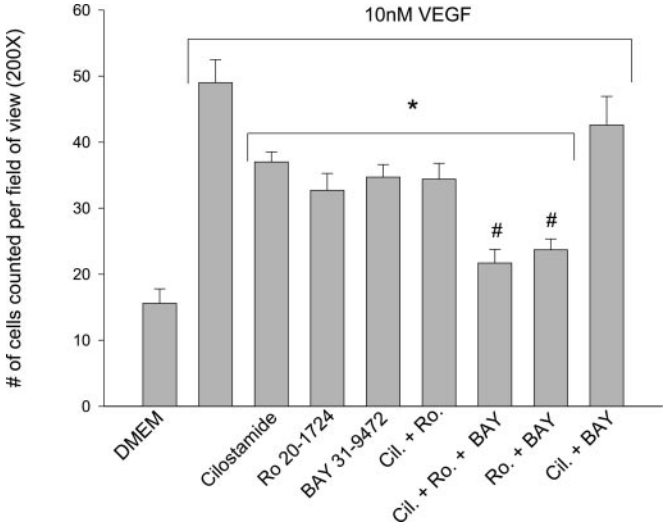

Fig. 5. Effect of PDE inhibitors on bAEC migration in 2-D. bAECs (30,000) were seeded on top of the Transwell membrane and allowed to migrate through 8-μm pores for 4 h. PDE inhibitors included PDE2 [100 nM BAY 31-9742 (BAY)], PDE3 [1 μM cilostamide (Cil)], and PDE4 (10 μM Ro) and were assessed for their ability to inhibit VEGF-induced migration, alone or in various combinations. Asterisk (*) indicates statistical significance ($P < 0.05$) against 10 nM VEGF induced migration; # indicates statistical significance ($P < 0.05$) against 10 μM Ro 20-1724.

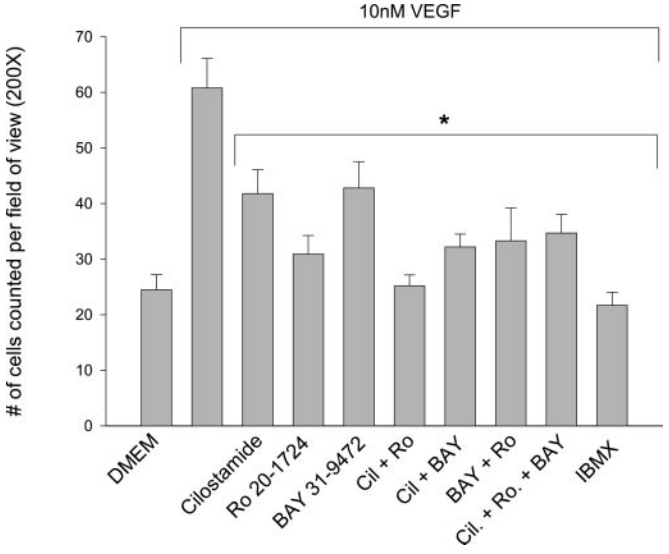

Fig. 6. Effect of PDE inhibitors on HAEC migration in 2-D. HAECs (50,000) were seeded on top of the Transwell membrane and allowed to migrate through 8-μm pores for 4 h. PDE inhibitors included PDE2 [100 nM BAY 31-9742 (BAY)], PDE3 [1 μM cilostamide (Cil)], and PDE4 (10 μM Ro) and were assessed for their ability to inhibit VEGF-induced migration, alone or in various combinations. Asterisk (*) indicates statistical significance ($P < 0.05$) against 10 nM VEGF-induced migration.

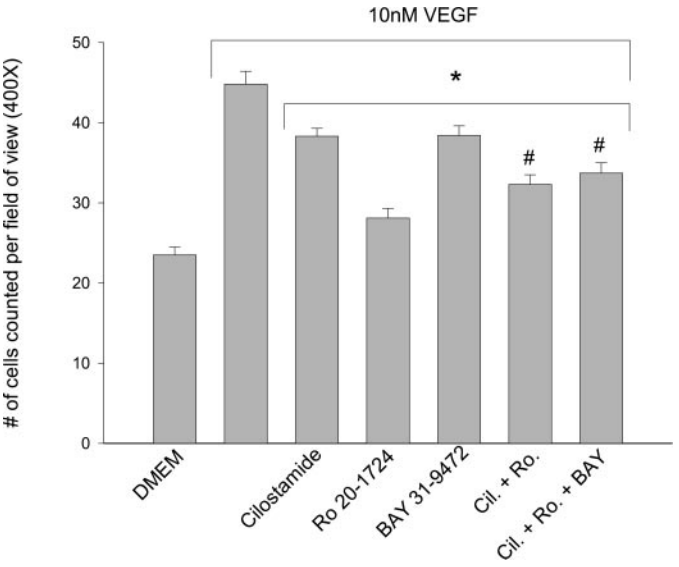

Fig. 7. Effect of PDE inhibitors on HUVEC migration in 2-D. HUVECs (75,000) were seeded on top of the Transwell membrane and allowed to migrate through 8-μm pores for 4 h. PDE inhibitors included PDE2 [100 nM BAY 31-9742 (BAY)], PDE3 [1 μM cilostamide (Cil)], and PDE4 (10 μM Ro) and were assessed for their ability to inhibit VEGF-induced migration, alone or in various combinations. Asterisk (*) indicates statistical significance ($P < 0.05$) against 10 nM VEGF-induced migration; # indicates statistical significance ($P < 0.05$) against 10 μM Ro 20-1724.

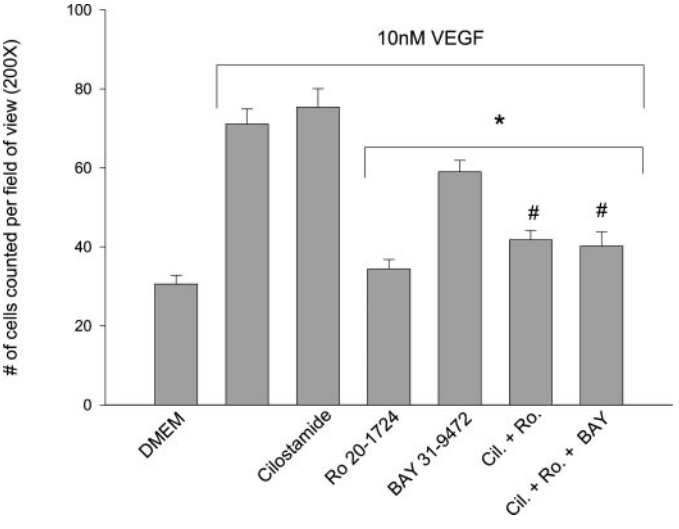

Fig. 8. Effect of PDE inhibitors on HMVEC migration in 2-D. HMVECs (35,000) were seeded on top of the Transwell membrane and allowed to migrate through 8-μm pores for 4 h. PDE inhibitors included PDE2 [100 nM BAY 31-9742 (BAY)], PDE3 [1 μM cilostamide (Cil)], and PDE4 (10 μM Ro) and were assessed for their ability to inhibit VEGF-induced migration, alone or in various combinations. Asterisk (*) indicates statistical significance ($P < 0.05$) against 10 nM VEGF-induced migration; # indicates statistical significance ($P < 0.05$) against 10 μM Ro 20-1724.

(Whelan and Senger, 2003), and interleukin-4-mediated stimulation of urokinase-type plasminogen activator between large and small blood vessel-derived VECs have been reported (Wojta et al., 1993). Because increased microvascular permeability is crucial in angiogenesis (Dvorak, 1986), and cAMP mediates several processes involved in this event, different cAMP signaling in VECs may be functionally important. As shown by others, increases in cAMP can inhibit VEGF- and bFGF-induced proliferation (D'Angelo et al., 1997), promote cell-cell, and cell-matrix association, promote focal adhesion complex formation (Stevens et al., 1999) as well as decrease microvascular permeability to proteins. In the context of PDE differences in VECs, and the impact that these differences might play in selective regulation of VEC functions, this work reports findings that may have therapeutic applicability.

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Address correspondence to: Dr. Donald H. Maurice, Department of Pharmacology and Toxicology, Botterell Hall, Queen's University, Kingston, ON K7L 3N6, Canada. E-mail: mauriced@post.queensu.ca