Both Protein Kinase A and Exchange Protein Activated by cAMP Coordinate Adhesion of Human Vascular Endothelial Cells

Stuart J. Netherton,* Jayda A. Sutton,* Lindsay S. Wilson, Rhonda L. Carter, Donald H. Maurice

Abstract—cAMP regulates integrin-dependent adhesions of vascular endothelial cells (VECs) to extracellular matrix proteins, their vascular endothelial cadherin–dependent intercellular adhesions, and their proliferation and migration in response to growth and chemotactic factors. Previously, we reported that cAMP-elevating agents differentially inhibited migration of human VECs isolated from large vascular structures (macro-VECs, human aortic endothelial cells [HAECs]) or small vascular structures (micro-VECs, human microvascular endothelial cells [HMVECs]) and that cAMP hydrolysis by phosphodiesterase (PDE)3 and PDE4 enzymes was important in coordinating this difference. Here we report that 2 cAMP-effector enzymes, namely protein kinase (PK)A and exchange protein activated by cAMP (EPAC), each regulate extracellular matrix protein–based adhesions of both macro- and micro-VECs. Of interest and potential therapeutic importance, we report that although specific pharmacological activation of EPAC markedly stimulated adhesion of micro-VECs to extracellular matrix proteins when PKA was inhibited, this treatment only modestly promoted adhesion of macro-VECs. Consistent with an important role for cAMP PDEs in this difference, PDE3 or PDE4 inhibitors promoted EPAC-dependent adhesions in micro-VECs when PKA was inhibited but not in macro-VECs. At a molecular level, we identify multiple, nonoverlapping, PKA- or EPAC-based signaling protein complexes in both macro- and micro-VECs and demonstrate that each of these complexes contains either PDE3B or PDE4D but not both of these PDEs. Taken together, our data support the concept that adhesion of macro- and micro-VECs is differentially regulated by cAMP and that these differences are coordinated through selective actions of cAMP at multiple nonoverlapping signaling complexes that contain PKA or EPAC and distinct PDE variants. (Circ Res. 2007;101:768-776.)

Key Words: endothelium ■ adhesion ■ PKA ■ EPAC ■ phosphodiesterase

The vascular endothelial cells (VECs), which line luminal surfaces of mature blood vessels, control blood pressure, inflammation, hemostasis, and thrombosis and also serve as a barrier to diffusion of plasma proteins, solutes, and liquids to perivascular spaces.1,2 Each of these events are dependent on integrin-based interactions of VECs with extracellular matrix (ECM) proteins and on the stability of interendothelial cell junctions.1,2 In contrast, during vasculogenesis or angiogenesis, motile and proliferative VECs interact with ECM proteins through dynamic, rather than stable, integrin-based processes and form few interendothelial cell junctions.1,2

cAMP differentially regulates integrin- and interendothelial cell junction–based adhesions in VECs derived from large (macro-VECs) or smaller vascular structures (micro-VECs), but the basis of these differences remains unclear.3 cAMP alters cellular functions by activating protein kinase A (PK)A4,5 and/or by stimulating guanine nucleotide exchanger at Rap1 through EPAC1 and EPAC2 (exchange proteins activated by cAMP).6,7 Although PKA inhibitors do not antagonize fully cAMP stimulation of VEC adhesive events,3 and specific EPAC activators promote VEC adhesions,8,9 the relative importance of PKA or EPAC in these events remains unknown. PKA interacts with intracellular tethers, including AKAPs (A-Kinase Anchoring Proteins), and these interactions allow subcellular targeting of PKA.11,12 Although EPAC was identified in an AKAP-based complex in rodent cardiomyocytes, the generality of this finding is unknown. In addition, recent evidence identifies an important role for cyclic nucleotide phosphodiesterases (PDEs) in coordinating cAMP effects in cells, and reports indicate that tethering of PDEs to AKAP-based complexes allow localized cAMP activation of either PKA or EPAC in cells.10,12,13 Compelling evidence of selective regulation of individual signaling complexes by specific PDEs was offered recently when catalyti-
cally inactive variants of individual PDEs were used as tethering “dominant negatives” in cells.14,15 Numerous enzymes of the PDE2, PDE3, and PDE4 family of PDEs catalyze cAMP hydrolysis in human VECs,12 but their relative roles in coordinating PKA or EPAC signaling in these cells is unknown.

Here we report that activation of either PKA or EPAC in human VECs stimulates their adhesion to ECM-coated surfaces and that EPAC plays a more significant role in micro- than macro-VECs and could represent a novel therapeutic avenue to regulate distinct populations of macro- and micro-VECs and could represent a novel therapeutic avenue to regulate distinct populations of VECs.

Fundus and Methods

Materials

N'-Benzoyladenosine-3',5'-cyclic monophosphate (6-BzCAMP), and 8-[(4-chlorophenylthio)-2'-O-methyl-cAMP (8CPT-2'-OMe-cAMP) were from BioLog (Bremen, Germany). ECM proteins and the polycarbonate (6.5 mm diameter Canada), and tissue culture reagents were from Life Technologies stearated PKI (St-PKI) were from Promega (Madison, Wis). Ro 20-1724 (Ro 20-1724) is a novel therapeutic avenue to regulate distinct populations of VECs. We propose that our data provide a mechanism whereby cAMP may act differently in macro- and micro-VECs and could represent a novel therapeutic avenue to regulate distinct populations of VECs.

Materials and Methods

Materials

N'-Benzoyladenosine-3', 5'-cyclic monophosphate (6-BzCAMP), and 8-[(4-chlorophenylthio)-2'-O-methyl-cAMP (8CPT-2'-OMe-cAMP) were from BioLog (Bremen, Germany). ECM proteins and the polycarbonate inserts (polycarbonate inserts Canada), and tissue culture reagents were from Life Technologies stearated PKI (St-PKI) were from Promega (Madison, Wis). Ro 20-1724, cilostamide, H89, indomethacin, and forskolin were from Calbiochem-Novacachem Corp or Sigma-Aldrich (Mississauga, ON, Canada), and tissue culture reagents were from Life Technologies Inc. Cell migration chamber inserts (polycarbonate inserts 6.5 mm diameter, 3.0 μm pore size) were from Corning (Acton, Mass), and PKA assay kits were from Upstate (Lake Placid, NY). Other chemicals were from Fisher Scientific (Ontario, ON, Canada).

Cellular cAMP and cAMP Effector Levels, PKA or Rap1 Activation, and VEC Migration and Adhesion

cAMP levels were determined at 15 minutes, as described previously.16 Membrane and cytosol fractions were prepared as described previously,17 and their PKA activities were measured using Kemp tide (50 μM) in a Tris buffer (50 mmol/L, pH 7.5) supplemented with 5 mmol/L EDTA, protease inhibitors, and cAMP (20 μmol/L). GTP-Rap1 was isolated with GSH–Sephadex–immobilized glutathione S-transferase–RalGDS and detected using an anti-Rap1 antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, Calif). Artin and nuclei immunostains were shown, similar data were obtained in multiple experiments, indicated by n. Effects of test agents on VEC migration or adhesion was measured in quadruplicate determinations. Adhesion assays were used as described.16 Forskolin (1 to 100 μmol/L) or 6-BzCAMP maximally inhibited cell migration by 40±6% or 38±5%, respectively (n=6). Although 8CPT-2'-OMe-cAMP was without effect on VEC migration, PKA inhibition with H89 (10 μmol/L) blunted 6-BzCAMP inhibition in HAECs and HMVECs by 88±12% and 78±10%, respectively (n=6). Activation might impact VEC adhesions.

Both PKA and EPAC Coordinate Human VEC Adhesion to ECM Proteins

Forskolin (1 to 100 μmol/L) did not stimulate adhesion of HAECs or HMVECs to fibronectin-coated surfaces (Figure 2A and 2C). In contrast, isoproterenol (0.1 to 10 μmol/L) promoted adhesion of HAECs and HMVECs to fibronectin by 20±6% and 19±11%, respectively (n=7, P≤0.05). When used alone, the PDE3 inhibitor cilostamide (1 μmol/L) and the PDE4 inhibitor Ro 20-1724 (10 μmol/L) each significantly promoted VEC adhesion to fibronectin, and these effects were augmented with either forskolin (Figure 2A and 2C) or isoproterenol (not shown). In contrast to its inability to influence migration, 8CPT-2'-OMe-cAMP (10 μmol/L) increased adhesion of HAECs and HMVECs by 42±10% and 37±7%, respectively (n=4 to 6, P≤0.05). Similarly a maximally effective concentration of 6-BzCAMP (30 μmol/L) promoted adhesion of HAECs, or HMVECs, by 32±5% or 30±8%, respectively (n=4 to 6, P≤0.05). When combined, 8CPT-2'-OMe-
cAMP and 6-BzCAMP subadditively stimulated adhesion of HAECs and HMVECs to 55±10% or 59±9% of control, respectively (n = 3, P≤0.05). Treatment of HAECs and HMVECs with H89 (10 μmol/L) or St-PKI (10 μmol/L) reduced adhesion to fibronectin-coated surfaces by 50±2% and 65±3%, respectively, and 58±4% and 54±6%, respectively, of control (n = 7, P≤0.05). Similarly, H89 and St-PKI reduced the proadhesive effects of 6-BzCAMP by 90±5% and 83±6%, respectively, and 92±4% and 85±9%, respectively, in HAECs or HMVECs (n = 8, P≤0.05). In contrast to the case with 6-BzCAMP, 8CPT-2′OMe-cAMP promoted adhesion of HAECs and HMVECs in the presence of PKA inhibitors, with this effect being more marked in HMVECs than in HAECs (Figure 2B through 2D). Although PKA inhibition alone but abolished the proadhesive effect of cilostamide and Ro 20-1724 in HAECs, both PDE inhibitors retained the capacity to promote HMVEC adhesion in the presence of PKA inhibitors (Figure 2B through 2D). Overall, these data support the idea that PKA coordinates cAMP-dependent adhesion of HAECs to fibronectin and that EPAC activation and PDE inhibition each promote HMVEC adhesion to fibronectin even with PKA inhibition.

Interestingly, the test agents acted differently when vitronectin- or gelatin-coated surfaces were used. Thus, H89 (10 μmol/L) inhibited binding of HAECs and HMVECs to vitronectin-coated surfaces by 73±6% and 76±3%, respectively (n = 10), and 8CPT-2′OMe-cAMP (10 μmol/L) promoted adhesion of HAECs and HMVECs by 37±17% and 36±12%, respectively, under these conditions (n = 10). Adhesion of HMVECs to vitronectin-coated surfaces was promoted by Ro 20-1724 (45±16% at 10 μmol/L), but not cilostamide (1 μmol/L) (not shown), in the presence of H89 (n = 10). PKA inhibition with H89 (10 μmol/L) completely abolished adhesion of either HAECs and HMVECs to gelatin-coated surfaces, and neither 8CPT-2′OMe-cAMP nor PDE inhibitors could overcome this effect (not shown).

**Figure 1.** Impact of cAMP-elevating agents or selective activators of PKA or EPAC on HAEC (A) and HMVEC (B) migration or Rap1 activation (C). Effects of forskolin (Fsk) (10 μmol/L) or 8CPT-2′OMe-cAMP (8CPT) (10 μmol/L) on migration of HAECs (A) or HMVECs (B) in response to vehicle (control) or vascular endothelial growth factor (VEGF) (10 nmol/L). Cells that migrated to the underside of the chamber in 4 hours were counted. *Significant inhibitory effect of forskolin on vascular endothelial growth factor–induced migration (P<0.05). C, Impact of incubation of HAECs or HMVECs with vehicle (control) (dimethyl sulfoxide, 0.1% vol/vol) or 8CPT-2′OMe-cAMP (8CPT) (10 μmol/L) on Rap1 activation.

**Amount and Subcellular Localization of cAMP Effectors in Human VECs**

HAECs and HMVECs each expressed EPAC1, PKA, PDE3B, and PDE4D, albeit at different levels. Thus, although HAECs and HMVECs each expressed EPAC1, this cAMP effector was 5±1 times (n = 3) more abundant in HMVECs (Figure 3). In contrast, HMVECs expressed only 37±10% (n = 3) of much RIIβ as was expressed by HAECs (Figure 3). Levels of PKA C subunit were not significantly different between the VECs (not shown). Whereas PDE3B was ∼15 times (n = 3) more abundant in HAECs than in HMVECs (Figure 3), levels of PDE3A in these cells were not different (not shown). Consistent with our previous report,16 immunoblot analysis of HAECs or HMVECs for PDE4D identified 2 immunoreactive bands. Although both HAECs and HMVECs each expressed roughly equivalent amounts of the ∼115-kDa anti-PDE4D species, significantly more of the 98-kDa species was present in HMVECs. As reported previously,19 the ∼115-kDa band could represent PDE4D5 and/or PDE4D7 and the ∼98-kDa band could constitute 1, or several, of PDE4D3, PDE4D8, and PDE4D9. At present, reagents that would allow analysis of the individual variants are lacking.

When analyzed at a subcellular level in actively adhering HAECs and HMVECs, endogenous RII was present throughout the cell body and within actin- and vinculin-rich regions of membrane ruffling; structures known to represent sites of membrane adhesion (Figure 4).20 Although EPAC1 was also detected throughout adhering HAECs and HMVECs, EPAC1 did not localize to the actin- and vinculin-rich membrane ruffles (Figure 4). Addition of forskolin, isoproterenol, 6-BzCAMP, 8CPT-2′OMe-cAMP, cilostamide, or Ro 20-1724 did not significantly alter the subcellular distribution of RII or EPAC1 in these cells (not shown). Although endogenous PDE3B staining was punctate and largely perinuclear in both VECs studied, endogenous PDE4D staining was detected throughout the cell body, as well as within cortical actin structures (Figure 4). Again, the addition of the cAMP-
Elevating test agents did not significantly impact the intracellular staining of either PDE3B or PDE4D in these cells (not shown). Interestingly, peripheral PDE4D staining in adhering cells was excluded from regions of cell–cell contact, even when this PDE was overexpressed (Figure 4).

**Global Intracellular cAMP Levels Are Not Predictive of VEC Adhesive Events**

Overall, our data were consistent with the idea that cAMP-induced activation of PKA and/or EPAC promoted HAEC or HMVEC adhesion. However, although the adenylyl cyclase activators tested increased cAMP significantly in both HAECs and HMVECs when measured after 15 minutes (Table), their impacts on cellular adhesion were at best modest. In contrast, even though the PDE inhibitors tested did not significantly increase global cAMP levels (Table), these agents markedly stimulated VEC adhesions. Consistent with their impact on global cAMP, forskolin (10 μmol/L), but neither cilostamide (1 μmol/L) nor Ro 20-1724 (10 μmol/L), promoted phosphorylation of several PKA substrates, including CREB and the PDE4D long-form variants expressed in these cells (not shown). Taken together, these data were inconsistent with the idea that global VEC cAMP or global activation of PKA was correlated with cellular adhesion but, rather, suggested that localized changes in cAMP more likely

---

**Figure 2.** Impact of cAMP-elevating agents and of selective PKA or EPAC agonists on HAEC or HMVEC adhesion to fibronectin-coated surfaces. HAECs (A and B) or HMVECs (C and D) were allowed to adhere to fibronectin-coated surfaces in the presence of vehicle (dimethyl sulfoxide, 0.1% vol/vol), forskolin (Fsk) (10 μmol/L), cilostamide (Cil) (1 μmol/L), Ro 20-1724 (Ro) (10 μmol/L), 8CPT-2′-OMe-cAMP (8CPT) (10 μmol/L), or combinations of these agents for 15 minutes (see Material and Methods). Data are means±SEM of 10 experiments in which the percentage increase in adhesion was measured. Vehicle adhesion values ranged between 4000 and 6000 disintegrations per minute. *Statistically significant differences compared with adhesion with dimethyl sulfoxide; P<0.05. In some experiments (B and D), cells were preincubated with H89 (10 μmol/L) for 10 minutes before assay. #Statistically significant differences compared with H89 (10 μmol/L); P<0.05.
was also seen in HMVECs (not shown). These data were consistent with the idea that particulate PKA likely plays an important role in human VEC adhesion to ECM proteins.

**Association of cAMP PDEs Within PKA- or EPAC-Based Complexes**

To isolate PKA- and/or EPAC-based signaling complexes, we adopted the cAMP–agarose adsorption method (herein referred to as cAMP–agarose pull downs). Based on immunoblot analyses of RIIβ and EPAC1 in post cAMP–agarose pull-down lysates, ≈90% of these proteins were recovered using this approach (Figure 6A and 6B). Consistent with the idea that PDE3B and/or PDE4D were associated with PKA and/or EPAC in human VECs, immunoblot analysis of proteins eluted from cAMP–agarose pull downs identified both PDE3B and PDE4D (Figure 6A and 6B). Indeed, compared with total levels of HAEC PDE3B and PDE4D, roughly 5% to 8% of each of these PDEs were recovered in these cAMP–agarose complexes (n = 10). Similar levels of PDE4D were observed in signaling complexes in other cells. Results of experiments in which cilostamide (1 μmol/L) and Ro 20-1724 (10 μmol/L) were included during pull downs obviated the possibility that PDEs associated with cAMP–agarose through their catalytic domains (not shown). The anti-EPAC and anti-RII antisera used in these studies immunoprecipitated these proteins poorly from human VECs (not shown). For this reason, components of EPAC-based complexes were studied in FLAG-tagged EPAC1-expressing cells by selective immunoprecipitation with anti–FLAG(M2)–agarose (herein called “M2-agarose pull downs”). Consistent with the idea that both PDE3B and PDE4D could associate with EPAC1 in HAECs, immunoblot analysis of HAEC EPAC1 isolates identified both these cAMP PDEs (Figure 6B and 6C). Overall, these data demonstrated for the first time that HAECs and HMVECs likely contain PKA- and/or EPAC-based complexes and that these signaling complexes were populated with PDE3B and/or PDE4D. Consistent with the novel idea that these cells contained distinct, nonoverlapping, PKA- and EPAC-containing cellular signaling complexes, EPAC1 isolates recovered from either HAECs or HMVECs were devoid of both the RII (Figure 6D) and C (not shown) subunits. In addition to showing that human VECs expressed nonoverlapping PKA and EPAC1 complexes, our data are also consistent with the idea that PDE3B and PDE4D are not present in the same complexes. Thus, M2-agarose pull downs from FLAG-tagged PDE3B-expressing cells were devoid of PDE4D whether the endogenous PDE4D was studied (not shown) or whether PDE4D3 was overexpressed (Figure 6E). Analogously, anti-VSV immunoprecipitates generated from VSV-PDE4D3-expressing HAECs cells were devoid of both endogenous PDE3B (not shown) and FLAG-tagged PDE3B when this protein was overexpressed (Figure 6E). Taken together, these data are most consistent with the idea that human VECs can contain a matrix of at least 4 distinct populations of cAMP-signaling complexes in which unique combinations of cAMP effector proteins (PKA or EPAC) and cAMP-PDEs (PDE3B or PDE4D) are present.
Discussion

In developed vascular structures, VEC lines the luminal surfaces of mature blood vessels and control blood pressure, inflammation, and thrombosis. During developmental vasculogenesis, or during angiogenesis in mature cardiovascular systems, motile VECs dynamically coordinate blood vessel expansion. It is generally accepted that VECs within microvascular structures (micro-VECs) more readily participate in angiogenesis than those within larger vascular structures (macro-VECs). VEC migration involves extension of a protrusion in the direction of movement, formation of protrusion-stabilizing adhesions, and translocation of the cell body and disassembly of adhesion at the cell rear. Because interference with adhesion molecules inhibits VEC spreading on ECM proteins, adhesion is widely thought to trigger migration. Although cAMP-elevating agents are known to regulate VEC adhesion to ECM proteins, few have studied the individual contributions of PKA or EPAC in this event. PKA activation has been reported to promote human VEC adhesion in an integrin-specific manner; PKA activity can be increased or decreased on integrin engagement. Also, previous studies suggested that PKA or EPAC could regulate and, in turn, be regulated by, VEC-integrin signaling.

In our work, we compared the cAMP-signaling events coordinating adhesion of HAECS (macro-VECs) and HMVECs (micro-VECs) to ECM proteins and demonstrated that PKA and EPAC both coordinate this event. To our knowledge, ours is the first report to comprehensively analyze the contributions of these 2 cAMP effectors in adhesion of human VECs and to compare phenotypically distinct VECs. Briefly, we show that cAMP-elevating agents can have...
Table. Impact of cAMP-Elevating Agents on HAEC or HMVEC cAMP

<table>
<thead>
<tr>
<th>Agent</th>
<th>HAECs</th>
<th>HMVEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.04±0.00</td>
<td>0.06±0.01</td>
</tr>
<tr>
<td>forskolin (10 μmol/L)</td>
<td>0.28±0.01*</td>
<td>0.32±0.01*</td>
</tr>
<tr>
<td>iso (1 μmol/L)</td>
<td>0.19±0.01</td>
<td>0.28±0.01</td>
</tr>
<tr>
<td>cilostamide (1 μmol/L)</td>
<td>0.03±0.02</td>
<td>0.06±0.02</td>
</tr>
<tr>
<td>Ro 20-1724 (10 μmol/L)</td>
<td>0.07±0.01</td>
<td>0.09±0.01</td>
</tr>
<tr>
<td>forskolin-cilostamide</td>
<td>0.34±0.02*</td>
<td>0.36±0.02*</td>
</tr>
<tr>
<td>forskolin-Ro 20-1724</td>
<td>0.42±0.02*†</td>
<td>0.52±0.02*†</td>
</tr>
</tbody>
</table>

*P<0.05 when compared with control; †P<0.05 vs 10 μmol/L forskolin treatment.

profound effects on VEC adhesion to several ECM proteins and that the relative contribution of PKA and/or EPAC in these events is different in macro- and micro-VECs. Moreover, although not a central focus of our work, we also show that the impact of PKA and EPAC is ECM dependent. Thus, our studies identify differences in the molecular mechanisms by which cAMP coordinates macro-VEC and micro-VEC adhesions, which are likely physiologically relevant and may be therapeutically important. Although most of the cAMP-elevating agents tested in HAECs could promote adhesion when PKA could be activated, when PKA was inhibited with either H89 or St-PKI, only the EPAC-selective activator promoted adhesion. In marked contrast, after PKA inhibition in HMVECs, the EPAC-selective agonist and either PDE3 or PDE4 inhibitors could still promote adhesion. Based on these findings, we propose that we have identified the existence of a functional link between EPAC and PDE3B- and PDE4D-catalyzed cAMP hydrolysis in HMVECs that is either missing, or less potent, in HAECs. The lack of an 8CPT or PDE inhibitor effect in the presence of H89 when cells were allowed to adhere to gelatin, in contrast to fibronectin, likely indicates that different cAMP-signaling pathways may be used depending on the ECM. Further studies will be needed to formally test this conclusion.

Another interesting finding relates to the observation that there was no direct correlation between total VEC cAMP levels and adhesion. Indeed, the adenylyl cyclase activators, which significantly increased VEC cAMP, had only modest effects on adhesion, whereas the PDE inhibitors, which did not increase total VEC cAMP, had marked proadhesive effects. Although we had initially hypothesized that the adenylyl cyclase activators would have the largest impact on VEC adhesion, recent advances inform our interpretation of these findings.10–15 Put simply, it is now generally accepted that macromolecular complexes containing each PKA or EPAC and some cAMP-PDE activity exist in cells and that tethering of these complexes to selected intracellular sites allows spatial and temporal control of cAMP-mediated events in a manner independent of global cAMP levels. We propose that our data are consistent with the proposition that such macromolecular complexes exists in human VECs and that these complexes allow for the “disconnect” between global cAMP and the effects of cAMP-elevating agents observed in our studies. More specifically, we suggest that our data are consistent with the idea that

![Figure 5. Localized cAMP signaling regulates human VEC adhesion. A, Effect of the PKA-AKAP disruptor on HAECs adhesion. HAECs, prepared as described in Materials and Methods, were incubated with PBS (control), stearated Ht31 (St-Ht31) (10 μmol/L), or an inactive peptide, stearated Ht31(P) [St-Ht31(P)] (10 μmol/L) for 30 minutes.; after which, they were allowed to adhere to fibronectin-coated surfaces at 37°C for 15 minutes in the absence of test agent or in the presence of dimethyl sulfoxide (0.02% vol/vol) (1); cilostamide (10 μmol/L) (2); Ro 20-1724 (10 μmol/L) (3); (6-BzcAMP) (30 μmol/L) (4); 8CPT-2′OMe-cAMP (10 μmol/L) (5); and 8CPT-2′OMe-cAMP-10 (μmol/L) (6). Adherent cells were counted as described in Materials and Methods. B, PKA activity in suspension or adhering HAECs. HAECs were either incubated in suspension at 37°C for 15 minutes or allowed to adhere to fibronectin-coated surfaces at 37°C for 15 minutes in the presence or absence of St-PKI (10 μmol/L). Following these incubations, cells were lysed in a buffer without Triton X-100. Following removal of cellular debris, HAEC pellet and cytosol fractions were resolved by centrifugation (100 000 g, 1 hour, 4°C) and independently resuspended in sodium dodecyl sulfate loading buffer (see Materials and Methods). Amounts of PKA-phosphorylated RIIβ ([P]-RIIβ) (top blot) or total RIIβ (RIIβ) (bottom blot) in these fractions were detected using an antisera directed against PKA-phosphorylated RIIβ (top) or an antisera directed against native RIIβ (bottom). Representative immunoblots from 1 experiment are shown, and similar data were obtained in three separate experiments.](http://circres.ahajournals.org/lookup/doi/10.1161/CIRCRESAHA.107.186045)
Macromolecular complexes containing either PKA or EPAC and PDE3B or PDE4D are present in both macro- and micro-VECs and that these complexes determine the impact of cAMP-elevating agents on VEC adhesion.

Evidence consistent with the idea that cAMP regulated VEC functions in a manner consistent with the existence of distinct cAMP "pools" was proposed before our studies. Indeed, Stevens and colleagues reported that cAMP synthesis within membrane or cytosolic cellular compartments allowed selective regulation of distinct effects in rat pulmonary artery VECs.28 However, these earlier studies were mute on issues related to the number and the complexity of the signaling complexes that operationalized these pools. In this work, we confirm the existence of multiple distinct nonoverlapping cAMP-signaling complexes in human VECs and provide mechanistic details of the manner by which they regulate VEC functions, such as adhesion. Thus, we show that multiple distinct PKA- or EPAC-based signaling complexes are present in both HAECs and HMVECs and that these complexes contain either PKA or EPAC but not both. Before this work, the only EPAC-containing complex identified was isolated from rodent cardiomyocytes and found to contain both EPAC and PKA.10,11 In addition, and of most interest to us, was the observation that whereas immunoblot EPAC-
based complexes isolated from human VECs were shown to contain both PDE3B and PDE4D, immunoprecipitates of PDE3B or PDE4D from these cells were completely inconsistent with the idea that these 2 families of PDEs could be found in the same complexes. To our knowledge, this is the first report to demonstrate the existence of multiple EPAC-based complexes in cells and to show that these are devoid of PKA. In addition, this is also the first report to demonstrate that the multiple distinct EPAC-based complexes expressed in cells can be segregated based on the identity of the PDE that they contain. Interestingly, although not tested directly, our data are also consistent with the idea that PKA-based complexes that contain either only PDE3B or PDE4D also exist in these cells. Because it is now recognized that targeting of distinct PDE4 enzymes is dependent on their unique amino-terminal regions, and that several cell types express multiple PDE4 proteins, analyses of the type performed here will likely be required to assess the true multiplicity of complexes expressed by cells. In addition, because PDE3A did not associate within the complexes identified in these cells, the molecular basis for the PDE3B associations noted here will likely be related to its unique amino-terminal region. Future studies should address these issues.

Sources of Funding
This work was supported by Heart and Stroke Foundation of Ontario grant T5426 (to D.H.M.). D.H.M. is a Heart and Stroke Foundation of Ontario Career Investigator.

Disclosures
None.

References
Both Protein Kinase A and Exchange Protein Activated by cAMP Coordinate Adhesion of Human Vascular Endothelial Cells

Stuart J. Netherton, Jayda A. Sutton, Lindsay S. Wilson, Rhonda L. Carter and Donald H. Maurice

Circ Res. 2007;101:768-776; originally published online August 23, 2007; doi: 10.1161/CIRCRESAHA.106.146159

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/101/8/768

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/