THE ROLE OF COMPARTMENTED cAMP-SIGNALLING IN THE
REGULATION OF VASCULAR ENDOTHELIAL CELL PERMEABILITY

BY

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A thesis submitted to the Department of Pathology and Molecular Medicine in
conformity with the requirements for the degree of Master of Science

Queen’s University
Kingston, Ontario, Canada
August 2009

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ABSTRACT

Vascular endothelial cells (VECs) maintain vascular integrity by regulating the passage of solutes, macromolecules, and cells between the vascular and perivascular space and are critical in a wide number of physiological processes, such as the delivery of nutrients and oxygen to surrounding tissues, leukocyte trafficking, angiogenesis, and tissue repair. VEC permeability is regulated, at least in part, by VE-cadherin-based adherens junctions (AJs) that coordinate inter-VEC contacts and communicate the strength of these interactions to the cell via the actin cytoskeleton. Although the ubiquitous second messenger, cyclic adenosine 3’, 5’-monophosphate (cAMP), has been shown to reduce VEC permeability the molecular basis of this effect is currently unclear. Herein, we report that cAMP and its two effectors, cAMP-dependent protein kinase A-II (PKA-II) and exchange protein activated by cAMP-1 (EPAC1), improve barrier function and differentially coordinate this effect through both VE-cadherin and actin cytoskeleton structures. We have also identified cyclic nucleotide phosphodiesterase (PDE) 4 as the major PDE regulating VEC barrier function. Furthermore, we show that PKA, EPAC1 and PDE4D are integral to VE-cadherin-based structures. Through the use of cAMP-elevating agents and RNAi-mediated knockdown of PKA-Cα, EPAC1 and PDE4D, we have identified a dominant role for EPAC1 in VEC permeability as well as recognized PDE4D as a potential adaptor protein in the VE-cadherin complex. Our results are consistent with previous reports of a role for each PKA and EPAC1 in controlling VE-cadherin mediated barrier function and additionally provide novel insight into the differential roles that PKA, EPAC1 and PDE4D play in stabilizing VEC barrier.
CO-AUTHORSHIP

Experiments outlined in this thesis were performed by Sarah Rampersad, Jefferey Ovens and Bibiana Umana, under the supervision and guidance of Dr. Donald Maurice. Permeability assays (Section 3.1), fc-VE-cadherin-based pulldowns and related PDE activity measurements (Section 3.9) were performed by Jefferey Ovens, as identified within the results section of the thesis. Immunocytochemistry preparation, staining and visualization, cAMP measurements (Sections 3.2 and 3.8) and verification of RNAi-mediated knockdown of PKA-Cα, EPAC1 and PDE4D (Section 3.5) were performed by Bibiana Umana.
ACKNOWLEDGEMENTS

The time I have spent at Queen’s University completing my Master’s degree has been a stimulating, challenging and overall very rewarding experience which has provided me with invaluable skills and knowledge that I will carry with me in my future endeavors. And for this, I have many people to thank.

Firstly, I would like to thank my parents who have always provided me with unconditional love and support and who continue to encourage me in the pursuit of my goals. I also have to thank my brother, Greg, who has influenced me in so many ways and is always there to lend an ear or provide helpful advice. Finally, I am very grateful for my friends in Ottawa, who have always been very supportive and encouraging throughout.

I would also like to thank the members of the Maurice lab who have all contributed positively to my Master’s experience, both inside and out of the lab. Sandra, Bibi, Lindsay, Lisa and Andrew, thank you for all of your help in the lab, for creating a wonderful working atmosphere and for being such great labmates and friends. I truly could not have asked for a better lab. Special thanks to Bibi, with whom I worked very closely with, for working so hard, always being available to help and for taking such great care of our cells! Finally, I must especially thank Don for taking me into his lab and for continually challenging and supporting me over the course of my work. You have been an excellent mentor and I have sincerely enjoyed working with you. I wish the very best of luck to you all.
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<tr>
<td>TCF</td>
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<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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# LIST OF DRUGS

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<td>PKA activator</td>
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<td>8-CPT</td>
<td>EPAC activator</td>
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<td>pan-PDE4 inhibitor</td>
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<td>Cil</td>
<td>pan-PDE3 inhibitor</td>
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CHAPTER 1

INTRODUCTION

1.1 Role of VECs in blood vessels

Vascular endothelial cells (VECs) form the inner most layer of tissue surrounding the lumen of all blood vessels in the body (Figure 1.1). This tissue layer, called the tunica intima, consists of a thin monolayer of endothelial cells that regulates the passage of gases, fluids, small solutes, macromolecules, plasma proteins and inflammatory mediators that cross the barrier (Mehta and Malik., 2006). Surrounding the tunica intima is an elastic lamina which is essential for blood vessel flexibility and allows shape changes to occur as a result of physiological responses. The elastic lamina is encircled by the tunica media which is composed of a layer of elastic fibre, connective tissue and polysaccharides. In the majority of blood vessels the tunica media is rich in vascular smooth muscle cells (VSMCs) whose role is to contract or relax to alter blood vessel diameter and control local and systemic blood pressure. Finally, the third and outer most layer of all blood vessels, the tunica adventitia, is composed almost entirely of connective tissue but may also contain nerves to innervate underlying VSMCs, adventitial fibroblasts and, in particularly large vessels, nutrient capillaries (Alberts et al., 2002). Some very small vessels, such as capillaries, have no VSMCs at all but consist entirely of an endothelial monolayer and a fine basal lamina. Hence, VECs act as the protective barrier between the blood and all surrounding tissues in the body. This barrier function is highly regulated by various growth factors and signals that circulate both in the blood, such as
Figure 1.1: Schematic cross-section of a blood vessel. The inner most layer of a blood vessel is the tunica intima (endothelial lining), consisting of a monolayer of endothelial cells which mediate the passage of macromolecules from the lumen to surrounding tissue by regulating inter-cellular adhesion. Surrounding the endothelial lining is the elastic lamina and then the tunica media which contains vascular smooth muscle cells. Finally, the outer layer, the tunica adventitia, is composed of loose connective tissue. (Adapted from Alberts et al, 2002)

thrombin of the coagulation cascade, and histamine, which is involved in local immune responses, and from the underlying tissue, like vascular endothelial growth factor (VEGF), which is an important pro-angiogenic mediator. All of these factors increase permeability and disrupt barrier function in VEC monolayers (Baumer et al., 2009, Doyle and Haas, 2009). As such, controlling VEC barrier function is critical in regulating tissue/fluid homeostasis, blood clotting, and the inflammatory response and is a necessary
process in blood vessel proliferation, migration and angiogenesis, without which tissue
growth and repair would be impossible. Notably, the importance of VECs are most
clearly illustrated in the event of VEC dysfunction which is characterized by increases in
vascular permeability leading to edema as well as inflammatory or even metastatic cell
infiltration into the vasculature.

1.2 Regulating adhesion in VECs

Vascular endothelial cells maintain barrier function by regulating the passage of
cellular molecules into the interstitial and intravascular space which can occur via either trans- or
para-cellular pathways. The endothelial monolayer acts as a restrictive barrier where,
under normal physiological conditions, only solutes with radii of up to 3nm, such as
water, hexoses and urea, can travel passively across the endothelium by way of the
paracellular pathway (Hu et al., 2008). This pathway is mediated by inter-endothelial
junctions, tight junctions (TJs) and adherens junctions (AJs), which provide cell-cell
adhesion and actinomyosin molecular motors that provide counter-adhesive forces. All
together, these elements act to regulate barrier function. Normally, macromolecules with
a radius greater than 3nm, such as albumin, can cross the barrier via a transcellular
vesicular pathway (Mehta et al., 2006). However, when exposed to inflammatory
mediators such as histamine, thrombin and VEGF, inter-endothelial junctions reorganize
and barrier junction opens to allow the passage of albumin and other plasma proteins and
fluid through the monolayer in an unrestricted manner. Leukocytes can traverse the
endothelial barrier by both para- and trans-cellular pathways although paracellular
transport is more common (Muller, 2009). As such, the inflammatory response heavily relies on the strength of inter-endothelial cell adhesions as well (Vestweber et al., 2008).

TJs are made up of occludens, claudins and junctional adhesion molecules (JAMs). Extracellularly, they adhere to one another through homotypic interactions and, intracellularly, interact with proteins that connect them to the actin cytoskeleton. For example, occluden-containing TJs bind zona occludens protein ZO-1 in the cytoplasm and link occludens to $\alpha$-catenin which in turn binds the actin cytoskeleton. Increasing amounts of occludens expressed in different endothelial beds are correlated with enhanced barrier properties (Fanning et al., 2009). Interestingly, in vitro, deletion of the N-terminal portion of occludens in endothelial cells results in leaky junctions. However, occluden -/- mice show no alteration in intestinal barrier function (Mehta et al., 2006). This may be in part due to a compensatory role of AJ’s, but requires further investigation. There is evidence that AJs may be required for the proper organization of TJs in VECs and recently, and one such mechanism was elucidated where AJ bound vascular endothelial (VE)-cadherin, was shown to upregulate expression of TJ adhesive protein claudin-5 (Taddei et al., 2008).

AJs are known to play a major role in VEC barrier function and are primarily composed of members of the cadherin family of adhesion proteins (Dejana, 2008). Endothelial cells express high levels of VE-cadherin and neuronal (N-) cadherin, which is also present in neural and smooth muscle cells, as well as other non-cell-type specific cadherins such as P- and T-cadherin which are variably expressed in different types of endothelial cells (Bazzoni and Dejana, 2004). Despite the expression of various cadherins in VECs and equally high expression levels of VE- and N-cadherin, AJs are composed primarily of VE-cadherin. In fact, previous work has demonstrated that N-cadherin shows...
diffuse staining on the membranes of endothelial cells and are poorly clustered at intercellular junctions (Navarro et al., 1998). As such, VE-cadherin plays a major role in regulating barrier integrity in VEC monolayers.

VE-cadherin forms Ca$^{2+}$-dependent interactions with the extracellular domain of other VE-cadherin molecules on neighbouring cells (Hewat et al., 2007). Intracellularly, its short cytosolic tail binds with β-catenin, p120-catenin and plakoglobin, also known as γ-catenin (Dejana, 2008). β-catenin and plakoglobin can associate with α-catenin which links the VE-cadherin complex to the actin cytoskeleton, creating strong adhesion throughout the endothelial monolayer (Figure 1.2). VE-cadherin null mice display an embryonic lethal phenotype due to immature vascular development and gene inactivation of VE-cadherin has comparable effects (Carmeliet et al., 1999). Furthermore, when a VE-cadherin mutation in its extracellular domain is expressed in a human dermal microvascular cell line and mouse endothelial cells, the cells exhibit decreased barrier function (Venkiteswaran et al., 2002). Recent literature has characterized the importance of other members of the VE-cadherin AJ complex, such as β- and γ-catenin, which are also functionally very important in maintaining barrier function. For example, the expression of a mutated or truncated VE-cadherin lacking β-catenin or γ-catenin binding sites has been shown to cause a decrease in barrier function and interfere with binding of AJs to the actin cytoskeleton (Navarro et al., 1995, Venkiteswaran et al., 2002).
Figure 1.2: Illustration of a VE-cadherin-based adherens junction. VE-cadherin’s extracellular domain homotypically binds neighbouring VE-cadherin through calcium dependent interactions. VE-cadherin’s cytoplasmic domain bind p120-, β- and γ-catenins. α-catenin interacts with β- and γ-catenins. (Adapted from Dejana, 2008)

1.3 VE-cadherin-based AJ dynamics and signalling

VE-cadherin-based adherens junction complexes are important in that they not only mediate cell-cell adhesion but they are also dynamic structures involved in several important signalling pathways. VE-cadherin can be internalized and recycled back to the membrane upon stimulation with various growth factors. One important signal, as mentioned previously, is VEGF. In VECs, VEGF is the ligand of VEGFR-2, which interacts with VE-cadherin to promote cell survival and decrease proliferation. Recently, studies in human aortic endothelial cells (HAECs) have shown that VEGF reduces the
expression of VE-cadherin at the cell membrane through the following signalling pathway. VEGF binds VEGFR-2 which activates Rac, a small GTPase, through Src-dependent phosphorylation of the guanine exchange factor (GEF), Vav-2. Activated Rac promotes p21-activated kinase (PAK)-mediated phosphorylation of VE-cadherin at Ser 665. Upon phosphorylation, β-arrestin2 is recruited and promotes the internalization of VE-cadherin into clathrin-coated vesicles (Gavard and Gutkind, 2006). In addition to this specific pathway, VEGF and several other growth factors such as histamine, tumour necrosis factor-α (TNF-α) and platelet activating factor (PAF) induce the tyrosine phosphorylation at numerous sites on VE-cadherin and various components of the AJ complex such as p120- and β-catenin which subsequently leads to the disassembly and internalization or degradation of the VE-cadherin complex and an increase in vascular permeability (Andriopoulou et al., 1999, Hudry-Clergeon et al., 2005, Angelini et al., 2006, Nottebaum et al., 2008). Induction of VEGFR2 with VEGF also promotes VEC migration and proliferation. In another study, VEGF was shown to promote VEGFR2 internalization into clathrin-coated vesicles. When VEGFR2 is internalized, the receptor is phosphorylated and localizes with phospholipase C–γ and p44/42 mitogen-activated protein kinase to promote cell proliferation (Kamiyama et al., 2008). However, DEP-1, a phosphatase whose activity increases with VEC monolayer confluence, associates with VE-cadherin and maintains its engagement at cell junctions. When VEC monolayers are at high confluency and VE-cadherin is active at cell junctions, VEGFR2 is retained at the membrane and cannot internalize into its signalling compartments (Lampugnani et al., 2006). As such, VE-cadherin can also exert anti-proliferative effects and control VEGF signalling, depending on VEC monolayer confluency (Figure 1.3).
Figure 1.3: *VEGF-mediated internalization of VE-cadherin*. At high VEC confluency, stable VE-cadherin AJ structures inhibit VEGF-mediated internalization of VEGFR-2 into clathrin-coated vesicles. At lower confluency, VEGF-signalling induces VEGFR-2 activation and recruitment of Src. Src activates Vav2, which then acts as a GEF for Rac. Once GTP-bound, Rac activates PAK which goes on to phosphorylate VE-cadherin and leads to its subsequent internalization. (Adapted from Gavard and Gutkind, 2006)

VE-cadherin stability at the membrane is also regulated by other members of the AJ complex. One such molecule is β-catenin, which binds VE-cadherin’s cytoplasmic tail. Previously, it was believed that β-catenin interacted directly with α-catenin, however recent research shows that α-catenin cannot bind both β-catenin and actin (Weis and Nelson, 2006). Although it has been shown that increases in endothelial permeability are mediated by contraction of actomyosin that is anchored to AJs through β-catenin and
plakoglobin, there may be various mediator molecules involved in this interaction and further work at the molecular level is necessary (Dudek and Garcia, 2001, Weis and Nelson, 2006). β-catenin does however play an important role in regulating VE-cadherin stability at the membrane. For example, tyrosine phosphorylation of VE-cadherin at position 731 via ICAM1 upon neutrophil adhesion to endothelial cells disrupts β-catenin binding to VE-cadherin and impairs barrier function (Allingham et al., 2007, Turowski et al., 2008). Additionally, in many systems, direct phosphorylation of β-catenin reduces its ability to bind VE-cadherin and increases AJ turnover at the membrane (Huber and Weis, 2001, Lilien and Balsamo, 2005). β-catenin is also involved in the Wnt signalling pathway, where high levels of stabilized β-catenin in the cytoplasm can be recruited to the nucleus and act as a transcription factor with TCF to promote cell proliferation and differentiation. One review by Nelson and Nusse explores the potential crosstalk between Wnt and cadherin pathways. Overall, data indicate that high tyrosine phosphorylation levels of β-catenin parallel low degrees of cell-cell adhesion, higher amounts of cytoplasmic β-catenin, and increased cell migration (Nelson and Nusse, 2004). Again, this demonstrates the intricate levels of regulation that encompass cell-cell adhesion through VE-cadherin complexes.

Similarly, p120-catenin also associates with the cytoplasmic tail of VE-cadherin and stabilizes AJs at intercellular borders (Chiasson et al., 2009). Tyrosine phosphorylation of VE-cadherin at position 658 causes p120 dissociation from VE-cadherin and the destabilization of barrier function (Potter et al., 2005). Furthermore, tyrosine phosphorylation of p120, like β-catenin, has been shown in many cases to disrupt barrier function as well (Lilien and Balsamo, 2005). Additionally, several studies
have shown that the presence of p120 at cadherin-based AJs prevents cadherin degradation through a clathrin-dependent pathway (Chiasson et al., 2009).

As such, it is evident that the regulation of VE-cadherin-based AJs is very complex and may be modified by numerous factors such as the tyrosine phosphorylation states of VE-cadherin and interacting molecules. Additionally, this regulation is very dynamic and important in that it interacts with various other pathways to mediate more complex cellular functions, such as the migration and proliferation of VECs.

1.4. Actin cytoskeleton organization in VEC barrier function

Actin dynamics are also an essential process in maintaining VEC barrier function since association of AJs with actin through α-catenin provides adhesive strength spanning across the entire endothelial monolayer. Essentially, cortical actin bands stabilize AJs whereas reorganization of actin into contractile stress fibres disrupts them (Prasain et al., 2008). VEC contraction is partly mediated though MLC phosphorylation by MLCK which propagates cross-bridge cycling of myosin-actin chains. RhoA-ROCK signalling further propagates this state by inhibiting MLC phosphatase (MLCP) and this contraction pulls VE-cadherin inward, producing intercellular gaps (Mong et al., 2009).

G-proteins are also important players in actin cytoskeleton organization. In fact, numerous studies have identified a correlation between cadherin engagement and the activation of Rho family GTPases, which are well known regulators of actin dynamics. For example, inhibition of Rac leads to endothelial junction destabilization and its activation is thought to promote actin polymerization and couple VE-cadherin complexes to the actin cytoskeleton (Waschke et al., 2004). Interestingly, although Rac has been
reported to aid in AJ stability at membranes, it has also been implicated in AJ
destabilization through the VEGF-mediated internalization, for example, as mentioned
previously. Similarly, RhoA is regulated by VE-cadherin which can transmit signals
through the actin cytoskeleton to adhesive proteins regulating cell-matrix interactions
(Kouklis et al., 2004, Tzima, 2006). Cdc24 is another example of Rho GTPases involved
in VE-cadherin mediated actin organization. It has been demonstrated to be involved in
attenuating thrombin induced permeability, as well as in controlling the binding of α-
catenin to the β-catenin/VE-cadherin complex (Kouklis et al., 2004, Broman et al., 2007).
Finally, Rap1, another Rho GTPase, is activated upon VE-cadherin engagement through a
mechanism dependent on MAGI, an adaptor protein which is associated with Rap1
activator, PDZ-GEF (Sakurai et al., 2006). These proteins are hypothesized to be
recruited to the VE-cadherin complex by β-catenin (Wallez et al., 2008).

1.5 cAMP signalling

A notable second messenger molecule influencing barrier function in VECs is
3’,5’ cyclic adenosine monophosphate (cAMP). cAMP is generated by adenylyl cyclase
(AC) which is mostly found at the cell membrane. There are in fact, 10 AC isoforms. ACs
1-9 are membrane spanning and AC10 is soluble. However, VECs only express AC3,
AC7 and AC6, which is the most active isoform in these cells (Sunahara and Taussig,
2002, Cioffi et al., 2002). cAMP elevating agents such as isopreterenol (Iso),
adrenomedullin (AM), prostacyclin (PGL2), prostaglandin E2 (PGE2) receptors, and β-
adrenergic agonists of Gs protein-coupled receptors (GPCR) agonize their respective
transmembrane receptors and release Gαs-proteins which stimulate AC and promote the
rapid production of cAMP in the cell. Subsequently, cAMP activates three principal effector molecules, cAMP-dependent protein kinase A (PKA), exchange protein activated by cAMP (EPAC), and cyclic nucleotide gated ion-channels (CN channels). Herein, we will focus primarily on PKA and EPAC as they are known to regulate VEC barrier function (Figure 1.5) (Fukuhara et al., 2005, Birukova et al., 2007).

**Figure 1.5:** cAMP-signalling cascade. GPCR activation by an agonist causes the release of a GTP-bound G\(_{\alpha S}\) G-protein. This G-protein activates AC and promotes cAMP synthesis. cAMP can then go on to activate two of its effector proteins, EPAC and PKA (consisting of R- and C-subunits). PDEs regulate the cAMP signal by hydrolysing cAMP to 5’AMP. (Adapted from Wong and Scott, 2004)
PKA, in its inactive form, is a heterotetramer consisting of two regulatory (R-) and two catalytic (C-) subunits. Upon localized increases in cAMP, two tandem cAMP-binding domains situated on the C-terminus of each PKA R-subunit bind cAMP, resulting in a conformational change and subsequent release of the C-subunits (Weber et al., 1987). These catalytic subunits act as serine/threonine kinases and phosphorylate substrates containing the -R-R/K-X-S/T- motif. There are two forms of PKA, type-I (PKAI) and type-II (PKAII). There exists three C-subunit variants, Cα, Cβ and Cγ, which associate with both PKAI and PKAII. However, the R-subunits of PKA, which dictate its localization in the cell, are type specific. Thus, PKAI consists of R-subunit variants RIα or RIβ, and is cytosolic, whereas PKAII is made up of R-subunits RIIα or RIIβ, and is membrane-bound (Taylor et al., 2004). Additionally, A-kinase anchoring proteins (AKAPs) bind the 50 first amino acids in the N-terminal region of PKA R-subunits, which varies widely between RI and RII forms. Although the majority of AKAPs reportedly bind RII, some can bind both RI and RII subunits and few preferentially bind RI (Aye et al., 2009). This binding to AKAPs thus allows PKA to be localized in discreet signalling domains to allow signal transduction to specific effectors and substrates.

Next, cAMP also activates guanine nucleotide exchange protein EPAC, another known key player in regulating VEC barrier function (Fukuhara et al., 2005, Kooistra et al., 2005). There are two isoforms, EPAC1, which is ubiquitously expressed in all tissues, and EPAC2, whose expression is less ubiquitous and limited to select tissues such as adrenal glands, liver, pancreatic islets of Langerhans, and areas of the brain (Kawasaki et al., 1998., Ozaki et al., 2000, de Rooij et al., 1998, Xiaodong et al., 2008). Both contain an N-terminal regulatory domain containing a Dishevelled/Egl-10/pleckstrin (DEP) domain followed by a cAMP-binding domain (CBD), which is homologous to those
found on PKA-R subunits, as well as a C-terminal catalytic domain. Interestingly, EPAC2 contains an additional low affinity CBD adjacent to the DEP domain, whose regulatory function remains unclear (de Rooij et al., 2000). Once bound by cAMP, EPAC proteins activate the Ras superfamily of small GTPases Rap1 and Rap2.

Although PKA was once believed to be the only effector of cAMP signaling, within the past decade, EPAC’s discovery and involvement in several related processes have been described. In some instances, PKA and EPAC play antagonizing roles in similar signaling processes, such as in the PKB/AKT pathway (Mei et al., 2002). In other cellular contexts, they act synergistically such as in the regulation of endothelial cell adhesion to the extracellular matrix (Netherton et al., 2007). In the past, PKA was perceived to be the sole regulator of VEC barrier function. However, when PKA activity was inhibited, cAMP-mediated barrier function was not completely abolished thus indicating the presence of PKA-independent pathways (Cullere et al., 2005) and a role for EPAC in VEC permeability was identified.

1.6 Cyclic nucleotide phosphodiesterases

Key components of cAMP-signalling are cyclic nucleotide phosphodiesterases (PDEs), the only molecules known to regulate and degrade second messenger cAMP. Given the numerous cellular processes governed by cAMP, it is unsurprising that there are 11 distinct PDE families, categorized by sequence, catalytic ability and regulatory function, and over 50 distinct PDE isoforms as a result of alternate promoters and mRNA splice variants (Netherton et al., 2005). Catalytically, PDE isoforms are generally very similar, but highly divergent throughout their amino- and carboxy-termini and these
differences are most relevant in the differential regulation and localization of each unique isoform (Maurice et al., 2003). VECs express four PDE isoforms: a cGMP-stimulated dual cAMP/cGMP PDE (PDE2), a cGMP-inhibited cAMP PDE (PDE3), a cAMP-specific PDE (PDE4) and a cGMP-specific PDE (PDE5) (Surapisitchat et al., 2007). In HAECs, which are the main focus the studies herein, PDE3 and PDE4 are the most abundant, representing 36% and 45% of total cAMP PDE activity, respectively (Netherton et al., 2007). However, relative levels of these PDEs differ between VECs isolated from distinct vasculatures which might allow for tissue specific regulation of subcellular pools of cAMP.

1.7 Compartmentation of cAMP signalling via PDEs

cAMP is produced upon AC activation and results in the rapid diffusion of cAMP into the cytoplasm (Houslay et al., 2007). As such, to ensure distinct signal transduction and activation of specific effectors, it is necessary to mediate and restrict the diffusion of cAMP in the cell. This is in part done through the localization of PDEs. Specific localization of PDEs is important in that it creates cAMP sinks in specific areas of the cell to provide a multitude of microenvironments where specific cAMP effectors and signalling molecules may act accordingly (Houslay et al., 2007). PDEs can be tethered by A-kinase anchoring proteins (AKAPs) to various parts of the cell or be recruited by scaffolding molecules. One example of a specific cAMP-signalling microdomain is in β2-adrenergic receptor (β2ARs) desensitization in cardiomyocytes. The β2AR is a GPCR which is activated upon stimulation by epinephrine. When activated, β2ARs couple to G\textsubscript{as} protein which stimulates AC and produces cAMP. This particular signal is localized in
part by PKA tethering to the \( \beta_2 \)AR via an AKAP, AKAP79 (Fraser et al., 2000). When cAMP activates PKA, it phosphorylates the \( \beta_2 \)AR and leads to recruitment of another scaffolding protein, \( \beta \)-arrestin. \( \beta \)-arrestin blocks further downstream signalling of the \( \beta_2 \)AR and also recruits specific PDE isoform 4D5 (PDE4D5) to the receptor which terminates the localized cAMP signal (Lynch et al., 2005). The recruitment of this \( \beta \)-arrestin-PDE4D5 complex to \( \beta_2 \)ARs also results in receptor internalization into clathrin-coated vesicles (Mundell et al., 2002). As such, precise scaffolding of cAMP-mediated components is crucial in localizing specific cAMP signalling to defined compartments or microdomains in the cell.

1.8 Role of cAMP signalling in VEC barrier function

Increases in cAMP, induced by receptor-selective agonists or cAMP elevating agents acting directly at AC, such as forskolin, increase basal barrier function and consequently decrease VEC permeability and also reverse vascular leakage caused by inflammatory mediators (Baumer et al., 2009, Schlegel et al., 2009). Furthermore, activation of PKA and EPAC has also been shown to increase barrier function through various mechanisms but characterization of their relative roles is still in its infancy.

PKA is a known mediator of actin dynamics and can attenuate vascular leakage induced by inflammatory mediators such as thrombin, phorbol myristate acetate, pertussis toxin, and bacterial wall lipopolysaccharide (Birukova et al., 2004) There are numerous mechanisms through which PKA exerts its effects. For example, activation of PKA phosphorylates MLCK thereby reducing its ability to phosphorylate MLC and reduce cell contraction. PKA also phosphorylates and inhibits RhoA and promotes RhoA association
with Rho-GDP dissociation inhibitor (Rho-GDI), thus stabilizing cortical actin (Birukova et al., 2004). Interestingly, a study by Lorenowicz et al. showed that PKA activation cannot improve endothelial barrier without integrin function (Lorenowicz et al., 2008). However, there is still no direct evidence to date of PKA interacting with a VE-cadherin complex nor a known mechanism through which PKA modulates VE-cadherin-complex stability.

Conversely, EPAC affects barrier function through both VE-cadherin-dependent and -independent mechanisms. Several studies in HUVECs have demonstrated that EPAC1 promotes the formation of VE-cadherin based junctions and is involved in cortical actin organization (Cullere et al., 2005, Fukuhara et al., 2005). Activation of EPAC can also attenuate thrombin-induced VEC permeability by down regulating Rho-GTPase activity (Cullere et al., 2005). Additionally, one study has shown that EPAC activation improves barrier function in wild type mice and not in VE-cadherin null mice, however actin remodelling is independent of AJ formation (Kooistra et al., 2005). In HPAECs, EPAC1/Rap1 signalling activates Rac by stimulating Rac-specific GEFs Tiam and Vav2, thus leading to cortical actin and AJ improvement when treated with prostaglandin E2, prostacyclin and atrial natriuretic peptide. Interestingly, PKA activation stimulates Rac under the same treatment conditions and thus converges with EPAC in this signaling pathway (Birukova et al., 2008).

As such, evidence shows that both PKA and EPAC are important mediators of VEC barrier function, yet further identification of their relative roles remain to be seen. Furthermore, the specific identification of PDE involvement in cAMP-mediated regulation of VEC barrier function has yet to be elucidated as well.
RESEARCH RATIONALE

VE-cadherin-based AJs do not simply regulate cell-cell adhesion but also act as signalling structures which can communicate important messages for cell position, limiting growth, angiogenesis, and general homeostasis of blood vessels. Our study seeks to clarify the relationship between VE-cadherin-based barrier stability and the involvement of cAMP-regulated effectors, such as PKA and EPAC, in regulating VEC barrier function and to identify a role for PDEs. Although PKA and EPAC have been implicated in the regulation of both actin-mediated and VE-cadherin-based intercellular adhesion, their relative roles have not been fully characterized. As such, we will further explore their actions on VEC barrier function through the use of cAMP-elevating agents and RNAi-mediated knockdown of the major players involved. This study will provide us with regulatory information and perhaps novel therapeutic targets for human diseases such as in the case of VEGF-induced permeability, which is often seen in tumor cell penetration and metastasis of cancer, and edema, which can lead to increased ischemic tissue injury in conditions such as stroke and myocardial infarction. Furthermore, manipulation of VEC permeability may also have practical pharmacological implications with respect to increasing drug accessibility to different tissues in the body.
RESEARCH HYPOTHESIS AND OBJECTIVES

We hypothesize that components of the cAMP-signalling system, specifically PKA, EPAC and PDEs are involved in regulating VEC barrier function through VE-cadherin and actin-mediated mechanisms. Furthermore, we predict that identified cAMP-signalling members interact at the membrane in a macromolecular complex containing VE-cadherin.

Firstly, we intend to investigate a role for PDEs in VEC barrier function by observing the effects of relevant PDE inhibitors on HAEC monolayer permeability through the use of permeability assays. Secondly, we will characterize the relative roles of PKA- and EPAC-activation on HAEC permeability by observing their effects on HAEC monolayer permeability as well as their impact on important intercellular adhesion molecules, VE-cadherin and actin, using immunofluorescence imaging. We will additionally use siRNA to reduce levels of PKA, EPAC and relevant PDEs to further characterize their roles in HAEC monlayer permeability as well as how they regulate VE-cadherin- and actin-based structure stability. Finally, we will utilize fc-VE-cadherin and GST-β-catenin pulldown methods to eludicate the potential interaction of PKA, EPAC and PDEs with VE-cadherin. Given the presence of such a complex, we will then test the dynamics of these interactions using a displacing peptide.
CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

Human aortic endothelial cells (HAECs), endothelial basal media (EBM-2) and endothelial growth media bullet kit (EGM-2) were purchased from Lonza (Walkersville, MD, USA). Lipofectamine 2000 and several siRNA targeting dimer constructs (PDE4D - 5’GAC AAG CAC AAU GCU UCC GUG GAA A 3’, PRKACA - 5’GGA AGC UCC CUU CAU ACC AAA GUU U 3’, EPAC1, 5’AUU GAG AUU CUU CUG CUC CUU GAG G 3’ and a high GC universal negative control) were all from Invitrogen (Carlsbad, CA, USA). Tissue culture flasks were obtained from Life Technologies, Inc. (Gaithersburg, MD, USA). Permeability assay plates, 6.5mm diameter transwell inserts (0.4um pore size, tissue culture treated polyester membrane) and polystyrene 24 well plates were from Costar (Corning, NY, USA), while fluorescein isothiocyanate (FITC)-tagged dextran (70 kDa) was from Sigma-Aldrich (Mississauga, ON, Canada). A plasmid encoding a chimeric protein composed of the extracellular domain of VE-cadherin and immunoglobulin Fc (Fc-VE-cadherin) was a gift from Dr. M. Shasby (University of Iowa College of Medicine, Iowa City, Iowa, USA). A FLAG-tagged EPAC1 expression vector was a gift from Dr. X Cheng (University of Texas Medical Branch, Galveston, TX, USA). A GST-β-catenin bacterial expression plasmid was a gift from Dr. L. Mulligan (Queen’s University, Kingston, ON, Canada). Anti- human VE-cadherin (BV6 clone) was purchased from Alexis Biochemicals (San Diego, CA, USA). Anti-β-catenin, anti-PKA-RIIβ, and anti-PKA-C were from Transduction Laboratories (Lexington, KY, USA).
Anti-p120-catenin (6H11) was from Santa-Cruz Biotechnology (Santa Cruz, CA, USA) and anti-PDE3B and anti-PDE4D were generously provided by ICOS Corporation (Bothell, WA, USA). Anti-VE-cadherin (CD144), Anti-FLAG, Phalloidin-TRITC P1951, and 4’,6’-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (Mississauga, ON, Canada). N6-Benzoyleadenosine-3’, 5’-cyclic monophosphate (6-Bz-cAMP) and 8- (4-Chlorophenylthio)-2’-O-methyl-cAMP (8-CPT) were from BioLog (Bremen, Germany). Fibronectin was from Promega (Madison, WI, USA). Ro 20-1724 (Ro), Cilostamide (Cil), and Forskolin (Fsk) were from Calbiochem-Novachem Corporation (Mississauga, ON, Canada). Other chemicals used in these experiments were from ICN Biomedicals Incorporated (Montreal, QC, Canada) or Fisher Scientific (Ottawa, ON, Canada).

2.2 Cell culture

HAECs were cultured in endothelial basal medium (EBM-2) supplemented with endothelial growth media bullet kit (EGM-2) in a 95% air/5% CO₂ humidified environment. Cells were sub-cultured at 1:3 dilutions and when used in experiments had been sub-cultured between 2 and 10 times as described previously (Netherton and Maurice, 2005 and Netherton et al., 2007)
2.3 RNAi transfections

Human vascular endothelial cells were transfected at 80% confluence using lipofectamine 2000 and one of the above siRNAs (2.1), as per manufacturer’s directions. In all experiments, the impact of RNAi in the cells was assessed 48 h post transfection.

2.4 Permeability Assay

HAECs (3.5 x 10^5 cells/mL), control cells or cells transfected with individual siRNA constructs, were seeded and allowed to adhere on the upper surface of fibronectin (5μg/μl)-coated transwells with 0.4 μm pores. After cells had formed contiguous monolayers on the upper chambers of the transwells (18-20 hours), experiments were initiated by the addition of FITC-labelled dextran (500μg/mL) (controls) or FITC-labelled dextran and one of the several test-agents used in our experiments. Permeability of monolayers to FITC-dextran, and the impact of treatments on this permeability, was measured by sampling the lower chamber of the transwell after 1h. Pharmacological agents tested included, Ro 20-1724 (Ro, 10μM, a selective PDE4 inhibitor), Cilostamide (Cil, 3μM , a selective PDE3 inhibitor), Forskolin (Fsk, 0.1-10μM, an activator of adenylyl cyclases), 8-(4-Chlorophenylthio)-2’-O-methyl-cAMP, (8-CPT, 10μM, an EPAC selective activator) or N6-Benzoyladenosine-3’, 5’-cyclic monophosphate (6-Bz-cAMP, 3-30μM a PKA-selective activator). A vehicle control was also used, dimethyl-sulphoxide (DMSO, 0.1% v/v). In some experiments the impact of selective peptides on permeability were studied. In those instances the peptides (1μM final concentration) were added to cells 3 hrs prior to addition of FITC-labelled dextran. Fluorescence was
measured using a Molecular Devices Spectra MAX Gemini X 5 and quantified using SoftMax Pro 4.8 software. All experiments were carried out in triplicate and repeated at least 3 times. Statistical differences were determined by one-way analysis of variance (ANOVA) with Neuman post-hoc test with significance considered at p < 0.05. % barrier function = (average fluorescence reading of controls) – (average fluorescence reading of test agent) / (average fluorescence reading of control) X 100.

2.5 Assay of VE-cadherin-, and actin-based structure dynamics

Control, or HAECs subjected to selective RNAi, were grown to confluence on 20μg/ml fibronectin-coated coverslips and incubated with endothelial growth media supplemented with either DMSO (0.1 %v/v), Ro (10μM), 8-CPT (10μM), 6-BzcAMP (3μM), Fsk (1-10 μM) or combinations of these agents for 20 min. Cells were rinsed twice with PBS (1%) prior to incubation with EGTA (5mM) for 20 min at 37°C in a 5% CO2. Cells were fixed with paraformaldehyde, (4% w/v final) either immediately after drug treatments, following drug treatments and EGTA incubation, or after a 2h post EGTA recovery in 1mM Ca2+.

For VE-cadherin disassembly experiments, VEGF (100ng/ml) was added for 1h after treatment with indicated reagents as above. Cells were stained with anti-human VE-cadherin (BV6 clone) antibody, Phalloidin-TRITC P1951(Sigma-Aldrich. ON, Canada), 4’,6’-diamidino-2-phenylindole - DAPI - (Sigma-Aldrich. ON, Canada). Fluorescent images were acquired using a Zeiss Axiovert S100 microscope. Images were converted to a binary type and skeletonized using Image J. Lengths were analyzed by counting the number of contiguous structures longer than 100 pixels in length.
2.6 Isolation and identification of constituent components of, VE-cadherin-, or β-catenin-based cellular complexes.

Isolation of VE-cadherin-based cellular complexes was carried by adsorption of cell lysate proteins to an immobilized Fc-VE-cadherin chimeric protein on protein A-sepharose. The Fc-VE-cadherin chimeric protein was expressed in 293E cells transiently transfected with a plasmid encoding this protein and purified from conditioned media as described previously (Chen YT and Nelson WJ, 1996). Isolation of β-catenin-based complexes was accomplished using a column of GST-β-catenin which had been immobilized on glutathione (GSH)-sepharose.

- VE-cadherin-based complexes: HAECs were lysed in a Tris (50 mM, pH 7.4)-based lysis buffer supplemented with 1% Triton-X-100, 150 mM sodium chloride, 10 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, 10mM sodium fluoride, 1 µg/ml pepstatin A, 1 µg/ml E-64, 20ug/ml bestatin, 100 µg/ml phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml aprotinin, 1 µg/ml leupeptin, 5 mM benzamidine, 10mM sodium orthovanadate. Prior to addition to immobilized Fc-VE-cadherin, HAEC lysates (1-2mg of protein/ml) were incubated with 100µL (packed volume) of Protein A/G sepharose for 1 h and centrifuged (1000 x g, 5 min) to remove insoluble and non-specific sepharose binding proteins. These pre-cleared HAEC cell lysates were incubated with immobilized Fc-VE-cadherin-beads in the presence of CaCl₂ (2 mM final concentration) on a rotating platform at 4°C for 16-24 h. Following this incubation, beads were collected by centrifugation and extensively washed in the Ca²⁺-containing buffer. Fc-VE-cadherin-binding proteins were eluted from the beads by
incubation with EGTA (20 mM) and subsequently concentrated (~10-fold) by centrifugation at 18 000 x g for 15 min using IEC Centra CL3R centricons.

**β-catenin-based complexes**: HAECs were lysed in a lysis buffer identical to that described above for VE-cadherin-complex isolation, except that TritonX100 was substituted for 0.1% sodium dodecyl sulfate, 1.0% igepl, and 0.5% sodium deoxycholate. Proteins specifically bound to β-catenin were removed from the beads by incubation with an SDS-based electrophoresis loading buffer (Netherton et al., 2007).

Proteins isolated from either of these pull-down assays were resolved by SDS PAGE, transferred to nitrocellulose membranes and immunoblotted for the proteins of interest. The following antisera were used in our studies, anti-VE-cadherin (CD144) (1:1000), anti-β-catenin (1:500), anti-p120 (1:200), anti-PKA-RIIβ (1:500), anti-PKA-C (1:1000), anti-PDE3B (1:4000), anti-PDE4D (1:4000), or anti-FLAG (1:10 000).

### 2.7 Measurements of cAMP PDE activities

cAMP PDE activities in HAEC lysates or in eluates from pulldowns, were determined as described previously by Netherton et al. using 1 μM [³H]cAMP as substrate (Netherton et al., 2007). Family specific PDE activities were determined by inclusion of the family-selective inhibitors Ro, 20−1724 (10 μM, PDE4 inhibitor) or cilostamide (1 μM, PDE3 inhibitor).
2.8 Measurement of cAMP in Cultured HAECs

Basal and drug-induced increases in levels of cAMP in control HAECs, or in siRNA transfected HAECs were measured as described in Palmer et al., 1999.
CHAPTER 3
RESULTS

3.1 Effect of the cAMP-signalling system in regulating HAEC monolayer permeability

Changes in HAEC monolayer permeability were measured upon the addition of various cAMP-elevating agents and activators of cAMP effectors, PKA and EPAC, both alone and in combination by Jeffrey Ovens, a previous student in the laboratory, and have subsequently been reproduced. Specifically, β-adrenergic receptor agonist, isoproterenol (ISO, 0.1-10 μM), adenylyl cyclase (AC) activator, forskolin (Fsk, 1-10 μM), selective PKA-activating cAMP analogue, 6-benzoyladenosine-3’, 5’-cyclic monophosphate (6BzcAMP, 3-30 μM) and selective EPAC-activating cAMP analogue, 8-(4-chlorophenylthio)-2’-O-methyl-cAMP (8CPT, 1-10 μM) were shown to individually reduce the permeability of HAEC monolayers as compared to untreated cells (Figure 3.1A). Inhibition of PDE4 using the PDE4 family-selective inhibitor Ro201724 (Ro, 10 μM) also significantly reduced the permeability of HAEC monolayers. In contrast, inhibition of PDE3 using PDE3-selective inhibitor cilostamide (Cil, 1 μM), which was previously shown to promote adhesion of these cells to fibronectin-coated surfaces (Netherton et al., 2007), did not have a significant effect on HAEC permeability (Figure 3.1A). When drugs were added in combination, Ro potentiated Fsk-mediated reduction of HAEC permeability, but Cil did not (Figure 3.1B), suggesting a leading role for PDE4 in regulating VEC barrier function. However, PDE3 inhibition did further decrease
permeability when added with Fsk and Ro, suggesting some overlap between PDE4- and PDE3-regulated pools of cAMP in these cells (Figure 3.1B).

Selective PKA-, or EPAC-activating, cAMP analogues reduced permeability of HAECs (Figure 3.1A). Thus, incubation of HAECs with 6BzcAMP, or 8CPT, reduced permeability by 16 ± 6%, or 18 ± 4%, respectively. When both agents were added to HAECs they reduced permeability by 49 ± 7% (means ± S.E.M, n=4). Overall, our data were consistent with the idea that some (ISO, Fsk, and Ro), but not all (Cil), agents acting on the cAMP-signaling system reduced human VEC permeability and that PKA and EPAC impact distinct, but complementary, cellular effects.
Figure 3.1A: Impact of cAMP-elevating agents and selective activators of PKA or EPAC, on HAEC Permeability. Impact of Iso (10 μM), Fsk (10μM), 6BzcAMP (3μM), 8CPT (10μM), Ro (10μM), Cil (1μM) on HAEC barrier function (i.e. permeability) was determined by measuring the transit of FITC-dextran across confluent monolayer cultures of HAECs during 1 h incubations (see Materials & Methods). Values are increases in barrier function expressed as means ± S.E.M. of seven experiments conducted in quadruplicate. * indicates a significant difference between the treatment and control (p<0.05). (Ovens, Msc Thesis 2007)
Figure 3.1B: Impact of combinations of cAMP-elevating agents on HAEC Permeability. Impact of Iso (10 μM), Fsk (10μM), 6BzcAMP (3μM), 8CPT (10μM), Ro (10μM), Cil (1μM) on HAEC barrier function (i.e. permeability) was determined by measuring the transit of FITC-dextran across confluent monolayer cultures of HAECs during 1 h incubations (see Materials & Methods). Values are increases in barrier function expressed as means ± S.E.M. of seven experiments conducted in quadruplicate. * indicates a significant difference between the treatment and control (p<0.05) and @ indicates a significant difference between the treatment and forskolin alone (p<0.05). (Ovens, Msc Thesis 2007)
3.2 Effects of PDE4 inhibition on global cAMP levels in HAECs

Increases in cAMP have been shown to be responsible for improved barrier function in VECs (Schlegel et al., 2009). Given the leading role for PDE4 in regulating HAEC permeability, levels of cAMP were measured in HAECs upon treatment with Ro (10μM), Fsk (1 μM or 10 μM) or a combination of Fsk (1 μM) and Ro (10 μM). Interestingly, although Ro (10 μM) significantly reduced permeability in HAECs, only Fsk (10 μM) or a combination of Ro (10 μM) and Fsk (1 μM) significantly increased cAMP levels (Table 3.2), thus showing only a weak correlation between increases in total HAEC cAMP and permeability in our studies. This suggests that relevant changes in cAMP impacting permeability may be taking place in more discreet sub-cellular compartments and are not represented by changes in global levels of this cyclic nucleotide.

<table>
<thead>
<tr>
<th>Additions</th>
<th>[3H]cAMP (% total [3H])</th>
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<tbody>
<tr>
<td>None</td>
<td>0.08 +/- 0.01</td>
</tr>
<tr>
<td>Forskolin (1 uM)</td>
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</tr>
<tr>
<td>Forskolin (10 uM)</td>
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</tr>
<tr>
<td>Ro (10 uM)</td>
<td>0.08 +/- 0.00</td>
</tr>
<tr>
<td>Forskolin (1 uM) + Ro (10 uM)</td>
<td>0.13 +/- 0.00*</td>
</tr>
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</table>

Table 3.2: Effect of cAMP elevating agents on global cAMP in HAECs. Basal cAMP levels and cAMP levels in response to Fsk (1μM or 10μM), Ro (10 μM) or both Fsk (1 μM) and Ro (10 μM) in HAECs are shown. [3H] cAMP values are percentages of total [3H] and individual treatments were performed in quadruplicate in three different experiments. * indicates a significant difference between the treatment and control (p<0.05).
3.3 Impact of PKA, EPAC and PDE4 on the stability of VE-cadherin-, and actin-based, structures in human VECs.

Changes in endothelial permeability are influenced by a combination of two principal mechanisms. The first is dependent on the stabilization or destabilization of VE-cadherin-based complexes at cell membranes and the second is based on actin cytoskeleton dynamics (Vandenbroucke et al., 2008). Since our previous experiments showed that PKA, EPAC and PDE4 each significantly reduced HAEC permeability as compared to controls, the ability of 6BzcAMP, 8CPT or Ro to inhibit, firstly, barrier destabilization through Ca2+ chelation (EGTA 5mM, 20 min) and, secondly, barrier reformation through Ca2+ supplementation (EGM-2, 2hrs) of confluent HAECs monolayers, was visualized using cell staining of VE-cadherin and actin (Figure 3.3A).
Figure 3.3A: *EGTA-induced VE-cadherin internalization and barrier reformation upon Ca2+ supplementation.* Curve demonstrates recycling of VE-cadherin to and from the membrane. This can be observed experimentally by treating cells with EGTA (a Ca2+-chelating agent) for 20 minutes. At this point, cells are rounded up due to lack of adhesion between adjacent cells. EGTA is replaced with normal growth media for 2hrs. During this time VE-cadherin is recycled back to the membrane and cell borders are reformed. Arrow (i) indicates the time point at which cells are in a confluent monolayer, unchallenged by EGTA, arrow (ii) denotes the time point at which HAECs are fixed post EGTA treatment, and arrow (iii) denotes the time point at which HAECs are fixed after Ca2+ re-supplementation with EGM-2. *i, ii and iii* arrows correspond to the panels identified in figures 3.3B-H.
Measurements of VE-cadherin staining of HAEC monolayers treated with 6BzcAMP prior to the addition of EGTA, was not significantly different than control untreated cells (Figure 3.3Bi,Ci). However, in the presence of 8CPT and Ro, VE-cadherin-based structures were protected by 30 ± 8% and 22 ± 4% (mean ± S.E.M., n=4), respectively (Figure 3.3Bi, Di, Hi). When EGTA was added to control cells, the actin cytoskeleton collapsed, promoting cell rounding, and destabilization of VE-cadherin intercellular borders occurred (Figure 3.3B-i,ii). Actin staining showed that both activation of PKA or EPAC with 6BzcAMP or 8CPT respectively attenuated EGTA-mediated cell rounding. However, treatment with 6BzcAMP reduced actin-mediated cell rounding to a greater extent. In contrast, VE-cadherin staining in monolayers treated with 6BzcAMP appeared to be localized primarily around the nucleus whereas 8CPT-treated HAECs exhibited VE-cadherin staining primarily at cell peripheries and was thus able to attenuate EGTA-induced VE-cadherin internalization (Figure 3.3Cii, Dii). Furthermore, simultaneous addition of these two cAMP-analogs additively stabilized both VE-cadherin-, and actin-based, structures in HAECs (Figure 3.3Eii). This is consistent with the idea that PKA and EPAC each regulate separate, but complementary, border-stabilizing effects. A role for each PKA and EPAC in coordinating the border-stabilizing effects of cAMP was confirmed using Fsk which concentration-dependently inhibited the loss of actin- and VE-cadherin-based structures in response to Ca2+ chelation (Figure 3.3Fii, Gii). Consistent with its effect on HAEC permeability (Figure 3.1A), PDE4 inhibition with Ro also attenuated the impact of Ca2+ chelation on actin-, and VE-cadherin-based structures (Figure 3.3Hii). Ro inhibited EGTA-induced loss of contiguous VE-cadherin staining at cell peripheries in a manner similar to 8CPT and also attenuated actin cytoskeleton breakdown as seen with 6BzcAMP. Overall, the result of PDE4
inhibition was comparable to the effects of Fsk (1 μM) (Figure 3.3Fii, Hii). Finally, consistent with PDE4’s role as the major cAMP-PDE regulating cAMP-mediated HAEC permeability, Ro synergized with Fsk (1 μM) (Figure 3.3Iii) to promote stabilization of VE-cadherin-based structures to a level similar to that seen with Fsk (10 μM) (Figure 3.3Gii).

The degree of VE-cadherin border re-establishment in control, 6BzcAMP, 8CPT, Ro and Fsk treated HAECs after EGTA treatment was measured (see Materials and Methods). In control cells, Ca2+ supplementation for 2hrs only promoted reestablishment of 49 ± 12% (mean ± S.E.M., n=5) of the VE-cadherin intercellular staining that was present prior to EGTA treatment. In the presence of 6BzcAMP, barrier re-establishment compared to pre-EGTA VE-cadherin intercellular staining was insignificant as compared to control (52 ± 10%, mean ± S.E.M., n=5). In contrast, treatment with EPAC activator, 8CPT, significantly promoted VE-cadherin reestablishment upon Ca2+ supplementation when compared to control (78 ± 11%, mean ± S.E.M., n=4, p<0.05) (Figure 3.3Biii,Diii) and thus promoted VE-cadherin-mediated barrier formation by ~30% compared to untreated cells.
Figure 3.3B: Effect of EGTA treatment and Ca\textsuperscript{2+} re-supplementation on barrier-stabilizing structures in control HAECs. HAECs grown to confluence on fibronectin-coated coverslips were treated under the indicated conditions as described on the individual panels in the figure and fixed as described in Materials & Methods. VE-cadherin-, and actin-based, structures were visualized following staining for VE-cadherin (BV6 clone) (green), actin (TRITC-phalloidin) (red) and nuclei (DAPI) (blue). Fluorescent images were acquired using a Zeiss Axiovert S100 microscope and processed as described in Materials & Methods. Panel \textit{i} depicts untreated HAECs (no drug and no EGTA). Panel \textit{ii} depicts rounded HAECs after treatment with EGTA for 20 min. Panel \textit{iii} depicts HAEC VE-cadherin and actin re-stabilization after supplementing EGTA treated HAECs with serum containing Ca\textsuperscript{2+}.
**Figure 3.3C:** Effect of EGTA treatment and Ca\(^{2+}\) re-supplementation on barrier-stabilizing structures in 6BzcAMP-treated HAECs. HAECs treated with 6-BzcAMP display attenuated cell rounding compared to control cells in figure 3.3B, yet VE-cadherin is internalized and no longer at cells periphery (panel ii). VE-cadherin-, and actin-based, structures were visualized following staining for VE-cadherin (BV6 clone) (green), actin (TRITC-phalloidin) (red) and nuclei (DAPI) (blue). Fluorescent images were acquired using a Zeiss Axiovert S100 microscope and processed as described in Materials & Methods.

<table>
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<th>Overlay</th>
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<tbody>
<tr>
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</tr>
<tr>
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<td><img src="image9" alt="3uM 6Bz - EGTA 20’ – 2h serum" /></td>
</tr>
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</table>
Figure 3.3D: Effect of EGTA treatment and Ca2+ re-supplementation on barrier-stabilizing structures in 8CPT-treated HAECs. HAECs display attenuated cell rounding (panel ii) as compared to controls in figure 3.3B but to a lesser degree than cells treated with 6-BzcAMP (figure 3.3C). VE-cadherin staining is more pronounced at cell periphery (panel ii) demonstrating attenuated VE-cadherin internalization. Reestablishment of VE-cadherin borders post-Ca2+ supplementation (panel iii) is significantly greater as compared to control HAEC (figure 3.3B). VE-cadherin-, and actin-based, structures were visualized following staining for VE-cadherin (BV6 clone) (green), actin (TRITC-phalloidin) (red) and nuclei (DAPI) (blue). Fluorescent images were acquired using a Zeiss Axiovert S100 microscope and processed as described in Materials & Methods).
Figure 3.3E: Effect of EGTA treatment and Ca2+ re-supplementation on barrier-stabilizing structures in 6BzcAMP and 8CPT-treated HAECs. HAECs treated with 6BzcAMP + 8CPT display both significantly attenuated cell rounding and VE-cadherin internalization (panel ii). VE-cadherin-, and actin-based, structures were visualized following staining for VE-cadherin (BV6 clone) (green), actin (TRITC-phalloidin) (red) and nuclei (DAPI) (blue). Fluorescent images were acquired using a Zeiss Axiovert S100 microscope and processed as described in Materials & Methods.)
**Figure 3.3F:** Effect of EGTA treatment and Ca²⁺ re-supplementation on barrier-stabilizing structures in Fsk (1µM) –treated HAECs. VE-cadherin-, and actin-based, structures were visualized following staining for VE-cadherin (BV6 clone) (green), actin (TRITC-phalloidin) (red) and nuclei (DAPI) (blue). Fluorescent images were acquired using a Zeiss Axiovert S100 microscope and processed as described in Materials & Methods).
Figure 3.3G: Effect of EGTA treatment and Ca$^{2+}$ re-supplementation on barrier-stabilizing structures in Fsk (10μM)–treated HAECs. Phenotype is similar to that seen in 6-BzCAMP + 8CPT drug treatment condition (Figure 3.3E). VE-cadherin-, and actin-based, structures were visualized following staining for VE-cadherin (BV6 clone) (green), actin (TRITC-phalloidin) (red) and nuclei (DAPI) (blue). Fluorescent images were acquired using a Zeiss Axiovert S100 microscope and processed as described in Materials & Methods).
**Figure 3.3H:** Effect of EGTA treatment and Ca2+ re-supplementation on barrier-stabilizing structures in Ro-treated HAECs. Inhibition of PDE4 with Ro shows attenuated cell rounding and VE-cadherin internalization (panel ii). Phenotype is similar to HAECs treated with Fsk (1μM) (Figure 3.3F). VE-cadherin-, and actin-based, structures were visualized following staining for VE-cadherin (BV6 clone) (green), actin (TRITC-phalloidin) (red) and nuclei (DAPI) (blue). Fluorescent images were acquired using a Zeiss Axiovert S100 microscope and processed as described in Materials & Methods).
**Figure 3.3I: Effect of EGTA treatment and Ca2+ re-supplementation on barrier-stabilizing structures in Fsk (1μM) and Ro-treated HAECs.** VE-cadherin-, and actin-based, structures were visualized following staining for VE-cadherin (BV6 clone) (green), actin (TRITC-phalloidin) (red) and nuclei (DAPI) (blue). Fluorescent images were acquired using a Zeiss Axiovert S100 microscope and processed as described in Materials & Methods).
3.4 Impact of PKA, EPAC and PDE4 on VEGF-mediated internalization of VE-cadherin and actin cytoskeleton remodelling

The role of PKA, EPAC and PDE4 on HAEC barrier stability was evaluated through the use of VEGF, a known permeabilizing factor in VECs that induces the internalization of VE-cadherin as well as actin rearrangements (Gavard and Gutkind 2006, Abraham et al., 2009). As expected, decreased levels of contiguous VE-cadherin intercellular staining was observed in control cells when VEGF was added (compare Figure 3.3Bi vs Figure 3.4A). Similarly to when Ca2+ chelation was used to destabilize VE-cadherin-based intercellular structures, 8CPT more effectively antagonized VEGF-induced VE-cadherin fragmentation than 6BzcAMP (Figure 3.4B,C). With respect to PDE4, inhibition with Ro protected intercellular VE-cadherin-based structures against the destabilizing effects of VEGF and potentiated the protective effect of Fsk (1μM) on these structures (Figure 3.4D,E,G).

Overall, these data were consistent with the idea that both PKA and EPAC influenced the integrity of VE-cadherin-, and actin-based, structures in HAECs and that their differential impact on the stability of these structures could allow for their additive effects on VEC permeability. In addition, the marked effects of PDE4 inhibition on the stability of these structures, without significant increases in total cellular levels of cAMP, again, indicate that PDE4 family enzymes likely regulate the HAEC pool of cAMP that activates these two effectors to regulate permeability and that this effect is compartmented.
**Figure 3.4:** Ability of HAECs treated with 6BzcAMP, 8CPT, Ro or Fsk to attenuate VEGF-mediated increases in permeability. HAECs grown to confluence on fibronectin-coated coverslips were treated under the indicated conditions as described on the individual panels. VEGF (100ng/mL) was added after drug-treatment of HAECs for 1hr and fixed as described in Materials & Methods. Staining recapitulates observations in EGTA experiments. VE-cadherin-, and actin-based, structures were visualized following staining for VE-cadherin (BV6 clone) (green), actin (TRITC-phalloidin) (red) and nuclei (DAPI) (blue). Fluorescent images were acquired using a Zeiss Axiovert S100 microscope and processed as described in Materials & Methods.)
3.5 Knock-down of PKA, EPAC or PDE4D in HAECs

To substantiate the effects of PKA, EPAC and PDE4 on HAEC permeability we next used an RNAi-based approach. Before assessing any functional effects on HAECs, appropriate knockdown of each siRNA was confirmed. Immunoblot analysis of HAEC lysates indicated that transfection with individual siRNAs targeting either the catalytic domain of PKA (PRKACA, PKA-Cα), EPAC1, or PDE4D, the dominant PDE4 expressed in these cells (Netherton et al., 2007), robustly and selectively reduced their levels in HAECs (Figure 3.5). Transfection of HAECs with a control siRNA was without effect on levels of the proteins of interest, or on β-actin.
Figure 3.5: Selective RNAi-based knock-down of PKA-Cα, EPAC1 or PDE4D in HAECs. HAECs transfected at 80% confluence with PKA-Cα, EPAC1 or PDE4D siRNAs, or a control siRNA, using lipofectamine 2000 were allowed to grow for 48 h after which they were either directly used for immunoblotting (A) or for permeability experiments (B) as described in Materials & Methods. Protein levels of all relevant targets were significantly reduced.
3.6 Knock-down of PKA, EPAC or PDE4D differentially alter HAEC permeability and barrier-stabilizing structures.

To further identify the effects of PKA, EPAC and PDE4, the permeability of PKA-Cα, EPAC1 and PDE4D siRNA- transfected HAECs were evaluated. Experiments demonstrated that selective knock-down of PKA-Cα, EPAC1 and PDE4D differentially impacted HAEC permeability (Figure 3.6A). PKA-Cα knockdown did not significantly impact HAEC permeability when compared to control cells, however, RNAi-induced knock-down of EPAC1 or of PDE4D increased HAEC permeability by 65 ± 9%, or 49 ± 6%, respectively (means ± S.E.M., n=7). These effects were internally consistent with the relative effects of 6BzcAMP, or 8CPT, on permeability in control HAECs (Figure 3.1A), as well as their abilities to stabilize VE-cadherin-, or actin-based, structures in response to the destabilizing effects of EGTA, or VEGF described previously (Figure 3.3C,D and 3.4B,C). Taken all together, our data suggests EPAC1 as the dominant cAMP-effector involved in coordinating cAMP-regulated permeability in HAECs.

Again, immunofluorescence images of PKA-Cα, EPAC1 and PDE4D knockdown cells were analysed to determine changes in barrier stabilizing structures. Images showed more pronounced VE-cadherin intercellular structures in PKA-Cα knockdown HAECs, but also a modest decrease in cortical actin when compared to negative controls. In contrast, EPAC knockdown cells displayed high levels of intracellular VE-cadherin staining and discontinuous intercellular borders, but also a modest increase in cortical actin (Figure 3.6B). When PKA-Cα and EPAC1 knockdown cells were treated with VEGF, the effects on cell staining were more pronounced (Figure 3.6C). This data is consistent with our previous EGTA experiments in HAECs treated with 6BzcAMP and

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8CPT in which 8CPT was able to attenuate EGTA-induced VE-cadherin internalization to a greater extent than 6BzcAMP. Interestingly, cell staining in PDE4D knockdown cells was very similar to EPAC1 siRNA HAEC. In addition to the decreased intercellular VE-cadherin staining and modest increase in cortical actin, both EPAC and PDE4D phenotypes showed an increase in intercellular “gaps”, and this too was augmented upon VEGF treatment (Figure 3.6B and C).
Figure 3.6A: Effect of selective RNAi-based knockdown of PKA-Cα, EPAC1 or PDE4D on HAEC monolayer permeability. HAECs were transfected at 80% confluence with PKA-Cα, EPAC1 or PDE4D siRNAs, or a control siRNA, using lipofectamine 2000 as described in Materials and Methods. HAEC barrier function (i.e. permeability) was determined by measuring the transit of FITC-dextran across confluent monolayer cultures of HAECs during 1 h incubations (see Materials & Methods). Values are increases in barrier function expressed as percentages of control ± S.E.M. of seven experiments conducted in triplicate. * indicates a significant difference between the treatment and control (p<0.05).
**Figure 3.6B: Effect of selective RNAi-based knockdown of PKA-Cα, EPAC1 or PDE4 on barrier stabilizing structures in HAEC monolayers.** Control, or HAECs subjected to selective RNAi, grown to confluence on fibronectin-coated coverslips and fixed as described in Materials & Methods. VE-cadherin-, and actin-based, structures were visualized following staining for VE-cadherin (BV6 clone) (green), actin (TRITC-phallloidin) (red) and nuclei (DAPI) (blue). Fluorescent images were acquired using a Zeiss Axiovert S100 microscope and processed as described in Materials & Methods.

<table>
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<th>Actin</th>
<th>Overlay</th>
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</tr>
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<td>EPAC1 SiRNA</td>
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<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
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</tbody>
</table>
**Figure 3.6C:** Effect of selective RNAi-based knockdown of PKA-Cα, EPAC1 or PDE4 on barrier stabilizing structures in HAEC monolayers in the presence of VEGF. Control, or HAECs subjected to selective RNAi, grown to confluence on fibronectin-coated coverslips, treated with 100ng/mL VEGF for 1hr and fixed as described in Materials & Methods. VE-cadherin-, and actin-based, structures were visualized following staining for VE-cadherin (BV6 clone) (green), actin (TRITC-phalloidin) (red) and nuclei (DAPI) (blue). Fluorescent images were acquired using a Zeiss Axiovert S100 microscope and processed as described in Materials & Methods.)
3.7 Effects of 6Bz6AMP, 8CPT and Ro on PKA-Cα, EPAC1 and PDE4D knockdown HAECs

Additional analysis of the roles of PKA, EPAC and PDE4 in the regulation of HAEC barrier function was performed by evaluating the effect of 6BzcAMP, 8CPT, Ro or Fsk on HAEC permeability in PKA-Cα, EPAC1 and PDE4D knockdown HAECs. Firstly, in cells with reduced PKA-Cα, treatment with 8CPT, Ro or Fsk significantly reduced permeability in a manner similar to control cells (Figure 3.7A). However, 6BzcAMP had no effect in these cells, as was expected. Next, although 6BzcAMP reduced permeability in control HAECs (Figure 3.1A), this PKA-selective cAMP analogue was ineffective in HAECs expressing reduced levels of EPAC1 (Figure 3.7B). Maximally effective concentrations of Fsk (10μM) showed decreased permeability in EPAC1 knockdown cells, however, its effect was less pronounced compared to cells expressing reduced levels of PKA-Cα. The effect of Ro in EPAC1 siRNA HAECs was similar to control cells and EPAC inhibition with 8CPT was without effect (Figure 3.7B). Finally, in PDE4D knockdown cells, 8CPT was shown to significantly improve barrier function and thus decrease permeability compared to the control. Neither, 6BzcAMP or Ro were able to significantly impact permeability in PDE4D knock-down cells. However, the effect of Fsk on HAEC permeability was markedly increased when compared to the effect of Fsk in PKA-Cα and EPAC1 siRNA HAECs (Figure 3.7C).
Figure 3.7A: Effect of 8CPT, 6BzcAMP, Fsk or Ro on HAECs transfected with PKA-Cα siRNA. HAECs transfected at 80% confluence with PKA-Cα siRNA using lipofectamine 2000 were allowed to grow for 48 h after which they were used for permeability experiments. 8CPT (10μM), 6BzcAMP (3μM), Fsk (10μM), or Ro (10μM) were added in combination with FITC-dextran and permeability was measured after 1hr as described in Materials and Methods. * indicates a significant difference between the treatment and control (p<0.05).
Figure 3.7B: Effect of 8CPT, 6BzcAMP, Fsk or Ro on HAECs transfected with EPAC1 siRNA. HAECs transfected at 80% confluence with EPAC1 siRNA using lipofectamine 2000 were allowed to grow for 48 h after which they were used for permeability experiments. 8CPT (10μM), 6BzcAMP (3μM), Fsk (10μM), or Ro (10μM) were added in combination with FITC-dextran and permeability was measured after 1hr as described in Materials and Methods. * indicates a significant difference between the treatment and control (p<0.05).
Figure 3.7C: Effect of 8CPT, 6BzcAMP, Fsk or Ro on HAECs transfected with PDE4D siRNA. HAECs transfected at 80% confluence with PDE4D siRNA using lipofectamine 2000 were allowed to grow for 48 h after which they were used for permeability experiments. 8CPT (10μM), 6BzcAMP (3μM), Fsk (10μM), or Ro (10μM) were added in combination with FITC-dextran and permeability was measured after 1hr as described in Materials and Methods. * indicates a significant difference between the treatment and control (p<0.05).
3.8 Global cAMP levels in PKA-Cα, EPAC1 and PDE4D knockdown HAECS treated with Ro and Fsk.

Next, cAMP levels in HAECs transfected with PKA-Cα, EPAC1 and PDE4D siRNA were determined to evaluate cellular cAMP fluxes during their response to Ro (10μM), Fsk (1 μM and 10 μM) or a combination of Fsk (1 μM) and Ro (10 μM) treatments. Again, as was the case in control HAECs in section 3.2, the effect of PDE4 inhibition with Ro in the knockdown of PKA-Cα, EPAC1 or PDE4D did not significantly alter global levels of cAMP in HAECs when compared to untreated knockdown controls (Table 3.8). In each case, basal cAMP levels, and the increases caused by incubation with Fsk (1 μM) or Ro (10 μM) were statistically indistinguishable, whereas combinations (Fsk, 1 μM and Ro, 10 μM) and maximal concentrations of Fsk (10 μM) caused increases in global cAMP as seen previously in control cells. However, in PDE4D siRNA HAECs, Fsk (1 μM) was able to significantly increase cAMP levels as well. Again, this suggests that changes observed in barrier function upon drug treatment in siRNA knockdown cells are most likely due to compartmented effects.
<table>
<thead>
<tr>
<th>Conditions</th>
<th>Negative siRNA [(^3)H]cAMP (% total [(^3)H])</th>
<th>PKA-(\alpha) siRNA [(^3)H]cAMP (% total [(^3)H])</th>
<th>EPAC1 siRNA [(^3)H]cAMP (% total [(^3)H])</th>
<th>PDE4D siRNA [(^3)H]cAMP (% total [(^3)H])</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0.06 ± 0.00</td>
<td>0.09 ± 0.00</td>
<td>0.08 ± 0.00</td>
<td>0.09 ± 0.00</td>
</tr>
<tr>
<td>Ro (10 (\mu)M)</td>
<td>0.07 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.10 ± 0.00</td>
</tr>
<tr>
<td>Fsk (1 (\mu)M)</td>
<td>0.07 ± 0.01</td>
<td>0.10 ± 0.00</td>
<td>0.10 ± 0.01</td>
<td>0.12 ± 0.01*</td>
</tr>
<tr>
<td>Fsk (1 (\mu)M)/Ro (10 (\mu)M)</td>
<td>0.11 ± 0.00*</td>
<td>0.15 ± 0.00*</td>
<td>0.13 ± 0.00*</td>
<td>0.13 ± 0.00*</td>
</tr>
<tr>
<td>Fsk (10 (\mu)M)</td>
<td>0.09 ± 0.00*</td>
<td>0.17 ± 0.02*</td>
<td>0.15 ± 0.01*</td>
<td>0.18 ± 0.00*</td>
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</table>

**Table 3.8: Effect of cAMP elevating agents on global cAMP in PKA-\(\alpha\), EPAC1 and PDE4D siRNA HAECs.** Basal cAMP levels and cAMP levels in response to Fsk (1\(\mu\)M or 10\(\mu\)M), Ro (10 \(\mu\)M) or both Fsk (1 \(\mu\)M) and Ro (10 \(\mu\)M) in PKA-\(\alpha\), EPAC1 and PDE4D siRNA HAECs are shown. [\(^3\)H] cAMP values are percentages of total [\(^3\)H] and individual treatments were performed in quadruplicate in three different experiments. * indicates a significant difference between the treatment and control (p<0.05).
3.9 Multiple components of the cAMP-signaling system associate with a VE-cadherin-based macromolecular complex in HAECs.

The pharmacological and RNAi-based studies reported here yielded data consistent with the idea that activation of PKA or EPAC1, and the inhibition of PDE4D, could regulate HAEC permeability and the barrier protecting structures in these cells, independent of global changes in cAMP. In order to assess if PKA, EPAC1 or PDE4D could be acting locally in a VE-cadherin-defined cellular locale, we isolated VE-cadherin-based signaling complexes from confluent HAEC cultures and investigated the possibility that they contained these cAMP-regulated proteins. Interestingly, when analyzed for their component proteins, HAEC-derived VE-cadherin-based cellular complexes contained several of the predicted proteins, including VE-cadherin, β-catenin and p120-catenin, as well as several cAMP-signaling proteins, including the regulatory (RII) and catalytic (C) subunits of PKA, EPAC1 as well as PDE4D (Figure 3.9A and B). In contrast, our analysis failed to identify the presence of PDE3B in the complex (Figure 3.9A and B). Consistent with the immunoblotting data, cAMP PDE assays conducted on the isolated VE-cadherin-based complex contained significant PDE4 inhibitor-, but not PDE3 inhibitor-sensitive, cAMP PDE activity (Table 3.9). Initial identification of VE-cadherin containing complexes were performed by Jeffrey Ovens (Ovens, MSc Thesis 2007) by adsorption chromatography using Fc-VE-cadherin as bait (Figure 3.9A) and similar results were obtained when these complexes were isolated using a completely different bait protein, namely, GST-β-catenin (Figure 3.9B), thus validating the presence of PKA, EPAC and PDE4D but not PDE3B in a VE-cadherin containing macromolecular complex. These findings are consistent with initial permeability assay data (Figure 3.1A)
distinguishing the leading role for PDE4 in regulating barrier function in HAECs as opposed to PDE3.
Figure 3.9A: Fc-VE-cadherin pulldown identifying a VE-cadherin based macromolecular complex containing PKA, EPAC and PDE4D, but not PDE3. Isolation of VE-cadherin-based cellular complexes was carried by adsorption of total cell lysate proteins (T) to an immobilized Fc-VE-cadherin chimeric protein on protein A-sepharose. A fraction of unbound of cell lysate (U) post Fc-VE-cadherin bead incubation is shown. The FC-VE-cadherin chimeric protein was expressed in 293E cells transiently transfected with a plasmid encoding this protein and purified from conditioned media as described previously (Chen YT and Nelson WJ, 1996). (Ovens, Msc Thesis 2007)
**Figure 3.9B:** Validation of VE-cadherin based macromolecular complex using GST-β-catenin pulldown method. Isolation of β-catenin-based complexes was accomplished using a column of GST-β-catenin which had been immobilized on glutathione (GSH)-sepharose as described in Materials and Methods. Total (T) and unbound (U) cell lysates are shown as in Figure 3.9A.
Table 3.9: Testing the presence of Ro- or Cil-sensitive cAMP PDE activity in total HAEC lysates and fc-VE-cadherin-pulldown eluates. PDE assays were performed, as described in Materials and Methods, on fractions of total HAEC lysate and protein specifically bound to fc-VE-cadherin-proteinA sepharose beads. The effects of no drug (none), Ro (10μM) and Cil (1 μM) were assessed to determine the relative contribution of PDE4 and PDE3 in these fractions. Bracketed values denote the percentage of PDE3 or PDE4 activity remaining in untreated fraction after inhibition with either Ro or Cil, respectively. * indicates a significant difference between the treatment and control (p<0.05). (Adapted from Ovens, MSc Thesis 2007)

<table>
<thead>
<tr>
<th>Additions</th>
<th>HAEC Lysate pmol/min/mg protein/(% inhibition)</th>
<th>VECAD-Pulldown pmol/min/μl/(% inhibition)</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>232.1 +/- 8.0</td>
<td>12.8 +/- 0.5</td>
</tr>
<tr>
<td>Ro,20-1724 (10 μM)</td>
<td>67.0 +/- 23.2*/(71)</td>
<td>4.3 +/- 3.0*/(66)</td>
</tr>
<tr>
<td>Cilostamide (1 μM)</td>
<td>155.4 +/- 23.4*/(33)</td>
<td>13.1 +/- 3.5*(0)</td>
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3.10 Effect of EPAC1-PDE4D disrupting peptide on cAMP-regulated HAEC permeability and the VE-cadherin-based barrier protecting cellular structures

Based on our identification of a VE-cadherin-based complex containing PKA (RII and C), EPAC1 and PDE4D, and our findings that EPAC1 and PDE4D exhibit similar effects on HAEC permeability, we subsequently asked whether EPAC1-PDE4D interactions in HAECs could directly influence HAEC permeability and/or the integrity of the barrier protecting structures of these cells. Consistent with the idea that a direct EPAC1-PDE4D interaction allows PDE4D-mediated control of HAEC permeability, incubation of HAECs with an EPAC1-based peptide, but not a scrambled control peptide, reduced HAEC permeability (Figure 3.10A) and protected VE-cadherin-, and actin-based, structures in these cells against the destabilizing effects of VEGF (Figure 3.10B). Both EPAC1-PDE4D disrupting and scrambled control peptides were synthesized and validated in the laboratory of Dr. Miles Houslay at the University of Glasgow and were provided to us through collaborative efforts. As such, incubation of HAECs with the EPAC1-PDE4D disrupting peptide provided similar effect on cellular permeability as HAEC treatment with Ro. To further assess the impact of the EPAC1-PDE4D disrupting peptide on barrier dynamics, its effects on VE-cadherin based complex stability was observed through GST-β-catenin pulldowns. Immunoblotting for PDE4D in HAECs incubated with this disrupting peptide appeared to have no significant effect compared to cells incubated with the scrambled control peptide, suggesting that this peptide does not displace PDE4D from the complex, but may be inhibiting interaction of EPAC and PDE4D from within the complex instead (Figure 3.10C).
Figure 3.10A: Effect of EPAC-PDE4D displacing peptide on HAEC permeability. HAECs were grown to confluence on transwells and pre-treated with EPAC-PDE4D displacing peptide (1μM) or non-displacing peptide (1μM) for 3hrs, then permeability of monolayer to FITC-dextran was assessed after 1hr, as described in Materials and Methods. Values are increases in barrier function expressed as percentages of control ± S.E.M. of three experiments conducted in triplicate.* indicates a significant difference between the treatment and control (p<0.05)
**Figure 3.10B:** Effect of EPAC-PDE4D disrupting peptide on VEGF-induced barrier destabilization. Confluent HAEC monolayers were incubated with EPAC-PDE4D disrupting peptide (1 μM) or negative peptide (1 μM) for 4 hrs and then were fixed and stained, as described in Materials and Methods. VE-cadherin-, and actin-based, structures were visualized following staining for VE-cadherin (BV6 clone) (green), actin (TRITC-phalloidin) (red) and nuclei (DAPI) (blue). Fluorescent images were acquired using a Zeiss Axiovert S100 microscope and processed as described in Materials & Methods.
Figure 3.10C: *Effect of EPAC-PDE4D disrupting peptide on VE-cadherin-based macromolecular complex.* HAECs were grown to confluence and incubated with EPAC-PDE4D disrupting peptide or control scrambled peptide (see Materials and Methods). GST-β-catenin pulldown performed on these cell lysates indicated no apparent disruption of PDE4D in the VE-cadherin-based complex compared to control.
4.1 Summary of findings

cAMP signalling plays an important role in the regulation of VEC permeability. Previously, two important components of this pathway have been identified in maintaining barrier function, namely cAMP effectors, PKA and EPAC (Baumer et al., 2009). Our study is consistent with reports which state that the activation of both PKA and EPAC cause a decrease in VEC permeability, yet there has been much debate surrounding their relative effects. This study further defines the roles of PKA and EPAC in HAECs in terms of their ability to impact barrier function as well as specifically alter two main barrier stabilizing structures, actin and VE-cadherin. Here, our EGTA- and VEGF-based experiments suggest a role for PKA in stabilizing the barrier via the actin cytoskeleton whereas EPAC appears to have a greater effect on VE-cadherin-mediated barrier stability. Collectively, our data suggests a more prominent role for EPAC on permeability as compared to PKA. Furthermore, for the first time, PDE4D was identified as the prominent PDE responsible for regulating permeability in HAEC monolayers, despite approximately equal PDE3 and PDE4 activity in these cells (Netherton and Maurice, 2005). This suggests a role for compartmentation of cAMP signalling in regulating cell-cell adhesion and identifies cAMP hydrolysis as a critical component of this compartmentation. As such, we identified a novel VE-cadherin-containing macromolecular complex which includes cAMP signalling components, PKA, EPAC as well as PDE4D, but not PDE3B, thus providing additional evidence for the leading role of
PDE4 and not PDE3 on barrier function and the importance of both PKA and EPAC as well. Finally, despite significantly reduced permeability in cells treated with PDE4 inhibitor Ro, knockdown of PDE4D in HAECs significantly increased monolayer permeability, thus indicating a potential role for PDE4D as a scaffold in the complex which will be discussed in more detail in the sections to come.

### 4.2 Differential roles for PKA and EPAC in HAEC permeability

Consistent with previous reports, we demonstrated that activation of PKA or EPAC, with 6BzcAMP or 8CPT respectively, improved HAEC barrier function. When added in combination, these two agents enhanced the barrier in a supra-additive manner. Consistently, cell staining of HAECs treated with activators for both cAMP effectors altered barrier function in EGTA and VEGF treatments in a manner similar to that achieved with a maximal concentration of Fsk (10μM). Combined, these data suggest that PKA and EPAC regulate distinct and overlapping pathways to facilitate improved barrier function regulating the formation of cortical actin as well as VE-cadherin-based AJ stability, which PKA and EPAC are known to regulate. HAEC cell staining experiments showed that PKA activator, 6BzcAMP, attenuated EGTA and VEGF-induced barrier destabilization through an effect prominently exerted through actin stabilization. This is consistent with the fact that PKA is a known regulator of actin dynamics through many distinct pathways such as its ability to phosphorylate MLCK to prevent cell contraction (Liu et al., 2001), regulate Rho GTPases which stabilize cortical actin (Qiao et al., 2008, Birukova et al., 2008) or phosphorylate vasodilator-stimulated phosphoprotein (VASP) which directly causes the relaxation of actin cytoskeleton linked to tight junctions and
leads to increased intercellular adhesion (Schlegel et al., 2008). However, 6BzcAMP did not significantly attenuate VE-cadherin destabilization after EGTA and VEGF treatments, suggesting a minimal role for PKA in specifically regulating VE-cadherin assembly at cell borders. Next, activation of EPAC with 8CPT attenuated EGTA-induced cell rounding and stabilized the actin cytoskeleton, but this was less pronounced than in cells treated with 6BzcAMP. 8CPT also significantly reduced VE-cadherin internalization in response to both EGTA and VEGF. These data are also consistent with evidence of cortical actin stabilization by EPAC/Rap1 signalling through Rac and Rho GTPases, as well as the significant impact of EPAC activation on VE-cadherin border stability through Rap1 (Fukuhara et al., 2005, Kooistra et al., 2005, Adamson et al., 2008). Furthermore, a recent study in human pulmonary artery endothelial cells demonstrated that PKA and EPAC/Rap1 signalling can converge through Rac, a Rho GTPase, leading to increased cortical actin and improved AJs upon induction by beraprost, a PGI2 analogue, and PGE2 treatment (Birukova et al., 2007). Although it is evident that PKA and EPAC have overlapping roles, our data suggests that PKA plays a dominant part in actin regulation whereas EPAC is largely involved in VE-cadherin stability and thus mediate complimentary roles in VEC barrier function.

4.3 EPAC plays a dominant role in regulating VEC barrier function

Although we previously established that both PKA and EPAC impact VEC permeability, several experiments herein identify EPAC as the dominant player and are consistent with various other reports (Fukuhara et al., 2005, Kooistra et al., 2005, Cullere et al., 2005). Firstly, siRNA-mediated knockdown of EPAC1 in HAECs significantly
increased permeability, while cells in which PKA-Cα was knocked down exhibited no significant loss of regulated permeability when compared to negative siRNA controls. EPAC1 knockdown-mediated increases in HAEC permeability have also been reported in studies performed in HUVECs (Lorenowicz et al., 2008) and correlate with the fact that EPAC activation in these cells leads to significant decreases in permeability.

Interestingly, although activation of PKA with 6BzcAMP significantly decreased permeability as well, the depletion of PKA-Cα did not increase permeability, as might be expected. This may be in part due to the fact that the regulatory subunits of PKA are still present. While PKA RIIβ is most notably a scaffolding subunit of PKA (Jarnaess et al., 2008), its presence in the VE-cadherin complex identified in our study may indicate that it also has a major scaffolding function in cells, though this has not been investigated.

Additionally, basal global cAMP levels measured in PKA-Cα knockdown cells were significantly higher than those found in cells incubated with the negative control siRNA, an effect that perhaps masked any loss of barrier function in this phenotype. This would suggest, however, that the lack of barrier destabilization in PKA-Cα depleted HAECs is due to EPAC’s ability to rescue barrier function through either PKA-independent, or shared signalling pathways. To support this idea, cell staining of PKA-Cα siRNA transfected HAECs demonstrated significantly greater intercellular VE-cadherin staining, a dominant feature of EPAC signalling, as described in the previous section. It is also possible that barrier function was not affected in PKA-Cα depleted cells due to incomplete (~90%) knockdown whereby the remaining 10% of PKA in the cells might still be modulating barrier function, as normal. To further test this possibility, addition of PKI, a PKA inhibitor, might be added to PKA-Cα depleted cells to determine the effect of complete inactivation of PKA on HAEC permeability.
Secondly, EPAC knockdown cells displayed a substantial increase in permeability compared to controls despite higher basal levels of global cAMP. Still, in spite of normal levels of PKA in EPAC1 depleted HAECs, PKA was unable to maintain barrier function. Furthermore, activation of EPAC with 8CPT in PKA-Cα depleted cells facilitated additional improvements on barrier function, whereas PKA activation in EPAC1 depleted cells had no significant effect. Interestingly however, Lorenowicz et al. performed similar studies in HUVECs where EPAC1 siRNA transfected cells were treated with 6BzcAMP (200μM) and showed restoration of barrier function (Lorenowicz et al., 2008). However, since concentrations of 6BzcAMP used in our experiments (3-30 μM) were more in keeping with the known concentration-dependent selectivity of 6BzcAMP, this discrepancy will need to be further studied. Nonetheless, given that the EPAC1 knockdown phenotype is very leaky, subtle increases in PKA activity may not impact the barrier, but higher doses may. Maximal concentrations of Fsk (10μM) were able to improve barrier function in all knockdown conditions as well, yet this effect was less prominent in EPAC1 knockdown cells. Collectively, this evidence supports the idea that EPAC1 is the dominant cAMP effector in mediating HAEC barrier function.

4.3 A key role for PDE4D in the regulation of VEC barrier function

PDEs are the only known enzymes capable of hydrolyzing intracellular cyclic nucleotides and of terminating their signalling (Maurice et al., 2007). Previous reports have identified PDE3 and PDE4 as the main cAMP hydrolyzing PDEs in HAECs, accounting for approximately 36% and 45% of total cAMP hydrolyzing activity, respectively (Netherton and Maurice, 2005). Interestingly, however, our reports have
shown that inhibition of PDE3 with Cil (1μM) had no significant impact on permeability whereas inhibition of PDE4 with Ro (10μM) showed a significant decrease in permeability when compared to control cells. Furthermore, fc-VE-cadherin and GST-β-catenin pulldown methods both confirmed the presence of PDE4D, and not PDE3B, in a macromolecular complex containing VE-cadherin through immunoblotting and cAMP-hydrolysing PDE activity assays. Interestingly, despite the fact that PDE4 inhibition with Ro increased barrier function in HAECs, RNAi-mediated knockdown of PDE4D, which one would *a priori* hypothesize would increase barrier function, in marked contrast increased permeability to a significant degree when compared to control cells. Notably, knocking down PDE4D levels in the cell is quite different than inhibiting its catalytic activity with competitive small molecule inhibitors. Indeed, while the latter occurs in the continued presence of the enzyme, the former does not. The disparate findings between treatment with Ro and PDE4D siRNA on HAEC permeability suggests that the presence of PDE4D in the cell is structurally important and in fact is consistent with the idea that PDE4D itself serves as a scaffold in these cells. Specific PDE4D isoforms have been documented to be tethered to various scaffolding molecules through their widely varying N-terminal regions, such as PDE4D3 to AKAPs and PDE4D5 to RACK1 or β-arrestin, thus providing a means for compartmentalized and specifically regulated cAMP signalling (Terrenoire *et al.*, 2009, Lynch *et al.*, 2005, Bolger *et al.*, 2006). Although PDE4 isoforms are more commonly known to be tethered to scaffolding molecules, one study in cardiomyocytes identifies PDE4D3 as an adaptor molecule for EPAC (Dodge-Kafka *et al.*, 2005). More recently, a common binding site for EPAC on PDE4 isoforms has been identified, suggesting that PDE4D isoforms in general may be able to act as scaffolds, as discussed in a review by Houslay *et al.*, 2007. Our data is also consistent
with these reports as not only is the presence of PDE4D essential to barrier function, but when levels of EPAC1 are reduced, permeability is impacted in an identical manner. Furthermore, cell staining of PDE4D and EPAC1 siRNA transfected cells display similar phenotypes consisting of poor VE-cadherin membrane staining and the presence of intercellular “gaps”. Finally, the identification of a VE-cadherin-based complex containing both PDE4D and EPAC further supports the idea that PDE4D interacts with and may be structurally important for EPAC. Taken all together, our data is the first to identify PDE4D as the major regulatory PDE in regulating barrier function and provides novel evidence for the role of PDE4D acting as an adaptor molecule (figure 4.3).

**Figure 4.3:** Schematic of VE-cadherin-based macromolecular complex. Our studies have shown that VE-cadherin interacts with PKA, EPAC and PDE4D. Based on our studies, we predict that without PDE4D, EPAC cannot be recruited to the complex to take part in VEC intercellular adhesion, whereby PDE4D acts as a scaffold for EPAC in the complex.
4.4 Specific VEC barrier regulation though compartmentation of cAMP signalling molecules.

In recent years, evidence for the subcellular localization of PDEs in regulating distinct pools of cAMP has grown and the idea of compartmentation has become more widely accepted (Lynch et al., 2005, Maurice et al., 2007, Vandecasteele et al., 2008). Similarly, our study identifies a role for PDE4D in the localized regulation of cAMP pools responsible for VEC barrier function. Firstly, we have demonstrated that inhibition of PDE4 with Ro significantly increases barrier function, but does not change global cAMP levels (Netherton and Maurice, 2005). This suggests that cAMP changes upon PDE4 inhibition must be taking place in distinct intracellular domains within cells. Our identification of a novel macromolecular complex containing VE-cadherin, its known associated partners, β-catenin and p120 and the cAMP signalling proteins, EPAC1, PKA- Ca and -RIIβ and PDE4D, provide additional evidence for localized cAMP regulation of PKA and EPAC1 by PDE4D in a localized pool with VE-cadherin at the membrane. Consistently, several studies have demonstrated the ability of PDE4 to form a cAMP diffusion barrier localized at the cell membrane (Barnes et al., 2005, Rich et al., 2007, Creighton et al., 2008) and thus its identification with membrane-bound VE-cadherin, reported here, is perhaps unsurprising. Additionally, similarities in cell staining of HAECs treated with Ro and Fsk (1μM) are thus expected as subtle increases in global cAMP by Fsk could presumably be mimicked by localized increases in cAMP surrounding the membrane to activate PKA and EPAC by Ro and directly influence permeability.
The absence of PDE3B in the complex is consistent with reports that demonstrate a lack of co-localization between PDE3B and PDE4D. Instead, PDE3B was shown to appear in punctuate structures throughout the cytosol and participate in cell adhesion to the extracellular matrix (Netherton et al., 2007). Differences in function and localization of these two enzymes, despite similar cAMP-hydrolyzing activity shown here and in previous reports, further demonstrate the importance of cAMP signal compartmentation through the use of PDEs to impart distinct and specific responses in cells. However, the role of PDE3 may indirectly impact permeability by inhibiting intracellular pools of cAMP from reaching the membrane. Thus, in the event of large influxes of cAMP, the distinction between these pools may overlap as we saw when cells are treated with Fsk, Cil and Ro combined.

The presence of complexes in HEK 293T cells containing either PKA or EPAC, but not both, has been reported previously in our lab. Additionally, PKA was shown to preferentially bind to PDE4D and EPAC to PDE3B (Netherton et al., 2007). Although our identified complex containing both PKA and EPAC may be an exception to the rule, it is possible that there are in fact two pools of VE-cadherin, one which associates with PKA and the other with EPAC, but both containing PDE4D. Given the higher affinity of PKA for cAMP as compared to EPAC (Das et al., 2009), their abilities to regulate differential pools of VE-cadherin might offer more sensitive and time-dependent regulation of permeability, enabling different levels of intercellular adhesion depending on the strength and length of stimuli provided. Another possibility is that PKA associates with the VE-cadherin complex which is regulated by PDE4D. Consistently, studies have identified a binding site on cadherins for AKAPs (Gorski et al., 2005), suggesting that PKA may be tethered to VE-cadherin in this manner. Pools of EPAC, however, which
may preferentially bind PDE3B, can be recruited by PDE4D, as we provided evidence for previously, and act in VE-cadherin based complexes under specific stimuli which maintain or increase intercellular adhesion. Otherwise, EPAC may bind PDE3B in the cytosol under basal levels or states requiring increased cellular adhesion to the extracellular matrix, for example, thus providing an avenue for dynamic crosstalk between different systems in the cell. In fact, many reports have identified a dynamic interplay between AJs and integrins in the regulation of more complex cellular processes such as migration, where increases in cell-ECM adhesion and decreases in cell-cell adhesion have been shown to increase migration (Nelson et al., 2004, Tan et al., 2008).

4.5 Future studies

a) Identify the specific PDE4D isoform present in VE-cadherin based macromolecular complex.

In our studies, immunoblot analysis of PDE4D in the VE-cadherin-based complex gave rise to two immunoreactive bands. The most prominent, top band at ~115kDa represents the presence of PDE4D5 and/or PDE4D7 isoform variants in the complex and the lower band at ~95kDa represents PDE4D3, PDE4D8 and/or PDE4D9 (Richter et al., 2005). By narrowing down the specific PDE4D variants present, we may be able to elucidate the specific function that this PDE is playing in the VE-cadherin complex. As described earlier, PDE isoforms vary widely, yet their conservation over evolutionary time suggests important non-redundant roles in distinct cellular processes. As such, PDEs are excellent targets for therapeutic drugs as they are highly specialized (Lugnier, 2006).
In the context of VEC permeability, elucidating the particular PDE4 variant involved herein might similarly provide excellent pharmacological targets for pathologies involving vascular leakage such as edema and the metastasis of cancer cells.

b) **Further investigate a role for PDE4D as an EPAC1 adaptor protein in VE-cadherin complex.**

The studies herein present ample evidence suggesting a role for PDE4D as a scaffold for EPAC1, however molecular evidence has yet to be provided. To do so, identification of a VE-cadherin complex in PDE4D depleted HAECs may further characterise PDE4D-EPAC1 interactions. As such, in a VE-cadherin complex lacking PDE4D, one would predict the absence of EPAC1 as well. Unfortunately, there is currently no EPAC1 antibody able to detect endogenous levels of EPAC in HAECs. An alternative approach may be to use a fluorescence resonance energy transfer (FRET)-based analysis to further explore PDE4D-EPAC1 interactions.

c) **Explore roles for PKA and PDE4D5 in VEGF-induced VE-cadherin internalization.**

As described previously, studies have shown that VEGF reduces the expression of VE-cadherin and β-catenin at the cell membrane. VEGF binds VEGFR-2 which activates Rac, a small GTPase, through Src-dependent phosphorylation of guanine exchange factor (GEF), Vav-2. Activated Rac promotes p21-activated kinase (PAK)-mediated
phosphorylation of VE-cadherin. Next, β-arrestin2 is recruited to the site of phosphorylation and this promotes internalization of VE-cadherin into clathrin-coated vesicles. (Gavard and Gutkind, 2006) β-arrestin2 is also a known mediator of β2AR internalization. Furthermore, β-arrestin2 has been identified as a scaffolding molecule for PDE4D5 and is important in the desensitization of β2AR signalling (Lynch et al., 2005). Interestingly, the novel VE-cadherin-containing complex described in our studies contains PDE4D as well. As such, it is possible that the PDE4D in the novel complex described earlier interacts with β-arrestin2 in a similar manner to β2AR internalization. Additionally, PKA, another component of the macromolecular complex, is also known to interact with PAK whereby PKA phosphorylates PAK and inactivates it (Howe and Juliano, 2000). In this case, is it possible that PKA inhibits PAK phosphorylation of VE-cadherin, and consistent with previous data, stabilizes the barrier while preventing VE-cadherin internalization. To elucidate how PKA and PDE4D act to coordinate PAK-induced effects on VE-cadherin internalization, HAECs may be transfected with a series of PAK expressing constructs and determine the efficiency with which cAMP-elevating agents alter their effects on VE-cadherin internalization. For these experiments, we have obtained myc-tagged wild type PAK1 (wt PAK), as well as constitutively active and “kinase-dead” PAK expression constructs. Differences in VE-cadherin internalization of PAK-expressing cells can be visualized by immunofluorescence. Similarly, the levels of PKA or PDE4D will be reduced using siRNA-based methods in PAK-expressing HAECs and VE-cadherin internalization can visualized by immunofluorescence to elucidate their roles in PAK-dependent internalization.
REFERENCES


Ovens J., Protein Kinase A, Exchange Protein Activated by cAMP1 and Phosphodiesterase 4D all associate with VE-cadherin to Regulate Endothelial Barrier Function. 2005-2007 (M.Sc. Thesis, Queen’s University, 2007)


APPENDIX

Figure I: GST-β-catenin pulldown on negative and PDE4D siRNA transfected HAEC lysate. Pulldown shows that in PDE4D depleted HAECs, EPAC1 is no longer present in the complex, providing evidence for PDE4D as a scaffold for EPAC1. This data was obtained after the date of submission for MSc defense.