Emerging themes of cAMP regulation of the pulmonary endothelial barrier

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Sayner SL. Emerging themes of cAMP regulation of the pulmonary endothelial barrier. Am J Physiol Lung Cell Mol Physiol 300: L667–L678, 2011. First published February 18, 2011; doi:10.1152/ajplung.00433.2010.—The presence of excess fluid in the interstitium and air spaces of the lung presents severe restrictions to gas exchange. The pulmonary endothelial barrier regulates the flux of fluid and plasma proteins from the vascular space into the underlying tissue. The integrity of this endothelial barrier is dynamically regulated by transitions in cAMP (3′,5′-cyclic adenosine monophosphate), which are synthesized in discrete subcellular compartments. Cyclic AMP generated in the subplasma membrane compartment acts through PKA and Epac (exchange protein directly activated by cAMP) to tighten cell adhesions, strengthen cortical actin, reduce actomyosin contraction, and decrease permeability. Confining cAMP within the subplasma membrane space is critical to its barrier-protective properties. When cAMP escapes the near membrane compartment and gains access to the cytosolic compartment, or when soluble adenylyl cyclases generate cAMP within the cytosolic compartment, this second messenger activates established cytosolic cAMP signaling cascades to perturb the endothelial barrier through PKA-mediated disruption of microtubules. Thus the concept of cAMP compartmentalization in endothelial barrier regulation is gaining momentum and new possibilities are being unveiled for cytosolic cAMP signaling with the emergence of the bicarbonate-regulated mammalian soluble adenylyl cyclase (sAC or AC10).

Emerging Concepts of cAMP Signaling

In 1957, while studying adrenaline regulation of glycogen metabolism, Sutherland and coworkers isolated a heat-stable factor generated in membrane fractions of liver homogenates. Subsequently, the factor was identified as cAMP (150). These seminal findings led to the emergence of the second messenger concept: extracellular hormone stimulation, adrenaline, generates an intracellular mediator, cAMP (149). Currently, cAMP has been implicated in the regulation of many physiological responses and cellular processes, including modification of the immune response, cardiac contractility, cell cycle control, and endothelial barrier regulation. Specificity of the physiological response was linked to hormone-specific receptor expression on the particular tissue or cell. However, it soon became apparent that individual cells express multiple hormone-specific adenylyl cyclase-linked receptors, combinations of G protein subunits, and several adenylyl cyclase isoforms to catalyze cAMP production. The finding that two different agonists generated a cAMP signal yet imparted a different physiological response gave rise to the idea that cAMP signals are spatially segregated into isolated subcellular compartments within a single cell (72, 79, 118, 170). Thus, although our interpretation of cAMP signaling specificity began at the tissue level, we now consider cAMP signaling specificity at the subcellular compartment level.

In 1968, Krebs identified a kinase whose activity was dependent on cAMP, thus providing a mechanism whereby cAMP exerted its cellular actions; cAMP-dependent protein kinase (PKA) provided a link between stimulation of adenylyl cyclase and activation of downstream effectors (162). Yet PKA does not account for all physiological effects of cAMP. In the search to explain PKA-independent cAMP signals, exchange protein directly activated by cAMP (Epac) was identified, which acts as a cAMP-activated guanine nucleotide exchange factor (cAMP-GEF) for Rap1 and Rap2 (36, 80). Whereas downstream functions of cAMP are mediated through PKA and Epac, temporal changes in cAMP are modulated by phosphodiesterase activity, which hydrolyzes cAMP to 5′-AMP halting activation of cAMP effectors (29, 95, 148).

Although cAMP is a highly hydrophobic second messenger with the potential to rapidly diffuse throughout the cytosolic environment creating a homogenous signal, probes that track the spatial migration of cAMP suggest that it is unevenly distributed and concentrates in microdomains (41, 107, 112, 120, 121, 178). It is possible that the perimeters of the microdomains are lined by obstacles, such as the endoplasmic reticulum, to physically block cAMP diffusion, and phosphodiesterases, which hydrolyze cAMP before it escapes the microdomain (120). Thus barricades encircling the microdomain restrict cAMP diffusion and create a subcellular compartment. This nonuniform rise in cAMP creates gradients, with elevated near membrane cAMP in close proximity to the adenylyl
cAMP IN THE PULMONARY ENDOTHELIUM

The pulmonary vasculature is lined by a nonfenestrated endothelium characterized by its continuous basement membrane and closely apposed, caveolae-rich endothelial cells. The endothelial monolayer has multiple homeostatic functions including acting as semipermeable barrier. In quiescent cells, this barrier functions as a molecular sieve permitting diffusion of low molecular weight molecules across the vessel wall and into the underlying tissue via paracellular transport, whereas larger molecules, such as proteins, traverse the endothelial barrier via the transcellular pathway in the process of transcytosis (101, 109). Multiple agonists perturb the normal homeostasis of the barrier, expanding the repertoire of molecules and volume of fluid that can gain access to the underlying tissue. The route of increased permeability is primarily through the formation of intercellular gaps or paracellular transport (46, 101, 102). Endothelial hyperpermeability leads to flooding of the underlying tissue and alveoli leading to life-threatening pulmonary edema due to hindered gas exchange. This review addresses cAMP signals that regulate the paracellular permeability pathway.

Endothelial barrier integrity is determined by endothelial cell shape, which was described by the tensility model as a balance between competing forces: barrier-protective adhesive forces impart centrifugal tension whereas barrier-disruptive antiadhesive forces provide centripetal tension (77). Adhesive forces are dependent on transmembrane cell adhesion molecules that anchor adjacent cells to each other as well as anchoring cells into the underlying matrix. To perform their adhesive function and maintain endothelial barrier integrity, adhesion molecules are tethered into the cortical actin cytoskeleton by adaptor molecules, such as α-, β-, and γ-catenin, plakoglobin, and α-actinin, which, together with p120, form junctional complexes (37, 167). This cortical rim (or dense peripheral band) appears as the cell monolayer reaches confluence and is composed of F-actin bundles stabilized by actin-binding proteins (171). Spatially, the dense peripheral band lies just beneath the spectrin cytoskeleton and circumnavigates the entire cell (114). In contrast to adhesive forces, barrier-disruptive, nonadhesive forces arise when cortical actin filaments disperse and stress fibers, extending throughout the cytoplasm, become more prominent. Actomyosin contraction of these stress fibers increases centripetal tension and is proposed to physically pull adjacent cells apart, changing cell shape and reducing endothelial barrier integrity (46, 105). Other cytoskeletal elements, such as microtubules, communicate with actin; thus microtubule dynamics also play an integral role in endothelial cell shape and vascular integrity (19, 91, 114, 160).

Barrier-protective or disruptive agents adjust the balance of adhesive vs. nonadhesive forces influencing endothelial cell shape and paracellular transport. In a quiescent endothelium and in endothelial monolayers with enhanced barrier properties, actin filaments distribute primarily in cortical actin with few stress fibers (10, 19, 114, 166). In contrast, barrier-disruptive agonists shift the balance of forces to reduced tethering of adhesion molecules, promote actin reorganization into stress fibers, and induce actin-myosin contraction leading to cell shape changes. Indeed, disruption of junctional complexes, stress fiber formation, and actomyosin contraction are hallmarks of endothelial barrier disruption. These events lead to retraction of cell-cell borders that permits the paracellular movement of fluid and macromolecules from the blood into the underlying tissue. Agents that reduce actomyosin contraction, stabilize cortical actin, and strengthen cell adhesions complexes improve the endothelial barrier integrity and/or facilitate repair. Multiple reports document that subplasma membrane cAMP, acting via PKA or Epac, plays a key role in modulating cytoskeletal dynamics and cell adhesions to improve endothelial barrier integrity (46, 103, 104, 114). Indeed, the use of PKA inhibitors (e.g., RpcAMPs) demonstrated the role of PKA in maintenance of basal endothelial monolayers as well as attenuating agonist-induced hyperpermeability (110, 147).

Pulmonary Edema and the cAMP Connection

The pathogenesis of edema associated with inflammation was of keen interest during the nineteenth century. In 1873, J. Cohnheim reported that blood vessels could become leaky during inflammation (as summarized in Ref. 97). Following his isolation of histamine from the fungus, ergot, Sir Henry Dale (35) revealed that histamine induced a “loss of blood volume owing to morbid permeability of the endothelium.” Over time, the concept that increased vascular permeability could initiate edema became more widely accepted. A few years after Sutherland and Rall’s description of cAMP (150), Majno and Palade (97) demonstrated histamine and serotonin induced vascular hyperpermeability involved “numerous endothelial openings…in blood vessels” and cellular contraction. They explained, “…endothelial leak was a result of endothelial cells [that] become partially disconnected.” It is now widely accepted that edema can be mediated by increased vascular permeability resulting from interendothelial cell gaps, which are generated by loss of cell adhesions, cytoskeletal contraction, and endothelial cell retraction (101, 114).

Within the lung, R. T. H. Laennec described the complications of edema as “an infiltration of serum into the pulmonary tissue, carried to a degree such that it significantly diminishes its permeability to air” (as summarized in Ref. 144). Indeed, the pathogenesis of pulmonary edema, provoked by various insults such as bacterial induced sepsis, air embolism, acid injury, and inflammatory mediators, is a multifactorial disease including increased pulmonary vascular permeability (144, 145, 164). Agents that increase cAMP, such as isoproterenol, prostaglandin, forskolin, and cholera toxin, attenuate edema in injury, and inflammatory mediators, is a multifactorial disease.

The Pulmonary Endothelial Barrier

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Targets of Barrier-Protective cAMP Mediated by PKA

In certain models of endothelial barrier disruption actomyosin contraction is triggered by phosphorylation of the regulatory subunit of myosin light chain (MLC) (46). Indeed, both thrombin and histamine elevate MLC phosphorylation, which is reversed by elevated cAMP (60, 104). When phosphorylated, MLC interacts with actin to promote cross-bridge cycling and tension development. In turn, the level of MLC phosphorylation is a balance between phosphorylation by calcium-calmodulin-activated MLC kinase (MLCK) and dephosphorylation by MLC phosphatase (Fig. 1) (161, 173). Thus decreasing MLCK activity, increasing MLC phosphatase activity, or easing MLC phosphatase inhibition can attenuate endothelial cell contraction and alleviate endothelial barrier disruption (51, 60). As the signaling molecules implicated in regulation of MLC phosphorylation were elucidated, they became potential targets to explain the barrier-protective roles of cAMP signaling.

The nonmuscle MLCK isoform identified in endothelial cells by Garcia et al. (61) exhibits a unique amino terminus and multiple conserved phosphorylation sequences including a conserved PKA phosphorylation sequence adjacent to the calcium-calmodulin binding region. Treatment of bovine pulmonary artery endothelial cells with cholera toxin, to elevate cAMP levels, increased MLCK phosphorylation with associated decrease in kinase activity (61). Subsequent studies using phosphatase inhibitors supported that MLCK phosphorylation reduced its kinase activity although the specific kinase was not identified (160). In bovine pulmonary artery endothelial cells, loss of PKA activity by RpcAMPS or overexpression of the PKA peptide inhibitor (PKI) promoted stress fiber formation and decreased electrical resistance across the monolayer, implicating PKA in cytoskeletal reorganization necessary to maintain baseline barrier integrity (110). However, in these studies PKA inhibition failed to increase MLC phosphorylation under basal or thrombin treated conditions, suggesting that PKA does not play a role in regulating MLCK activity, yet the possibility still remains for other endothelial cell types or models of elevated permeability (94, 110).

As stated above, another mechanism to reduce MLC phosphorylation and decrease contraction is to promote MLC dephosphorylation. MLC phosphatase is a trimeric molecule composed of a regulatory/targeting subunit, MYPT1, a catalytic subunit, PP1, and a subunit of unknown function, M20. PKA signaling modulates MLC phosphatase activity through the regulatory and catalytic subunits. First, Rho kinase phosphorylation of the regulatory subunit, MYPT1, inhibits MLC phosphatase activity. The small GTPase, Rho, which relays signals for cytoskeletal reorganization, activates Rho kinase. In its active GTP-bound state, Rho translocates to the membrane where it activates Rho kinase, whereas in the inactive GDP-bound state it is sequestered in the cytoplasm. The level of active Rho is a balance between Rho-guanine nucleotide exchange factors (Rho-GEFs) and Rho-GTPase activating proteins (Rho-GAPs). Rho-GEFs activate Rho by promoting GDP for GTP exchange, whereas Rho-GAPs catalyze the slow intrinsic GTPase activity of Rho hydrolyzing Rho-GTP to inactive Rho-GDP. In addition, Rho-GDI (guanosine nucleo-
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Filamin expression at the plasma membrane fluctuates (163). Filamin, an actin binding protein, is linked to the actin cytoskeleton. Its phosphorylation by PKA at serine 2152, lies within the protein-binding domain (106, 163). Loss of filamin is embryonically lethal with dramatic decreases in endothelial barrier permeability (106). PKA-dependent filamin phosphorylation increases RhoA phosphorylation at serine 188 and corresponds to decreased RhoA activity, decreased MLC phosphorylation, and protection against thionin-induced hyperpermeability (65). Another level of PKA barrier protection comes through Rho-GDI phosphorylation, which negatively regulates RhoA activity (115); however, the significance of PKA-mediated Rho-GDI phosphorylation in regulation of the endothelial barrier has not been resolved. Thus PKA phosphorylation negatively regulates Rho through direct phosphorylation of Rho or its inhibitor, Rho-GDI. PKA phosphorylation renders Rho unable to activate Rho kinase thereby improving MLC phosphatase activity and attenuating MLC phosphorylation (65, 116). In addition to MLC phosphatase, Rho kinase targets Lim kinase, which subsequently phosphorylates and inactivates the actin binding protein, cofilin. In its active unphosphorylated state, cofilin promotes actin depolymerization and F-actin severing. Currently, how active, unphosphorylated cofilin affects the actin cytoskeleton related to endothelial barrier dysfunction is not clear (65). Thus there are multiple implications of PKA-induced inhibition of Rho and downstream Rho kinase activity, including release of MLC phosphatase inhibition to promote MLC dephosphorylation and attenuate actomyosin stress fiber contraction. Collectively, cAMP-PKA-mediated inhibition of Rho promotes protection against endothelial barrier disruption via inactivation of the contractile machinery.

A second mechanism whereby cAMP-PKA signaling modulates MLC phosphatase activity is through regulation of the catalytic subunit, PP1, by CPI-17 (PKC-potentiated inhibitory protein for PP1 of 17 kDa). Following phosphorylation, CPI-17 forms an inhibitory complex with PP1. Thrombin- and histamine-induced hyperpermeability is mediated in part by phosphorylation of CPI-17 (84), whereas forskolin activation of adenyl cyclase activity promotes CPI-17 dephosphorylation, loss of CPI-17-PP1 complex formation, and improved PP1 activity (5). Thus elevated cAMP-PKA activity releases MLC phosphatase from its inhibitor to decrease MLC phosphorylation and promote endothelial cell relaxation leading to endothelial barrier enhancement.

Stabilization of cortical actin by actin binding proteins attenuates agonist induced endothelial barrier disruption. The actin binding protein filamin facilitates high-angle F-actin branching and localizes to cell-cell borders in endothelial cells (106, 163). Loss of filamin is embryonically lethal with dramatic increases in vascular permeability (55). Through its protein-binding domain, filamin binds transmembrane proteins, such as integrins, thus anchoring membrane-tethered proteins into the cortical actin rim (56). The PKA phosphorylation site, serine 2152, lies within the protein-binding domain (78). Filamin expression at the plasma membrane fluctuates with the level of endothelial monolayer confluence, with greatest membrane distribution at confluence and increased cytosolic expression in pre- and postconfluent monolayers (163). Barrier-disruptive agonists such as bradykinin and hydrogen peroxide induce filamin translocation into the cytosol. However, when pretreated with cAMP-PKA stimulating agents, which protect against agonist-induced barrier disruption, filamin translocation is prevented (69, 163). Furthermore, increasing subplasma membrane cAMP with isoproterenol increases filamin phosphorylation at a consensus PKA phosphorylation site, serine 2152, while decreasing subplasma membrane cAMP with thrombin decreases phosphorylation at this site (27, 129, 168). Thus cAMP-PKA retains filamin at the periphery and protects against endothelial barrier disruption.

Vasodilator-stimulated phosphoprotein (VASP) is also an actin filament-associated protein involved in regulation of actin dynamics, cell adhesions, cell migration, and regulation of the endothelial permeability (73, 132). VASP is a target for PKA, PKG, and AMP kinase phosphorylation at serine 157, serine 239, and threonine 278, respectively (18, 66, 141). VASP localizes to stress fibers, cell-cell contacts, and focal adhesions by its NH2- and COOH-terminal Ena/VASP homology domains, which includes threonine 278, whereas the central proline-rich region has been linked to F-actin polymerization and includes serine 157 and serine 239 residues (28). PKA phosphorylation of VASP at serine 157 has been implicated in adenosine-mediated endothelial barrier protection (28) and attenuation of LPS hyperpermeability by transelectrical resistance measurements (20). PKA-induced VASP phosphorylation attenuates LPS-induced VASP translocation from the cell periphery to focal adhesions and suppresses stress fiber formation (20). The mechanisms of PKA-induced VASP endothelial protection have not been fully resolved, yet emerging evidence suggests that VASP is involved in cAMP-mediated activation of the small GTPase, Rac (131–133).

Not all permeability-inducing agonists promote stress fiber formation. Although MLCK-mediated phosphorylation of MLC. Indeed, phorbol 12-myristate 13-acetate (PMA) activates a PKC-Raf-1 pathway leading to ERK activation and phosphorylation of the regulatory cytoskeletal protein, caldesmon, which is involved in restructuring the actin cytoskeleton (46, 94). Elevations in cAMP attenuate the PMA-induced permeability and gap formation. Furthermore, PKA inhibitors (PKI and Rp-cAMP) transiently activate Raf-1 and ERK to promote caldesmon phosphorylation that was inhibited by the upstream ERK kinase, MEK. Thus these data suggest a role for cAMP-PKA in restricting caldesmon phosphorylation to enhance endothelial barrier integrity, although the target of PKA has not been resolved (94).

The microtubule cytoskeleton is tightly linked to endothelial permeability, such that disassembly of peripheral microtubules increases endothelial permeability whereas microtubule stabilization attenuates permeability (11, 142, 159). Indeed, treatment with microtubule-disrupting, anticancer vinca alkaloids leads to sudden pulmonary edema (24). During TNF-α-induced endothelial cell barrier injury, disruption of microtubules precedes actin cytoskeletal reorganization suggesting interplay between the cytoskeletal elements (111). Microtubules originate at the microtubule-organizing center where they have the greatest density of fibers, with lower fiber density as they emanate toward the cell periphery (142). Functionally, microtubule filaments stabilize cell shape, transport intracellular membrane-bound organelles and act as storage sites for signaling proteins, such as the Rho family GTPases. For example, GEF-H1 becomes active upon release from microtubules whereby it promotes Rho activation and increases MLC...
phosphorylation and actin–myosin contraction (Fig. 1) (8). Thus GEF-H1 provides cross talk between microtubule dynamics, actomyosin contraction, and endothelial barrier disruption (8, 14, 87, 157). However, under these experimental conditions, forskolin- or cholera toxin-mediated stimulation of PKA activity attenuates nocodazole-induced stress fiber formation and increase in transelectrical resistance (14).

Thus the second messenger cAMP signals through PKA-mediated phosphorylation of RhoA, RhinoGDI, and CPI-17 to attenuate actomyosin contraction and endothelial tension whereas phosphorylation of filamin and VASP stabilizes cortical actin. Furthermore, PKA-mediated regulation of RhoA activity also modulates coflin. Currently, it is unclear whether all these phosphorylation events occur simultaneously or independently to attenuate agonist-induced endothelial barrier disruption. For example, subcellular compartments could separate some events from others such that only a subset of PKA targets is activated at any one time.

**Targets of Barrier-Protective cAMP Mediated by Epac**

In 1998, Epac was identified as a novel cAMP target that directly activates Rap1 and Rap2, small GTPases of the Ras superfamily, and the only downstream effectors of Epac that have currently been identified (36, 80). Rap1 had been shown to suppress Ras oncogenic transformation by maintenance of cell adhesions (81). By utilizing the Epac-specific agonist 8-pCPT-2′-O-Me-cAMP (also known as 007), the barrier-protective properties of Epac-mediated Rap1 activation have been linked to improved cell-cell contacts through enhanced VE-cadherin expression at cell-cell borders, enrichment of cortical actin, and decreased RhoA activation (1, 32, 59, 85) and explained cAMP-mediated, PKA-independent events that regulate vascular permeability (21, 64, 122). Currently, the mechanisms of Epac-Rap1 enhanced VE-cadherin accumulation at cell-cell borders and improved adhesive properties are unresolved, although recent studies suggest that forskolin-inhibited stabilization of VE-cadherin to cortical actin bundles is dependent on α- and β-catenin (108). Indeed, Epac has been shown to form a complex at cell-cell junctions with phosphodiesterase 4, VE-cadherin, and β-catenin to regulate endothelial permeability (119). Furthermore, Epac localizes to the plasma membrane, nuclear membrane, mitochondria, and microtubules (96, 117). In human umbilical vein endothelial cells, Epac association with microtubules has been linked to microtubule growth in a Rap1-independent but AKAP9-dependent manner (137, 138). Although a second Epac isoform 2 has been identified, Epac 1 is the isoform abundant in blood vessels (64).

Atrial natriuretic peptide (ANP), PGE2, PGJ2, isoproterenol, and forskolin in combination with the phosphodiesterase 4 inhibitor rolipram (or forskolin and the pan phosphodiesterase inhibitor, IBMX) elevate subplasma membrane cAMP to activate Epac-GEF function in the endothelium (7, 16, 17, 32, 59). Although Epac promotes Rap1 activation through GDP for GTP exchange, GAPS, such as Spa-1, enhance intrinsic GTP to GDP hydrolysis and inactivate Rap1 (88) (Fig. 2). A growing body of evidence implicates Epac in reduced baseline endothelial permeability as well as attenuated thrombin (7, 32, 85), PAF (1) and ventilator-induced lung injury (VILI) (13) hyperpermeability in a Rap1-dependent manner. In turn, Rap1 activates the Rac-specific GEFs, Vav2 and Tiam1, promoting Rac and Cdc42 activation (17). Rac-GTP acts on downstream targets, p21-activated kinase 1 (PAK1), p190-Rho-GAP and cortactin, to enhance endothelial barrier integrity (15). The Rac downstream target, PAK1, inhibits p115Rho-GEF, thus repressing RhoA activity (9, 123). In fibroblasts, Rac also stimulates p190Rho-GAP to further promote Rho downregulation and attenuate actomyosin contraction (169). Indeed, PGE2, PGJ2, and ANP induced human pulmonary artery or microvascular endothelial barrier enhancement and protection from thrombin-induced hyperpermeability is mediated through Rap-Vav2 and -Tiam1 activation of Rac (13, 16, 17). Loss of any of these signaling molecules leads to diminished Rac and PAK1 activation, whereas small interfering RNA (siRNA) to either Rap1 or Rac leads to loss of PGE2- and iloprost-induced VE-cadherin recruitment to cell-cell borders, whereas small interfering RNA (siRNA) to either Rap1 or Rac leads to loss of PGE2- and iloprost-induced VE-cadherin recruitment to cell-cell borders (13, 17). The PGJ2 analog, iloprost, acts through a similar cAMP activated Epac-Rap1 pathway to protect against VILI (13). Using the Rac and Cdc42 agonist, CNF-1, Waschke et al. (165) demonstrated Rac and Cdc42 enhanced cortical actin by recruiting cortactin and VASP and reduced PAF-mediated increase in hydraulic conductivity in perfused mesenteric microvessels. IQGAP, which lacks GTPase activity, binds and stabilizes Rac and Cdc42 in their active GTP-bound form (22, 68). Furthermore, Rac and Cdc42 binding, prohibits IQGAP from binding β-catenin. Thus Rac and Cdc42 strengthen cell-cell junctions by release of β-catenin from IQGAP, promoting β-catenin association with junctional complexes.

A recently identified target of Epac-Rap1 signaling is Krit1 (Krevl interaction trapped gene), a protein lost in the endothelial junction disease cerebral cavernous malformations (45).
Active Rap1 enhances Krit1 association with β-catenin at cell-cell junctions and Krit1 expression at junctions is essential for Rap1 barrier stabilization (63). Krit1 depletion abolishes Epac-mediated protection against thrombin hyperpermeability (63).

Recently, ANP has been implicated in protection against thrombin hyperpermeability. ANP elevates cAMP to activate both Epac-Rap1 and PKA signaling cascades leading to decreased MLC phosphorylation (16). Subsequent studies reveal Rac integrates cAMP-Epac and -PKA-mediated endothelial barrier protection (12). Thus, although Epac and PKA events are described independently here, their actions are intertwined. Both cAMP effectors modulate similar pathways and indeed both effectors can be integrated into the same subcellular complexes. However, the signaling events described above implicate cAMP in improved barrier integrity, yet cAMP has similarly been linked to endothelial barrier disruption.

**Bacterial Soluble Adenylyl Cyclases Disrupt the Endothelial Barrier**

It is well known that microorganisms are a risk factor for endothelial cell dysfunction (91). Early reports describe a toxin produced by the bacterium *Bacillus anthracis*, which increases endothelial permeability (31, 140, 143). This edema-inducing agent, later named edema factor, is a calcium- and calmodulin-dependent adenylyl cyclase, which elevates intracellular cAMP when delivered into eukaryotic cells, increases endothelial permeability, and impairs host immunity (4, 25, 92). The cytoplasmic actions of edema factors are mediated through both PKA and Epac to alter the cytoskeleton and cell shape, leading to cell rounding (75, 76). Although transmembrane adenylyl cyclase activity generates cAMP gradients with decreasing concentrations away from the subplasma membrane space toward the cell center, edema factor-cAMP was focused around the cell center and diminished toward the cell periphery (33, 34). Thus reversing the endogenous cAMP gradient by localizing adenylyl cyclase to the cytosolic compartment leads to edema.

Other bacteria have evolved strategies to transfer exotoxins into eukaryotic cells, such as the type three-secretion system (T3SS) of *Pseudomonas aeruginosa* (176). The T3SS uses an injection type needle to deliver exotoxins across bacterial and host membrane barriers into the cytosol of eukaryotic cells (70). *P. aeruginosa* is a ubiquitous pathological bacterium associated with acute infections in patients with sepsis, in postoperative and burn patients, and in ventilator-associated pneumonia and cystic fibrosis patients (49). Strains of *P. aeruginosa* expressing a functional T3SS are correlated with severity of disease, increased mortality, poor disease outcomes in patients with ventilator-associated pneumonia, and bacterial persistence (47, 71, 124, 158). In general, 89% of strains that possess the T3SS have the *exoy* gene, which encodes for ExoY, a soluble adenylyl cyclase (53, 177). To avoid unregulated cAMP production within the pathogen, ExoY is stimulated by an as yet unknown soluble eukaryotic factor (177). ExoY lacks membrane localization domains expressed on other exotoxin proteins, and in pulmonary microvascular endothelial cells it is excluded from the subplasma membrane space but localizes to the cytosolic compartment (38, 86, 130). Thus T3SS-competent, *exoY*-encoded *P. aeruginosa* deliver ExoY directly into the cytosol of infected cells where it manipulates the host cellular machinery to elevate cytosolic cAMP concentrations (130, 156, 177). This cytosolic cAMP pool leads to endothelial cell gaps as cell-cell borders retract and increases permeability in the isolated lung (126, 130). However, a criticism of these studies was that the genetically engineered strains of *P. aeruginosa* used to deliver ExoY permitted the accumulation of nonphysiological levels of cAMP. Toward this end, we exploited a forskolin-sensitive mammalian adenylyl cyclase chimera (sACI/II), which generated significantly lower cytosolic cAMP levels compared with plasma membrane cAMP levels (128).

The central cytosolic loop and carboxy terminus of eukaryotic transmembrane adenylyl cyclase merge to create the catalytic domain of the enzyme. The catalytic domains of adenylyl cyclase I and II have been excised from the membrane-spanning regions and linked to form a functional forskolin-sensitive, soluble adenylyl cyclase chimera (sACI/II) (40, 151). Forskolin stimulation of pulmonary endothelial cells expressing the sACI/II chimera activates not only sACI/II but also transmembrane adenylyl cyclase, thus elevating cAMP in both the cytosolic and membrane compartments (128). These elevations in cAMP provoke retraction of cell-cell borders and endothelial cell gaps despite simultaneous production of barrier-protective subplasma membrane cAMP. Thus even low levels of cytosolic cAMP are sufficient to disrupt the endothelial barrier and, indeed, this minimal cytosolic cAMP overwhelms barrier-protective plasmalemma cAMP. Cytosolic cAMP barrier disruption appears to be mediated through reorganization of microtubules.

Microtubule-associated proteins (MAPs) bind and stabilize microtubule structures and provide docking sites for enzymes (155). Indeed, MAPs promote microtubule assembly from tubulin subunits and create a more stable structure that resists disassembly (39, 44). In turn, phosphorylation...
events regulate the association of MAPs with microtubules. Nonneuronal tau is a MAP expressed in pulmonary endothelium with a PKA phosphorylation target at serine 214 (30, 152). In the phosphoserine 214 configuration, tau dissociates from microtubules and promotes microtubule disassembly (98, 136, 155). Although forskolin activation of transmembrane adenylyl cyclase did not phosphorylate tau at serine 214, the cytosolic cAMP generated by either ExoY or sAC/II increased tau phosphoserine 214 (30, 113, 129) (Fig. 3). In contrast, whereas isoproterenol increased filamin phosphorylation at serine 212, ExoY did not, demonstrating that cytosolic cAMP cannot traverse into the near membrane compartment (129). Thus tau is a target of cytosolic cAMP, which initiates microtubule disruption and endothelial barrier disruption. Surprisingly, microtubule disruption did not instigate remodeling of actin filaments into stress fibers as has been described for other mechanisms of microtubule-related endothelial barrier disruption, suggesting the possibility of two independent mechanisms of microtubule induced endothelial barrier disruption (113).

The inability of transmembrane adenylyl cyclase activity to induce tau phosphorylation suggests that spatially restrictive cAMP compartments exist within the pulmonary endothelium. cAMP elevations in each distinct compartment are linked to endothelial barrier-protective (membrane cAMP) or disruptive outcomes (cytosolic cAMP). Phosphodiesterases play a key role in defining cAMP signaling microdomains functioning as a barricade to prevent cAMP diffusion between near plasma membrane and cytosolic compartments and shaping intracellular cAMP gradients (58, 120, 121). Interestingly, in pulmonary endothelial cells, phosphodiesterase 4D4 is anchored to the cell periphery through its association with the spectrin membrane skeleton. By use of a dominant-negative peptide to inhibit phosphodiesterase 4D4 activity, forskolin stimulation of plasmalemma adenylyl cyclase induced PKA-mediated tau serine 214 phosphorylation that was accompanied by microtubule reorganization and endothelial barrier disruption (30). Interestingly, these effects were analogous to the barrier-disruptive effects of soluble ExoY or sAC/II adenylyl cyclase activity. When tau serine 214 is mutated to alanine, loss of phosphodiesterase D4D activity failed to reorganize microtubules and disrupt the barrier (179). Thus, in pulmonary endothelium, phosphodiesterase 4D4 localizes to the subplasma membrane space to spatially limit migration of the cAMP signal into the cytosol. Furthermore, cytosolic cAMP leads to phosphorylation of tau and decreased endothelial barrier integrity (30, 179).

Thus accumulating evidence supports the idea that subplasma membrane cAMP is critically retained within near membrane compartments to maintain the endothelial barrier. Once cAMP escapes into the cytosolic compartment, it activates cytosolic cAMP signaling cascades that disrupt the barrier. These observations raise several intriguing questions. First, if cAMP is highly compartmentalized to the subplasma membrane compartment, then why are cAMP targets localized to the cytosol? Indeed, the cAMP signaling machinery for microtubule-mediated endothelial barrier disruption is in place, but the endogenous cAMP source to activate these targets has not been defined. Second, these findings bring into question the impact of the endogenous soluble adenylyl cyclase (sAC or AC10) on endothelial permeability.

Emerging Story of Endogenous Soluble Adenylyl Cyclase Isoform 10

Unlike transmembrane adenylyl cyclases, sAC lacks transmembrane domains and is expressed in the cytosol (Fig. 4). sAC is insensitive to G protein signaling and forskolin, yet activated by physiological transitions in bicarbonate, which is potentiated by calcium (26, 67, 93, 154). In contrast to membrane localization, sAC localizes to discrete compartments in the cell such as microtubules, mitochondria, the microtubule-organizing center, and the nucleus (54, 62, 174, 180, 181). Expression of sAC was initially found in sperm, yet distribution in somatic tissues is now evident (139). Currently, sAC activity has been implicated in biological processes such as sperm motility, cilia beat frequency, apoptosis, regulation of the electron transport chain, cell migration, and gene transcription (50, 74, 134, 135, 175). Thus sAC is involved in regulation of microtubule functions, i.e., sperm motility and cilia beat frequency, providing the possibility that bacterial adenylyl cyclase hijack the endogenous sAC signaling systems to regulate endothelial microtubule dynamics.

We have identified sAC in pulmonary microvascular endothelial cells where it generates a bicarbonate-sensitive cAMP pool. Unlike the ExoY- and forskolin-stimulated sAC/II-cAMP pool, the sAC-cAMP pool is regulated by phosphodiesterase 4. Furthermore, this sAC-cAMP pool appears to increase endothelial permeability. These data have been published in abstract form (127). The bicarbonate transitions that induce increased permeability are relevant to bicarbonate therapy in the management of ventilator induced hypercapnic acidosis. The ARDSnet (http://www.arl.org/clinicalnetwork/) suggests sodium bicarbonate infusion to correct acidosis when pH falls below pH 7.3 (3, 83, 158a). Yet within the clinic bicarbonate infusion to correct acidosis is controversial, with some reports suggesting it actually worsens outcomes (89, 153). The role of bicarbonate-stimulated sAC activity in this setting is currently under investigation.
cAMP IN THE PULMONARY ENDOTHELIUM

Summary

Understanding of the mechanisms of cAMP regulation of the endothelial barrier has evolved significantly over the last 15–20 years. The emergence of the new cAMP effector, Epac, has recruited many new players into regulation of endothelial barrier. Facilitated by the development of PKA vs. Epac selective analogs, these two cAMP signaling branches have emerged independently only to finally be reunited through common effectors. Yet, still new players are emerging. From the organ level to the tissue level and now to the cellular level, all aspects of cAMP signaling are highly compartmentalized such that the physiological and cellular response to cAMP depends on where it is made. This has been eloquently demonstrated within the endothelium, where plasma membrane cAMP protects the endothelial barrier whereas cytosol cAMP disrupts the barrier. To facilitate signaling specificity, cAMP signaling platforms are localized throughout the cellular environment and are not restricted to the plasma membrane. Thus it appears that the signaling components are already in place for cytosolic cAMP production. One of the future challenges to resolve the physiological function of compartmentalized cAMP signals is to understand the cytosolic bicarbonate-stimulated sAC.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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