Control of cAMP in lung endothelial cell phenotypes. Implications for control of barrier function

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Stevens, Troy, Judy Creighton, and W. Joseph Thompson. Control of cAMP in lung endothelial cell phenotypes. Implications for control of barrier function. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L119–L126, 1999.—Pulmonary microvascular endothelial cells (PMVECs) form a more restrictive barrier to macromolecular flux than pulmonary arterial endothelial cells (PAECs); however, the mechanisms responsible for this intrinsic feature of PMVECs are unknown. Because cAMP improves endothelial barrier function, we hypothesized that differences in enzyme regulation of cAMP synthesis and/or degradation uniquely establish an elevated content in PMVECs. PMVECs possessed 20% higher basal cAMP concentrations than did PAECs; however, increased content was accompanied by 93% lower ATP-to-cAMP conversion rates. In PMVECs, responsiveness to β-adrenergic agonist (isoproterenol) or direct adenyl cyclase (forskolin) activation was attenuated and responsiveness to phosphodiesterase inhibition (rolipram) was increased compared with those in PAECs. Although both types of endothelial cells express calcium-inhibited adenyl cyclase, constitutive PMVEC cAMP accumulation was not inhibited by physiological rises in cytosolic calcium, whereas PAEC cAMP accumulation was inhibited 30% by calcium. Increasing either PMVEC calcium entry by maximal activation of store-operated calcium entry or ATP-to-cAMP conversion with rolipram unmasked calcium inhibition of adenyl cyclase. These data indicate that suppressed calcium entry and low ATP-to-cAMP conversion intrinsically influence calcium sensitivity. Adenyl cyclase-to-cAMP phosphodiesterase ratios regulate cAMP at elevated levels compared with PAECs, which likely contribute to enhanced microvascular barrier function.

adenyl cyclase; phosphodiesterase; signal transduction; calcium; pulmonary edema

MORPHOLOGICAL AND BIOPHYSICAL assessment of microvessels illustrates that endothelial cells form a semipermeable barrier to fluid and protein transudation in the noninflamed lung that limits accumulation in interstitial spaces (44). However, lung inflammation is associated with endothelial cell disruption that causes fluid and protein accumulation in the underlying tissue. Inflammation can be compartmentalized to either extraalveolar or alveolar vessels (7, 26). Stimuli such as microthrombi and neurohumoral inflammatory mediators increase pulmonary macrovascular permeability, accompanied by perivascular cuffing (7, 46). Fluid, protein, and inflammatory infiltrates present in perivascular (arterial and venular) cuffs may be cleared through lymphatics without directly impacting the functional gas-exchange area. However, stimuli such as endotoxin- and oxidant-mediated lung injury from ischemia and reperfusion or activated neutrophils increase pulmonary microvascular permeability, accompanied by interstitial edema in the lung parenchyma (6, 18, 24, 25). Such accumulation of fluid, protein, and inflammatory infiltrates directly impacts the functional gas-exchange area, resulting, in severe cases, in refractory hypoxemia.

Because refractory hypoxemia represents a clinical complication lacking effective therapy (5), significant emphasis has recently been placed on elucidating the cellular and molecular mechanisms governing the pulmonary microvascular endothelial cell (PMVEC) compared with the pulmonary arterial endothelial cell (PAEC) response to inflammatory stimuli (27). Constitutive protein flux is greatly attenuated in PMVECs compared with that in PAECs (11, 12, 20, 34, 36). This enhanced barrier property is associated with increased expression of focal adhesion complexes that promote cell-cell tethering (36). Furthermore, whereas PAECs respond to inflammatory calcium agonists with a decrease in cell-cell and cell-matrix tethering and an increase in centripetal tension that produces intercellular gaps and protein permeability (14, 23, 27), PMVECs exhibit neither a visible change in surface morphology nor an increase in macromolecular permeability (7, 20). Thus, compared with conduit endothelial cells, PMVECs express a unique phenotype characterized by increased cell-cell and cell-matrix tethering and decreased centripetal tension development.

Given this unique phenotype of PMVECs, important questions remain as to what intracellular signals regulate focal adhesion complexes that govern enhanced barrier properties. cAMP, by activating protein kinase A, directly impacts focal adhesion complex formation (1, 15–17, 31, 35, 48). Elevated cAMP promotes cell-cell and cell-matrix association, ostensibly by phosphorylation of key complex-associated proteins (1, 15–17, 31, 35, 48). Therefore, increases in cAMP may account for enhanced barrier properties of PMVECs. Also, strategies aimed at elevating cAMP may reduce inflammatory permeability by promoting cell-cell contact (2, 8, 27, 28, 37, 41). Current studies were therefore undertaken to determine whether PMVECs possess elevated cAMP content compared with that in PAECs and to critically evaluate the mechanisms regulating cAMP production and hydrolysis in the two cell types.

METHODS

Isolation and culture of lung endothelial cells. Main stem PAECs were isolated from the pulmonary truncus as previ-
ously described (26). Briefly, male Sprague-Dawley rats (CD strain, 350–400 g; Charles River) were anesthetized with pentobarbital sodium (50 mg intraperitoneally), a sternotomy was performed, and the heart and lungs were excised en bloc. The pulmonary artery was isolated, cut, and inverted, and the heart and lungs were excised en bloc. Experiments were routinely performed with a physiological salt solution containing physiological relevant extracellular calcium concentrations unless otherwise noted and pH balanced to 7.4, with osmolality equal to 285–305 mosM. Agonists were added for 5 min, the cells were washed and DMEM, and the cells were solubilized (reactions were stopped) with 1 M NaOH. After assessment of cAMP concentrations, the results were standardized to cell counts (10^6 cells). Analysis of dose-response curves was performed with Prism software utilizing nonlinear regression (“Equation 1”). Derivatives were evaluated with Richardson’s method.

Measurement of cAMP content. Assessement of cAMP content was performed with a standard radiomimnoassay (Immunolog, Stoughton, MA). The cells were seeded onto 2-cm^2 24-well plates, PAECs at 150,000 cells/ml and PMVECs at 40,000 cells/ml, and grown to confluency over 3–4 days. Experiments were conducted with DMEM with physiological extracellular calcium concentrations unless otherwise noted and pH balanced to 7.4, with osmolality equal to 285–305 mosM. Agonists were added for 5 min, the cells were washed and DMEM, and the cells were solubilized (reactions were stopped) with 1 M NaOH. After assessment of cAMP concentrations, the results were standardized to cell counts (10^6 cells). Analysis of dose-response curves was performed with Prism software utilizing nonlinear regression (“Equation 1”). Derivatives were evaluated with Richardson’s method.

Measurement of ATP-to-cAMP conversion. ATP-to-cAMP conversion was performed as described previously by Kelly et al. (19). Briefly, the cells were seeded onto 35-mm six-well plates, PAECs at 150,000 cells/ml and PMVECs at 40,000 cells/ml, and grown to confluence over 3–4 days. Experiments were conducted in DMEM with physiological extracellular calcium concentrations unless otherwise noted and pH balanced to 7.4, with osmolality equal to 285–305 mosM. The cells were incubated with 2 µl of [3H]adenine at a concentration of 2.0 µCi/ml for 1 h at 37°C to radiolabel the ATP pool for measurement of ATP-to-cAMP conversion. [3H]adenine was aspirated, and the cells were rinsed with DMEM. Agonists were added at the concentrations and for the time periods indicated. Fifteen microliters of [14C]at a concentration of 0.01 mCi/ml was diluted in 100 ml of 5% ice-cold TCA, and 1 ml was added to stop the reactions. The wells were scraped, samples were collected, and the wells were washed with 200 µl of distilled H2O (dH2O). Two hundred microliters of sample were transferred to a scintillation vial containing 6 ml of scintillation fluid to evaluate total [3H]adenine and [14C] counts. The remaining samples were centrifuged for 5 min, and the supernatants were poured over Dowex columns. The Dowex columns were then washed with 4 ml of dH2O to remove ATP from the column and then rewashed with 4 ml of dH2O to transfer cAMP onto alumina. To elute cAMP from alumina, 4 ml of 0.1 M imidazole (pH 7.1) were poured over the columns. Effluent containing cAMP was collected into scintillation vials, and 6 ml of scintillation fluid were added to the vials. Counts from purified samples were standardized to total counts. These studies were designed to evaluate the independent and combined effects of adenylyl cyclase and phosphodiesterase activity on cAMP accumulation. Studies of adenylyl cyclase turnover rates are not reported because our studies did not completely inhibit either phosphodiesterase activity or cAMP extrusion. Thus the value of percent ATP-to-cAMP conversion represents an estimate of cAMP accumulation.

Assessment of cytosolic calcium concentration. Cells were seeded onto glass coverslips and grown to confluence as described in Measurement of cAMP content were loaded for 20 min with 3 µM Ca2+-sensitive fluorophore fura 2-AM (Molecular Probes, Eugene, OR) and 10% pluronic acid. The cells were rinsed with a physiological salt solution and incubated in a CO2-controlled incubator (37°C) to allow fluorophore deesterification. The cells were again washed and utilized to evaluate cytosolic calcium concentration ([Ca2+]i) as previously described (26). Experiments were routinely performed with a physiological salt solution containing physiological relevant extracellular calcium concentrations unless otherwise noted.

RESULTS

Constitutive pulmonary endothelial cell cAMP. Constitutive PMVEC and PAEC cAMP contents were assessed in six independent experiments. cAMP content was 20% higher in PMVECs than in PAECs (Fig. 1). Intact cell prelabeling was used to probe the mechanisms responsible for elevated cAMP content in PMVECs. cAMP conversion studies showed that constitutive ATP-to-cAMP conversion in PMVECs was 7% of the conversion rate seen in PAECs.

Stimulation of adenylyl cyclase. Stimulation of cAMP accumulation was tested with the nonselective β-adrenergic agonist isoproterenol over a range of doses. Intrinsinc activity of isoproterenol was higher in PAECs than...
in PMVECs, although the sensitivity to isoproterenol was greater in PMVECs (EC\textsubscript{50} 73 nM; \(R^2 = 0.97\)) than in PAECs (EC\textsubscript{50} = 3 \text{ M}; \(R^2 = 0.89\); Fig. 2). To determine whether increased agonist stimulation in PAECs was an intrinsic feature of the adenylyl cyclase complex, studies were repeated in response to the direct adenylyl cyclase activator forskolin. Intrinsic activity was increased in PAECs compared with that in PMVECs, whereas sensitivity was greater in PMVECs (EC\textsubscript{50} = 200 nM; \(R^2 = 0.97\)) compared with that in PAECs (EC\textsubscript{50} = 9.6 \text{ \mu M}; \(R^2 = 0.97\); Fig. 3). Therefore, the data indicate that PMVEC \(\beta\)-adrenergic and direct adenylyl cyclase stimulation of cAMP production is suppressed. Further experiments were conducted to determine whether suppression was due to an intrinsic feature of the adenylyl cyclases or an enhanced activity of phosphodiesterases.

Inhibition of phosphodiesterase. PMVECs have been shown to express predominantly phosphodiesterase 4 family isoforms absent of cGMP-hydrolyzing activities (27). Therefore, regulation of PAEC versus PMVEC cAMP accumulation was studied with rolipram over a range of doses. The intrinsic activity of rolipram was increased in PMVECs compared with that in PAECs, whereas the sensitivity was greater in PAECs (EC\textsubscript{50} = 230 nM; \(R^2 = 0.99\)) than in PMVECs (EC\textsubscript{50} = 1.7 \text{ \mu M}; \(R^2 = 0.99\); Fig. 4). These data indicated that low constitutive and reduced \(\beta\)-adrenergic- and adenylyl cyclase-stimulated cAMP accumulation in PMVECs could be due to high phosphodiesterase 4 activity.

To address this issue, forskolin stimulation of cAMP content was assessed after rolipram treatment of the cells to inhibit phosphodiesterase 4 activity in PMVECs and PAECs. Rolipram synergistically increased forskolin responses in both cell types (\(P < 0.05\) vs. Figs. 3 and 4). The intrinsic activity of forskolin was similar among the cells, although the sensitivity to forskolin was greater in PAECs (EC\textsubscript{50} = 3.2 \text{ \mu M}; \(R^2 = 0.96\)) than in PMVECs (EC\textsubscript{50} = 30 \text{ \mu M}; \(R^2 = 0.99\); Fig. 5). Thus whereas stimulation of cAMP content in PAECs is principally regulated by receptor-coupled G protein activation of the adenylyl cyclase activity, stimulation of cAMP accumulation in PMVECs is more effectively regulated by phosphodiesterase 4 inhibition.

Calcium regulation of adenylyl cyclase and cAMP. Stevens and colleagues (40, 41) have previously established that physiological rises in [Ca\textsuperscript{2+}], especially due to activation of store-operated calcium entry, inhibit PAEC adenylyl cyclase activity and reduce global cellular cAMP content. To assess the regulation of PMVEC adenylyl cyclase activity by calcium, thapsigargin was administered to both cell types to activate store-operated calcium entry, and ATP-to-cAMP conversion was measured. Activation of store-operated calcium entry inhibited cellular cAMP, ranging from 20 to 50% in PAECs in six separate experiments, but had no discernable effect on cAMP in PMVECs (Fig. 6).

The inability of calcium to regulate PMVEC cAMP could be due to the insensitivity of adenylyl cyclase to calcium, a reduced calcium response to thapsigargin, or an intrinsic feature of the activation state of adenylyl cyclase. A prior study by Stevens et al. (40) indicated that PMVECs express type VI calcium-inhibited adenylyl cyclase. We therefore next tested whether store-operated calcium entry was suppressed in rat PMVECs compared with that in PAECs. Thapsigargin was applied over a range of doses to measure the [Ca\textsuperscript{2+}]i, especially due to inhibition of store-operated calcium entry. A maximal response to isoproterenol was higher in PAECs than in PMVECs (\(n = 4\) experiments). *Significant difference between cell types at corresponding dose, \(P < 0.05\).
Responsiveness and sensitivity to thapsigargin were suppressed in PMVECs (EC\textsubscript{50} = 65 nM; R\textsuperscript{2} = 0.85) compared with those in PAECs (EC\textsubscript{50} = 31 nM; R\textsuperscript{2} = 0.95; Fig. 7). Thus it was possible that decreased calcium entry accounted for the inability of thapsigargin to decrease constitutive ATP-to-cAMP conversion in PMVECs.

To further address this issue, thapsigargin was added to PAECs and PMVECs in the presence of 100 nM extracellular calcium, and then extracellular calcium was replenished. On readdition of extracellular calcium, this protocol represented an efficient manner of increasing [Ca\textsuperscript{2+}]\textsubscript{i} in PMVECs to equal to or greater than that observed in PAECs (Fig. 8). Sensitivity to the readdition of extracellular calcium was similar in both cell types (PMVECs: EC\textsubscript{50} = 260 µM, R\textsuperscript{2} = 0.94; PAECs: EC\textsubscript{50} = 195 µM, R\textsuperscript{2} = 0.94). With the use of this protocol to equilibrate the [Ca\textsuperscript{2+}]\textsubscript{i} responses to thapsigargin, activation of store-operated calcium entry decreased constitutive cAMP content, ranging from 18 to 35% in PMVECs (Fig. 9), indicating that reduced store-operated calcium entry uncouples calcium from regulation of adenylyl cyclase activity in PMVECs.

Because constitutive ATP-to-cAMP conversion in PMVECs is only 7% of that observed in PAECs and adenylyl cyclase sensitivity to regulation by cytosolic mediators depends on the activation state of the enzyme, we next examined whether low constitutive turnover contributed to insensitivity of adenylyl cyclase to calcium. Rolipram was applied to increase ATP-to-cAMP conversion, and then the cells were challenged with thapsigargin. Inhibition of phosphodiesterase 4 activity increased the ATP-to-cAMP conversion in PMVECs, and unmasked calcium inhibition of adenylyl cyclase (Fig. 10).

**DISCUSSION**

Although collectively lung endothelium forms a semipermeable barrier to fluid and macromolecules, extra-alveolar endothelial cells are phenotypically distinct from alveolar endothelial cells. When compared with PAECs, PMVECs are constitutively less permeable and resistant to agonist-evoked macromolecular permeability largely due to increased expression of cell-cell adhesion proteins (11, 12, 20, 34, 36). Intracellular signal transduction mechanisms responsible for the maintenance of enhanced focal adhesion complexes regulating cell-cell adhesion proteins in PMVECs are unknown, although focal adhesion complex formation is promoted by elevated cAMP. The present studies were therefore undertaken to examine the enzymatic regulation of cAMP in PMVECs and PAECs.

Assessment of cAMP content in the two cell types indicated higher constitutive levels in PMVECs. However, the elevated content was accompanied by decreased ATP-to-cAMP conversion. Measurements of the conversion were made over a fixed time period, and although the turnover rates were not evaluated, the data are interpreted to indicate that the balance of cAMP synthesis versus degradation was higher in PAECs than in PMVECs. Thus low adenylyl cyclase...
and/or high phosphodiesterase activity establishes a set point for elevated cAMP content in PMVECs. cAMP concentrations typically differ between tissues and cell types. cAMP accumulation and turnover rates do not directly relate to content, indicating enzymatic regulation of cAMP production, and degradation establishes cell-specific set points. The biochemical mechanisms underlying establishment of constitutive set points are poorly understood. Nonetheless, increased PMVEC cAMP content is consistent with the hypothesis that increased constitutive cAMP promotes focal adhesion complex formation in these cells.

β-Adrenergic stimulation of adenylyl cyclase is utilized clinically to reverse airway inflammation in asthma (29, 32, 45) and peripheral edema in urticaria (30, 47) and experimentally to prevent or reverse pulmonary edema (2, 8, 27, 28, 37, 41). β-Adrenergic agonists elevate endothelial cell cAMP to promote cell-cell apposition that decreases macromolecular permeability. Although inflammatory agonists of different etiologies target selective segments in the pulmonary circulation, e.g., macrovascular versus microvascular (7), it is unclear whether the biochemical response to β-adrenergic agonists differs between macrovascular...
Fig. 8. Maximal activation of store-operated Ca\(^{2+}\) entry in elevation of cytosolic [Ca\(^{2+}\)] in PMVECs and PAECs. Ratiometric shifts in fluorophore fura 2-AM were used to detect changes in cytosolic [Ca\(^{2+}\)]. TG was added for 5 min to cells bathed in physiological salt solution containing 100 nM extracellular Ca\(^{2+}\), then Ca\(^{2+}\) was readded at indicated concentrations. On readdition of extracellular Ca\(^{2+}\), PMVECs (representative traces in A and summary data in C) reached higher maximal cytosolic [Ca\(^{2+}\)] levels than PAECs (n = 4 experiments; representative traces in B and summary data in C). *Significant difference between cell types at corresponding dose, P < 0.05. D: both cell types displayed similar sensitivity to readdition of calcium (n = 4 experiments).

Fig. 9. Effects of maximal activation of store-operated Ca\(^{2+}\) entry on cAMP content in PMVECs. TG was added for 5 min to cells bathed in physiological salt solution containing 100 nM extracellular Ca\(^{2+}\), then Ca\(^{2+}\) was readded for 2.5 min at indicated concentrations. Readdition of Ca\(^{2+}\) to microvascular cells decreased cAMP content (n = 4 experiments/group). *P < 0.05.

Fig. 10. Effect of phosphodiesterase 4 inhibition in conjunction with activation of store-operated Ca\(^{2+}\) entry on ATP-to-cAMP conversion in PMVECs. Rolipram (40 µM) was added to PMVECs for 17.5 min followed by application of TG (1.5 µM) for 2.5 min. Stimulation of ATP-to-cAMP conversion by rolipram unmasked Ca\(^{2+}\) inhibition of ATP-to-cAMP conversion induced by activation of store-operated Ca\(^{2+}\) entry (n = 30 experiments). *P < 0.05.
and microvascular endothelial cells. Our data indicate that PAECs, which are sensitive to calcium agonist-evoked increases in permeability (20), are highly responsive to β-adrenergic stimulation of cAMP, whereas PMVECs, which are insensitive to calcium agonist-evoked increases in permeability (20), are unresponsive to β-adrenergic stimulation of cAMP.

To assess whether decreased PMVEC responsiveness to β-adrenergic stimulation of cAMP was due to receptor function or altered adenylyl cyclase activity, studies were performed with forskolin. Responsiveness to forskolin was decreased in PMVECs, indicating either reduced intrinsic activity of PMVEC adenylyl cyclase complexes or increased phosphodiesterase activity. Both cell types express similar isoforms of adenylyl cyclase, including types II, IV, VI, and VII (8, 40). Expression of types VIII and IX have not been rigorously tested, and quantitative estimates of cyclase abundance have yet to be performed. Thus it is possible that PMVECs express a lower abundance of enzyme or, alternatively, possess unique regulatory properties that directly decrease enzyme intrinsic activity.

However, increased responsiveness to rolipram in PMVECs suggests that phosphodiesterase activity importantly dictates cAMP content in these cells to a greater extent than in PAECs and may contribute to the apparent lack of responsiveness to adenylyl cyclase activation in PMVECs. Indeed, when responsiveness to the combination of rolipram and forskolin was assessed, similar synergistic increases in cAMP were observed in both cell types. Activation of adenylyl cyclase, therefore, most effectively increases PAEC cAMP, whereas inhibition of phosphodiesterase activity most effectively increases PMVEC cAMP. It is unclear why phosphodiesterase 4 activity may be constitutively higher or more responsive to increases in cAMP in PMVECs than in PAECs (3, 21, 22, 38). Phosphodiesterase 4 consists of A–D subfamilies with 15–20 splice variants; each exhibits Michaelis–Menten kinetic behavior (13). Molecular characteristics and the associated biochemical properties of these related products are poorly characterized in endothelial cells and, in particular, in phenotypically distinct endothelial cells.

Physiological rises in [Ca2+]i, inhibit type VI adenylyl cyclase in PAECs and reduce global cAMP to promote intercellular gaps and increase macromolecular permeability (9, 10, 41). Although PMVECs express the type VI enzyme, activation of store-operated calcium entry did not reduce constitutive cAMP. Because [Ca2+]i responses were suppressed in PMVECs relative to those in PAECs, we determined whether suppressed calcium entry uncoupled calcium from the inhibition of adenylyl cyclase. Studies were designed to increase calcium entry in PMVECs to levels similar to those achieved in PAECs. Under these conditions, stimulation of calcium entry inhibited cAMP in PMVECs. We also evaluated whether sensitivity of adenylyl cyclase to calcium could be enhanced in PMVECs by increasing the activation state of the enzyme. Prior studies indicated conditional regulation of certain adenylyl cyclase isoforms based on the presence of cytosolic regulators like Gsα and protein kinase C (42, 43). Because rolipram increased ATP-to-cAMP conversion, we determined whether such increased conversion rates would unmask calcium regulation of adenylyl cyclase, and, indeed, increased conversion was associated with calcium inhibition of ATP-to-cAMP conversion. Thus calcium conditionally inhibits PMVEC cAMP accumulation when either adenylyl cyclase activity is increased or calcium entry is elevated.

Taken together, cAMP is regulated by distinct mechanisms in PMVECs and PAECs. PMVECs are unresponsive to adenylyl cyclase stimulation and highly responsive to phosphodiesterase inhibition, whereas PAECs are highly responsive to adenylyl cyclase stimulation and unresponsive to phosphodiesterase inhibition. Because inflammatory mediators of different etiologies selectively target macrovascular versus microvascular segments within the pulmonary circulation, it is conceivable that rational pharmacological therapies could be developed to selectively increase cAMP in macrovascular (e.g., adenylyl cyclase activation) versus microvascular (e.g., phosphodiesterase inhibition) endothelial cells to serve an adjunctive anti-inflammatory therapy. In support of this notion, rolipram reverses microvascular dysfunction in reperfusion pulmonary edema (4) but is only partially effective at reducing macrovascular dysfunction in thapsigargin-induced pulmonary edema (P. M. Chetham, unpublished observations).

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