

Paradoxical cAMP-Induced Lung Endothelial Hyperpermeability Revealed by *Pseudomonas aeruginosa* ExoY

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Abstract—Mammalian transmembrane adenylyl cyclases synthesize a restricted plasmalemmal cAMP pool that is intensely endothelial barrier protective. Bacteria have devised mechanisms of transferring eukaryotic factor-dependent adenylyl cyclases into mammalian cells. *Pseudomonas aeruginosa* ExoY is one such enzyme that catalyzes cytosolic cAMP synthesis, with unknown function. *Pseudomonas aeruginosa* genetically modified to introduce only the ExoY toxin elevated cAMP 800-fold in pulmonary microvascular endothelial cells over 4 hours, whereas a catalytically deficient (ExoY^{K81M}) strain did not increase cAMP. ExoY-derived cAMP was localized to a cytosolic microdomain not regulated by phosphodiesterase activity. In contrast to the barrier-enhancing actions of plasmalemmal cAMP, the ExoY cytosolic cAMP pool induced endothelial gap formation and increased the filtration coefficient in the isolated perfused lung. These findings collectively illustrate a previously unrecognized mechanism of hyperpermeability induced by rises in cytosolic cAMP. (*Circ Res.* 2004;95:196-203.)

Key Words: adenylyl cyclase ■ phosphodiesterase ■ signal transduction ■ edema ■ *Bacillus anthracis*

Since its discovery by Sutherland and Rall in the 1950s,¹ cAMP has been recognized for its essential role in cellular functions that include proliferation, control of cell shape, excitability, gene expression, metabolism, differentiation, apoptosis, and ion homeostasis. However, nowhere have the functional consequences of cAMP been better defined than in control of the endothelial barrier.²⁻⁴ In vivo, in situ, and in vitro observations all demonstrate that cAMP elevating agents reduce endothelial cell permeability.⁵⁻⁷ Precise molecular targets that mediate such barrier-enhancing effects are poorly understood. Increased cAMP may decrease myosin light chain kinase-dependent myosin light chain phosphorylation and decrease centripetally directed tension.⁸⁻¹¹ It also polymerizes F-actin and increases tight junction complexes at cell-cell borders.^{3,5,12} Functionally, these cytoskeletal alterations reduce or prevent intercellular gap formation induced by inflammatory agents and speed the rate of recovery once the endothelial cell barrier has been disrupted.^{11,13} Interestingly, global rises in cAMP are not required to confer such barrier enhancing actions of cAMP.¹⁴ Mammalian adenylyl cyclases (ACs) are transmembrane proteins, some enriched in lipid rafts and caveolae,^{15,16} which generate restricted cAMP pools near the site of synthesis.^{17,18} In endothelial cells, the predominant isoform is the calcium inhibited type 6 AC (AC6). Submicromolar calcium concentrations reduce membrane associated cAMP sufficient to disrupt the endothelial

barrier.^{19,20} Indeed, heterologous expression of the calcium-stimulated type 8 AC (AC8) converts calcium inhibition into calcium stimulation of membrane-delimited cAMP, and prevents thrombin-induced gap formation without changing global cAMP concentrations.¹⁴ Hence, the physiological transitions in membrane-associated cAMP exert a dominant, protective control over endothelial cell barrier function. Recent advances have demonstrated that only small global changes in cAMP critically localized to plasma membrane microdomains are required to confer these barrier protective properties.¹⁴

Interestingly, pathogenic bacteria have devised two mechanisms for increasing cAMP in mammalian cells. Firstly, certain bacterial toxins ADP-ribosylate components of the host G-protein signal transduction apparatus (eg, cholera and pertussis toxins), thereby activating endogenous ACs and elevating intracellular cAMP.^{11,21-24} Secondly, bacteria transfer proteins with AC activity directly into the cytosol of target cells. One such protein is edema factor of *Bacillus anthracis*, an AC that catalyzes high-level synthesis of cAMP when transferred into target cells by protective antigen.²⁵ CyaA of *Bordetella pertussis* is another bacterial AC²⁶ as is ExoY, a recently described AC toxin of *Pseudomonas aeruginosa*.²⁷

Subcutaneous injection of edema factor causes edema.^{28,29} Tissue edema occurs in response to an increase in hydrostatic pressure, a decrease in lymph flow, a decrease in fluid

Original received January 8, 2004; revision received May 21, 2004; accepted May 27, 2004.

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DOI: 10.1161/01.RES.0000134922.25721.d9

reabsorption, or commonly, a breach of the endothelial cell barrier; indeed, interendothelial cell gap formation is a well-recognized mechanism of hyperpermeability and tissue edema. The effect of ExoY on permeability is unknown, but prior studies indicate it causes cell rounding of Chinese hamster ovary (CHO) cells.³⁰ The direct actions of bacterial ACs have never been examined in endothelial cells. In particular, it is not clear how a rise in cAMP could actually disrupt—rather than strengthen—the barrier. We specifically addressed this issue using three strains of *P. aeruginosa*: one that transfers an active form of ExoY into eukaryotic cells, a second that transfers a catalytically inactive form of ExoY, and a third strain that expresses ExoY but cannot transfer it into eukaryotic cells. Using these strains, our results indicate that ExoY synthesizes a cAMP pool that is distinct from the endogenous pool in its location and regulation by phosphodiesterases. In contrast to cAMP generated by mammalian adenylyl cyclases, ExoY-derived cAMP induces intercellular gap formation in pulmonary microvascular endothelial cells (PMVECs) and disrupts the endothelial barrier in the isolated perfused lung.

Materials and Methods

Isolation and Culture of PMVECs

PMVECs were isolated, cultured, and routinely passaged as described in detail by Stevens et al.³¹

Bacterial Strains and Growth Conditions

Bacteria were taken from frozen explants, grown overnight on solid agar/carbenicillin (400 $\mu\text{g}/\text{mL}$) media, and resuspended in PBS to OD₅₄₀ 0.25, for which it had previously been determined equalled 2×10^8 bacteria/mL. Dilutions were made in PBS to attain the appropriate MOI. Generation of bacterial strains PA103 $\Delta\text{exoUexoT}::\text{Tc pUCPexoY}$, PA103 $\Delta\text{exoUexoT}::\text{Tc pUCPexoY}^{\text{K31M}}$,²⁷ and PA103 $\Delta\text{pCrV pUCPexoY}^{\text{S2}}$ have been described previously. Bacteria were provided by D.W.F.

Western Blot Analysis

P. aeruginosa strains were grown in trypticase soy broth supplemented with 100 mmol/L monosodium glutamate, 1% glycerol, and 10 mmol/L nitrilotriacetic acid.³³ Supernatant fractions, obtained after centrifugation of cultures at 10 000g, 4°C, were precipitated by addition of a saturated solution of ammonium sulfate to 55%. Protein precipitates were suspended in SDS-sample buffer, separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide gels), transferred to nitrocellulose paper and subjected to Western blot analysis using rabbit anti-ExoY (1:20 000),²⁷ mouse monoclonal anti-PcrV, mab166 (1:20 000),³⁴ or U29F8 a mouse monoclonal antibody to ExoU (D.W.F., unpublished data, 2004). Primary antibody binding was detected using horseradish peroxidase labeled rabbit anti-mouse or goat anti-rabbit IgG and chemiluminescence.

Determination of cAMP Cell Lysates

cAMP content was assessed using a standard radioimmunoassay (Biomedical Technologies). Vehicle control, bacteria (MOI ranging from 0.2 to 20:1), or forskolin (100 $\mu\text{mol}/\text{L}$) were added to cells as indicated. Ten minutes before terminating the experiment, rolipram (10 $\mu\text{mol}/\text{L}$) was added as indicated.

Time-Lapse Microscopy and Intercellular Gap Determination

Endothelial cells were incubated with bacterial strains (MOI 20:1) or PBS for 2 hours, and images (40 \times oil immersion) acquired at 1-minute intervals for an additional 2 hours using Metamorph and Spot Software (Diagnostic Instruments). For forskolin (100 $\mu\text{mol}/\text{L}$)

and rolipram (10 $\mu\text{mol}/\text{L}$) studies, images were acquired at 1-minute intervals for 30 minutes.

Fluorescence and Confocal Microscopy

Cells were grown to confluence on 25-mm coverslips, incubated for 4 hours with bacteria or PBS at 37°C, and further prepared at room temperature. Initially, coverslips were washed three times in PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.1% triton-X 100 in PBS each for 10 minutes. After PBS wash, nonspecific binding sites were blocked by 3% goat serum in PBS for 20 minutes. Fixed cells were washed, incubated with primary antibodies for 30 minutes [rabbit anti-ExoY 1:200 in 3% goat serum or β catenin (BD Biosciences), 1:100 in 1.5% goat serum], and washed in PBS before incubating with secondary antibody conjugated to fluorescent markers [TRITC-conjugated swine anti-rabbit IgG (DAKO) or Alexa Fluor 488 monoclonal antibody (Molecular Probes)] at 1:150 in 1.5% goat serum for 20 minutes. After a final PBS rinse, coverslips were mounted with fluorescent mounting media (DAKO) and incubated overnight at 4°C. Fluorescent images were acquired on a Leica TCS SP2 confocal laser-scanning microscope with a 63 \times oil immersion objective at excitation wavelength 488 nm (Alexa-488) or 543 nm (TRITC) and emission bands 500 to 535 nm (Alexa-488) or 555 to 560 nm (TRITC). Images consisted of 15 to 18 slices at 0.3- μm sections spanning the entire depth of the cell and reconstructed to produce 3-dimensional images using the Leica software.

Cytochemical Localization of Adenylyl Cyclase

Studies were based on those described by Howell and Whitfield³⁵ and adapted by Els and Butterworth.³⁶ Briefly, confluent cells grown on filter supports were incubated for 30 minutes at 30°C in medium containing 0.5 mmol/L 5'-adenylyimidodiphosphate (AMP-PNP), an AC substrate readily hydrolyzed to cAMP and imidodiphosphate, which forms an electron-dense precipitate. The AMP-PNP incubation medium consisted of 5 mmol/L MgCl₂ and 80 mmol/L Tris-maleate buffer. Osmolarity was balanced to 285 to 305 mOsm by adding glucose. Levamisole (1 mmol/L) was used to inhibit nonspecific phosphatases, dithiothreitol to inhibit ATP-phosphatases, and 5 mmol/L g-strophanthin to block Na⁺/K⁺ ATPase activity. The capturing agent is 5 mmol/L CeCl₃.

Lung Isolation and Perfusion

The lungs and heart were removed en bloc from adult male Sprague-Dawley rats (250 to 350 g).²⁰ After the first filtration coefficient (K_f) recording, bacteria were added to the perfusate to a concentration of 1×10^7 bacteria/mL perfusate and K_f measured.²⁰

Statistical Analysis

One-way ANOVA followed by Newman-Keuls posttest or two-way ANOVA followed by Bonferroni posttest was used for comparisons. Significance was accepted as $P < 0.05$.

Results

ExoY Generates a Restricted cAMP Pool

Previous pathology reports and in vivo studies indicate *P. aeruginosa* infection can lead to disruption of the pulmonary microvasculature.^{37–39} The type III secretion system transfers four known effector proteins from the cytosol of *P. aeruginosa* into the cytosol of eukaryotic cells, including the AC ExoY.^{27,30} The goal of this study was to determine whether ExoY-elevated cAMP is sufficient to induce pulmonary microvascular gap formation.

The effects of ExoY on pulmonary microvascular cells were studied using various strains of *P. aeruginosa*. PA103 $\Delta\text{exoUexoT}::\text{Tc}$ is a natural *exoS* deletion strain and is unable to express ExoY.³⁰ This strain serves as a platform

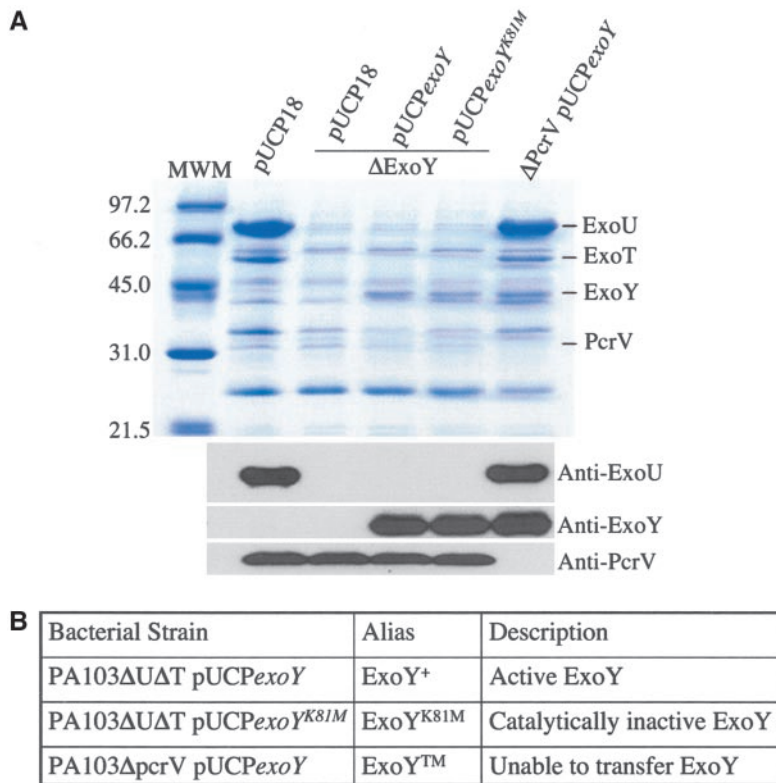


Figure 1. Extracellular protein profiles and expression analysis of *P aeruginosa* strains used in this study. A, *P aeruginosa* strains were grown as described in the Materials and Methods to induce the type III secretion system. Spent medium was collected, concentrated (20-fold), and analyzed for protein expression by SDS-PAGE (top panel) and Western blot analysis using specific antibodies to ExoU, ExoY, or PcrV (bottom 3 panels). Parental strain, PA103, makes the type III toxins ExoU and ExoT but does not make ExoS or ExoY. This parental strain was subjected to allelic replacement to construct PA103ΔexoUexoT::Tc (ΔExoUT) to eliminate ExoU and ExoT expression. This strain is still capable of secreting and translocating type III effectors encoded on separate plasmids (pUCP18) such as ΔExoUT pUCPexoY, which expresses ExoY. *exoY^{K81M}* is a site-specific mutation introduced into the *exoY* gene generating a protein devoid of adenyl cyclase activity. ΔPcrV is a deletion in *pcrV*, which is required to inject effector proteins into eukaryotic cells. PA103ΔpcrV pUCPexoY is able to express and secrete chromosomally encoded ExoU and ExoT in addition to plasmid-encoded ExoY but is unable to transfer these proteins into eukaryotic cells. B, Nomenclature and description of *P aeruginosa* strains.

into which expression plasmids encoding one or more of the effectors [ExoS, ExoT, ExoU, or ExoY (ExoY⁺)] (Figure 1) or noncatalytic enzyme (ExoY^{K81M}) (Figure 1) can be transformed, allowing the exclusive expression and delivery of one or more of the *Pseudomonas* type III toxins. To ensure type III-mediated delivery and to act as a control for effects of the presence of bacteria, an additional strain is used that has a deletion of *pcrV* (PA103Δ*pcrV*).³² *pcrV* encodes a protein necessary for the translocation of effectors from the bacterium directly into eukaryotic cells. The *pcrV* deletion strain engineered to express ExoY is secretion competent but unable to inject any of the toxins (ExoYTM)³² (Figure 1). We confirmed the phenotype of each strain through analysis of the extracellular protein pattern after growth under inducing conditions for type III secretion (Figure 1A).

When engineered strains of *P aeruginosa* are used to infect PMVECs at a MOI 20:1, a rise in intracellular cAMP is detectable within 3 hours (Figure 2A, ExoY⁺ versus PBS). cAMP does not accumulate within cells when a catalytically inactive expression strain is used (Figure 2B, ExoY⁺ versus ExoY^{K81M}) or when the bacterium is unable to deliver the toxin because of a defect in *pcrV* (Figure 2C, ExoY⁺ versus ExoYTM). All control groups maintained basal intracellular cAMP concentrations at all time points tested as summarized at the 4-hour time point (Figure 2D). These data demonstrate that *P aeruginosa* can intoxicate PMVECs via its type III system. Delivery of a catalytically active form of ExoY (ExoY⁺) results in supraphysiological levels of intracellular cAMP that accumulate in a time-dependent fashion.

Prior studies indicated that PMVECs predominantly express transmembrane AC6,¹⁹ which is directly activated by forskolin. Increasing concentrations of forskolin elevated

intracellular cAMP over a 20-minute time period (Figure 3A); however, the EC₁₀₀ concentration was not sufficient to elevate intracellular cAMP to concentrations attained by the ExoY⁺ bacteria.

PMVECs have high phosphodiesterase 4 activity so that cAMP accumulation is tightly regulated.^{31,40} We sought to determine whether phosphodiesterase 4 inhibition in the

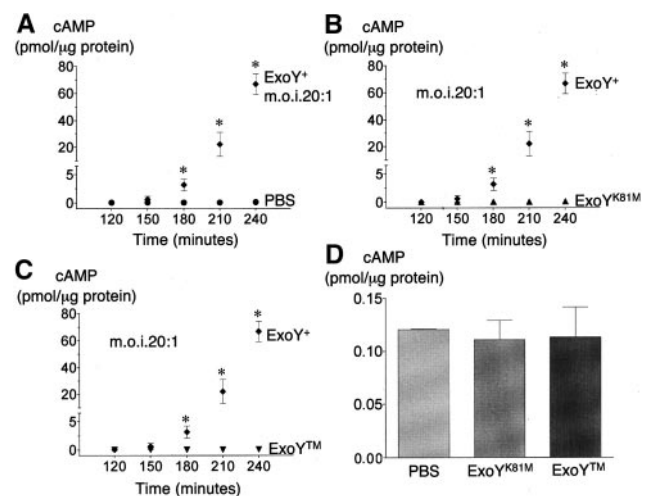


Figure 2. ExoY elevates intracellular cAMP in PMVECs. A, Inoculation of PMVECs with *P aeruginosa* expressing ExoY (ExoY⁺) results in a time-dependent increase in intracellular cAMP. Neither catalytically inactive ExoY strain (ExoY^{K81M}) (B), nor the translocation deficient strain ExoYTM (C), increased cAMP. D, All control groups (PBS vehicle control, ExoY^{K81M}, and ExoYTM) show no increase intracellular cAMP above basal levels; note difference in y-axis scale between A through C and D. *Significant increase in cAMP with ExoY⁺ strain at corresponding time points *P*<0.05.

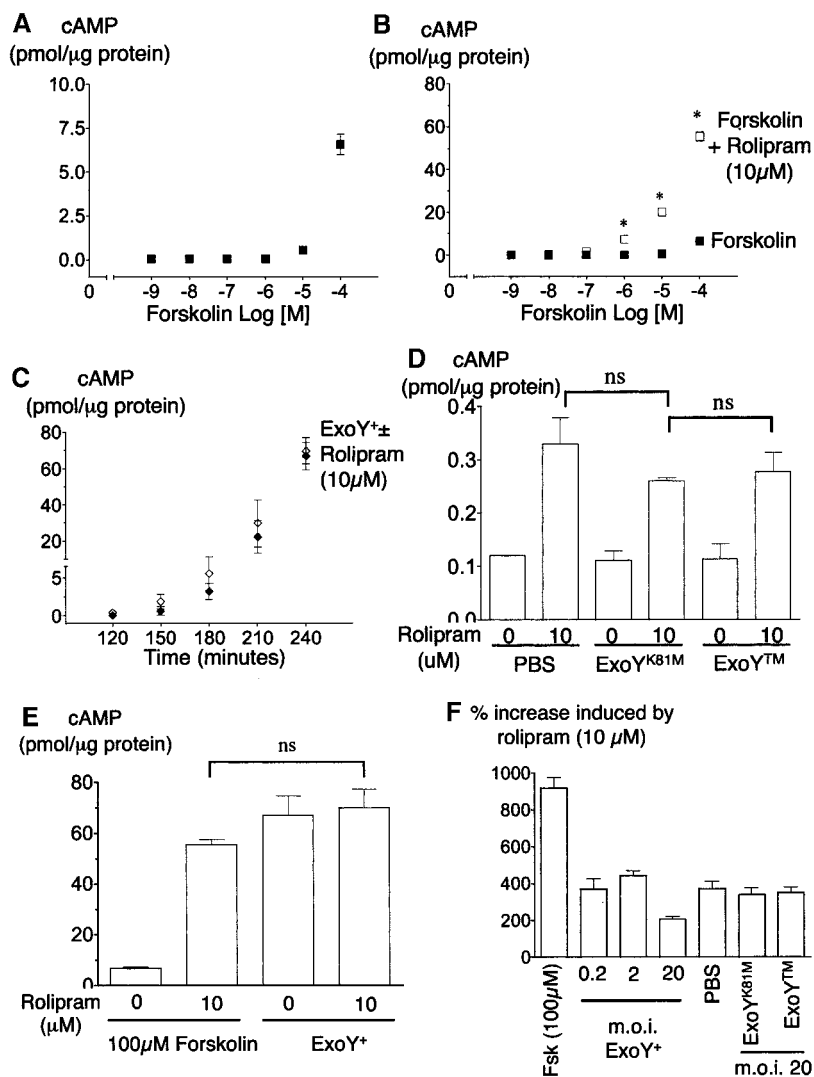


Figure 3. Endogenous cAMP synthesis is regulated by phosphodiesterase 4, in contrast to ExoY⁺ cAMP. **A**, Forskolin dose-dependently activates endogenous ACs, elevating intracellular cAMP. **B**, Inhibition of phosphodiesterase 4 activity with rolipram synergistically potentiates the forskolin elevated intracellular cAMP. Further, rolipram unmasked the forskolin activation of endogenous ACs at lower forskolin concentrations. **C** through **E**, PMVEC monolayers were inoculated with strains of *P. aeruginosa* or PBS vehicle control and rolipram added before terminating the experiment. Cellular lysates were assayed for cAMP content and normalized to protein concentration. **C**, ExoY⁺ strain elevates intracellular cAMP (solid diamond) but inhibition of phosphodiesterase activity with rolipram (open diamond) does not further increase this cAMP-pool. **D**, PBS or the mutant bacterial strains, ExoY^{K81M} and ExoYTM, do not elevate cAMP at this 4-hour time point. Basal cAMP concentrations are increased in the presence of rolipram with all control groups suggesting that mutant ExoY protein (ExoY^{K81M} strain) does not inhibit phosphodiesterase activity. **D**, Forskolin elevates intracellular cAMP in PMVECs, which is further synergistically increased with rolipram. ExoY⁺ dramatically increases intracellular cAMP 4 hours after inoculation with the bacteria, but this ExoY cAMP pool is not further elevated in the presence of rolipram. Forskolin and rolipram cAMP response is quantitatively similar to the 4-hour ExoY⁺-induced elevation in cAMP, which is unaffected by rolipram. **F**, Rolipram increases the forskolin cAMP-pool 900%, whereas the rolipram-induced increase after 4-hour inoculation with various MOI of ExoY⁺ is only 300%. The rolipram-induced increase of ExoY⁺ is similar to the rolipram-induced increase of controls PBS, ExoY^{K81M} and ExoYTM. *Significant increase in cAMP with rolipram at corresponding forskolin dose $P < 0.05$. ns, no significant difference between groups.

presence of increasing forskolin concentrations was sufficient to elevate intracellular cAMP to levels attained by ExoY⁺ strain of bacteria. The combination of rolipram (10 μmol/L) with forskolin (EC₁₀₀ 100 μmol/L) produced a profound synergistic increase in intracellular cAMP concentrations (Figure 3B) over those achieved by forskolin alone and unmasked the activation of endogenous ACs at lower forskolin concentrations. These data illustrate the endogenous, membrane delimited cAMP pool in PMVECs is rapidly synthesized and subsequently rapidly degraded by high phosphodiesterase activity. We next sought to determine whether the ExoY⁺-cAMP was similarly regulated.

Confluent PMVEC monolayers were inoculated with either bacteria (MOI 20:1) or PBS. Ten minutes before terminating the experiment, 10 μmol/L rolipram was added to the cells; however, rolipram did not further increase the ExoY⁺-cAMP pool (Figure 3C). The reason for this apparent lack of sensitivity to phosphodiesterase activity is unclear. We reasoned that the ExoY protein might inhibit phosphodiesterase 4 either directly or indirectly in a complex with endogenous proteins. However, in the presence of the ExoY^{K81M} strain of bacteria (just one amino acid change in the ATP binding

region of the protein compared with wild type) basal cAMP is increased with rolipram (Figure 3D), suggesting the ExoY protein does not directly inhibit phosphodiesterase 4. Alternatively, cAMP synthesized as a result of ExoY activity may be restricted to a subcellular compartment that expresses minimal phosphodiesterase activity. In addition, forskolin (EC₁₀₀) together with rolipram elevated cAMP to levels similar to those achieved by 4-hour incubation with ExoY⁺ bacteria (Figure 3E). To address whether the magnitude of ExoY-induced rise in cAMP may contribute to the relative insensitivity to phosphodiesterase inhibition, studies were repeated over a concentration range of bacteria (MOI 0.2 to 20). Four-hour incubation at the lowest MOI produced a time-dependent increase in cAMP that was less than 2-fold, whereas the highest concentration produced a time-dependent increase in cAMP that was 800-fold. Rolipram increased cAMP approximately 300% at all ExoY⁺ concentrations tested, equal to the cAMP rise observed with just PBS treatment and in ExoY^{K81M} and ExoYTM strains (Figure 3F). In summary, ExoY-derived cAMP is not regulated by phosphodiesterase activity, and although forskolin alone does not elevate cAMP to levels achieved by ExoY⁺, the combination

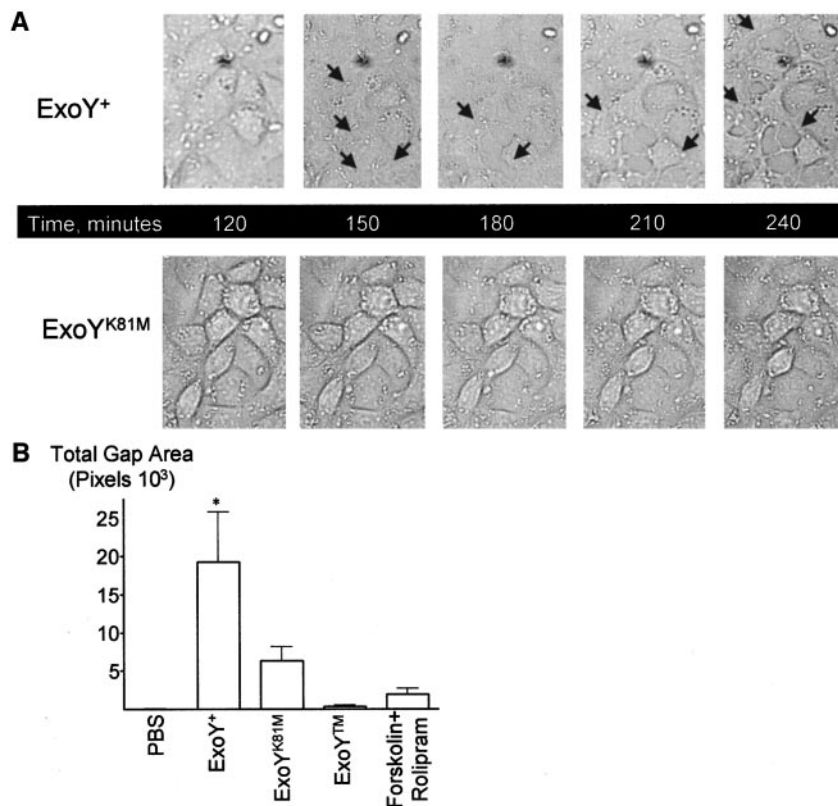


Figure 4. ExoY induces intercellular gaps in PMVEC monolayers. PMVECs were grown to confluence on glass coverslips and incubated with strains of bacteria. Cells were placed under the microscope and images taken every minute for 2 hours. A, No intercellular gaps were visible 120 minutes after inoculating cells with either ExoY⁺ or ExoY^{K81M} strains. However, gaps (seen by the translucent area at cell-cell borders and indicated by the arrows) begin to appear 150 minutes after infection with the ExoY⁺ strain. Cells remain healthy as widespread gaps continue to expand over the 240-minute time course. No such gaps appear with ExoY^{K81M} bacteria. See online Movie 1, which demonstrates the ExoY⁺ bacteria induced progressive gap formation initiated 150 minutes after inoculation and which continued to expand to the 240-minute time point. PMVECs inoculated with ExoY^{K81M} bacteria maintain a complete monolayer (A, bottom; online Movie 2), one representative of three experiments. B, PMVECs inoculated with ExoY⁺ bacteria induce dramatic paracellular gaps over 4 hours (* $P < 0.05$, $n = 3$); no effect is seen with PBS control, the translocation mutant strain, ExoYTM, or in cells treated with forskolin (100 $\mu\text{mol/L}$) and rolipram (10 $\mu\text{mol/L}$). ExoY^{K81M} strain transfers catalytically inactive ExoY protein into eukaryotic cells but did not induce significant intercellular gap formation.

of forskolin-rolipram does increase cAMP to levels achieved by the ExoY⁺ strain of bacteria.

ExoY Induces PMVEC Gap Formation

Having established that ExoY⁺ elevates intracellular cAMP in PMVECs, we sought to determine whether this activity was sufficient to induce intercellular gap formation in confluent PMVEC monolayers. Four days after seeding, cells were inoculated with bacteria (MOI 20:1) and images taken every 60 seconds for 2 to 4 hours. Progression of gap formation over this time course in the presence of ExoY⁺ bacteria was evident by the appearance of translucent areas between cell-cell borders (Figure 4A). Images were compressed into a time-lapse movie (online Movie 1, available in the online data supplement at <http://circres.ahajournals.org>). Gaps were not typically evident with the ExoY^{K81M} strain (Figure 4A and online Movie 2). These experiments were repeated in triplicate and Metamorph software used to measure the total area of gaps formed for each experimental condition at the 4-hour time point. Neither the PBS vehicle control nor the ExoYTM strain of bacteria altered integrity of the monolayer in any replicate study. Similarly, cells treated with forskolin (100 $\mu\text{mol/L}$) and rolipram (10 $\mu\text{mol/L}$), which produced a rise in cAMP equal to ExoY⁺, did not exhibit intercellular gap formation. ExoY^{K81M}-induced periodic insignificant ($P = \text{NS}$) gap formation (summarized in Figure 4B). The reason for this minimal ExoY^{K81M}-induced gap formation is not clear, although it could be associated with the presence of an exogenous protein, sequestration of a cytosolic factor, or insertion of the translocation apparatus

into the eukaryotic cell membrane that may affect the integrity of the plasmalemma inducing intercellular gaps.

In the presence of ExoY⁺ strain, total gap area increased over the 2- to 4-hour time course (Figure 5A), while intracellular cAMP also progressively increased over the same time course (Figure 5B). Regression analysis reveals that increases in gap formation and elevations in cAMP were correlated with a $R^2 = 0.8$ (Figure 5C), further implicating increased cAMP in the formation of intercellular gaps in PMVEC monolayers.

ExoY Localizes to the Cytosol

We have hypothesized that ExoY⁺-cAMP is not regulated by phosphodiesterases, which may be attributed to its subcellular localization. We compared the subcellular localization of endogenous ACs versus that of ExoY. Cytochemical localization studies using 5'-adenylylimidodiphosphate, an AC substrate that produces an electron-dense product, demonstrated that in PMVECs, the endogenous ACs synthesize cAMP restricted to lipid rafts and caveolae along the luminal plasma membrane and at sites of cell-cell adhesion (Figure 6A). These electron micrograph images are consistent with our previous fluorescent localization of heterologously expressed AC8,¹⁴ which revealed a punctate staining pattern localized to cell-cell borders at the plasma membrane.

We used indirect immunofluorescence to determine the subcellular localization of the ExoY protein after type III secretion system-mediated transfer into PMVECs. We compared the localization of ExoY to β -catenin, a protein known to reside at the plasma membrane in association with the cytoplasmic tail of cadherins.⁴¹ After 4-hour inoculation of

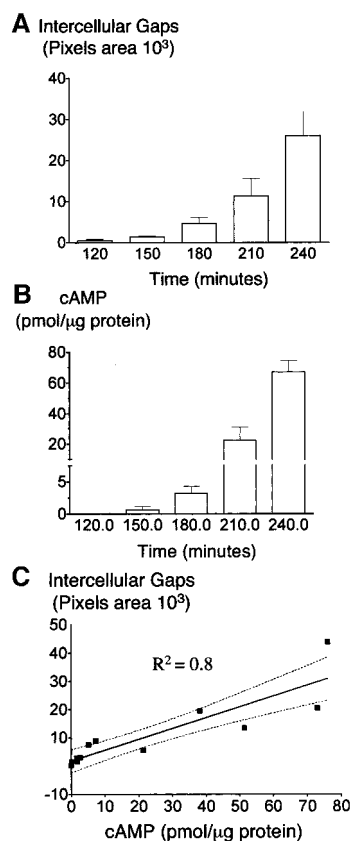


Figure 5. Elevated ExoY-cAMP is associated with increasing gap formation. A, Metamorph software was used to measure the area of the monolayer that developed gaps after inoculation with ExoY⁺ bacteria and demonstrates the progressive increase in the total size and/or number of gaps. B, PMVEC intracellular cAMP concentrations increase in the presence of ExoY⁺ bacteria as demonstrated at 30-minute intervals over the 240-minute time course. C, Elevations in intracellular cAMP correlate with increasing gap formation ($R^2=0.8$), indicating that the ExoY-elevated cAMP is associated with gap formation.

PMVEC confluent monolayers with strains of bacteria, dual labeling immunofluorescence and confocal microscopy showed that the translocated ExoY protein is localized to the cytosol, whereas endogenous β -catenin resides predominantly at the plasma membrane. Three-dimensional reconstruction of infected cells demonstrates that β -catenin is restricted to cell-cell borders, whereas ExoY is evenly distributed throughout the cytosol. Although the ExoY antibody did not appear to be exclusive for ExoY, any protein that it recognized clearly resided in the cytosolic compartment and not at the plasma membrane where endogenous transmembrane-ACs are found. In all experiments, control cells processed in parallel showed no signal if the primary ExoY- or β -catenin-specific antibodies were omitted (data not shown). The ExoY and β -catenin dual staining reveal no spectral overlap indicating these two proteins are not colocalized. Thus, our findings reveal that although endogenous ACs reside at the plasma membrane, ExoY resides in the cytosol. Therefore, ExoY synthesizes a predominantly cytosolic cAMP-pool, whereas the endogenous cAMP-pool is restricted to the plasma membrane.

ExoY Increases Lung Endothelial Cell Permeability

To examine whether observations in cell culture were relevant to the intact pulmonary circulation, we next tested whether ExoY⁺ bacteria was sufficient to increase K_f in the isolated perfused lung model. ExoY⁺ bacteria were applied to perfusate at a concentration of 1×10^7 bacteria per mL. ExoY⁺ increased K_f from 0.15 ± 0.02 to 0.87 ± 0.16 mL/min per cmH₂O per 100g after 180 minutes of perfusion (Figure 7), whereas permeability in ExoY^{K81M}-treated and time control lungs were not changed. The absence of such an effect in studies using the ExoY^{K81M} strain demonstrates nonspecific bacterial signaling, such as LPS, was not responsible for increased K_f . In fact, ExoY^{K81M} bacteria did not increase K_f above control. Therefore, these studies demonstrate ExoY is responsible for increasing endothelial permeability in the isolated perfused lung.

Discussion

It is widely recognized that cAMP-elevating agents ameliorate hyperpermeability caused by inflammation.² However, these studies focused on agents that either activate endogenous transmembrane ACs or deliver membrane permeable cAMP analogues to membrane-localized compartments. To date, few studies have addressed the role of soluble ACs from pathogenic bacteria on endothelial barrier integrity. Our present findings demonstrated that once ExoY is transferred into the target cell, it is not localized to the plasma membrane, but is dispersed throughout the cytosolic compartment; subsequent elevations in cAMP promote pulmonary microvascular gap formation, in stark contrast to the prevailing paradigm.

Pathogenic bacteria have devised a unique mechanism of producing cAMP in eukaryotic target cells. A type III secretion/translocation system introduces ExoY of *P. aeruginosa* into eukaryotic cells.^{27,42} Once inside the cell, association of ExoY with an unknown eukaryotic factor is required for AC activity.²⁷ Indeed, elevations in cAMP were not observed until 3 hours after inoculation with the bacteria, reflecting the time necessary for bacteria to establish contact with the target cell, initiate the secretion/translocation system, insert ExoY into the target cell, and associate with an unknown eukaryotic factor to induce AC activity. The AC activity of edema factor (*Bacillus anthracis*) is similarly dependent on a eukaryotic factor (calmodulin) for activity.⁴³ Whereas edema factor elevates cAMP 200-fold in CHO cells,²⁵ ExoY increases cAMP 800-fold in PMVECs, a physiologically relevant target cell. Neither the edema factor nor ExoY cAMP-pools appear to be sensitive to hydrolysis by phosphodiesterases. At higher edema factor doses tested, phosphodiesterase inhibitors approximately doubled cAMP.²⁵ We have similarly demonstrated that the ExoY-cAMP pool is only minimally regulated by phosphodiesterase activity. Therefore, bacteria have devised a mechanism of manipulating the mammalian cell to synthesize unregulated cAMP.

Nine of the mammalian ACs are transmembrane proteins. In endothelial cells, calcium-inhibited AC6 is the predominant isoform,^{19,20} which is enriched in caveolae and lipid raft microdomains.¹⁵ Rich et al^{17,18} have proposed that cAMP

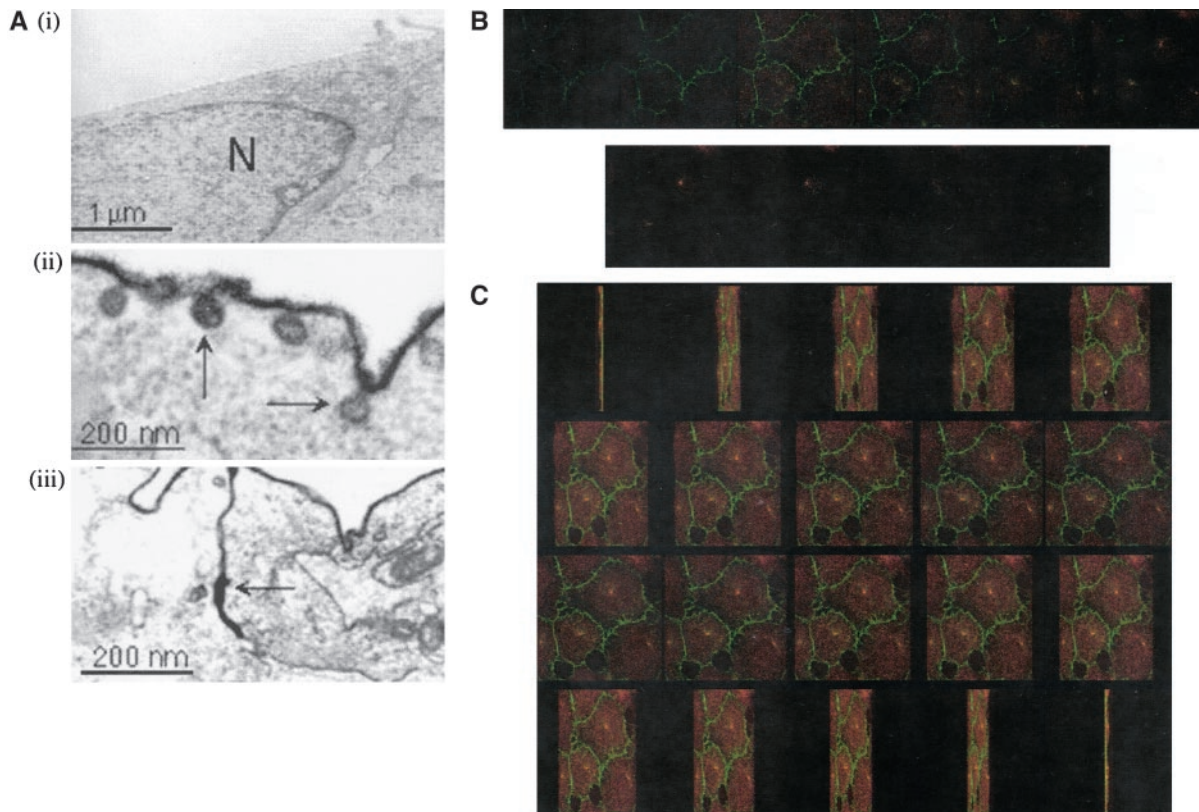


Figure 6. Endogenous adenylyl cyclases produce membrane-localized cAMP, whereas ExoY protein is injected into the cytosol of PMVECs. A, AC activity is localized to caveolae and along the luminal plasma membrane of PMVECs. Uranyl acetate control experiments (i) reveal that the buffer and fixation procedure does not produce an electron-dense staining at cell membranes. In adenylylimidodiphosphate-treated cells, forskolin (EC_{100}) and rolipram (EC_{100}) stimulate AC activity prominent in caveolae or caveolae-like structures (ii) and at sites of cell-cell tethering (iii). B and C, PMVECs were grown on coverslips to confluent monolayers and infected with strains of bacteria for 4 hours. Cells were dual labeled with anti-ExoY followed by TRITC-conjugated goat anti-rabbit IgG and anti- β -catenin followed by Alexa-488-labeled goat anti-mouse IgG. Merged images were acquired by confocal microscopy of abluminal-to-luminal cell slices at $0.3 \mu\text{mol/L}$ resolution. B, Cells incubated with the ExoY⁺ strain demonstrate the abluminal-to-luminal cytosolic expression pattern of ExoY in contrast to the localization of β -catenin to sites of cell-cell borders. C, Three-dimensional reconstruction of confocal images taken in B. Online Movie 3 shows the lateral rotation of this 3-dimensional image.

gradients exist within a single cell. Such gradients are due to physical constraints, such as organelles, which create 3-dimensional compartments that limit diffusion of cAMP, and biochemical constraints due to phosphodiesterase activ-

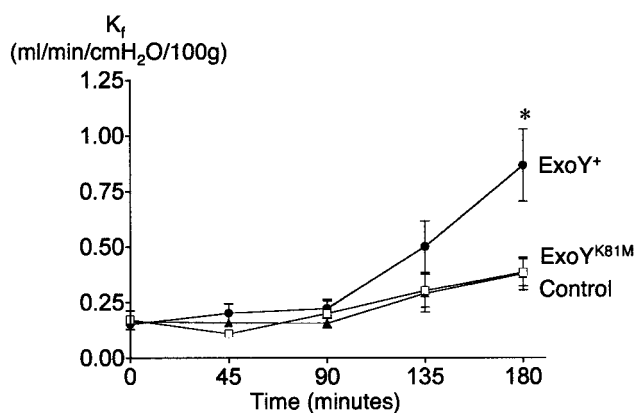


Figure 7. ExoY increases the K_f of the isolated perfused rat lung. Isolated lungs were perfused for 30 minutes between consecutive K_f measurements. ExoY⁺ significantly increased K_f over 180 minutes ($*P < 0.05$, $n = 6$), but K_f in vehicle or ExoY^{K81M} did not increase.

ity, which hydrolyze cAMP to further confine diffusion of this second messenger. Restraints on membrane-generated cAMP give rise to elevated cAMP concentrations in plasma membrane microdomains with a decreasing cAMP gradient in the cytosol. In contrast to endogenous transmembrane ACs, we have demonstrated by immunofluorescence that ExoY occupies the bulk cytosolic compartment. Therefore, this ExoY-cAMP does not generate a membrane-to-cytosol gradient, but rather generates a predominantly cytosolic pool. Under these conditions, centrally localized cAMP alters the balance of cytoskeletal forces sufficient to induce gap formation. We have demonstrated ExoY increases filtration coefficient (eg, permeability) in the intact pulmonary circulation, illustrating such gap formation results in hyperpermeability.

The barrier-disrupting effect of ExoY-cAMP is not simply due to supraphysiological cAMP concentrations. Indeed, ExoY increases cAMP 800-fold. However, forskolin and rolipram also increase cAMP approximately 800-fold, and this treatment is widely recognized to enhance the endothelial cell barrier in isolated organs and cell culture experiments.² Thus, it is most likely that cytosolic cAMP targets – usually protected from endogenously produced cAMP pools – activated by ExoY-cAMP are necessary to trigger gap formation.

In summary, we have demonstrated that the subcellular localization of elevated cAMP is a critical determinant of its downstream effects. ExoY induced elevations in intracellular cAMP are endothelial barrier disruptive, in contrast to the endogenous synthesis of cAMP which is barrier protective.

Acknowledgments

This work was supported by NIH grants HL-60024 and HL-66299 (T.S.), grants AI49577 and HL59239 (D.W.F.), and by American Heart Association Southeast Consortium Predoctoral Fellowship 0315134B (S.L.S.). This article is dedicated to the memory of Dr Alan Tucker. We thank Ingrid Durcova for her assistance in immunocytochemistry.

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Circ Res. 2004;95:196-203; originally published online June 10, 2004;

doi: 10.1161/01.RES.0000134922.25721.d9

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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ONLINE MOVIE LEGENDS

Supplemental Movie 1: PMVECs develop increasing size and number of intercellular gaps 2-4 hours after inoculation with ExoY⁺ bacteria.

Supplemental Movie 2: PMVEC gaps do not form after inoculation with ExoY^{K81M} bacteria.

Supplemental Movie 3: Lateral rotation of three-dimensional dual labeled PMVECs following inoculation with ExoY⁺ bacteria. TRITC labeled ExoY and Alexa-488 labeled β -catenin.