

# Soluble Adenylyl Cyclase Reveals the Significance of cAMP Compartmentation on Pulmonary Microvascular Endothelial Cell Barrier

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**Abstract**—Subtle elevations in cAMP localized to the plasma membrane intensely strengthen endothelial barrier function. Paradoxically, pathogenic bacteria insert adenylyl cyclases (ACs) into eukaryotic cells generating a time-dependent cytosolic cAMP-increase that disrupts rather than strengthens the endothelial barrier. These findings bring into question whether membrane versus cytosolic AC activity dominates in control of cell adhesion. To address this problem, a mammalian forskolin-sensitive soluble AC (sACI/II) was expressed in pulmonary microvascular endothelial cells. Forskolin stimulated this sACI/II construct generating a small cytosolic cAMP-pool that was not regulated by phosphodiesterases or  $G_{\alpha s}$ . Whereas forskolin simultaneously activated the sACI/II construct and endogenous transmembrane ACs, the modest sACI/II activity overwhelmed the barrier protective effects of plasma membrane activity to induce endothelial gap formation. Retargeting sACI/II to the plasma membrane retained AC activity but protected the endothelial cell barrier. These findings demonstrate for the first time that the intracellular location of cAMP synthesis critically determines its physiological outcome. (*Circ Res.* 2006;98:675-681.)

**Key Words:** phosphodiesterase ■ signal transduction ■ ExoY ■ second messenger ■ *Pseudomonas aeruginosa*

The discovery of an adenine ribonucleotide (cAMP) as an intracellular signaling messenger, and the recognition of its highly hydrophilic nature, led to the initial idea that this molecule rapidly and equivalently accesses all cellular compartments. However, as studies advanced, it became unclear how different external stimuli, which similarly elevate cAMP, selectively control different physiological processes.<sup>1,2</sup> These and related studies have given rise to the evolving hypothesis that cAMP signaling occurs within spatially and temporally confined microdomains.<sup>2-5</sup>

Whole cell cAMP concentration is established by the activities of ACs that synthesize cAMP and phosphodiesterases that hydrolyze it. AC1 to -9 are all transmembrane proteins,<sup>6,7</sup> and therefore cAMP synthesis emanates from the plasma membrane. Phosphodiesterases are distributed discretely throughout the cell where they inactivate cAMP before it escapes into physiologically inappropriate domains. Indeed, the coordinated activities of ACs and phosphodiesterases are responsible for generating cAMP gradients within a single cell, where high concentrations arise at the plasma membrane and lower concentrations exist in the bulk cytosol.<sup>8-12</sup> Yet it is unclear how cAMP is targeted to its physiologically appropriate effectors. Hall and colleagues have proposed that molecules of the signaling cascade may be orientated within a molecular complex such that cAMP is steered directly to its relevant effectors.<sup>13</sup> Thus, the prevailing

theory is that signaling fidelity is derived from spatial and temporal cAMP transitions generated within functional sub-cellular compartments where downstream targets are similarly located.<sup>2,3,14-16</sup>

These paradigms presume that cAMP synthesis occurs exclusively at the plasma membrane. However, recent evidence suggests human soluble AC (hsAC) exists in a wide range of cells and tissues,<sup>17-20</sup> where it is tethered to mitochondria, nucleus, microtubules, and the microtubule organizing center,<sup>17</sup> generating localized cAMP in response to bicarbonate fluctuations.<sup>20</sup> This finding brings into question the role hsAC plays in the activation of downstream cytosolic cAMP targets. Although many cAMP effectors (eg, protein kinase A [PKA] and Epac) localize to nonplasma membrane cellular compartments,<sup>21,22</sup> the current paradigm is that these targets are activated by cAMP that is generated at the plasma membrane, paradoxical to emerging evidence that free cAMP diffusion is quite limited. Currently, the physiological significance of cytosolic cAMP synthesis is not known.

Opposing actions of membrane versus cytosolic cAMP synthesis have been previously described in endothelial cells,<sup>23</sup> providing evidence for physiologically relevant cAMP compartments. Pulmonary microvascular endothelial cells (PMVECs) predominantly express the calcium inhibited AC6.<sup>24,25</sup> Calcium inhibition of AC6 cannot easily be resolved by standard whole cell cAMP measurements in PMVECs, yet 90% of its membrane

Original received September 13, 2005; resubmission received January 19, 2006; accepted January 26, 2006.

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DOI: 10.1161/01.RES.0000209516.84815.3e

activity is inhibited by submicromolar calcium concentrations.<sup>26</sup> Heterologous expression of calcium stimulated AC8 reverses the calcium inhibition of AC6 but only modestly increases global cAMP concentrations. Despite such subtle shifts in the calcium regulation of cAMP, reversing the calcium inhibition to a stimulation of AC prevents thrombin from inducing interendothelial cells gaps.<sup>27</sup> Thus, a membrane delimited cAMP pool that is not reflected by global cAMP concentrations has powerful barrier enhancing properties. In stark contrast to this membrane delimited cAMP pool, *P aeruginosa* transfers a soluble AC (eg, ExoY) into the cytosol of PMVECs where it exhibits a time-dependent increase in AC activity that is restricted exclusively to the cytosol.<sup>23</sup> This cytosolic cAMP induces intercellular gaps in cultured PMVEC monolayers and increases the filtration coefficient of the isolated perfused lung.<sup>23</sup> These studies suggest that the disparate compartmentation of cAMP synthesis, eg, plasma membrane versus cytosol, exerts exact opposite effects on endothelial barrier strength, although the ExoY-dependent cAMP rise occurs progressively over 4 hours without a concomitant increase in membrane cAMP. Thus, the goal of this study was to determine whether simultaneous rises in plasma membrane versus cytosolic cAMP dominates in the regulation of endothelial barrier strength. Our findings indicate that when cAMP is simultaneously generated at the plasma membrane and within the cytosol, cAMP synthesis in the cytosolic compartment overwhelms the barrier protective properties of membrane-restricted cAMP to induce endothelial cell gaps, demonstrating for the first time that the subcellular localization of signal generation is an important determinant of its physiological outcome.

## Materials and Methods

### Isolation and Culture of PMVECs

PMVECs were isolated, cultured, and passaged in EC media (DMEM+10% FBS+1% PBS) as described.<sup>25</sup>

### Adenovirus Constructs and Infection

Adenovirus encoding soluble ACII<sup>28</sup> and green fluorescence protein (GFP) (Ad 889<sup>29</sup>) have been described elsewhere. Adenovirus was added to 70% confluent PMVECs in EC media at multiplicity of infection (moi) 10:1 for 40 to 48 hours.

### Retrovirus Constructs and Infection

The TM-AC8 fusion described previously,<sup>30</sup> was inserted between *Hind*III and *Xho*I sites of a hygromycin-encoding retroviral vector pBABA-Hygro derivative. The sACI/II terminal codon was removed and *Bam*HI sites attached. This was inserted between the N-terminal transmembrane domains of TM-AC8 to generate sACI/II-TM-AC8, and subcloned into the pBABA-Hygro derivative. Retrovirus media was added to 40% confluent PMVECs in 5.9  $\mu$ g/L polybrene, centrifuged for 1 hour at 750g at room temperature. Retrovirus media remained on the cells for 15 hours and was then replaced with EC media. Retroviral cell lines were passaged and plated for each experiment into hygromycin selection media (50  $\mu$ g/mL).

### Whole Cell cAMP Measurements

cAMP concentration was assessed by standard radioimmunoassay (Biomedical Technologies, Stoughton, Mass). Forskolin (100  $\mu$ mol/L) or isoproterenol (1  $\mu$ mol/L) with or without rolipram (10  $\mu$ mol/L) was added as indicated.

### Fractionation of Cellular Lysates

Confluent cells were rinsed in ice-cold buffer A (137 mmol/L NaCl, 2.7 mmol/L KCl, 10.14 mmol/L Na<sub>2</sub>HPO<sub>4</sub>, 10.76 mmol/L KH<sub>2</sub>PO<sub>4</sub>) followed by 10-minute incubation in ice-cold buffer B (40 mmol/L Tris-HCl, 1 mmol/L EDTA, 150 mmol/L NaCl). Cells were scraped using 10 mL ice-cold buffer C (20 mmol/L HEPES, pH 7.5, 5 mmol/L EDTA, 1 mmol/L EGTA, 2 mmol/L dithiothreitol, 200 mmol/L sucrose, protease inhibitor cocktail) and centrifuged for 10 minutes at 250g at 4°C. Supernatant 1 (media) was removed and the pellet resuspended in 2 mL buffer C using 20 strokes each of course and fine pestle with Dounce homogenizer. This suspension was centrifuged at 100 000g for 20 minutes at 4°C. Supernatant 2 (cytosolic fraction) was removed and the pellet (plasma membrane fraction) resuspended in 1.5 mL buffer C using 15 strokes of Duall homogenization. Fractions were stored at -80°C. Protein concentration was determined using the modified Lowry method (Pierce).

### Adenylyl Cyclase Activity Assay

Assays were performed using a 1-mL solution of 0.1 mmol/L ATP, 1  $\mu$ mol/L cAMP, 2.5 U/mL creatine phosphokinase, 12 mmol/L phosphocreatine, 1 U/mL adenine deaminase, 0.04 mmol/L GTP, 2 mmol/L MgCl<sub>2</sub>, and 25 mmol/L HEPES at pH 7.3 with physiological potassium solution (103.1 mmol/L KOH, 10 mmol/L KHCO<sub>3</sub>, and 26.9 mmol/L K<sub>2</sub>HPO<sub>4</sub>) and 100  $\mu$ mol/L calcium (determined using Fabiato free ion calculator).<sup>31</sup> Reactions contained [ $\alpha$ -<sup>32</sup>P]ATP (1 $\times$ 10<sup>6</sup> cpm) with or without 100  $\mu$ mol/L forskolin and initiated by addition of 0.15 mg of protein, proceeded for 30 minutes at 30°C and terminated using 200  $\mu$ L 30% trichloroacetic acid. Recovery was monitored using internal standard [2,8-<sup>3</sup>H]cAMP (15 000 cpm). Production of radio-labeled cAMP from substrate [ $\alpha$ -<sup>32</sup>P]ATP was determined following isolation of [<sup>32</sup>P]cAMP by Dowex cation exchange then alumina oxide chromatography.<sup>32</sup>

### Time-Lapse Microscopy

Images were acquired at 1-minute intervals for 2 hours as described previously.<sup>23</sup> Forskolin (100  $\mu$ mol/L) was added after 60 minutes.

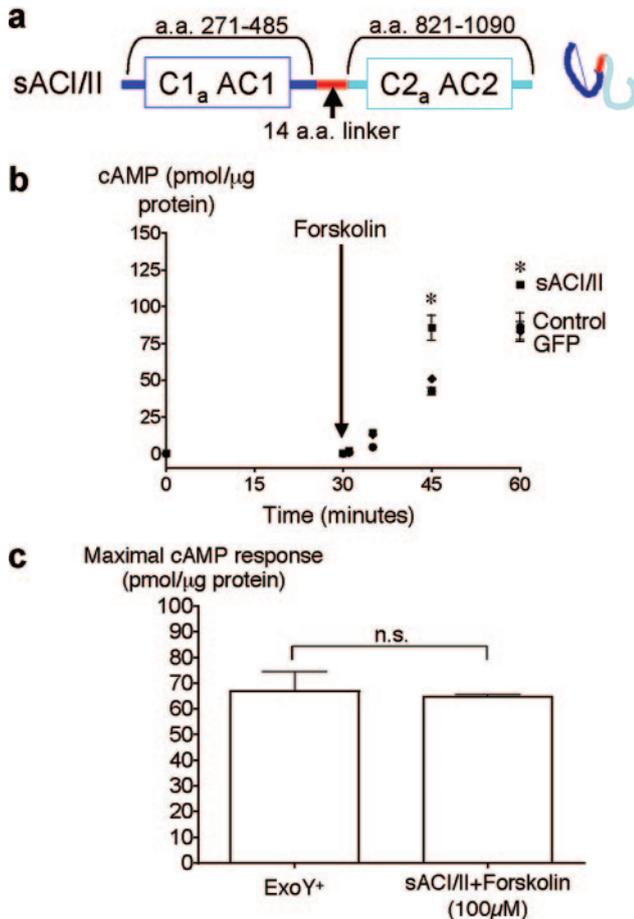
### Statistical Analysis

Values represent means $\pm$ SD. One- or 2-way ANOVA followed by Bonferroni post test or *t* test was used for comparisons. Significance was accepted as *P*<0.05.

## Results

### sACI/II Elevates cAMP in PMVECs

Previous studies demonstrate that plasma membrane AC activity is endothelial barrier protective, whereas cytosolic activity is barrier disruptive.<sup>23</sup> To determine which of these two sites of synthesis dominates in the regulation of endothelial permeability, we used a soluble forskolin-sensitive AC system that was generated by fusing the amino-terminal portion of the catalytic loops of mammalian transmembrane AC type I (C1a) and II (C2a) with a 14 amino acid linker<sup>33</sup> (sACI/II, Figure 1a). Loss of the C<sub>1b</sub> domain of ACI renders the chimera insensitive to calcium.<sup>28</sup> Dessauer and Gilman generated an adenovirus for expression of this soluble enzyme in mammalian cells.<sup>28</sup> When expressed in PMVECs, activation by forskolin generated a time-dependent increase in intracellular cAMP (Figure 1b). Interestingly, no increase in basal cAMP was observed before the addition of forskolin, which was attributable to the lack of constitutive enzymatic activity. To confirm the sACI/II adenoviral specificity of elevated cAMP, the forskolin response was compared with cells infected with a control soluble GFP adenovirus. Forskolin activates endogenous transmembrane ACs increasing intracellular cAMP in uninfected and GFP infected control



**Figure 1.** Forskolin stimulation of sACI/II increases cAMP in PMVECs. a, Schematic demonstrating the generation of the sACI/II chimera protein construct. C1a of AC1 (amino acids 271 to 484) is linked to C2a of AC2 (amino acids 821 to 1090) by a 14 amino acid linker. b, At adenovirus moi 10:1, the sACI/II chimera does not elevate basal cAMP levels until it is stimulated with forskolin (100  $\mu\text{mol/L}$ ), which significantly increases cAMP over time above adenovirus GFP and vehicle control groups. After statistical significance between sACI/II and control groups was determined by 2-way ANOVA ( $P < 0.0001$ ) significance of differences between sACI/II and control groups was determined by Bonferroni multiple comparisons test ( $*P < 0.001$ ,  $n = 6$ ). c, At moi 10:1 the maximal cAMP response 30 minutes following stimulation with forskolin (100  $\mu\text{mol/L}$ ) of PMVECs infected with adenovirus sACI/II was similar to the maximal 4-hour ExoY<sup>+</sup> response (moi 20:1).<sup>23</sup> After statistical significance between groups was determined by 1-way ANOVA ( $P < 0.0001$ ) significance of differences between groups was determined by Bonferroni multiple comparisons test. n.s. indicates not significant ( $n = 3$ ).

cells (Figure 1b). Expression of sACI/II increased the forskolin-stimulated intracellular cAMP above control groups, which was attributed to simultaneous activation of both endogenous transmembrane ACs and the sACI/II chimera (Figure 1b).

Previous studies have shown *P aeruginosa* competent for the type three secretion system transfers the AC ExoY into PMVECs, generating a cytosolic cAMP pool that is sufficient to increase endothelial permeability.<sup>23</sup> At a sACI/II adenoviral dose moi 10:1, the maximal forskolin-stimulated cAMP response mimicked concentrations achieved by ExoY expressing *P aeruginosa* (Figure 1c).

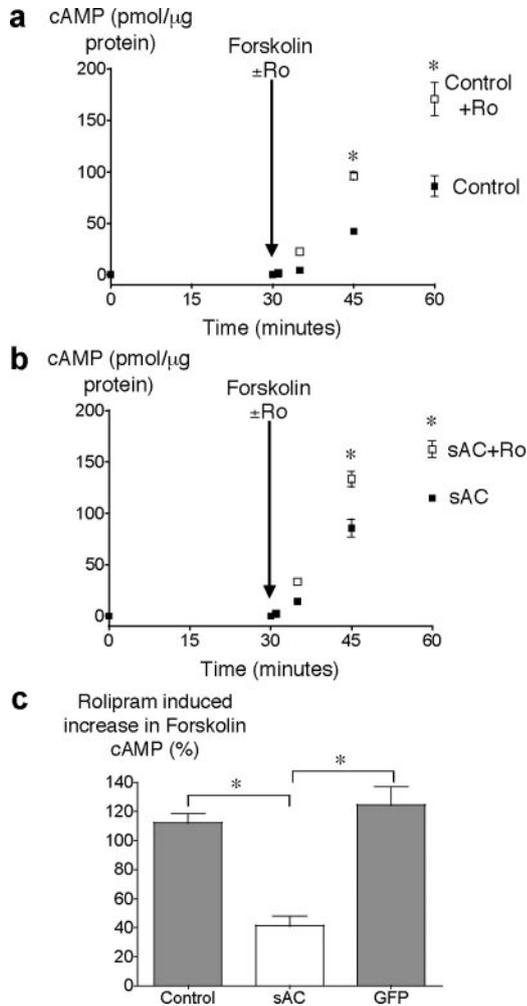
### Functional Compartments of Forskolin-Induced sACI/II-cAMP

Although these studies demonstrate sACI/II has activity in PMVECs, they provide no indication of the intracellular compartment where this activity resides. Phosphodiesterase activity is important to tightly restrict cAMP diffusion, thereby maintaining isolated cAMP pools<sup>8,10,34</sup> and downstream signaling specificity. PMVECs express the rolipram-sensitive phosphodiesterase isoform 4, which controls the plasmalemmal cAMP pool.<sup>25</sup> We sought to compare phosphodiesterase 4 regulation of the sACI/II-cAMP versus endogenously generated plasmalemmal cAMP. The forskolin-sensitive cAMP pool of uninfected control PMVECs is augmented by rolipram (Figure 2a). The substantial increase in cAMP generated by simultaneous forskolin and rolipram treatment was not sufficient to deplete total cellular ATP pools (data not shown). However, despite the fact that sACI/II expression increased the amount of cAMP produced in response to forskolin, rolipram did not significantly augment the forskolin-stimulated sACI/II pool (Figure 2b). Whereas rolipram induced a 110% increase in the forskolin-stimulated cAMP pool of uninfected and GFP infected controls, rolipram induced only a 40% rise in the forskolin-stimulated cAMP pool of sACI/II expressing cells (Figure 2c). This relative insensitivity of the sACI/II-dependent cAMP pool to hydrolysis by phosphodiesterase 4 suggests sACI/II and endogenous AC activity reside in different compartments.

To further determine compartmentation of sACI/II, we examined its integration into endogenous signaling pathways. Rolipram [10  $\mu\text{mol/L}$ ,  $EC_{100}^{25}$ ], used throughout this study to amplify global cAMP levels for detection purposes, doubles the intracellular cAMP concentrations of all treatment groups (data not shown). Isoproterenol activation of  $\beta$ -adrenergic receptors is coupled via the trimeric G protein,  $G_{\alpha_s}$ , to endogenous transmembrane ACs. In PMVECs, isoproterenol (1  $\mu\text{mol/L}$ ,  $EC_{100}^{25}$ ) increases intracellular cAMP above rolipram alone (Figure 3). Interestingly, although in vitro kinetic analyses have demonstrated that  $G_{\alpha_s}$  can stimulate sACI/II,<sup>28</sup> activation of  $G_{\alpha_s}$  in PMVECs expressing the sACI/II does not increase cAMP above control groups (Figure 3). Collectively, these data suggest that sACI/II resides in a compartment not readily accessible by  $G_{\alpha_s}$ .

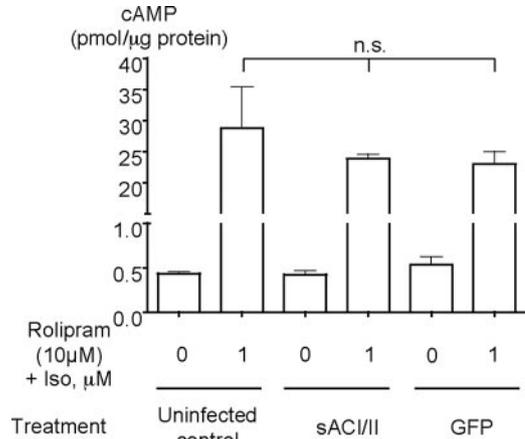
### Subcellular Localization of the sAC Activity

To determine the subcellular localization of sACI/II activity in PMVECs, cells were lysed into plasma membrane and cytosolic fractions and the AC activity measured. Similar basal- and forskolin-stimulated AC activity was detected in the plasma membrane fraction of all treatment groups (uninfected control, sACI/II and GFP infected cells) representative of the endogenous transmembrane ACs (Figure 4a). The plasma membrane fraction of sACI/II expressing cells did not possess elevated forskolin-sensitive AC activity above controls, demonstrating the chimera did not have activity at the plasmalemma. As the predominant endogenous AC in PMVECs is calcium sensitive isoform 6,<sup>26</sup> calcium inhibition of forskolin activity further supports the plasma membrane composition of this fraction (Figure 4b). Although this is not the 90% calcium inhibition of AC activity in the lipid- and caveolin-enriched fraction,<sup>26</sup> it demonstrates the presence of a



**Figure 2.** The sACI/II cAMP pool is not regulated by phosphodiesterase activity. a, Forskolin (100 μmol/L) increases cAMP in PMVECs (solid squares), which is further increased with simultaneous addition of rolipram (10 μmol/L [open squares]). After statistical significance between control and control+rolipram for each time point was determined by 2-way ANOVA ( $P < 0.0001$ ), significance of differences between control and control+rolipram was determined by Bonferroni multiple comparisons test ( $*P < 0.001$ ,  $n = 6$ ). b, Forskolin (100 μmol/L) increases cAMP in PMVECs infected with adenovirus expressing sACI/II (solid squares) with a further increase in cAMP with rolipram (10 μmol/L [open squares]). After statistical significance between sACI/II and sACI/II+rolipram for each time point was determined by 2-way ANOVA ( $P < 0.0001$ ), significance of differences between sACI/II and sACI/II+rolipram was determined by Bonferroni multiple comparisons test ( $*P < 0.001$ ,  $n = 6$ ). However, in PMVECs expressing sACI/II the forskolin-stimulated cAMP pool is less responsive to rolipram compared with vehicle or adenoviral GFP control groups. c, After statistical significance between groups was determined by 1-way ANOVA ( $P < 0.0001$ ), significance of differences between groups was determined by Bonferroni multiple comparisons test ( $*P < 0.001$ ,  $n = 6$ ).

calcium regulated AC in this bulk plasma membrane fraction. Within the cytosolic fraction, no basal AC activity was detected in any treatment group (Figure 4c); however, forskolin revealed 6 times more AC activity in the cytosolic fraction of sACI/II expressing cells compared with uninfected or GFP infected cells. These data demonstrate the unique cytosolic compartmentalized AC activity of sACI/II infected PMVECs. Interestingly, the modest level of forskolin-



**Figure 3.** sACI/II does not respond to isoproterenol. PMVECs treated with rolipram (100 μmol/L) with or without isoproterenol for 30 minutes demonstrate that signaling through the β-adrenergic receptor does not activate sACI/II in PMVECs. After statistical significance between groups was determined by 1-way ANOVA ( $P < 0.0001$ ), significance of differences between groups following treatment with isoproterenol was determined by Bonferroni multiple comparisons test. n.s. indicates not significant ( $n = 3$ ).

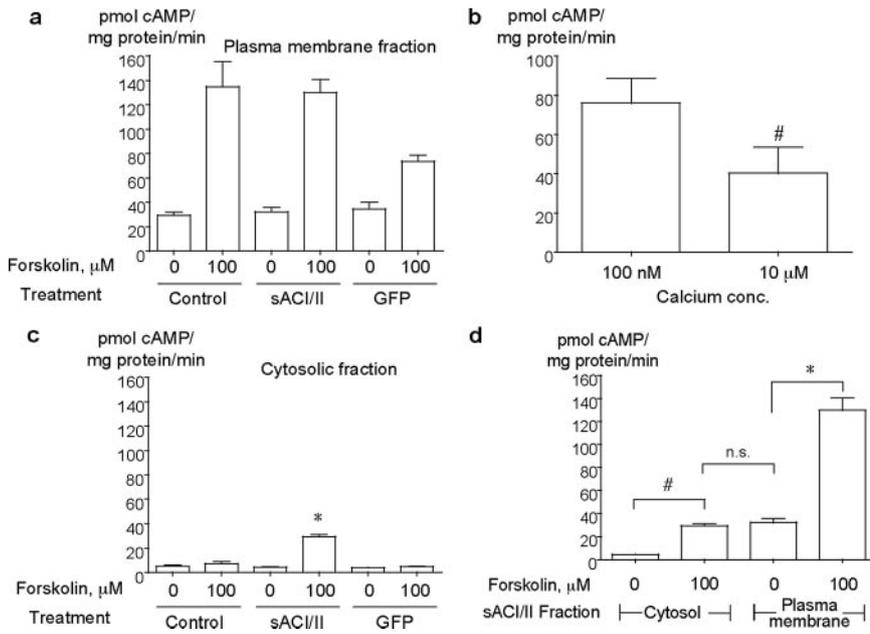
sensitive cytosolic sACI/II activity was comparable to the “basal” plasma membrane activity, but significantly less than the forskolin-stimulated membrane activity (Figure 4d). Therefore, when sACI/II expressing cells are stimulated by forskolin, there is 4.5 times more AC activity at the plasma membrane than in the cytosol, but 6 times more activity in the cytosolic compartment compared with basal conditions.

### Forskolin-Stimulated sACI/II Activity Induces PMVEC Gap Formation

We next sought to determine whether plasma membrane or cytosolic AC activity would dominate in the regulation of the endothelial barrier. Forskolin was not sufficient to induce PMVEC gaps with the adenoviral GFP control. Whereas unstimulated sACI/II expressing PMVECs did not reveal gap formation, gaps rapidly formed with the addition of forskolin, as seen by the retraction of cell-cell borders (Figure 5; Movies 1 and 2 in the online data supplement available at <http://circres.ahajournals.org>). Thus, activation of the sACI/II chimera, although representing only a modest rise in cytosolic versus membrane activity, is sufficient to overwhelm the barrier protective effects of endogenous plasma membrane restricted AC activity to form endothelial cell gaps.

### Relocation of sACI/II to the Plasma Membrane

To further test the hypothesis that the subcellular localization of AC activity governs its barrier protective or disruptive properties, the sACI/II chimera was relocated to the plasma membrane. Gu et al<sup>30</sup> have demonstrated that YFP sandwiched between the 2 transmembrane domains of AC8 (TM-AC8) (Figure 6a, i and ii) is sufficient for plasma membrane targeting. Plasma membrane localization of TM-AC8 was also detected in PMVECs (Figure 6b). Insertion of sACI/II between the transmembrane domains of AC8 generated sACI/II-TM-AC8 (Figure 6a, iii). Retroviral expression of sACI/II-TM-AC8 also localized to the membrane in PMVEC (Figure 6c). Whereas plasma membrane fractions of



**Figure 4.** The cytosolic fraction of sACI/II expressing PMVECs contains forskolin-sensitive adenylyl cyclase activity. a, The plasma membrane fraction of all treatment groups has basal and forskolin stimulated AC activity. b, The control plasma membrane fraction has calcium-sensitive AC activity indicative of isoform 6. # $P < 0.05$ , compared with 100 nmol/L calcium group by paired t test ( $n = 3$ ). c, Only the cytosolic fraction of sACI/II expressing PMVECs has forskolin-sensitive adenylyl cyclase activity. After statistical significance between groups was determined by 1-way ANOVA ( $P < 0.0001$ ), significance of differences between groups was determined by Bonferroni multiple comparisons test ( $*P < 0.001$ ,  $n = 9$ ). d, Forskolin stimulates AC activity in sACI/II expressing PMVECs. The forskolin-stimulated sACI/II cytosolic fraction has the same AC activity as the basal plasma membrane fractions from all treatment groups, which all have significantly less activity than the forskolin-stimulated plasma membrane fraction. After statistical significance between groups was determined by 1-way ANOVA ( $P < 0.0001$ ) significance of differences between groups was determined by Bonferroni multiple comparisons test.  $*P < 0.001$ , # $P < 0.05$ . n.s. indicates not significant ( $n = 9$ ).

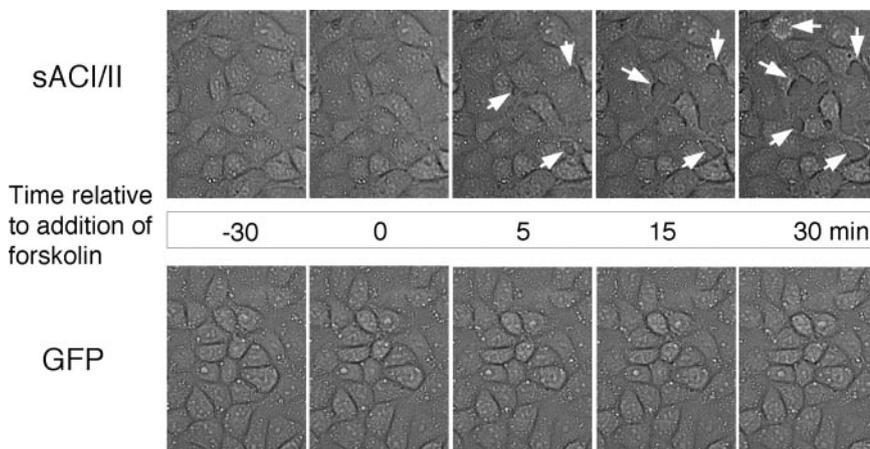
PMVECs expressing sACI/II-TM-AC8 exhibited elevated forskolin-dependent AC activity above controls (Figure 6d), no AC activity was detected in the cytosolic fraction (data not shown), illustrating its exclusive plasmalemma targeting. Forskolin stimulation of sACI/II-TM-AC8-expressing PMVECs did not lead to endothelial gap formation (Figure 6e and online data supplement, Movie 3), further demonstrating that AC activity exclusively restricted to the plasma membrane compartment with corresponding lack of activity in the cytosol does not disrupt the endothelial barrier.

**Discussion**

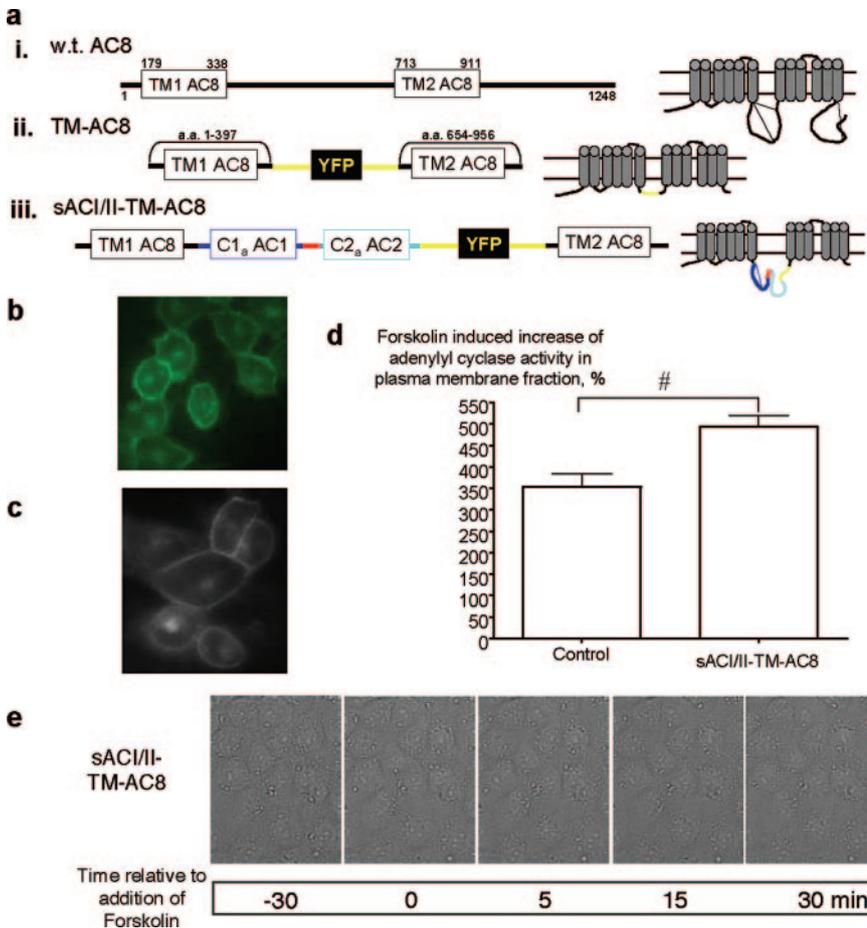
Elevated cAMP is widely recognized to be endothelial barrier protective.<sup>35</sup> More specifically, only small cAMP elevations critically localized to the plasma membrane are needed to protect the PMVEC barrier.<sup>27</sup> However, nature provides a paradox. Pathogenic bacteria have developed a mechanism to synthesize cAMP in the cytosol of eukaryotic cells, resulting in decreased

endothelial barrier function. For example, edema factor of *Bacillus anthracis* elevates intracellular cAMP in CHO cells yet induces edema when injected into the skin of rabbits and guinea pigs.<sup>36</sup> In addition, *P aeruginosa* inserts the adenylyl cyclase, ExoY, into the cytosol of PMVECs generating a barrier disruptive cytosolic cAMP pool.<sup>23</sup> As theories of cAMP compartmentation have evolved, it appears that within many cell types not all cAMP elevations produce equivalent physiological outcomes. However, the discrete role of AC activity within different cellular microdomains has never been directly tested owing to the lack of a suitable physiological outcome measurement. Using novel techniques to manipulate compartments of AC activity, our present findings demonstrate that cytosolic AC activity in PMVECs is sufficient to overwhelm the barrier protective effects of plasma membrane activity to promote endothelial gap formation.

The mammalian sACI/II chimera is a fusion of C1a-ACI with C2a-ACII that contains an AC catalytic site and forskolin



**Figure 5.** Forskolin induces gap formation in sACI/II expressing PMVECs. PMVECs expressing sACI/II (infected at moi 10:1) maintain a confluent monolayer until stimulated with forskolin (100  $\mu\text{mol/L}$ ) when gaps between endothelial cells rapidly form, whereas PMVECs expressing GFP (moi 10:1) are unresponsive to forskolin (100  $\mu\text{mol/L}$ ) and maintain a coherent monolayer. Arrows and green outline (30 minutes) highlight gaps ( $n = 6$ )



**Figure 6.** sACI/II-AC8-TM expressing PMVECs respond to forskolin but do not form gaps. **a**, Schematic to illustrate the design of constructs used in these studies: wild type AC8 (i), TM-AC8 (ii), and sACI/II-TM-AC8 (iii). **b**, Plasma membrane expression of TM-AC8 (c) and sACI/II-TM-AC8 (d). Expression of sACI/II-TM-AC8 in PMVECs leads to elevated forskolin-stimulated AC activity in the plasma membrane fraction above uninfected control cells demonstrating the localization and functional plasmalemma activity of the construct. # $P < 0.05$ , control compared with sACI/II-TM-AC8 group by paired  $t$  test ( $n = 3$ ). **e**, Forskolin does not induce gap formation in confluent PMVECs monolayers expressing sACI/II-TM-AC8 ( $n = 5$ ).

binding-pocket, thereby generating a conditionally active, soluble enzyme. Our findings that sACI/II has forskolin-dependent cytosolic AC activity in PMVECs that is not activated by  $G_{\alpha s}$  signaling in vivo support previous work of Dessauer and Gilman<sup>28</sup>; indeed sACI/II could not be activated by  $G_{\alpha s}$  signaling in vivo unless the enzyme was stimulated with forskolin.<sup>28</sup> In PMVECs, sACI/II-cAMP was insensitive to phosphodiesterase 4 activity, collectively suggesting AC activity resides in a unique cytosolic compartment. Although forskolin simultaneously activates both the cytosolic sACI/II and endogenous transmembrane AC6, the magnitude of sACI/II activity only contributes a small fraction of the global cAMP pool. Despite the small contribution of the cytosolic cAMP to this global cAMP-pool, it was sufficient to induce PMVEC gaps. Currently it is unclear whether a change in the plasma membrane-to-cytosolic cAMP gradient, or a small rise in cytosolic AC activity, is responsible for overwhelming the barrier protective effects of plasma membrane AC activity resulting in endothelial gap formation. However, we have recently shown that disrupting membrane phosphodiesterase 4 activity reveals forskolin-stimulated PMVEC gap formation suggesting that cAMP must remain within the membrane domain for it to confer barrier protection (T.S., unpublished data, 2006).

When sACI/II is sandwiched between the two transmembrane domains of AC8, this plasma membrane targeted sACI/II retained its forskolin-stimulated AC activity, indicating “free” amino and carboxy termini are not required for enzymatic

activity. Indeed, this is the first evidence that the entire AC catalytic pocket can reside in the C1 loop and still retain activity. When stimulated by forskolin, this chimeric protein generated small increases in cAMP similar to the level of activity achieved by sACI/II free in the cytosol. However, restriction of this sACI/II-dependent cAMP to the plasma membrane did not induce PMVEC gaps, definitively illustrating the site of cAMP synthesis critically mediates the physiological outcome.

The downstream cytosolic effectors that determine such diverse physiological responses of membrane and cytosolic cAMP transitions are unknown. Interestingly, mammalian, bicarbonate-stimulated, soluble ACs localize to mitochondria, nucleus, microtubules, and centrosomes where evidence is emerging that complete signalsomes exist. cAMP targets, such as PKA and Epac, also reside within the cytosol, some in association with complete signal transduction cascades.<sup>37</sup> For example, PKA compartmentalized through its interactions with A kinase anchoring proteins (AKAPs), is involved in phosphorylation events that lead to rearrangement of the cytoskeleton.<sup>38</sup> PKA phosphorylates microtubule associating proteins such as MAP2 and tau, which in turn reduces their ability to stabilize microtubules.<sup>39,40</sup> Within the endothelium, microtubule disassembly increases permeability.<sup>41</sup> An unregulated cytosolic cAMP pool could destabilize such cytoskeleton structures necessary to induce gap formation. Indeed, cAMP-signaling cascades are already in place within the cytosol, such that atypical cytosolic AC activity could lead to

their activation. Future studies are necessary to pursue the identification of these cytosolic targets.

Thus, the present data demonstrate that novel targeting of minimal AC activity to the cytosol of PMVECs dominates over substantial plasmalemmal AC activity to induce endothelial gap formation. Relocalizing this same AC construct to the plasma membrane substantiates endothelial barrier protective effects of plasma membrane AC activity. Our data presented here contribute to the idea that compartmentalized microdomains of cAMP signaling play a key physiological and pathophysiological role on the regulation of pulmonary endothelial barrier.

### Acknowledgments

This work was supported by NIH grants HL-60024 and HL-66299 (to T.S.) and by American Heart Association Southeast Consortium Predoctoral Fellowship 0315134B (to S.L.S.). We thank Dermot Cooper for providing the TM-AC8 plasmid construct and Judy Creighton for performing ATP studies.

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*Circ Res.* 2006;98:675-681; originally published online February 9, 2006;

doi: 10.1161/01.RES.0000209516.84815.3e

*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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Supplemental movie 1. **Forskolin induces gap formation in sACI/II expressing PMVECs.** Forskolin was added to sACI/II expressing PMVECs 1 hour after initiating the movie and continued for another hour.

Supplemental movie 2. **Forskolin does not induce gaps in GFP expressing PMVECs.** Following the addition of forskolin 1 hour after initiation of the movie, the monolayer remains intact.

Supplemental movie 3. **AC8-TM-sACI/II expressing PMVECs do not form gaps** Forskolin was added to AC8-TM-sACI/II expressing PMVECs 1 hour after initiating the movie and continued for another hour.