Soluble adenylate cyclase reveals the significance of compartmentalized cAMP on endothelial cell barrier function

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Abstract
Within pulmonary microvascular endothelial cells, activation of endogenous adenylate cyclase generates tightly regulated cAMP transitions in the subplasma membrane space. These cAMP fluxes strengthen contacts between adjacent cells to tighten their barrier function. However, pathogenic bacteria have devised a mechanism to transfer toxic adenylate cyclases into eukaryotic cells and generate a cytosolic pool of cAMP that disrupts the barrier. To determine whether membrane or cytosolic cAMP synthesis dominates in control of endothelial cell barrier function, we expressed a soluble mammalian adenylate cyclase chimaera. This chimaera is not constitutively active, but is activated by forskolin. Thus forskolin application increases cAMP in both the plasma membrane and cytosolic compartments. Forskolin induced inter-endothelial cell gaps in cells expressing the soluble adenylate cyclase, demonstrating that the cytosolic cAMP pool dominates over the plasma membrane cAMP pool in control of endothelial cell barrier strength. Indeed, when the soluble chimaera is relocalized to the plasma membrane, the forskolin-stimulated adenylate cyclase activity does not induce gaps. These results therefore support the growing paradigm that membrane and cytosolic cAMP pools target discrete effectors to control different physiological processes.

Subplasmalemmal cAMP protects the pulmonary endothelial cell barrier

Strongly adherent pulmonary microvascular endothelial cells interact to form the pulmonary capillary barrier, necessary to maintain efficient gas exchange in the lung. This barrier functions as a gatekeeper, limiting the transudation of blood components into the underlying interstitium and alveolar spaces. Tight cell–cell junctions between adjacent pulmonary capillary endothelial cells contribute to the integrity of this barrier and are dynamically regulated by transitions in cAMP [1].

The predominant transmembrane AC (adenylate cyclase) in pulmonary microvascular endothelial cells is the calcium–inhibited isoform 6 (AC6) [2]. Store-operated calcium entry pathways provide the calcium source necessary to inhibit AC6, as both store-operated calcium entry channels and AC6 are enriched within plasma membrane lipid rafts and caveolae [3]. Indeed, calcium inhibition of AC activity is readily detected in caveolin-enriched pulmonary microvascular endothelial cell membranes isolated on a 30–40% sucrose gradient. While calcium acts to regulate cAMP concentrations by negatively controlling enzymatic activity, the rolipram-sensitive phosphodiesterase isoform 4 regulates cAMP concentrations by hydrolysing this second messenger to 5′-AMP. Interestingly, rolipram-inhibited phosphodiesterase activity is also detected in this 30–40% sucrose gradient fraction.

Moreover, in addition to their role in terminating the second messenger signal, it has been proposed that phosphodiesterases, critically located within such microdomains, also act as diffusion barriers hydrolysing cAMP to restrict activation of non-specific targets in neighbouring compartments [4]. The subcellular localization of AC activity in pulmonary microvascular endothelial cells has been demonstrated using the exogenous substrate 5′-adenylylimidodiphosphate, which is hydrolysed to cAMP and imidodiphosphate. The imidodiphosphate is captured by cerium chloride to produce an electron-dense product and seen in pulmonary microvascular endothelial cells to be restricted to caveolae like structures, and at cell–cell borders [5]. Therefore, within pulmonary microvascular endothelial cells, AC6, calcium and phosphodiesterase 4 act to fine-tune cAMP concentrations critically localized to the subplasma membrane space of lipid raft compartments.

Thus activation of endogenous transmembrane AC signalling in pulmonary microvascular endothelial cells generates discrete spatial and temporal plasma membrane cAMP transitions. This highly compartmentalized cAMP enhances cell–cell contacts and tightens the endothelial cell barrier, thus optimizing gas exchange (Figure 1). However, pathogenic bacterial have devised mechanisms to induce cytosolic cAMP transitions that are barrier-disruptive.

Bacteria bypass the membrane cAMP signalling domain to generate a cytosolic cAMP pool

Various pathogenic bacteria have devised mechanisms that enable the direct transfer of bacterial proteins into the host cell.

Key words: adenylate cyclase, bacterium, cAMP, cytosol, endothelial cell, forskolin.

Abbreviations used: AC, adenylate cyclase; sACI/II, soluble ACI/II chimaera; YFP, yellow fluorescent protein.

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Upon access to the eukaryotic environment, these proteins are activated by a eukaryotic factor and utilize the available eukaryotic substrates to achieve enzymatic activity. One such bacterial protein is oedema factor of Bacillus anthracis, which is stimulated by calmodulin to achieve high-level AC activity [6]. This oedema factor-cAMP pool is minimally regulated by phosphodiesterase activity. In contrast with the idea that elevations in cAMP are barrier-protective, oedema factor induces oedema when injected into the skin of guinea-pigs or rabbits. Oedema can be induced by an increase in pressure, a reduction in lymph flow, a decrease in fluid reabsorption or an increase in fluid efflux from the blood. Indeed, a breach in the integrity of the endothelial cell barrier is a common mechanism for hyperpermeability and supports the idea that oedema factor induces endothelial gap formation.

ExoY of Pseudomonas aeruginosa is transferred into the cytosol of pulmonary microvascular endothelial cells where it has AC activity to generate a cAMP pool that is not regulated by phosphodiesterase activity. In contrast with endogenous transmembrane AC activity, the cytosolic AC activity of ExoY is associated with gap formation between adjacent pulmonary microvascular endothelial cells in culture as well as an increase in the filtration coefficient of the isolated perfused lung [5]. Collectively, these findings demonstrate that cytosolic AC activity disrupts the endothelial cell barrier (Figure 1), but does not indicate whether plasma membrane AC activity could rescue these barrier-disruptive effects.

**Cytosolic AC activity is endothelial cell barrier-disruptive**

Linking the first cytosolic loop of AC1 (C1a) to the second cytosolic loop of AC2 (C2a) with a 14-amino-acid linker generates a forskolin-activated mammalian soluble AC chimera (sACI/II) with minimal basal activity [7]. The chimera maintains forskolin-sensitive activity after adenoviral expression in pulmonary microvascular endothelial cells [8]. Indeed, in vivo, forskolin simultaneously activated AC activity at the plasma membrane (endogenous ACs) and within the cytosol (chimera). In contrast with plasma membrane AC activity, which is tightly regulated by phosphodiesterase activity, the cytosolic cAMP pool is not regulated by phosphodiesterase activity. Similarly, while isoprenaline stimulates endogenous transmembrane AC activity via Gαs, signalling, the cytosolic chimera was not activated, further illustrating its distinctive intracellular localization.

Forskolin activation of cytosolic and transmembrane AC activity rapidly induces retraction of adjacent endothelial cell borders. Therefore the cytosolic AC activity overwhelms the barrier-protective effects of transmembrane AC activity in the formation of endothelial cells gaps. Indeed, the cytosolic localization of sACI/II is required for endothelial cell barrier disruption.

**Relocalizing sACI/II to the plasma membrane**

Removal of the cytosolic loops of AC8 and reconnecting the two transmembrane domains with a YFP (yellow fluorescent protein) linker generates a catalytically inactive plasma membrane localization sequence [9]. Insertion of the soluble AC chimera between these two transmembrane domains and N-terminus to the YFP linker is sufficient to relocalize the soluble construct back to the plasma membrane. While this transmembrane AC chimera retains its forskolin-sensitive activity, it loses the ability to induce pulmonary microvascular gap formation. These findings therefore support the idea that membrane transitions in cAMP strengthen the endothelial cell barrier, whereas the production of cAMP within the cytosol (away from the membrane) disrupts the endothelial cell barrier.

**Summary**

Following the discovery of the second messenger cAMP, it was proposed that this highly hydrophilic second messenger would readily diffuse throughout the cellular environment to indiscriminately activate distant targets. However, Rall [10] has noted that greater signalling specificity must exist. Indeed, Buxton and Brunton [11] and others have corroborated this statement through observations in cardiomyocytes [12,13]. Indeed, it has been proposed that ‘microclimates’ of cAMP are maintained by phosphodiesterases and physical barriers like organelles [14], while spatiotemporal signal integration is established through the combinatorial assembly of
multiprotein complexes [e.g. AKAPs (A-kinase-anchoring proteins)] [15]. Currently, within pulmonary microvascular endothelial cells, emerging evidence establishes the opposing physiological outcomes of plasma membrane cAMP versus cytosolic cAMP. Whereas plasma membrane cAMP is endothelial cell barrier-protective, cytosolic cAMP is barrier-disruptive.

References

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