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Compartmentalized Regulation of CFTR Chloride Channel Function in Apical Epithelia

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Abstract

The cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel that is located primarily at the apical plasma membrane of polarized epithelial cells, plays a crucial role in transepithelial fluid homeostasis. Tight regulation of CFTR function is critical, as either hypofunctioning or hyperfunctioning of CFTR activity may result in life-threatening disorders. Accumulating evidence has suggested that the regulation of CFTR channel function is highly compartmentalized, i.e., CFTR function is tightly fine-tuned in localized microdomains, in which CFTR-containing multiprotein complexes play a critical role. A growing number of proteins has been reported to interact directly or indirectly with CFTR, implying that CFTR is coupled functionally and/or physically to a wide variety of interacting partners including ion channels, receptors, transporters, scaffolding proteins, enzyme molecules, signaling molecules, and effectors. In this review article, I summarized the most recent studies that characterize the compartmentalized regulation of CFTR function coupled to some interacting proteins such as receptors (i.e., adenosine receptor, beta adrenergic receptor, and lysophosphatidic acid receptor), transporters (i.e., cAMP efflux pump MRP4), and enzyme molecules (i.e., protein kinases, protein phosphatase, cAMP-specific phosphodiesterases, etc). These compartmentalized and dynamic interactions modulate not only the channel function, but also the localization and processing of CFTR protein within cells.

Keywords: CFTR regulation; Compartmentalized signaling; Protein interaction; Macromolecular complex; cAMP signaling; Epithelial apical membrane: Membrane protein transporter

Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is the product of the gene that is mutated in patients with cystic fibrosis (CF), the most common genetic disorder in Caucasians that affects approximately 1 in every 2,500 newborns [1]. CFTR is a plasma membrane, cAMP-regulated Cl^- channel that is responsible for transepithelial salt and fluid transport [2-4]. It is localized primarily to the luminal (apical) membranes of epithelial cells in multiple, functionally diverse tissues including the airway, intestine, pancreas, kidney, vas deferens, and sweat ducts. Two major human disorders resulting from altered function of CFTR chloride channel are CF and secretory diarrhea [5-7]. CF is a lethal genetic disease that is caused by the mutations of CFTR Cl^- channel, resulting in a decrease of either the protein biosynthesis or the function of the channel [8,9]. The absence or dysfunction of CFTR chloride channel leads to aberrant ion and fluid homeostasis at epithelial surfaces where it is normally expressed. The major clinical manifestation of CF is chronic lung disease, the main cause of morbidity and mortality for CF patients [1]. Other symptoms include exocrine pancreatic insufficiency and the resultant meconium ileus, elevated sweat electrolytes, male infertility, etc [1]. So far, close to 1900 CFTR mutations have been described, among which F508del-CFTR is the most common CF mutation [1]. F508del-CFTR is a defective gene product resulting from the deletion of a single phenylalanine (F) residue at position 508 on the protein level [10,11]. As an inefficiently folded and thus unstable CFTR protein [8,9], the F508del-CFTR fails to be correctly processed and delivered to the surface membrane, and is therefore trapped in the endoplasmic reticulum and subsequently targeted for degradation in lysosomal compartments [9,12]. Another major dysfunction involving CFTR is secretory diarrhea that is caused by excessive activation of this chloride channel in the gastrointestinal tract [13,14]. The involvement of CFTR in secretory diarrhea was first reported by Gabriel et al. who showed that bacterial toxins failed to induce secretory diarrhea in CF mice [14]. A major cause for acquired secretory and inflammatory diarrhea is the intestinal colonization by pathogenic microorganisms, including *E. coli, Vibrio cholerae*, *Yersinia enterocolitica, Shigella flexneri,* and *Salmonella typhimurium* [15,16].When the gut lumen is exposed to the various enterotoxins, excessive intracellular second messengers (cAMP and/or cGMP) are generated and subsequently activate PKA and/or PKG, which excessively phosphorylate luminal CFTR and lead to Cl^- secretion across the epithelium, consequently increasing the electrical and osmotic driving forces for the parallel flows of Na^+ and water, respectively [17,18]. Therefore, the net result is the robust secretion of fluid and electrolytes across the mucosal epithelium into the gut lumen, leading to secretory diarrhea and the resultant dehydration [19]. Normally, cholera toxin and heat-labile *E. coli* toxin induce intestinal fluid secretion by over-stimulating the production of intracellular cAMP, due to irreversible activation of adenylate cyclase by the toxins [20-22]. Other toxins, such as heat-stable *E. coli* toxin or *Y. enterocolitica* toxin, enhance intracellular cGMP and thus over-stimulate the apical membrane-associated cGMP-dependent protein kinase II that efficiently phosphorylates CFTR, and results in the activation of Cl^- secretion in crypts and intestine apical membranes [23-27].

CFTR belongs to the superfamily of the ATP-binding cassette

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(ABC) transporters, which bind ATP and use the energy to drive the transport of a wide variety of substrates across cellular membranes [28]. Structurally, CFTR consists of two repeated motifs, and each motif contains a hydrophobic membrane-spanning domain (MSD) containing six helices and a cytosolic hydrophilic region for binding with ATP (i.e., nucleotide binding domain, NBD) [28]. A cytoplasmic regulatory (R) domain links the two motifs, and this R domain is unique to CFTR among all ABC transporters and contains many charged residues and multiple consensus phosphorylation sites (substrates for various serine/threonine protein kinases, such as PKA, PKC, and cGMP-dependent protein kinase II). Activation of CFTR chloride channel requires both ATP binding to and hydrolysis by the NBD domains and phosphorylation of the R domain by protein kinases (PKA, PKC, PKG, etc) [29]. Both the amino (N) and carboxyl (C) terminal tails of CFTR channel are cytoplasmically oriented and mediate interactions with a growing number of binding proteins, and these physical interactions are crucial to the tightly modulated and highly compartmentalized regulation of CFTR function [5-7,30,31] as will be discussed in the following sections.

Regulation of CFTR function is highly compartmentalized in the apical membrane of epithelia

The cAMP pathway is one of the most versatile signal pathways in eukaryotic cells and is involved in regulating a plethora of cellular functions in virtually all mammal tissues [32]. In most cases, extracellular signals converge on the cAMP pathway through ligand occupancy of G protein-coupled receptors (GPCRs), and this signal pathway has been well documented to be tightly regulated at multiple levels to maintain specificity in the multitude of signal inputs [32]. Particular GPCRs are confined to specific domains of the cell membrane in association with intracellular organelles or cytoskeletal constituents, along with other signaling components and specific downstream effectors [33]. Ligand-induced changes in cAMP concentration vary in duration, amplitude, and extension into the cell, and it has been well documented that local cAMP gradients are dynamically shaped by specific adenylyl cyclases (ACs) in their proximity that generate cAMP, as well as phosphodiesterases (PDEs) that degrade cAMP into 5′-AMP and also serve as one of the cAMP downstream effectors, along with PKA, guanine nucleotide exchange factors (GEFs) known as exchange proteins activated by cAMP (EPACs), and cyclic-nucleotide-gated (CNG) ion channels [34]. Different PKA isozymes with distinct biochemical properties and cell-specific expression patterns contribute to cell and organ specificity. A structurally diverse but functionally related family of proteins that now includes more than 50 members, the so-called A kinase anchoring proteins (AKAPs), targets various PKA isozymes to specific substrates and distinct subcellular compartments, thus providing spatial and temporal specificity for cAMP-PKA pathway mediated biological processes [35]. AKAPs also serve as scaffold proteins that dynamically assemble specific PKA together with signal terminators (such as protein phosphatases and cAMP-specific PDEs), as well as elements of other signaling pathways into multiprotein signaling complexes to spatially and temporally control the cellular actions of cAMP at defined areas within the cell [35]. Targeting of PKA and integration of a wide repertoire of proteins involved in signal transduction into complex signal networks further increase the specificity required for the precise regulation of numerous cellular and physiological processes. This also provides a mechanism by which cAMP regulates cellular responses to external stimuli within specific subcellular regions and for limited time intervals [34].

The polarized distribution of ion channels and transporters in epithelia that enables vectoral solute transport has fostered great interest in investigations into the mechanisms underlying the signal transduction pathways that selectively regulate functions localized in the apical or basolateral cell membranes. Compartmentalization of the various signaling elements into distinctive microdomains colocalized at the apical or basolateral membranes has been suggested by many reports. It is now well accepted that the formation of multiprotein macromolecular signaling complexes at specialized subcellular microdomains increases the specificity and efficiency of signaling in cells [35,36]. One interesting feature of epithelial cells is that signals originating at either the apical or basolateral cell surface do not always lead to detectable changes in the concentration of specific second messengers (cAMP, cGMP, Ca^{2+}, etc.), although the cellular response is significantly altered [37-40]. An increasing number of studies are suggesting the same pattern for the regulation of CFTR function [41-43], as will be discussed in detail in the following sections.

CFTR-mediated Cl^- secretion across many epithelial cells is regulated through modulation of channel activity and total number of CFTR channels in the membrane. The compartmentalized modulation of CFTR channel activity, which is the focus of this review article, depends primarily on the dynamic regulation of the channel by various enzyme molecules including protein kinases (such as PKA), protein phosphatases (such as PP2A, PP2C), and cAMP-specific phosphodiesterases (such as PDE3, PDE4) [29]. The regulation of CFTR by PKA and other protein kinases has been well documented. PKA can phosphorylate the R domain of CFTR on multiple residues, which activates CFTR gating by destabilizing channel closed states, thus increasing CFTR open probability [29,44-46]. PKA-mediated phosphorylation may also enhance Cl^- transport through the insertion of additional CFTR channels into the plasma membrane [47]. The cellular machinery that is responsible for cAMP generation, including various GPCRs (such as adenosine receptor and beta-adrenergic receptor), as well as the membrane-bound ACs, are reported to be compartmentalized in proximity to CFTR in the apical membrane of secretory epithelia [41,48]. Localization of PKA by AKAPs restricts the action of this broad specificity kinase. At least one AKAP, the F-actin-binding protein (i.e., ezrin), has been reported to link type II PKA to protein complexes with CFTR [49-51], and the disruption of the PKA/AKAP interaction abolishes the ability of PKA to activate CFTR in response to physiologic stimuli [49]. In addition, it has been reported that the phosphodiesterase PDE4D is present with CFTR and forms a cAMP diffusion barrier at the apical plasma membrane [52]. Recently, another isozyme of PDEs, PDE3A, has also been demonstrated to bind directly to CFTR, which, through a synergistic mechanism with PDE4, contributes to the localized cAMP gradient in proximity to CFTR and regulates CFTR function in a compartmentalized fashion [53].

As CFTR is regulated by cAMP, which has been adequately demonstrated to be regulated in a compartmentalized fashion in various models and systems, it is conceivable to speculate that CFTR channel function could also be regulated in a compartmentalized manner. A growing body of evidence has suggested the existence of various physical and functional interactions between CFTR and an ever-increasing number of proteins, including transporters, ion channels, receptors, kinases, phosphatases, signaling molecules, and cytoskeletal elements, and these interactions between CFTR and its binding proteins have been shown to play an important role in regulating CFTR-mediated transepithelial ion transport *in vitro* and most probably *in vivo* [41-43,48,53-59]. In the following sections, I

will critically review recent studies and reports that investigated the compartmentalized regulation of CFTR channel activity in the apical surface membrane of secretory epithelia.

Compartmentalized regulation of CFTR function coupled to an adenosine receptor $A_{2b}AR$

The compartmentalized regulation of CFTR function was initially proposed by researchers from the University of North Carolina, Chapel Hill, who, using patch-clamp techniques, observed a substantial level of CFTR-mediated Cl^- conductance in models of airway epithelia in the absence of intentional stimulation of cAMP production [60]. This observation suggests that very low levels of cAMP activate CFTR and that there exist signaling pathways that produce small quantities of cAMP locally in response to tonic, low-level stimulation. In identifying the elements of cAMP signaling that were responsible for basal activity of CFTR in Calu-3 cells, a model system for exploring CFTR function in airway epithelia, they found that CFTR was selectively regulated by the membrane-associated isoform of PKA holoenzyme, PKA-II [61,62], and this regulation demonstrated a close physical and functional association between PKA-II and CFTR that is coupled by certain AKAPs, as the physical association of PKA and CFTR could be disrupted by an α-helical peptide (HT-31) that disrupts the anchoring of PKA-II to AKAPs [49]. They found that CFTR was no longer activated by a cAMP analogue (cpt-cAMP), while a control peptide (HT-31P) that contained prolines to disrupt the α-helical conformation of HT31 was completely ineffective [49]. The stimulation of CFTR in isolated membrane patches by a cAMP analogue and the inhibition of this effect by a peptide capable of dislodging PKA-II from preferred binding sites clearly indicated the existence of organized pathways for PKA-mediated regulation of CFTR, probably facilitated by AKAPs.

Using both protein interaction assays and functional measurements of CFTR activity, Sun et al. [50] further identified the CFTR-associated AKAP to be ezrin, an F-actin-binding protein present at the apical membrane domain of many epithelia cells. They found that CFTR is part of a regulatory complex that contains ezrin and both the catalytic and regulatory subunits of PKA. Disruption of these interactions blocks CFTR- and PKA-specific substrate phosphorylation. Ezrin was found to be present at the apical membrane domains of both airway (Calu-3 cells) and intestinal (T84 cells) secretory epithelia where CFTR is abundantly expressed, and it bound RII of PKA in protein overlay and co-immunoprecipitation experiments, suggesting that ezrin is a CFTR-associated AKAP in secretory epithelial cells [49].

Huang et al. [41] reported a compartmentalized regulation of CFTR channel activity by a physiological cAMP-elevating compound (i.e., adenosine, ADO). Using electrophysiological techniques, they elegantly demonstrated a compartmentalized signaling of extracellular ADO to CFTR at the apical membrane of polarized airway epithelial cells. In an Ussing chamber, they found that 1 μM ADO, the approximate half-maximal effective dose for adenosine-activated Cl^- secretion, barely increased cAMP production in Calu-3 cells. In contrast, 1 μM luminal forskolin stimulated no more Cl^- secretion than 1 μM ADO, but produced 9 times more cAMP. This efficient stimulation of chloride secretion by ADO (versus forskolin) but with little change in intracellular cAMP level suggests a localized regulation of CFTR function by adenosine receptor in the apical cell membrane. To better evaluate the localized regulation of CFTR and define the molecular identity of the signaling components, Huang et al. [41] used patch-clamp techniques to physically isolate the apical cell membranes into subcellular domains and monitored CFTR single-channel activity (channel open probability, NPo) within the subcellular domains, by applying different compounds such as adenosine receptor antagonist (8-SPT), PKA inhibitor (PKI), and AC inhibitor (SQ-22536) within the pipette. They observed that 1 μM exogenous ADO added to the pipette in the cell-attached recording elicited a dramatic stimulation of CFTR NPo, which was blocked by adenosine receptor antagonist (8-SPT), suggesting that $A_{2b}AR$ within an apical cell-attached membrane patch senses ADO and signals to CFTR contained within the same patch. This signaling between extracellular surface ADO and apical membrane CFTR was tightly compartmentalized, because even a large dose of ADO (100 μM) added to the bath outside the pipette had no effect on CFTR activity within cell-attached patches. However, CFTR in these same patches could be robustly stimulated by the cell-permeant forskolin (10 μM), which caused a large increase in total intracellular cAMP. To localize key cAMP-signaling elements, Huang et al. [41] excised apical membrane patches from polarized Calu-3 cells and applied various pharmacological interventions within the pipette. In outside-out and inside-out apical membrane patches, they demonstrated that functional PKA is downstream of $A_{2b}AR$, and cAMP-signaling between $A_{2b}AR$ and CFTR is functionally intact in isolated patches of apical cell membrane, which contains Gs protein and AC as well.

Based on the results, Huang et al. [41] proposed that, at the inner apical membrane surface, $A_{2b}AR$ is coupled to CFTR by means of G_s, AC, and PKA [41]. After release of ATP onto the extracellular surface by diverse physical stimuli (e.g., hypotonicity and shear stress), ADO is generated by ectonucleotidases. A_{2b} receptors bind ADO and activate AC present in the apical membrane by means of Gs. Sufficient cAMP is generated locally to activate PKA in a diffusionally restricted apical microdomain, but not in other cellular compartments. CFTR in the same microdomain is therefore activated in a highly compartmentalized fashion. In pursuing the question of what limits the lateral spread of apical membrane signaling, the same group extended their findings to demonstrate the involvement of PDEs [52], the enzymes that degrade and inactivate cAMP and have been found in complex with AKAPs and PKA [63,64], as discussed in detail in the following section.

Compartmentalized regulation of CFTR function coupled to phosphodiesterases PDE4D and PDE3A

The close proximity of cAMP-specific PDEs to PKA has been suggested to control the access of cAMP to binding sites on the regulatory subunit of the kinase [56,65]. PDEs are capable of regulating steady-state levels of cyclic nucleotides under basal conditions and can affect both amplitude and duration of cyclic nucleotide-dependent responses following hormonal stimulation [66]. O'Grady et al. [67] reported the involvement of certain PDEs in regulating transepithelial Cl^- secretion in cultured monolayers of T84 cells grown on membrane filters. They found that the most abundant PDE isoform in these cells was PDE4, accounting for 70-80% of the total cAMP hydrolysis within the cytosolic and membrane fractions from these cells, while the PDE3 isoform was also identified in both cytosolic and membrane fractions and accounted for 20% of the total cAMP hydrolysis in the cytosolic fraction and 15-30% of the total cAMP hydrolysis observed in the membrane fraction. Various PDE inhibitors produced significant increases in CFTR-dependent short-circuit current (I_{sc}) in confluent monolayers of T84 cells. The PDE3-selective inhibitors (terqinsin, milrinone and cilostamide) increased I_{sc} with EC50 values that were in close agreement with the IC_{50} values for cAMP hydrolysis [67]. The effects of the selective inhibitors for PDE1 (8-MM-IBMX) and

PDE4 (RP-73401) on I_{sc} were significantly less potent than for PDE3 inhibitors. The effects of 8-MM-IBMX and terqinsin on Cl^- secretion were additive, suggesting that inhibition of PDE1 also increases Cl^- secretion. Their results demonstrated that, although PDE4 is present in greatest abundance within T84 cells, PDE1 and PDE3 play a role in regulating Cl^- secretion [67]. By measuring CFTR-dependent transepithelial I_{sc} in Calu-3 monolayer, Cobb et al. [68] reported that PDE3 inhibitors (milrinone and cilostazol) and PDE4 inhibitor (rolipram) can elicit I_{sc}. They demonstrated that cilostazol and rolipram augment both the magnitude and the duration of I_{sc} after low-dose stimulation of adenosine receptor with ADO. Their results suggest that, in addition to PDE3, other PDEs including PDE4 may play roles in regulating CFTR in Calu-3 cells [68].

By directly monitoring the chloride secretion using ^{125}I as tracer in T84 monolayer, Liu et al. [69] reported that inhibitors of PDE3 and PDE4 (particularly the PDE4D) potently augmented CFTR-mediated iodide secretion with their efficacy coupled to the AC activation state. The iodide secretion from PDE3 or PDE4 inhibition was characterized by an initially prolonged efflux duration followed by progressively elevated peak efflux rates and reduced response time at higher inhibitor concentrations. The peak efflux from maximal PDE4 and PDE3 inhibition matched that from full AC activation. Dual suppression of PDE3 and PDE4 activity was synergistic, instead of additive, in stimulating the cAMP-mediated CFTR activation under the basal AC state with a limited cAMP turnover. These data suggest that PDE3 and PDE4 (mainly PDE4D) form the major cAMP diffusion barrier in T84 cells to ensure a compartmentalized CFTR signaling [69]. When analyzing the cAMP content of T84 cells in response to forskolin stimulation and PDE4 or PDE3 inhibition, they found that significantly elevated cAMP was detected only after a 10-min treatment with >1 μM forskolin [69]. In the absence of forskolin, treatment with different PDE inhibitors (1 μM) such as Cpd-A (PDE4), roflumilast (PDE4), and trequinsin (PDE3), separately or their combination, elicited a negligible global cAMP elevation. In the presence of 0.3 μM forskolin, elevated cAMP was detected only after treatment with more than 5 μM trequinsin, which is >1000-fold higher than the minimal dose capable of prolonging the efflux duration. Therefore, the global cAMP elevation from either forskolin stimulation or in combination with PDE3 or PDE4 inhibition all dissociated from their more potent activation of iodide secretion, suggesting a compartmentalized cAMP signaling by restricting cAMP diffusion through degradation by PDEs at natural physiological conditions. The abundant PDE4 and PDE3 expression in T84 cells compared well with that in airway epithelial cells (such as Calu-3 cells). Despite its lower abundance in T84 cells as reported by O'Grady et al. [67] as well, PDE3 inhibition by trequinsin seems to be a more effective activator of iodide efflux with a quicker response, compared with that from PDE4 inhibition by Cpd-A under an identical AC state [69]. They speculated that the enhanced PDE3/CFTR coupling may be due to their potential proximity or from the approximately 10-fold enhanced cAMP affinity of PDE3. Interestingly, most recently, studies from our lab revealed a direct physical and functional association between an isoform of PDE3 (PDE3A) and CFTR, which contributes to the generation of compartmentalized cAMP in proximity to CFTR [53], as discussed in detail in the following section.

To define the role of PDEs in signal compartmentalization in polarized airway epithelial cells, Barnes et al. [52] from UNC, Chapel Hill, extended their above-described observations with regard to local cAMP signaling in human airway epithelium and assessed the impact of PDEs in limiting apical cAMP signaling and the regulation of CFTR. Using a pharmacological approach, they determined which PDE gene families would modulate ADO-mediated activation of CFTR channel activity in cell-attached patches [52]. They found that only inhibitors of PDE3 and PDE4 affected CFTR currents, with PDE4 inhibitors being the more efficacious. They further demonstrated that PDE4D is a critical component of cAMP signaling at the apical membrane that controls the diffusion of cAMP from its site of generation and that this control is modulated through PKA activation. One other factor that could contribute to the finely tuned regulation of cAMP signaling downstream from apical A_{2b} receptors in airways is the ability of PKA to increase the activity of long isoforms of PDE4D, including PDE4D5 [70,71]. To test the hypothesis that subapical cAMP levels might be regulated by a negative feedback loop through PDE4, Barnes et al. [52] measured the effect of A_{2b} receptor stimulation on the activity of PDE4 in Calu-3 cells and found that luminal application of ADO or forskolin stimulated PDE4 activity in cell lysates, and this stimulation was mediated by PKA because it was blocked by the PKA inhibitor Rp-cAMP. Thus, in addition to the already well-established roles of PKA in transducing increases in cAMP, stimulation of PKA activity in the apical membrane also enhances the diffusional barrier provided by localized PDE4. This observation supports the presence of a mechanism that allows cells to coregulate activation and deactivation of a signal transduction pathway by using a feedback loop [52]. The data from Barnes et al. [52] provided physical and functional evidence for a major role of PDE4 as a diffusion barrier to cAMP signaling in the apical membrane of airway epithelia. This spatial barrier of PDEs was also shown to be sensitive and proportional to the levels of local cAMP [52]. However, it should be noted that the effectiveness of this barrier would also depend on the intensity and duration of cellular stimulation, because the excessive generation of cAMP in the microdomain may exceed the catalytic capacity of available PDEs. And this suggests that other pathways may exist to regulate cAMP dynamics in the microdomains. We recently reported a previouslyunidentified mechanism for cAMP regulation, an efflux pathway for cAMP via a cAMP pump, MRP4 [43], which will be discussed in detail in a later section.

Studies from Lee et al. [72] recently demonstrated that an apical PDZ scaffold protein, Shank 2 [5,6], was physically and functionally associated with PDE4D that precludes cAMP/PKA signals recruited by another PDZ scaffold protein, EBP50/NHERF1, in epithelial cells and mouse brains. Using surface plasmon resonance assay, they found that the dissociation constant of CFTR-Shank2 binding was similar to that of CFTR-NHERF1 binding and that both proteins apparently compete for binding at the same site [72]. Consecutive patch-clamp studies revealed that CFTR Cl^- channel activity was dynamically regulated by the competition of Shank2 and EBP50 binding. Notably, in contrast to the PKA/AKAP recruitment by NHERF1 [6,73], Shank2 was found to tether PDE4D to the CFTR complex, thus attenuating cAMP/PKA signals [72]. A thorough molecular characterization in their study revealed that Shank2 recruits PDE4D through a direct interaction between the proline-rich region of Shank2 and the UCR1/2 region of PDE4D [72]. Their results clearly suggested that CFTR activity is tightly and dynamically regulated by a competitive balance between CFTR-activating and CFTR-inactivating PDZ domain interactions. The results from Lee et al. [72] clearly show that opposite signals can be delivered to the same PDZ-binding motif of a given membrane protein (such as CFTR) by different adaptors, contributing to the fine-tuned regulation of signaling. Similarly, as will be discussed later, certain

AKAPs have also been reported to anchor counter-balancing enzymes such as protein kinases (e.g., PKA) and protein phosphatases (e.g., PP2A) in proximity to their enzyme substrate proteins (e.g., CFTR), thus coordinating the CFTR phosphorylation and dephosphorylation processes in a compartmentalized manner and providing efficient and timely regulation of signaling events.

As reviewed above, inhibition of PDE4 and/or PDE3 has been demonstrated to activate CFTR Cl^- channel function [52,67-69,72,74]. However, most of those studies were focused on functional association between PDEs and CFTR by using electrophysiological techniques. Most recently, Penmatsa et al. [53] reported a direct physical interaction between CFTR and PDE3A, and inhibition of PDE3A generates compartmentalized cAMP, which further clusters PDE3A and CFTR into microdomains at the plasma membrane and augments CFTR channel function including tracheal submucosal gland secretion. They also showed that disruption of CFTR-PDE3A-containing macromolecular complexes abolishes compartmentalized cAMP signaling [53]. Using the pig tracheal submucosal gland secretion model [53], they observed that a specific PDE3 inhibitor, cilostazol (100 μM), elicited a three-fold increase in mean mucosal secretion rate, which was inhibited by a specific CFTR channel inhibitor, CFTRinh-172. Consistent with the previous observation [74], they demonstrated that PDE3A inhibition increased CFTR-mediated currents in a dose-dependent manner (10 -100 μM cilostazol) [53]. PDE3A inhibition induced a smaller magnitude of I_{sc} response compared with that stimulated by forskolin (20 μM), an AC stimulator that elicits a global increase of cAMP and maximally stimulates CFTR function [42,43]. The cilostazol-induced I_{sc} can be further increased to a maximal level by forskolin (20 μM), suggesting that PDE3A inhibition generates localized cAMP rather than global cAMP [53]. They also observed that inhibition of PDE4 (by rolipram) led to a smaller increase in I_{sc} n Calu-3 cells when compared with PDE3 inhibitor (cilostazol), a finding that is consistent with the previous observation that PDE3 inhibition elicited a bigger and quicker CFTR-mediated iodide efflux than did PDE4 inhibition in T84 cells under an identical AC state [69]. Interestingly, inhibition of both PDE3 and PDE4 elicited a synergistic increase of CFTR currents [53]. A low dosage of ADO (1 μM), which stimulates CFTR channel function in a compartmentalized manner at the apical cell membranes [41,43], elicited a significant CFTR-mediated iodide efflux, and, PDE3A inhibition (cilostazol, 100 μM) demonstrated >50% additive effect in ADO-elicited CFTR-mediated iodide efflux [53]. They further observed an increased level of cAMP in Calu-3 cells upon PDE3A inhibition most prominently at the cell edge area, whereas forskolin elicited a global increase of cAMP. However, PDE4 inhibition induced a maximal increase in cAMP levels that was similar to the effect seen with forskolin stimulation (20 μM). These data suggest that PDE3A is probably involved in compartmentalized cAMP signaling.

Using a direct, sensitized emission FRET approach, Penmatsa et al. [53] observed microscopically that PDE3A and CFTR interact at the plasma membrane and this interaction increased by almost 70% upon treatment with a PKA-activating cocktail, suggesting that the interaction between CFTR and PDE3A is PKA-dependent

The physical interaction between PDE3A and CFTR was also detected via co-immunoprecipitation assay under both native (Calu-3 cells) and overexpression (HEK293 cells) conditions however, there is no detected interaction between PDE3B and CFTR [53].

Although dual inhibition of PDE4 and PDE3 was reported to demonstrate a synergistic action on activating CFTR function, there is no detected physical interaction between PDE4D and PDE3A via co-immunoprecipitation under native conditions [53]. However, the possibility of PDE3A and PDE4D being in proximity still cannot be ruled out. PDE4D was reported to directly interact with a PDZ scaffold Shank2 and Shank2 directly binds to the C-tail of CFTR via its PDZ-domain [6], thereby bringing PDE4D close to CFTR without direct contact [72]. This may explain why PDE4D was not co-precipitated with PDE3A in Calu-3 cells [53]. Actin cytoskeleton has been shown to be important for maintaining CFTR in highly restricted domains at the plasma membrane [75], as well as the activation and regulation of CFTR Cl^- channel function [76,77]. PDE3A demonstrated a confined diffusion pattern in the plasma membrane as monitored by single-particle tracking method [53], similar to that reported for CFTR [78]. Upon cytoskeleton disruption, the lateral diffusion of PDE3A was significantly increased, which suggests that actin cytoskeleton disruption uncouples PDE3A from the CFTR-containing complex, causes it to move freely, and compromises the integrity of multiprotein complex [53]. Similarly, cytoskeleton disruption led to a significant decrease (>90%) in cilostazol-activated and CFTR-dependent mean mucosal secretion and a reduced potentiating effect of PDE3A inhibition on adenosine-activated CFTR-mediated currents by almost 65–80%; however, the maximally stimulated I_{sc} by forskolin (increases global cAMP) remains unaffected by cytoskeleton disruption [53].

These data further support the notion that PDE3A functionally and physically interacts with CFTR in a compartmentalized fashion. Upon disruption of actin cytoskeleton, the CFTR–PDE3A interaction was reduced, and the functional coupling between PDE3A and CFTR was disrupted. As a result, the integrity of the CFTR-PDE3A–containing macromolecular complex was compromised and the compartmentalized cAMP signaling was abolished. Inhibition of PDE3A thus no longer potentiates CFTR Cl^- channel function in a compartmentalized manner.

Given the fact that PDEs convert cAMP into 5'-AMP and the resultant AMP can activate a serine/threonine kinase, AMP-dependent protein kinase (AMPK), which has been reported to directly bind CFTR and reduce CFTR currents [55], the GPCR (such as A_{2b} receptor) –driven stimulation of subapical PDE4D and PDE3A could initiate negative feedback on CFTR activity through AMPK. It has been reported that AMPK is localized to the apical membrane [55], down-regulates CFTR currents in Calu-3 cells [79], and may be stimulated by the locally increased 5′-AMP generated by PDE activity near the activated receptor/channel complex. It was observed that pretreating Calu-3 cell monolayers with an AMPK-activating compound (5-aminoimidazole-4-carboxamide riboside) to fully activate AMPK reduced CFTR-mediated Cl^- secretion induced by luminal ADO [52]. Furthermore, CFTR was also reported to have an intrinsic adenylate kinase activity that may regulate channel gating by directly binding AMP to the second nucleotide binding domain [80]. However, additional studies are required to elucidate conclusively the exact relationship between the 5′-AMP generated by PDE4 and AMPK activity in Calu-3 cells.

Compartmentalized regulation of CFTR function coupled to protein phosphatase PP2A

Once activated by PKA phosphorylation of the R-domain, CFTR channels were found to deactivate quickly when membrane patches were excised from Chinese hamster ovary cells [81] or airway cells [82]. This quick rundown presumably results from dephosphorylation of

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PKA sites, because it does not occur if PKA catalytic subunit is present, and channels can be fully r-stimulated by exposure to PKA during the first few minutes [81,83]. Rundown indicates that at least one of the phosphatases regulating CFTR is membrane-delimited and remains active in the excised patch.

How the serine/threonine phosphatases regulate CFTR activity or how they are compartmentalized with CFTR is still not fully understood. To date, no single phosphatase has been shown to be both necessary and sufficient to completely down-regulate CFTR channel activity, suggesting that CFTR is dephosphorylated by multiple phosphatases. So far, multiple phosphatases, including PP2A, PP2B, PP2C, and alkaline phosphatase, are reported to be involved in deactivating CFTR [59,84-90]. However, the specificity of CFTR regulation by phosphatases is poorly understood. Thelin et al. [59] recently reported a direct interaction between CFTR and PP2A, and this interaction involves the COOH terminus (residues 1451-1476) of CFTR and the PP2A B′ε regulatory subunit. PP2A localizes to the apical cell surface, where it negatively regulates CFTR channel activity in Calu-3 cells and intact mouse jejunum. Furthermore, PP2A inhibition increases the airway surface liquid in primary human bronchial epithelial (HBE) cells by a mechanism requiring CFTR channel activity. They concluded that PP2A functions as a relevant CFTR phosphatase in epithelial tissues [59].

In the presence of PP2A inhibitors, CFTR rundown was blocked in outside-out membrane patches from Calu-3 cells and intact mouse jejunum [59]. Consistent with the biochemical observations, Thelin et al. [59] found PP2A in the membrane patches containing CFTR, strongly suggesting that these proteins are closely compartmentalized [59]. In T84 and airway epithelial cells, PP2A inhibitors did not block the rundown of CFTR following maximal activation by forskolin [88,90]. They speculated that they measured the effect of PP2A inhibitors on basal CFTR currents as opposed to CFTR currents following maximal activation with forskolin or PKA [59]. It is possible that endogenous PP2A plays a role in regulating basal CFTR currents but that other phosphatases contribute to the deactivation of hyperphosphorylated CFTR. Future studies are needed to address the relative contribution of different phosphatases to the regulation of CFTR channel activity in epithelial tissues.

Most recently, it was reported that PP2A associated with a multimolecular signaling complex scaffolded by mAKAP, a muscle AKAP in cardiac myocytes [91], that also contains PDE4D3 and PKA, promotes PDE4D3 dephosphorylation, and serves to both inhibit PDE4D3 in unstimulated cells and mediate a cAMP-induced positive feedback loop following AC activation [63,92]. Each of these three enzymes is likely to play an important role in the temporal control of cAMP concentration in the vicinity of perinuclear mAKAP complex. In light of this finding, there is a possibility that PP2A may also interact directly with ezrin, the CFTR-associated AKAP [50] that functions to also scaffold PKA, PDEs, and even AC into a macromolecular signaling complex. Ezrin has been shown to bind the scaffold protein NHERF1/EBP50, and the latter binds directly to CFTR via a PDZ-based interaction [48]. It has been reported that ezrin can form homodimers or heterodimers with moesin, a microvillar protein that is closely related to ezrin [93], which raises a possibility that dimerization of ezrin may not only cluster the various enzyme molecules but also bring different substrate proteins and membrane receptors and channels in proximity. In addition to regulating channel activity by dephosphorylating CFTR, PP2A may also be important for mediating other signaling and trafficking events for CFTR. For example, AMPK, which directly binds to CFTR and negatively regulates channel activity, has been reported to also directly interact with PP2A via the A regulatory subunit [55,94]. Moreover, the direct interaction between CFTR and AMPK has been mapped to CFTR residues 1420-1457, adjacent to the residues where PP2A binds (residues 1451-1476) [55]. It is interesting to speculate that PP2A and AMPK may function to stabilize each other's interactions with CFTR or may compete for binding to the CFTR COOH terminus, which may contribute to the fine-tuning of CFTR function in a compartmentalized manner.

Compartmentalized regulation of CFTR function coupled to a beta adrenergic receptor β_2AR

Normal airway epithelium is composed of an absorptive epithelial surface and a secretory submucosal gland [95], and the secretory function of the submucosal gland is essential for maintaining the airway surface liquid and for mucociliary clearance, and for clearing mucus from the submucosal gland [96]. In individuals harboring mutant forms of CFTR, the secretory function of the submucosal gland is impaired and leads to the accumulation of mucus in the airway surfaces, in turn leading to serious respiratory complications. As the major adrenergic receptor isoform expressed in airway epithelial cells, β_2 adrenergic receptor (β_2AR) stimulated with eceptor agonist (isoproterenol) was reported to lead to activation of CFTR-dependent chloride transport *in vivo* [97]. Biochemical studies have demonstrated that the C-termini of CFTR and β_2AR possess PDZ binding motifs, which contribute to interactions between these membrane proteins [98-100] and cytoskeletal proteins [101] that are important for channel regulation [102,103] and trafficking [104,105]. Recently, Naren et al. [48] investigated the possible biochemical and functional association connecting CFTR and β_2AR. They observed that, in polarized Calu-3 epithelial cell monolayers, β_2AR was significantly expressed at or near the apical cell surface, where it was colocalized with CFTR [48]. β_2AR-selective agonists activate CFTR-dependent Cl^- currents at the apical surface of these epithelial cells. Both β_2AR and CFTR bind NHERF1/EBP50, a PDZ scaffold protein [5,6,98], through their PDZ motifs. Deletion of the PDZ binding motif of CFTR (last 3 aa and referred to as ΔTRL-CFTR) eliminated the physical interaction between the channel and receptor. Furthermore, removing the PDZ motif (ΔTRL-CFTR) specifically reduced CFTR-mediated iodide efflux after β_2AR stimulation. In contrast, activation of CFTR by agonists such as ADO or forskolin, both of which elevate cAMP without by using the β_2AR signaling pathway, was not affected by deletion of the CFTR PDZ motif. This result suggests that deleting the PDZ motif from CFTR uncouples the channel from the β_2AR receptor both physically and functionally, and this uncoupling is specific to the β_2AR receptor and does not affect CFTR coupling to other receptors (e.g., adenosine receptor pathway). They further demonstrated biochemically the existence of a macromolecular complex containing CFTR-NHERF1-β_2AR in a PDZ-dependent manner at the apical surface of airway epithelial cells, which is regulated by PKA-dependent phosphorylation [48]. Interestingly, PKA phosphorylation of CFTR inhibited formation of the macromolecular complex in a dose-dependent manner, while deleting the regulatory R domain of CFTR abolished PKA regulation of the complex assembly.

Accordingly, a model was proposed to depict the coupling of β_2AR signaling to CFTR function [48]. CFTR, NHERF1, and β_2AR form a macromolecular complex together with signaling molecules at the apical surfaces of airway epithelia. G proteins can be associated

with β_2AR and PKA anchored to AKAP (ezrin) and are likely to be in the complex. Upon agonist activation of the receptor, AC is stimulated through the Gs pathway, leading to an increase in highly compartmentalized cAMP. This increased local concentration of cAMP leads to the activation of PKA, which is in proximity to CFTR, and results in a compartmentalized and specific signaling from β_2AR to the CFTR channel. Phosphorylation disrupts the complex, leading to the receptor-based activation of CFTR. The macromolecular complex assembly is essential for full activation of CFTR channel by β_2AR pathway, which can be circumvented by using other pathways (e.g., ADO or forskolin). This interaction is critical for an efficient and specific signal transduction from the β_2AR to the CFTR channel in a compartmentalized fashion. Interactions of this sort may also be important to CFTR regulation of other ion channels, receptors, and transporter proteins, as will be reviewed in the following sections. These findings also suggest how certain defective forms of CFTR may lead to abnormal CFTR function in the context of receptor-based signaling and signal compartmentalization. For instance, in patients with F508del-CFTR and other mutations in which the mutant CFTR channel is either degraded or mislocalized, disruption in signal transduction may have effects broader than those predicted simply from the absence of a functioning CFTR chloride channel.

Compartmentalized regulation of CFTR function coupled to a lysophosphatidic acid receptor LPA_2

Among the major microorganisms that cause infectious diarrhea in humans are *Escherichia coli* and *Vibrio cholera*, whose secreted toxins, such as heat-stable or heat-labile toxin and cholera toxin (CTX), induce diarrhea by numerous mechanisms [18]. CFTR has been shown to play a pivotal role in CTX-induced secretory diarrhea in model cell systems and experimental animals [14]. Recently, we demonstrated that lysophosphatidic acid (LPA) [106], a naturally occurring phospholipid in blood and foods, significantly inhibited CFTR-dependent iodide efflux, short-circuit currents, and single-channel activity in a compartmentalized fashion [42]. This inhibition is mediated through a type 2 LPA receptor (LPA_2), which is localized to the luminal membrane of colonic epithelial cells and gut mucosal epithelia where CFTR and NHERF2 (a PDZ scaffold protein) also reside.

Activation of LPA receptors by LPA results in inhibition of the AC pathway, which, in turn, decreases cAMP levels [106]. Therefore, LPA might regulate CFTR Cl^- channel function, which is regulated by cAMP. We observed that LPA pretreatment reduced CFTR-mediated iodide efflux, in Calu-3 and HT29-CL19A cells in response to cAMP-elevating ligand ADO in a dose-dependent manner [42]. In addition, LPA inhibited CFTR-dependent I_{sc} on polarized epithelial monolayers and mouse intestine epithelia stimulated by ADO or cpt-cAMP in a dose-dependent fashion [42]. More interestingly, the inhibitory effect of LPA on CFTR Cl^- currents was more prominent when CFTR was activated at lower concentrations of ADO, whereas LPA failed to inhibit channel function significantly when CFTR was activated maximally by a cocktail agonist mixture, suggesting a localized LPA signaling of CFTR function. Similar findings were also observed in the study of CFTR single-channel activity at cell-attached patch-clamp configuration. The CFTR channel was activated when 2 μM ADO were added in the pipette, but not when it was added to the bath [42]. In LPA-pretreated cells, the stimulatory effect of ADO (2 μM, applied in the pipette) on channel activity was inhibited. In contrast, when 10 μM forskolin (leading to a global increase in cAMP) was used in the pipette or the bath, LPA no longer attenuated channel function. We observed similar results when cpt-cAMP was used to activate the channel. A lower concentration of cpt-cAMP (20 μM) stimulated localized CFTR activity that could be inhibited by LPA, whereas a higher concentration of cpt-cAMP (200 μM) led to global stimulation that could not be attenuated by LPA. These single-channel recordings clearly suggested that LPA inhibits CFTR-mediated Cl^- currents in a compartmentalized fashion.

In our study, we also observed that cAMP accumulation inside polarized epithelial cells (Calu-3), upon stimulation with a low concentration of ADO (2 μM), was almost indistinguishable from that of unstimulated cells, while CFTR was active under these conditions and LPA could significantly inhibit channel activity [42]. This is a very interesting observation, consistent with the results from Huang et al. [41] who demonstrated compartmentalized signaling from the receptor (A_{2b}) to the channel (CFTR) by using electrophysiological methods. We found that LPA efficiently inhibited CFTR function (in response to 2 μM ADO) without causing a decrease in the global cAMP accumulation in the cell [42]. Our results support the notion that cAMP is generated in a compartmentalized pocket upon stimulation by receptor-mediated agonists (ADO). However, when the ADO level was increased to 20 μM, we observed that CFTR function increased slightly compared to an increase of 2 μM ADO, but there was a significant increase in cAMP accumulation inside the cell (global cAMP accumulation). Although there was a significant decrease in cAMP accumulation with LPA treatment, the CFTR-mediated currents were not significantly different (in the presence or absence of LPA), suggesting that a global increase in cAMP may offset CFTR inhibition elicited by LPA. As both LPA_2 and CFTR interact with the scaffold protein NHERF2 [107,108], and LPA signaling was functionally coupled to CFTR activity [42], we further demonstrated biochemically that LPA_2 forms a macromolecular signaling complex with CFTR mediated by NHERF2 in a PDZ-motif dependent manner in native airway and gut epithelial cells [42]. We also observed that an LPA_2-specific peptide (last 11 a.a. of LPA_2 containing the PDZ motif), which could efficiently disrupt the LPA_2 and NHERF2 interaction, significantly prevented the LPA-elicited inhibition of CFTR-dependent Cl^- currents in polarized epithelial monolayers [42]. In addition, LPA treatment significantly reduced the fluid accumulation in the toxin-treated intestinal loops in a mouse model of secretory diarrhea [42].

Our study convincingly showed a macromolecular complex (CFTR-NHERF2-LPA_2) formed *in vitro*, which provides the anatomical basis for the compartmentalized regulation of CFTR function, and was also physiologically and functionally relevant *in vivo* [42]. LPA_2 and CFTR are physically associated with NHERF2, which clusters LPA_2 and CFTR into a macromolecular signaling complex at the apical plasma membranes of epithelial cells. Upon LPA stimulation of the receptor, AC is inhibited through the G_i pathway, leading to a decrease in cAMP level. This decreased local or compartmentalized accumulation of cAMP results in the reduced activation of Cl^- channel in the vicinity by CFTR agonists (e.g., ADO).

Compartmentalized regulation of CFTR function coupled to a cAMP efflux transporter MRP4

In epithelial cells lining the gut, kidney, and lung, cAMP plays key roles in extracellular regulation of fluid homeostasis [32]. Tight regulation of intracellular cAMP levels is critical, because excessive cAMP production within cells leads to overstimulation of certain secretory events, dysregulation of cell function, or even cell toxicity [32]. We recently identified a novel means of regulating cAMP levels in

a compartmentalized microdomain underneath the surface membrane, an efflux path for cAMP via MRP4 transporter in the close vicinity of CFTR-containing signaling complex [43].

The functional activity of CFTR Cl^- channel is regulated by PKA after a rise in the local concentration of cAMP. Inhibition of the plasma membrane cAMP efflux transporter MRP4 might, in theory, enhance the cAMP signal and thus magnify CFTR function. We observed, in basolateral-permeabilized cells pretreated with MK571, a potent MRP4 inhibitor [109], a low dose of cAMP (10 μM) elicited a near-maximal CFTR-mediated I_{sc}, which was not boosted further by a higher dose of cAMP (50 μM). Whereas in untreated (or vehicle-treated) cells, 10 μM cAMP induced only a very small I_{sc} response (only 30%–40% magnitude of the MK571-treated cells), the currents could be further increased to a maximal level by a higher dose of cAMP (50 μM) [43]. However, at a dose of 20 μM cAMP, maximal CFTR-dependent I_{sc} responses were observed both in the presence and absence of MK571. We consistently observed that, in MK571-pretreated cells, the cAMP-activated state of the CFTR Cl^- channel were sustained for a relatively longer period compared to untreated cells, i.e., the deactivation of the channel was relatively slower in the presence of MK571. This potentiation of CFTR function in response to exogenous cAMP by MRP4 inhibition raises the possibility that attenuated MRP4-mediated apical cAMP efflux may contribute to the accumulation of the second messenger locally in proximity to the CFTR Cl^- channel, thereby resulting in increased CFTR Cl^- currents, as opposed to what is seen with the untreated control [43]. MRP4 inhibition potentiated CFTR-mediated I_{sc} across intact (nonpermeabilized), polarized gut epithelial cell monolayers in response to ADO stimulation, and the potentiating effect was more prominent when CFTR was activated with a lower concentration of ADO (at 3 μM ADO, ~2-fold I_{sc} for MK571-treated cells compared to the control; at 50 μM ADO, ~1.4-fold I_{sc} for MK571-treated cells compared to the control). However, MRP4 inhibition failed to significantly potentiate the channel function when CFTR was maximally stimulated with a relatively higher dose of ADO (100 μM) or 20 μM forskolin, the AC stimulator, which causes a global increase of the intracellular cAMP level [41,42]. Interestingly, MRP4 inhibition can induce a very small I_{sc} (~3–5 μA/cm^2) even in the absence of ADO stimulation [43].

CFTR single-channel activity was significantly increased at concentrations of 1.5-10 μM ADO in the presence of MK571 compared to control. In contrast, at doses of ADO greater than 20 μM in the pipette (leading to a significantly higher increase in intracellular cAMP, which might activate CFTR function sub maximally or maximally), MK571 no longer potentiated CFTR channel function [43]. NPo of CFTR Cl^- channels was higher in MK571-pretreated cells compared to untreated cells even in the absence of ADO stimulation, a result consistent with the above-described observation that MK571 itself can induce a very small I_{sc} even in the absence of ADO stimulation. The potentiation of CFTR function by MRP4 inhibition also demonstrated a dose-dependence, with a linear increase range falling within 0-30 μM of MK571, when the CFTR channel was stimulated in a localized pattern by only 1.5 μM ADO [43]. All of the above data suggest that MK571 potentiates CFTR-mediated Cl^- currents in a locally restricted manner when CFTR is not maximally activated.

We further observed that MRP4 inhibition induced a small but substantial increase of cAMP level as assayed by a FRET-based cAMP indicator, CFP-EPAC-YFP [110], similarly to what was observed from I_{sc} measurements and CFTR single-channel recordings. Interestingly, the most prominent increase of intracellular cAMP, after blocking the cAMP efflux transporter MRP4, occurred at the edge of the cells which indicates a localized (spatially restricted) cAMP accumulation near the plasma membrane (i.e., subcellular cAMP heterogeneity). A lower dose of ADO (2 μM) also induced a small but substantial increase of cAMP. While MRP4 inhibition led to a significant further increase in cAMP level in addition to the cAMP increase caused by a lower dose of ADO (2 μM), a higher dose of ADO (100 μM) induced a much higher cAMP level. However, MRP4 inhibition failed to execute any significant additional increase of cAMP on top of the cAMP increase caused by a higher dose of ADO (100 μM). These results reveal a transition from compartmentalized cAMP elevation to global increase of intracellular cAMP (indicated by the uniform increase of cAMP in the entire cytoplasm). We observed a similar pattern of the cAMP change in the presence or absence of MK571 when cells were stimulated by 20 μM forskolin, which usually induces a global increase of intracellular cAMP. These results imply that the increased cAMP accumulation through inhibiting cAMP efflux (MRP4 inhibition) demonstrates the biggest magnitude only when it is happening in a compartmentalized, spatially restricted microdomain near the plasma membrane, suggesting a spatial cAMP heterogeneity within a single cell. In addition, MK571 treatment significantly increased the fluid accumulation in the toxin-treated intestinal loops in wild type mice but did not in *MRP4* knockout mice in a closed-loop model of secretory diarrhea [43]. Compared to wild type mice, PBS control loop in *MRP4* knockout mice showed a slightly and significantly higher level of basal fluid secretion. In addition, CTX-injected loops in *MRP4* knockout mice also demonstrated a significantly higher level of toxin-stimulated fluid accumulation compared to loops in wild type mice [43]. Therefore, our data demonstrated that *Mrp4*-deficient mice are more prone to CFTR-mediated secretory diarrhea.

Like CFTR, MRP4 also possesses a consensus PDZ motif at its C terminus [6]. It is possible that MRP4 can interact with CFTR via PDZ motif-based interaction. We identified a PDZ scaffold protein, PDZK1, which binds directly to MRP4 with high affinity (with an EC50 of 3.75 nM). PDZK1 has been reported to interact with CFTR through a PDZ-based interaction [6,103]. We demonstrated the formation of a macromolecular complex of MRP4, PDZK1, and CFTR *in vitro*, and the complex formation increased dose dependently with increasing amounts of the intermediary protein, PDZK1. Both MRP4 and PDZK1 could be coimmunoprecipitated with CFTR from cultured gut epithelial cells that endogenously express all these proteins [43]. These studies suggest that a macromolecular complex consisting of endogenous MRP4-PDZK1-CFTR is likely present on the apical surface of gut epithelial cells and that it forms the basis for the functional coupling of MRP4 transporter activity to CFTR channel function that we observed in this study

We disrupted the complex by using competitive MRP4 PDZ-motif peptides (containing the last 10 a.a., including the tripeptide PDZ-motif at the extreme C terminus), which have been shown to interfere specifically with the endogenous interaction between MRP4 and PDZK1 but not with the interaction of PDZK1 and other target proteins. The MRP4-specific peptides significantly attenuated the MK571-elicited potentiation of CFTR-mediated currents in gut epithelial cells- at low doses of ADO (3-15 uM).

In light of the literature and the data obtained from this study, we proposed a model to depict the spatiotemporal coupling of cAMP transporter to CFTR Cl^- channel function in the gut epithelia (Figure

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1) [43]. Under the apical plasma membrane there exist highly localized compartments that are composed of a series of signaling molecules such as adenosine receptor (A_{2b}); G protein (Gs); AC; PKA and its anchoring proteins AKAPs; CFTR; cAMP transporter (MRP4); and PDZ scaffolding proteins (in this case, PDZK1), which functions to physically connect CFTR to MRP4. This macromolecular signaling complex provides an anatomical basis for generating and modulating local cAMP compartments. Upon an agonist (such as ADO) binding the A_{2b} receptor, a series of G-protein-mediated reactions leads to activation of AC present in the apical membrane. Sufficient cAMP is locally generated in a diffusionally restricted apical microdomain. cAMP activates PKA, which is anchored also to the apical membrane by AKAP (i.e., ezrin), and phosphorylates CFTR Cl^- channel in close vicinity, resulting in an increase of Cl^- currents. The CFTR-mediated Cl^- currents can be further potentiated by increased local cAMP resulting from the reduced or blocked efflux via a neighboring apical membrane cAMP transporter (MRP4) in the same subcellular compartment. It is generally believed that PDEs provide the sole means for degrading cAMP in cells and play a vital role in shaping intracellular cAMP gradients [32,111]. Here, we identified additional means of regulating cAMP levels in a microdomain underneath the surface membrane, an efflux path for cAMP via MRP4 transporter in the close vicinity of CFTR-containing signaling complex [43]. The interaction between CFTR and MRP4 provides an additional layer of mechanism to regulate CFTR function in a compartmentalized fashion, which is important in maintaining epithelial and body homeostasis

Conclusions and Perspectives

Based on the published studies so far, the compartmentalized cAMP signaling that regulates CFTR function elicited by ligand binding to cognate GPCRs in various secretory epithelia can be summarized as follows.

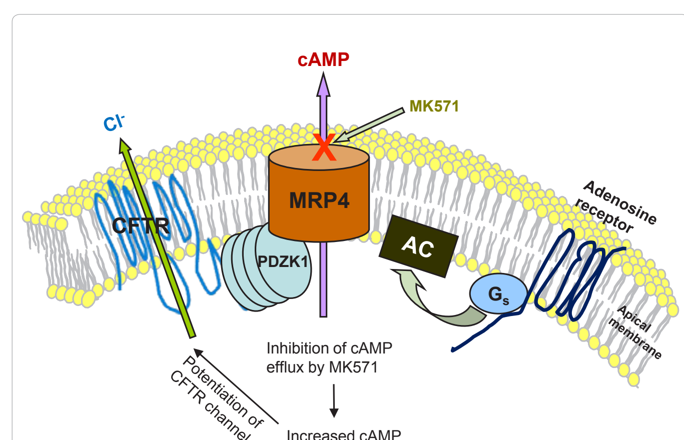

Figure 1: Compartmentalized regulation of CFTR function coupled to a cAMP efflux transporter MRP4. Under the apical plasma membrane in the gut epithelia, there exist highly localized compartments that are composed of a series of signaling molecules such as adenosine receptor (A_{2b}); G protein (Gs); AC; PKA and its anchoring protein AKAPs (not shown here); CFTR; cAMP transporter (MRP4); and PDZ scaffolding protein (PDZK1), which physically connects CFTR to MRP4. This multi-protein signaling complex provides an anatomical basis for generating and modulating local cAMP compartments. When an agonist (such as ADO) binds A_{2b}, a series of G-protein-mediated reactions leads to activation of AC present in the apical membrane. Sufficient cAMP is locally generated in a diffusionally restricted apical microdomain (but not in other cellular compartments). cAMP activates PKA, which is anchored also to the apical membrane by AKAP (i.e., ezrin), and phosphorylates CFTR Cl^- channel in close vicinity, resulting in an increase of Cl^- currents. The CFTR-mediated Cl^- currents can be further potentiated through the additional increase of local cAMP resulting from the reduced or blocked efflux via a neighboring apical membrane cAMP transporter (MRP4) in the same subcellular compartment.

There exist self-regulating mechanisms for cAMP generation and termination at the apical membrane of secretory epithelia, and both cAMP and its by-products can regulate CFTR activity in a compartmentalized fashion. Activation of the apical membrane GPCRs leads to $G\alpha_s$ stimulation (i.e., in the case of A_{2b} receptor and β_2AR) or $G\alpha_i$ inhibition (i.e., in the case of LPA_2) of tissue-specific membrane-associated AC (that is anchored to apical membrane via certain AKAPs). The resulting increase in cAMP triggers PKA that is anchored near CFTR by an AKAP (in this case, ezrin). CFTR open probability is increased by PKA phosphorylation leading to the chloride transport. PKA also phosphorylates and thus increases the activity of cAMP-specific PDEs (such as PDE3A and PDE4D) that is also anchored close to PKA by specific AKAPs (other AKAP isoforms, or probably also ezrin), and this results in an attenuation of the cAMP signal through degradation of cAMP into 5'-AMP, and also through an efflux pathway via a cAMP efflux transporter (i.e., in this case of MRP4) that is clustered in proximity to the cAMP generation site. The by-product of cAMP degradation can also activate AMPK that directly associates with CFTR close to its C terminus (at residues 1420-1457), leading to CFTR down-regulation. PKA counter-enzyme protein phosphatases (PP2A, PP2C, etc.) are also anchored by certain AKAPs to the subapical compartments where CFTR is located. Like PDEs, phosphatases can also be phosphorylated and activated by PKA and can dephosphorylate the phosphorylated CFTR, deactivating the channel function. Some phosphatases (such as PP2A) also bind directly to CFTR (at residues 1451-1476). Therefore, different or even the same AKAPs (probably through homo- or heterodimerization for ezrin) anchor probably almost all the enzyme molecules participating directly or indirectly in CFTR regulation to the apical membrane in proximity to their substrate molecules, to ensure the signal specificity and efficiency for the compartmentalized regulation of CFTR function. In the meanwhile, parallel mechanisms exist that contribute critically to the compartmentalized regulation of CFTR activity, i.e., the PDZ scaffold protein-mediated coupling of CFTR to a variety of other membrane proteins that function as receptors, transporters, and channels in many other important biological processes [42,43,48]. These physical associations mediated by various PDZ scaffold proteins provide an anatomical basis for the functional coupling of CFTR and its binding partners, contributing substantially to the compartmentalized regulation of CFTR chloride channel activity that is rendered by highly localized cAMP signaling described above. However, some of the above proposed regulations of CFTR function have been speculative or still remain controversial. More studies surely have to be conducted to conclusively define the detailed mechanisms that are responsible for compartmentalized CFTR signaling.

Compartmentalized regulation of CFTR function at the apical plasma membrane has significant physiological importance, as this channel not only transports Cl^- and HCO_3^- but also regulates the activities of many other transporters and channels [6]. The activity range of CFTR chloride channel can be altered dynamically by inputs from multiple regulatory networks that can be integrated or cross-talked with the channel. The physiological significance of these interactions is that they not only provide a means to link CFTR activity to various epithelial functions and processes but also coordinate the CFTR chloride channel function with the overall physiologic demands of epithelial cells. Although there is an emerging knowledge base

regarding elucidating the compartmentalized regulation of CFTR activity, it is clear that there remain other mechanisms of CFTR regulation yet to be identified. To understand completely the functional regulation of CFTR in a compartmentalized fashion will definitely help to identify novel targets and effective interventions for cystic fibrosis or secretory diarrhea therapies. It will be even more patho-physiologically significant if we can fully understand how CFTR mutations affect the formation and localization of macromolecular signaling complexes and whether alterations in signaling-complex formation account for the numerous phenotypic changes that are observed in patients with cystic fibrosis [31]. Toward these ends, a proteomic approach (such as mass spectroscopy) can be taken to identify the CFTR interactome for wild type and mutant forms of CFTR protein. The identification of novel CFTR interacting partners will not only reveal mechanisms as to how CFTR functions as an ion channel and a regulator for other transporters and channels, which will help to identify new drug targets for cystic fibrosis and other diseases resulting from CFTR dysfunction such as secretory diarrhea, but also can provide insights into the etiology of many diseases that are linked to other ABC transporters as similar dynamic macromolecular complexes may regulate the functions of other ABC transporters [31].

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