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Functional regulation of CFTR-containing macromolecular complexes: a small-molecule inhibitor approach

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SYNOPSIS

CFTR has been shown to form multiple-protein macromolecular complexes with its interacting partners at discrete subcellular microdomains to modulate trafficking, transport and signaling in cells. Targeting protein-protein interactions within these macromolecular complexes would affect the expression or function of the CFTR channel. We specifically targeted PDZ-based LPA₂-NHERF2 interaction within the CFTR-NHERF2-LPA₂-containing macromolecular complexes at airway epithelia and tested its regulatory role on CFTR channel function. We identified a cell-permeable small-molecule compound that preferentially inhibits LPA₂-NHERF2 interaction. We show that this compound can disrupt LPA₂-NHERF2 interaction in cells and thus compromises the integrity of macromolecular complexes. Functionally, it elevates cAMP levels in proximity to CFTR and upregulates its channel activity. Our results demonstrate that CFTR Cl[−] channel function can be finely tuned by modulating PDZ-based protein-protein interactions within the CFTR-containing macromolecular complexes. Our study might help to identify novel therapeutic targets to treat diseases associated with dysfunctional CFTR Cl[−] channel.

Keywords

CFTR; LPA₂ receptor; NHERF2; PDZ; protein-protein interactions; small-molecule inhibitors

INTRODUCTION

The cystic fibrosis transmembrane conductance regulator (CFTR) is the product of the gene mutated in patients with cystic fibrosis (CF), a lethal autosomal recessive genetic disease most common among Caucasians [1]. CFTR is a cAMP-regulated chloride (Cl[−]) channel primarily localized at the apical surfaces of epithelial cells lining airway, gut, exocrine

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AUTHOR CONTRIBUTION

The manuscript was written by W.Z. and supervised by A.P.N. and N.F. The project was designed and supervised by A.P.N. N.F. offered chemical library and supervised chemical synthesis; W.Z. performed AlphaScreen assays, chemical synthesis, and pig tracheal submucosal glands secretion experiments. H.P. conducted ratiometric FRET measurements; A.R. performed short-circuits measurements; A.P.N. conducted co-IP experiments. C.P. assisted in developing AlphaScreen assays. A.L. and B.Y. performed LC/MS/MS analysis. All authors discussed the results and commented on the manuscript.

COMPETING FINANCIAL INTEREST STATEMENT

The authors declare no competing financial interests.

glands *etc.*, where it is responsible for transepithelial salt and water transport [2,3]. CFTR function is also critical in maintaining fluid homeostasis, airway fluid clearance, and airway submucosal glands secretion in healthy and disease phenotypes [4,5].

Na⁺/H⁺ exchanger regulatory factor-2 (NHERF2) is a PSD95/Dlg/ZO-1 (PDZ) domain-containing protein that is primarily localized at the apical surfaces of epithelial cells. It contains two tandem PDZ domains and a C-terminal ERM (ezrin-radixin-moesin) domain, which mediates the association of NHERF2 with MERM proteins (merlin-ezrin-radixin-moesin) and links NHERF2 to the cytoskeleton [6]. NHERF2 has been shown to cluster signaling molecules into supramolecular complexes [7–11].

Lysophosphatidic acids (LPA) are growth factor-like phospholipids present in biological fluids and foods. LPA mediate diverse cellular responses such as cell proliferation, differentiation, migration, survival, angiogenesis, inflammation, and platelet aggregation [12,13]. At least eight G protein-coupled LPA receptors have been identified which couple to G_s, G_{i/o}, G_q and/or G_{12/13} protein to activate various signaling pathways [7,13]. Among these LPA receptors, LPA₂ (type 2 LPA receptor) belongs to the Endothelial Differentiation Gene (EDG) family and is structurally unique in the carboxyl-terminal (CT) tail, in which it contains a di-leucine motif and several putative palmitoylated cysteine residues in the proximal region that are responsible for binding to several zinc-finger proteins such as thyroid hormone receptor-interacting protein 6 (TRIP6). The last four amino acids of LPA₂ (DSTL; a class I PDZ-binding motif) mediate its interaction with several PDZ proteins, including NHERF2 [7,13,14]. Through the interaction with LPA₂, NHERF2 regulates the LPA-mediated phospholipase C-β3 signaling (PLC-β3) pathway and the activation of extracellular signal-regulated kinases (ERK) [15] and AKT [16]. It has also been reported that LPA induces the formation of a ternary complex containing LPA₂, TRIP6, and NHERF2 at microdomain on the plasma membrane and regulates the antiapoptotic signaling of LPA₂ [7].

A growing number of studies suggest that CFTR interacts directly or indirectly with other ion channels, transporters, scaffolding proteins, protein kinases, effectors, and cytoskeletal elements to form macromolecular complexes at specialized subcellular domains. These dynamic protein-protein interactions influence CFTR channel function as well as its localization and processing within cells [8,10,11,17–20]. Previously, we found that CFTR, LPA₂, and NHERF2 (along with other signaling molecules) form macromolecular complexes at the plasma membrane of gut epithelia, which functionally couple LPA₂ signaling to CFTR-mediated Cl[−] transport. We demonstrated that LPA inhibits CFTR-mediated Cl[−] transport through the LPA₂-mediated G_i pathway in a compartmentalized manner in cells, and that LPA inhibits CFTR-dependent cholera toxin-induced mouse intestinal fluid secretion *in vivo*. The formation of CFTR-NHERF2-LPA₂-containing macromolecular complexes and their importance in compartmentalized cAMP signaling are further supported by the observation that disruption of the integrity of the macromolecular complexes by using a cell-permeable LPA₂-specific peptide reversed LPA₂-mediated inhibition [8]. Most recently, Singh and colleagues investigated the roles of NHERF1/2/3 in regulating CFTR-dependent murine duodenal HCO₃[−] secretion in mice. They demonstrated that the absence of each NHERF protein resulted in distinct alteration in the regulation of HCO₃[−] secretion. NHERF1 ablation strongly reduced basal as well as forskolin (FSK)-stimulated HCO₃[−] secretory rates and blocked β₂-adrenergic receptor (β₂AR) stimulation. PDZK1 (NHERF3) ablation reduced basal but not FSK-stimulated secretion. As for NHERF2, the authors showed that FSK-stimulated HCO₃[−] secretion was significantly increased in *Nherf2* ^{−/−} mice and that NHERF2 is absolutely required for LPA₂-mediated inhibition of HCO₃[−] secretion [21], which is consistent with our previous findings [8].

These findings imply that targeting individual NHERF proteins might provide new approaches for therapeutic interventions of CFTR-associated diseases [8,21].

To study the formation of CFTR-NHERF2-LPA₂-containing macromolecular complexes at airway epithelia and their importance in regulating CFTR Cl⁻ channel function, and to explore the possibility of identifying new therapeutic targets (by perturbing PDZ-based protein-protein interactions within CFTR-containing macromolecular complexes) for treating diseases associated with a dysfunctional CFTR Cl⁻ channel, we identified a cell-permeable small-molecule compound that preferentially inhibits biochemical LPA₂-NHERF2 interaction. We demonstrate that this compound indeed disrupts LPA₂-NHERF2 interaction in cells and thus compromises the integrity of CFTR-NHERF2-LPA₂-containing macromolecular complexes. Functionally, it abolishes the inhibitory effect of LPA₂-dependent events on CFTR Cl⁻ channel (mediated by NHERF2), and thus augments CFTR Cl⁻ channel activity in Calu-3 cells and also in pig tracheal submucosal glands fluid secretion.

EXPERIMENTAL

Cell culture and transfection

Calu-3 cell line was purchased from ATCC and cultured in MEM medium (Invitrogen) containing 15% serum (v/v), 1% penicillin/streptomycin (w/v), 1 mM sodium pyruvate, and 1× nonessential amino acids. HEK293 cells overexpressing Flag-LPA₂ and HA-NHERF2 were cultured in DMEM-F12 medium (Invitrogen) containing 10% serum (v/v) and 1% penicillin/streptomycin (w/v). The cells were maintained in a 5% CO₂ incubator at 37 °C. The Flag (M2) tag was introduced into LPA₂ (Flag tag on N-terminal tail on the outer loop of the protein) by using a two-step Quickchange Mutagenesis kit (Invitrogen). The sequence was confirmed, and the Flag-LPA₂ was cloned into pcDNA3 vector (Invitrogen). Lipofectamine 2000 (Invitrogen) was used to transfect plasmids containing Flag-LPA₂, HA-NHERF2, Flag-CFTR, or Flag-PLC-β3 in HEK293 cells. Stable cell lines were generated by selection using 2 μg/ml of puromycin in the media. Lipofectamine was also used to transfect plasmids containing CFP-EPAC-YFP into Calu-3 cells. Pig tracheas were harvested less than 1 h postmortem from piglets that had been killed for projects unrelated to the present study.

Screening for potent inhibitors that disrupt LPA₂-NHERF2 interaction by using AlphaScreen™ assay

Biotin-LPA₂ peptide (10 μM final concentration) and GST-NHERF2 (100 nM final concentration) were mixed in the assay buffer [25 mM HEPES, 100 mM NaCl, 0.1% BSA (w/v), 0.05% Tween 20 (v/v), pH 7.4], into which the compounds were added, serially diluted (final concentration: 1 mM ~10 nM), and incubated at room temperature for 30 min. Each sample solution (15 μl) was transferred to a 384-well white opaque OptiPlate™ (PerkinElmer) in triplicate and anti-GST acceptor beads (5 μl, 20 μg/ml final concentration) were added and incubated for 30 min. Streptavidin donor beads (5 μl, 20 μg/ml final concentration) were then added and incubated for 1 h at room temperature. The plates were read on an EnVision plate reader (PerkinElmer). The binding curve and IC₅₀ value were generated using GraphPad Prism software.

Co-immunoprecipitation and immunoblotting

HEK293 cells stably expressing Flag-LPA₂ and HA-NHERF2 (HEK293-Flag-LPA₂-HA-NHERF2 cells) were treated with compound CO-068 (50 μM) or equal volume of DMSO for 1 h at 37 °C. The cells were washed with PBS (1×) and then lysed in lysis buffer [1× PBS, containing 0.2% Triton-X-100 (v/v) and protease inhibitors: phenylmethylsulfonyl

fluoride (1 mM), pepstatin A (1 μ g/ml), leupeptin (1 μ g/ml), and aprotinin (1 μ g/ml)]. The lysate was centrifuged at 16,000 *g* for 10 min at 4 °C. The protein concentration of the clear supernatant was determined by using the bicinchoninic assay (Pierce). The clear supernatant was subjected to immunoprecipitation by using α -Flag beads (Sigma). The immunoprecipitated beads were washed three times with lysis buffer, and the proteins were eluted from the beads by using Laemmli sample buffer [5 $\times$; containing 2.5% β -mercaptoethanol (v/v)]. The eluted proteins were denatured, subjected to SDS-PAGE on 4–15% gel (BioRad), transferred to PVDF membrane, and immunoblotted for NHERF2 and LPA₂ with α -HA monoclonal antibody (sigma) and α -LPA₂ monoclonal antibody (rabbit-2143, against the last 11 amino acids), respectively. The immunoreactive bands were visualized by ECL (Amersham Biosciences). HEK293 cells expressing HA-NHERF2 (HEK293-HA-NHERF2 cells) were also used as negative control.

Co-immunoprecipitation of NHERF2 and CFTR in the presence of 50 μ M compound CO-068 (or equal volume of DMSO) was performed in HEK293 cells stably expressing Flag-CFTR (HEK293-Flag-CFTR cells) using the method described above. Specific antibodies were used to detect CFTR (R1104 monoclonal mouse antibody) and NHERF2 (affinity purified rabbit polyclonal antibody) levels.

Co-immunoprecipitation of NHERF2 and PLC- β 3 in the presence of 50 μ M compound CO-068 (or equal volume of DMSO) was performed in HEK293 cells expressing Flag-PLC- β 3 (HEK293-Flag-PLC- β 3 cells) using the method described above. Anti-Flag monoclonal antibody was used to detect PLC- β 3. Affinity purified rabbit polyclonal antibody (α -NHERF2) was used to detect NHERF2 levels.

Short-circuit currents (I_{sc}) measurements (Ussing chamber experiments)

Polarized Calu-3 cell monolayers were grown to confluency on Costar Transwell permeable supports (Cambridge; filter area is 0.33 cm²) and then mounted in a modified Ussing chamber. Short-circuit currents mediated by CFTR Cl[−] channel were measured as previously described [19,20]. The cells were bathed in Ringer's solution (mM) (basolateral: 140 NaCl, 5 KCl, 0.36 K₂HPO₄, 0.44 KH₂PO₄, 1.3 CaCl₂, 0.5 MgCl₂, 4.2 NaHCO₃, 10 Hepes, 10 glucose, pH 7.2, [Cl[−]] = 149), and low Cl[−] Ringer's solution (mM) (apical: 133.3 Na-gluconate, 5 K-gluconate, 2.5 NaCl, 0.36 K₂HPO₄, 0.44 KH₂PO₄, 5.7 CaCl₂, 0.5 MgCl₂, 4.2 NaHCO₃, 10 Hepes, 10 mannitol, pH 7.2, [Cl[−]] = 14.8) at 37 °C and gassed with 95% O₂ and 5% CO₂. All reagents were added to the apical side of the cell monolayers. At the end of the experiments, A specific CFTR channel inhibitor, CFTRinh-172 (20 μ M), was added to the apical sides of both chambers to inhibit the Cl[−] currents to verify that the I_{sc} responses observed were CFTR-dependent.

Pig tracheal submucosal glands fluid secretion

Fluid secretion from pig tracheal submucosal glands was monitored as described by Wine's lab [22]. Pig tracheas were collected within 1 h of sacrifice, placed in ice-cold Krebs-Ringer's bicarbonate buffer (KRB buffer; composition: 120 mM NaCl, 25 mM NaHCO₃, 3.3 mM KH₂PO₄, 0.8 mM K₂HPO₄, 1.2 mM MgCl₂, 1.2 mM CaCl₂, 10 mM D-glucose, and 1 μ M indomethacin) and bubbled with 95% O₂ and 5% CO₂ gas. The tracheal ring was cut open along the dorsal fold in ice-cold KRB buffer. The mucosa with underlying glands was carefully dissected from the cartilage, and a 1-cm piece was mounted in a chamber with the mucosal side up. The mucosal side was blotted clean, further dried with a gentle stream of 95% O₂ and 5% CO₂ gas, and then partly covered with a thin layer of water-saturated mineral oil. To establish a baseline, KRB buffer was added to the serosal side, maintained at 37 °C and superfused with 95% O₂ and 5% CO₂. All pharmacological reagents were diluted to the final concentration in 37 °C appropriately gassed KRB buffer, and added to the

serosal side after monitoring basal secretion. Carbachol (10 μ M) was added at the end of the experiments to check for the viability of the submucosal glands. The tissues covered with water-saturated oil were obliquely illuminated to visualize the spherical droplets of secreted mucus within the oil. Digital images were collected at 1-min intervals with a digital camera attached to a stereoscopic microscope (National Optical) and controlled by Motic Images 2.0 ML software. ImageJ software (NIH) was used to analyze the fluid secretion. Briefly, a 1-mm $\times$ 1-mm grid was placed on the tissue in the last image to set the scales, which were used to measure the area of secreted bubbles. The volumes of the secreted bubbles were calculated from *Area* using the formula $V = 4/3\pi r^3$, where r is the radius. The fluid secretion rates were calculated as slopes of *Volume vs Time* plots using linear regression ($R^2 > 0.8$). For basal secretion, the fluid secretion rates were calculated from data over a 10-min period; for secretion induced by FSK (10 μ M), FSK (10 μ M) plus CFTRinh-172 (50 μ M), compound CO-068 (50 μ M), compound CO-068 (50 μ M) plus CFTRinh-172 (50 μ M), FSK(10 μ M) plus compound CO-068 (50 μ M), or FSK (10 μ M) plus compound CO-068(50 μ M) plus CFTRinh-172 (50 μ M), the secretion rates were calculated from data over a 25-min period; for carbachol (10 μ M), or carbachol (50 μ M) plus CO-068 (50 μ M) induced secretion, the secretion rates were calculated from data over a 5-min period.

Ratiometric FRET microscopy and data analysis

Calu-3 cells expressing a cAMP sensor, CFP-EPAC-YFP, were grown on 35-mm glass-bottom dishes (MatTek), washed twice with Hank's balance salt solution (HBSS), added in 1 ml HBSS, and mounted on an inverted Olympus microscope (IX51; the microscope is described in detail in Supplementary Methods). Cells were maintained in HBSS in the dark at room temperature. After establishing the baseline, compound CO-068 (50 μ M) or FSK (10 μ M) was added to the buffer, and ratiometric FRET imaging was performed as described previously [19,20]. Briefly, time-lapse images were captured with 100- to 300-ms exposure time and 1-min interval with a cooled EM-CCD camera (Hamamatsu) controlled by Slidebook 4.2 software (Intelligent Imaging Innovations Inc.). Following background subtraction, multiple regions of interest (10–20) were selected (3–5 cells) for data analysis with the ratio module of Slidebook 4.2 software. The emission ratio (CFP/FRET) was obtained from CFP and FRET emission of background subtracted cells.

Statistical Analyses

Data are represented as *mean $\pm$ s.e.m.* unless otherwise indicated. Student's *t*-test (2-tailed) was used to compare the data of different groups. $**P < 0.01$ or $*P < 0.05$ was considered significant.

RESULTS

Screening for small-molecule inhibitors that disrupt LPA₂-NHERF2 interaction

Development of small-molecule inhibitors for protein-protein interactions is of great importance. The compounds identified can be used as tools to study the cell physiology associated with the protein-protein interactions and may also have a therapeutic potential [23–25]. Generally, two sequential approaches are adopted to identify inhibitors for protein-protein interactions. One can produce chemical libraries of derivatives of a chemical scaffold that is rationally designed, and use high-throughput screening to identify the hit compounds. For this study, we used the AlphaScreenTM assay (Amplified Luminescent Proximity Homogeneous Assay) to screen a library of compounds that was previously designed to inhibit PDZ-based protein-protein interactions [26–29]. We first developed a direct binding assay between the purified full length GST-NHERF2 protein and a biotin-LPA₂ peptide (biotin-NGHPLMDSTL-COOH, which is derived from the carboxyl-terminal sequence of LPA₂ containing the PDZ motif DSTL; the schematic representation of this

assay is shown in Supplementary Figure 1A). As shown in Figure 1A, the biotin-LPA₂ peptide binds to GST-NHERF2 in a dose- and pH-dependent manner. At pH 6 and 8, the binding signals are weak, whereas at physiological pH range (7.4) the binding signals are strong. The strongest signal was observed when the concentration of biotin-LPA₂ peptide reached 10 μ M. Further increase of biotin-LPA₂ peptide concentration led to decreased signals (the typical 'Hook effect' usually observed in AlphaScreen™ assays). To verify if the observed signals were specific to LPA₂-NHERF2 interaction, we performed competition assays in which a non-biotinylated LPA₂ peptide was used to compete against biotin-LPA₂ peptide for binding to GST-NHERF2 (the schematic representation of this assay is shown in Supplementary Figure 1B). A dose-dependent inhibitory effect was observed which confirmed the specificity of the binding signals (Figure 1B). The IC₅₀ was determined to be 12 μ M.

The optimized assay conditions were then used to screen for small-molecule inhibitors. Among eighty (80) compounds screened so far, one compound (named as compound CO-068 in this paper; its structure is shown in Figure 1C) showed the best inhibitory effect (IC₅₀ = 63 μ M; Figure 1D). To check the selectivity of its inhibitory effect, we developed other AlphaScreen™ assays for protein-protein interactions that have been reported to get involved in CFTR-containing macromolecular complexes (such as CFTR-NHERF2, MRP4-PDZK1 and CFTR-NHERF1) [10,11], and then tested the inhibitory effects of compound CO-068 in these systems. Our results show that compound CO-068 did not preferentially perturb these PDZ-based protein-protein interactions (Table 1). For a proof-of-concept study, we used this compound to study the CFTR-NHERF2-LPA₂-containing macromolecular complexes in airway epithelia and their importance in regulating CFTR Cl⁻ channel function.

Synthesis of compound CO-068

Compound CO-068 was synthesized with high purity (> 97%) according to Scheme 1. The starting material (compound 1) is not commercially available; its synthesis has been published previously [26].

Compound CO-068 inhibits LPA₂-NHERF2 interaction in cells

To test whether compound CO-068 inhibits LPA₂-NHERF2 interaction in cells, we co-transfected HEK293 cells with Flag-LPA₂ and HA-NHERF2 constructs and generated a stable cell line (HEK293-Flag-LPA₂-HA-NHERF2 cells). Since the potency of compound CO-068 for inhibiting LPA₂-NHERF2 interaction is ~5 times weaker than that of LPA₂-peptide, we used this compound at 50 μ M concentration throughout the study. It is to be noted that this concentration (50 μ M) is below its IC₅₀ for LPA₂-NHERF2 interaction and well below its IC₅₀ for CFTR-NHERF2 interaction, which would minimize the possibility of its disruption on CFTR-NHERF2 interaction. We treated the cells with 50 μ M of compound CO-068 for 1 h at 37 °C and then lysed the cells. The protein complex was immunoprecipitated from clear supernatant by using α -Flag beads. The proteins were eluted from the beads, subjected to SDS-PAGE, and immunoblotted for LPA₂ and NHERF2 by using specific α -LPA₂ or α -HA antibody. Cells pretreated with equal volume of DMSO (solvent used to solvate compound CO-068) were used as control. HEK293 cells expressing HA-NHERF2 (HEK293-HA-NHERF2 cells) were also used as negative control. As shown in Figure 2, in immunoprecipitated protein complex, the Flag-LPA₂ levels remain the same for cells treated with compound CO-068 or DMSO. However, NHERF2 level decreases substantially (42%; as analyzed by quantifying the blots with Scion Image software) for cells treated with compound CO-068 compared to that from cells treated with DMSO, indicating that compound CO-068 disrupts LPA₂-NHERF2 interaction in these cells.

To further test whether compound CO-068 disrupts CFTR-NHERF2 interaction in cells at 50 μM concentration, we treated HEK293 cells stably expressing Flag-CFTR (HEK293-Flag-CFTR cells) with compound CO-068 (or DMSO) for 1 h at 37 $^{\circ}\text{C}$, lysed the cells, and immunoprecipitated the protein complex from clear supernatant by using α -Flag beads. The proteins were eluted from the beads, subjected to SDS-PAGE, and immunoblotted for CFTR and NHERF2 by using specific α -CFTR or α -NHERF2 antibody. HEK293 parental cells were also used as control. As shown in Figure 3A, in immunoprecipitated protein complex, both Flag-CFTR level and NHERF2 level remain the same for cells treated with compound CO-068 or DMSO, indicating that at 50 μM concentration, compound CO-068 does not disrupt CFTR-NHERF2 interaction in cells.

We also used a similar method to study whether CO-068 disrupts NHERF2-PLC- β 3 interaction in cells. HEK293 cells overexpressing Flag-PLC- β 3 (HEK293-Flag-PLC- β 3 cells) were treated with compound CO-068 (or DMSO) under the same conditions as described above. As shown in Figure 3B, in immunoprecipitated protein complex, both Flag-PLC- β 3 level and NHERF2 level remain the same for cells treated with CO-068 or DMSO, suggesting that at 50 μM concentration, compound CO-068 does not disrupt PLC- β 3-NHERF2 interaction in cells.

For these co-immunoprecipitation experiments, compound CO-068 was added externally at 50 μM concentration. The cell permeability of compound CO-068 was tested by using HEK293 cells. The cell numbers were counted and the cells were incubated with compound CO-068 (50 μM) for 1 h at 37 $^{\circ}\text{C}$. The culture medium was removed by centrifugation and the cells were pelleted. The cell pellets were washed twice with the RIPA buffer (without SDS), and then lysed in the RIPA buffer (without SDS). Cells treated with equal amount of DMSO were used as controls. LC/MS/MS was used to measure the concentrations of compound CO-068 in these cell lysates (Supplementary Table 1). As expected, samples from cells treated with DMSO did not show a compound CO-068 peak. For samples from cells treated with CO-068, a mean concentration of 28.74 μM in cell lysates was detected ($n = 5$, standard deviation = 1.74; the volume for cell lysates is 63.3 μl). Considering the volume of cell pellets is about 40 μl , the mean intracellular concentration of compound CO-068 was determined as $\sim 46 \mu\text{M}$, indicating it is quite cell permeable.

In conclusion, at 50 μM concentration, compound CO-068 seems to specifically disrupt LPA₂-NHERF2 interaction in cells.

Compound CO-068 augments CFTR Cl⁻ channel function in lung epithelial cells (Calu-3 cells)

Given the facts that: (1) LPA₂ has an inhibitory effect on adenylyl cyclase (AC) [8,13]. AC generates cAMP which regulates CFTR Cl⁻ channel function; (2) CFTR, LPA₂ and NHERF2 form macromolecular complexes at the plasma membrane of gut and lung epithelial cells (HT29-CL19A cells and Calu-3 cells), which forms the molecular basis for functional coupling between LPA₂-mediated signaling events and CFTR-mediated Cl⁻ transport [8]; and (3) compound CO-068 disrupts LPA₂-NHERF2 interaction and thus would disrupt the macromolecular complexes, we envisioned that disruption of LPA₂ from CFTR-NHERF2-LPA₂-containing complexes would increase CFTR Cl⁻ channel function. To test this hypothesis, we measured CFTR-mediated short-circuit currents (I_{sc}) in polarized Calu-3 monolayers mounted in an Ussing chamber with treatment of compound CO-068. DMSO was used as a control. Another compound, FJL-3-18 (its structure is shown in Supplementary Figure 2A) which has very weak potency to inhibit LPA₂-NHERF2 interaction ($\text{IC}_{50} = 850 \mu\text{M}$; AlphaScreen result), was also used as a control. Calu-3 cells are airway serous gland epithelial cells that endogenously express CFTR, LPA₂, and NHERF2 at the apical surfaces when polarized and have been used as a model system to study CFTR

channel function [8,30,31]. When polarized Calu-3 cells were treated with compound CO-068 (50 μ M), a significant increase in CFTR-dependent I_{sc} was detected (Figure 4A and 4B), whereas DMSO or compound FJL-3-18 did not induce significant I_{sc} responses (Figure 4A-B and Supplementary Figure 2B-C). A specific CFTR channel inhibitor, CFTRinh-172, was added toward the end of the experiments to verify that the observed I_{sc} responses were indeed CFTR-dependent. These results demonstrate that disruption of LPA₂-NHERF2 interaction by using compound CO-068 increases basal CFTR Cl⁻ channel function.

We further tested whether compound CO-068 could potentiate agonist-stimulated CFTR Cl⁻ channel function by using forskolin (FSK). As shown in Figure 4C-D, compound CO-068 indeed further potentiated FSK-induced CFTR Cl⁻ channel function. It is interesting to note that in the presence of compound CO-068, the FSK-activation rate was faster; possibly suggesting that compound CO-068 changed the 3-dimensional arrangement of signaling components within the CFTR-NHERF2-LPA₂-containing macromolecular complexes by disrupting LPA₂-NHERF2 interaction, which accounted for the changes in magnitude and kinetics of CFTR Cl⁻ channel activation.

In summary, these observations support our hypothesis that CFTR, NHERF2 and LPA₂ form macromolecular complexes at the plasma membrane of Calu-3 cells and disruption of LPA₂ from the macromolecular complexes augments CFTR Cl⁻ channel function. The data also imply that: (1) targeting PDZ-based protein-protein interactions within the CFTR-NHERF2-LPA₂-containing macromolecular complexes can locally regulate CFTR Cl⁻ channel function, which might provide potential therapeutic targets for treating CFTR-related diseases; and (2) compound CO-068 could be a seed compound for developing improved leads to augment CFTR function in CF patients who have CFTR mutants with impaired channel function such as G551D or R117H.

Compound CO-068 augments CFTR-dependent fluid secretion from pig tracheal submucosal glands

Submucosal glands secretion plays important roles in maintaining airway and lung health. It can be stimulated by cholinergic agonists or agonists that elevate cAMP or Ca²⁺ levels. CFTR is present in the apical membrane of gland serous cells and mediates at least part of the fluid secretion. Loss of CFTR function reduces the capacity of glands to secrete fluid and has been suggested to link to the airway pathology of CF [4,31,32]. In this study, we used the pig tracheal submucosal glands secretion model to investigate whether compound CO-068 could potentiate CFTR-dependent fluid secretion from submucosal glands. Pig is considered a closer model to human CF, and a CF pig model is available for studying CFTR function [33]. Because it has been reported that FSK can induce fluid secretion from pig tracheal submucosal glands [34], we first used FSK to validate the method. Our results showed that FSK induced a 5-fold increase in mean fluid secretion rate compared to basal secretion rate (Figure 5B). CFTRinh-172 markedly inhibited FSK-induced secretion, indicating that the observed secretion was CFTR-dependent. When compound CO-068 (50 μ M) was added in combination with FSK, we observed a 2.5-fold increase in mean secretion rate compared to FSK-induced secretion (Figure 5B), suggesting that compound CO-068 potentiated FSK-induced fluid secretion. This potentiation effect was inhibited by CFTRinh-172, indicating that it was CFTR-dependent. These results are consistent with the results from I_{sc} measurement (Figure 4C-D). We then tested the effect of compound CO-068 on basal CFTR-dependent fluid secretion. As shown in Figure 5A-B, compound CO-068 induced a 4-fold increase in mean fluid secretion rate compared to basal secretion, and the effect was reversed by CFTRinh-172. These findings are also consistent with the results from I_{sc} measurements (Figure 4A-B).

To further verify that the increased fluid secretion by using compound CO-068 is specific to CFTR and not through another mechanism, we investigated whether compound CO-068 could augment carbachol-induced fluid secretion. Our results demonstrate that when added in combination with carbachol, compound CO-068 did not increase the mean fluid secretion rate (Supplementary Figure 3).

In summary, compound CO-068 increases both basal and FSK-induced CFTR-dependent submucosal glands fluid secretion in pig, a finding that could be potentially useful to restore the impaired mucociliary clearance process in diseased airways due to a dysfunctional CFTR Cl^- channel such as G551D-CFTR mutant.

Compound CO-068 augments CFTR Cl^- channel function by means of elevating cAMP levels in cells

The results described above show that compound CO-068 disrupts LPA_2 -NHERF2 interaction and augments CFTR Cl^- channel function in Calu-3 cells and in pig tracheal submucosal glands. To gain direct evidence whether compound CO-068 acts through the cAMP pathway, we transfected a FRET-based cAMP sensor, CFP-EPAC-YFP, into Calu-3 cells and then performed ratiometric fluorescence resonance energy transfer (FRET) measurements to directly visualize cAMP dynamics in live cells. This highly sensitive cAMP sensor can be used to monitor cAMP dynamics in intact cells with very high temporal and spatial resolution [19,20,35]. After establishing the baseline, compound CO-068 (50 μM) was added into the buffer and ratiometric FRET signals were monitored. As shown in Figure 6A–C, cAMP levels (represented by CFP/FRET emission ratio) increased 1.2-fold upon treatment of the cells with compound CO-068. Forskolin (10 μM) was added at the end of the experiments as a positive control which elicited a further increase in cAMP levels. When FSK was added after the basal had been established, it induced a 1.5-fold increase in cAMP levels. Addition of compound CO-068 further increased the intracellular cAMP levels (Figure 6D). The data provide direct evidence that compound CO-068 indeed elevates cAMP levels and consequently augments CFTR Cl^- channel function, a finding that supports our hypothesis that compound CO-068 disrupts LPA_2 from the CFTR-NHERF2-containing macromolecular complexes and abolishes the inhibitory effect of LPA_2 on AC, and consequently increases cAMP levels.

DISCUSSION

Formation of multiple-protein complexes at discrete subcellular microdomains increases the specificity and efficiency of signaling (*e.g.*, cAMP-PKA signaling) in cells [19,36,37]. For polarized epithelial cells (*e.g.*, Calu-3 cells and HT-29 cells), it has been observed that the signals originating at cell surfaces do not always induce detectable changes for specific intracellular second messengers (*e.g.* cAMP, cGMP, or Ca^{2+}). However, the cellular response transduced by these specific second messengers is specifically and efficiently accomplished. These observations suggest that receptors, effectors, ion channels, transporters, and signaling intermediates form macromolecular complexes and compartmentalize into discrete subcellular microdomains that, at the molecular level, ensure that the right signaling components are localized at the right place (spatially) and at the right time (temporally), thus increasing the velocity of response and specificity of signaling [38].

PDZ domains are conserved protein-protein interaction modules of ~ 90 amino acids in length that fold to form a peptide-binding groove that binds to the specific short peptide motif (PDZ motif) found in the carboxyl-terminus or internal region of a variety of target proteins [39]. PDZ domain-containing proteins (PDZ proteins) often contain multiple PDZ domains and can interact simultaneously with multiple binding partners (*e.g.*, receptors, ion channels, or transporters) to assemble larger protein complexes at specific subcellular

compartments involved in signaling, trafficking, or subcellular transport in a variety of tissues [10,11,40,41]. Many PDZ proteins can interact with disease-associated proteins, and the regulation of disease-associated proteins by PDZ proteins gives them provisional roles in many disease states. The discrete properties of PDZ based protein-protein interactions make them promising candidates for modulation to understand cell physiology and to develop novel therapeutic agents against diseases [24,25,42]. Developing small-molecule inhibitors to compete against PDZ targets for binding to PDZ protein is a very attractive approach in formulating pharmaceutical agents [25–29,43,44].

CFTR has been shown to interact directly or indirectly with a wide variety of proteins and to form distinct multiprotein macromolecular complexes at different subcellular microdomains and tissues [10,11,18]. Previously, we reported the multiprotein macromolecular complex formation between CFTR, NHERF2, and LPA₂ (along with other signaling molecules) at the apical plasma membranes of gut epithelia, and their importance in compartmentalized cAMP signaling and in local regulation of CFTR Cl[−] channel function [8]. To further study the regulatory roles of PDZ-based protein-protein interactions within the macromolecular complexes on regulating CFTR Cl[−] channel function, and to explore potential therapeutic value of using such an approach to treat diseases associated with dysfunctional CFTR protein (*e.g.*, G551D-CFTR and R117H-CFTR), we screened a specially designed chemical library and identified a compound (compound CO-068) that preferentially disrupts LPA₂-NHERF2 interaction. Our results demonstrate that this compound does inhibit LPA₂-NHERF2 interaction in cells and consequently disrupts the integrity of the CFTR-NHERF2-LPA₂-containing macromolecular complexes, which leads to increased cAMP levels and augments CFTR Cl[−] channel function.

Based on our findings, we propose a model to depict the formation of multiple-protein macromolecular complexes at airway epithelia and the regulatory role of LPA₂-NHERF2 interaction on CFTR channel function (Figure 7). Since LPA₂ binds to only the PDZ2 domain of NHERF2 whereas CFTR can bind to both PDZ domains, one possible way to form the macromolecular complexes is for NHERF2 to self-associate through PDZ domains [45] and thereby bridge LPA₂ and CFTR. Other signaling intermediates such as ezrin and PKA should also be present in the macromolecular complexes [46]. Under basal conditions, LPA₂ exerts inhibitory effects on AC through the G_i pathway, which results in the reduced cAMP levels in proximity to CFTR and thus downregulates its channel function (Figure 7A). However, perturbing LPA₂-NHERF2 interaction within the macromolecular complexes would scatter LPA₂ from its binding partners, which would abolish the inhibitory effects of LPA₂ on AC, leading to cAMP generation and consequently augmenting CFTR Cl[−] channel function (Figure 7B).

Our observations support *in vivo* studies, which showed that, upon deletion of NHERF2 in mice, the basal CFTR-dependent murine duodenal HCO₃[−] secretion was slightly (but not significantly) higher than that in WT mice [21]. Deletion of NHERF2 would completely disrupt the macromolecular complex formation between CFTR, NHERF2 and LPA₂, and lead to the complete loss of compartmentalized cAMP signaling. In our study, we sought to disrupt NHERF2-LPA₂ interaction and leave CFTR-NHERF2 interaction intact, which would possibly contribute to the substantial increase in CFTR channel function at both the basal and agonist-induced levels.

The molecular assembly of CFTR with its interacting proteins is of great interest and importance because that: (1) in addition to serving as a channel to transport Cl[−] and HCO₃[−], CFTR also regulates a wide variety of other channels, transporters and processes [10,11,17]; (2) several human diseases are attributed to altered regulation of CFTR, among which CF and secretory diarrhea are two major disorders [1,17]. CF is caused by the loss or

dysfunction of CFTR Cl⁻ channel activity resulting from mutations that decrease either the biosynthesis or the ion channel function of the protein [47]. Secretory diarrhea is caused by excessive activation of the CFTR Cl⁻ channel in the gut [48]. It is therefore reasonable to propose that any reagent (or approach) that can specifically enhance CFTR Cl⁻ channel activity would be potentially beneficial in treating diseases like CF. Conversely, any reagent (or approach) that can specifically decrease CFTR Cl⁻ channel activity would be potentially beneficial in treating CFTR-mediated secretory diarrhea. CFTR itself has been targeted to develop inhibitors for therapy of secretory diarrheas, and activators for therapy in CF [49,50]. By using high-throughput screening, Verkman and colleagues have identified some small-molecule inhibitors and activators that show promising potential in the treatment of CF and CFTR-mediated secretory diarrhea [50]. In this study, we targeted the PDZ-based LPA₂-NHERF2 interaction within the CFTR-NHERF2-LPA₂-containing macromolecular complexes at airway epithelia and demonstrated that a synthetic cell-permeable inhibitor (compound CO-068) could specifically increase CFTR Cl⁻ channel activity at both basal and agonist-induced states. To our knowledge, this is the first study that specifically targeted one type of PDZ-based protein-protein interaction within the CFTR-containing macromolecular complexes and demonstrated that a small-molecule inhibitor could potentiate CFTR Cl⁻ channel function in cells and tissues. Our study implies that these macromolecular complexes can potentially be a new therapeutic target for treating CFTR-associated diseases. Moreover, our study suggests that by targeting different PDZ-based protein-protein interaction within the macromolecular complexes, we can modulate CFTR channel function on a use-dependent mode for treating different diseases, that is, targeting LPA₂-NHERF2 interaction to potentiate CFTR Cl⁻ channel function for drug development to treat CF (especially those due to the presence of G551D- or R117H-CFTR); and targeting CFTR-NHERF2 interaction to downregulate CFTR Cl⁻ channel function for drug development to treat CFTR-mediated secretory diarrhea.

It is to be noted that when cells were treated with compound CO-068 (50 μM), no side effects on cell viability or morphology were observed. Currently, development of more potent inhibitors for LPA₂-NHERF2 interaction, as well as more potent inhibitors for CFTR-NHERF2 interaction, is under way.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

AC	adenylyl cyclase
AlphaScreen™	amplified luminescent proximity homogeneous assay
CF	cystic fibrosis
CFTR	the cystic fibrosis transmembrane conductance regulator

EPAC	exchange protein activated by cAMP
FRET	fluorescence resonance energy transfer
FSK	forskolin
I_{sc}	short-circuit currents
LPA	lysophosphatidic acid
LPA₂	type 2 LPA receptor
NHERF2	Na ⁺ /H ⁺ exchanger regulatory factor-2
PDZ	PSD95/Dlg/ZO-1
PLC-β3	phospholipase C-β3
PKA	protein kinase A

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Figure 1. Screening for small-molecule inhibitors that disrupt LPA₂-NHERF2 interaction
(A) Development of AlphaScreen™ association assay between the biotin-LPA₂ peptide and the purified full-length GST-NHERF2 protein. The results show that biotin-LPA₂ peptide binds to GST-NHERF2 in a dose- and pH-dependent manner. AlphaScreen™ signals are presented as counts per second (cps). The data are shown as mean ± s.e.m. (n = 3). Trace a: pH = 7.4; Trace b: pH = 8; Trace c: pH = 6. **(B)** AlphaScreen™ competition assays show that a nonbiotinylated LPA₂ peptide competes against biotin-LPA₂ peptide for binding to GST-NHERF2 protein, verifying the specificity of the binding results observed in AlphaScreen™ association assays. The data are shown as mean ± s.e.m. (n = 3). The IC₅₀ was determined as 12 μM. **(C)** Structure of compound CO-068. **(D)** Compound CO-068 shows the best

inhibitory effect on LPA₂-NHERF2 interaction ($IC_{50} = 63 \mu M$). The data are shown as mean $\pm$ s.e.m. (n = 3).

Figure 2. Compound CO-068 inhibits LPA₂-NHERF2 interaction in cells

HEK293 cells stably expressing Flag-LPA₂ and HA-NHERF2 (HEK293-Flag-LPA₂-HA-NHERF2 cells) were treated with compound CO-068 (50 μM) or equal volume of DMSO. The protein complex was immunoprecipitated using α-Flag beads and immunoblotted for LPA₂ and NHERF2. HEK293 cells expressing HA-NHERF2 (HEK293-HA-NHERF2 cells) were also used as control. Please see details in text. The upper panel shows that LPA₂ levels remain the same in immunoprecipitated protein complex from cells treated with compound CO-068 or DMSO. The middle panel shows that NHERF2 level decreases (42%; analyzed by quantifying the blots using Scion Image software) in immunoprecipitated protein complex from cells treated with compound CO-068 compared to that from cells treated with DMSO, indicating that compound CO-068 disrupts LPA₂-NHERF2 interaction in cells.

Figure 3. Compound CO-068 does not disrupt NHERF2-CFTR interaction or NHERF2-PLC-β3 interaction in cells

(A) HEK293 cells stably expressing Flag-CFTR (HEK293-Flag-CFTR cells) were treated with compound CO-068 (50 μM) or equal volume of DMSO. HEK293 parental cells were also used as control. The protein complex was immunoprecipitated using α-Flag beads and immunoblotted for CFTR and NHERF2. The upper panel shows that CFTR levels remain the same in immunoprecipitated protein complex from cells treated with compound CO-068 or DMSO. The middle panel shows that NHERF2 levels also remain the same in immunoprecipitated protein complex from cells treated with compound CO-068 or DMSO, indicating that compound CO-068 does not disrupts CFTR-NHERF2 interaction in cells at this concentration. (B) HEK293 cells overexpressing Flag-PLC-β3 (HEK293-Flag-PLC-β3 cells) were treated with compound CO-068 (50 μM) or equal volume of DMSO. HEK293 parental cells were also used as control. The protein complex was immunoprecipitated using α-Flag beads and immunoblotted for PLC-β3 and NHERF2. The upper panel shows that Flag-PLC-β3 levels remain the same in precipitated protein complex from cells treated with compound CO-068 or DMSO. The middle panel shows that NHERF2 levels remain the same in precipitated protein complex both from cells treated with compound CO-068 and from cells treated with DMSO, indicating that compound CO-068 does not disrupts the interaction between PLC-β3 and NHERF2 in cells at 50 μM concentration.

Figure 4. Compound CO-068 augments basal and FSK-stimulated CFTR Cl⁻ channel function in Calu-3 cells

(A) Representative trace of CFTR-dependent short-circuit currents (I_{sc}) elicited by adding compound CO-068 to Calu-3 cell monolayers mounted in a Ussing chamber (top trace). DMSO was used as a control (bottom trace). The results show that compound CO-068 augments basal CFTR Cl⁻ channel function. CFTRinh-172 was used to verify that the responses were CFTR-mediated. The experiment was repeated 3 times. (B) Quantification of data from Ussing chamber experiments as represented in (A). The data are presented as the changes of CFTR-dependent I_{sc} after addition of CO-068 (or DMSO) compared to their corresponding basal levels (mean $\pm$ s.e.m, $n = 3$, $**P < 0.01$ as determined by two-tailed t test). (C) Representative trace of CFTR-dependent I_{sc} induced by compound CO-068 and forskolin (FSK). The results show that compound CO-068 augments FSK-stimulated CFTR-dependent I_{sc} . DMSO was used as a control. CFTRinh-172 was used to verify that the responses were CFTR-mediated. The experiment was repeated 3 times. (D) Quantification of data from Ussing chamber experiments as represented in (C). The data are presented as the changes of CFTR-dependent I_{sc} after addition of FSK compared to the I_{sc} levels after CO-068 (or DMSO) had been added (mean $\pm$ s.e.m, $n = 3$, $*P < 0.05$ as determined by two-tailed t test).

Figure 5. Compound CO-068 augments basal and FSK-stimulated CFTR-dependent pig tracheal submucosal glands fluid secretion

(A) Representative images of fluid secretion from pig tracheal submucosal glands before (basal) and after addition of 50 μ M compound CO-068 (time = 25 min), followed by addition of 10 μ M carbachol (time = 3 min). Scale bar (1 mm). (B) The mean secretion rate upon addition of compound CO-068 and/or FSK. CFTRinh-172 was used to verify that the augmentation effects were indeed CFTR-dependent (mean $\pm$ s.e.m. The results are from 11 pigs. n = 11–43 glands, ** P < 0.01 as determined by two-tailed t test).

Figure 6. Compound CO-068 elevates cAMP levels in Calu-3 cells

The cells were transiently transfected with a cAMP sensor, CFP-EPAC-YFP, and subjected to ratiometric FRET measurements. **(A)** Representative pseudocolor images of CFP/FRET emission ratio before (time = 0 min) and after addition of 50 μ M compound CO-068 (time = 10 min), followed by addition of 10 μ M FSK (time = 10 min). Look-up bars show the magnitude of emission ratio. **(B)** Representative line graph for the change of CFP/FRET emission ratio versus time after addition of compound CO-068 or FSK. **(C)** Quantification of the mean CFP/FRET ratio change after addition of compound CO-068, followed by addition of FSK (mean $\pm$ s.e.m.; $n = 4$ separate experiments, $*P < 0.05$ as determined by two-tailed t test). **(D)** Quantification of the mean CFP/FRET ratio change after addition of FSK, followed by addition of compound CO-068 (mean $\pm$ s.e.m.; $n = 5$ separate experiments, $**P < 0.01$ as determined by two-tailed t test).

Figure 7. Pictorial representation of the molecular mechanism underlying the disruption of LPA₂-NHERF2 interaction within CFTR-containing macromolecular complex and its regulatory effect on CFTR Cl⁻ channel function

Scheme 1. Reaction scheme for synthesis of compound CO-068

Reaction conditions: I) 3-phenylpropanoyl chloride, ZnCl₂ (in Et₂O), dry DCM, 4 °C, 3 h. II) first step: NaOH, 1,4-dioxane, methanol, 90 °C, 6 h; second step: HCl. The compound was synthesized with high purity (> 97%).

Table 1
IC₅₀ of compound CO-068 for inhibiting PDZ-based protein-protein interactions
important in CFTR-containing macromolecular complexes

AlphaScreen[™] association and competition assays were developed for these systems. Compound CO-068 was then used in competition assays to determine its inhibitory effects. The IC₅₀ values were calculated using GraphPad Prism software.

Interaction	IC ₅₀ (μM)
LPA ₂ -NHERF2	63
CFTR-NHERF2	122
MRP4-PDZK1	391
CFTR-NHERF1 (PDZ2)	329