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The role of SPCA2 in store-independent STIM1-ORAI1 activation and the regulation of basal CFTR activity in epithelial secretion

Ph.D. Thesis

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1. LIST OF PUBLICATION RELATED TO THE THESIS

I. Store-independent activation of STIM1-ORAI1 by SPCA2 determines the basal CFTR activity in secretory epithelial cells
Aletta Kata Kiss*, Árpád Varga*, Marietta Görög, Tamara Madácsy, Woo Young Chung, Petra Pallagi, Viktória Szabó, Petra Susánszki, Enikő Kúthy-Sutus, Dániel Varga, Péter Bíró, Ingrid Hegnes Sendstad, Tim Crul, Boldizsár Jójárt, Bálint Tél, Zsófia Horváth, Szintia Barnai, Anita Balázs, György Lázár, Miklós Erdélyi, Shmuel Muallem, József Maléth
Current Biology 35: 20 pp. 4970-4987.e7., 25 p. **(2025)**; MTMT ID: 36356148
IF: 7.5 (D1)

II. Human Pancreas-Derived Organoids with Controlled Polarity: Detailed Protocols and Experimental Timeline
Aletta Kiss, Attila Farkas, Ferhan Ayaydin, György Lázár, Árpád Varga, József Maléth
Current Protocols 4: 11 Paper: e70045, 25 p. **(2024)**; MTMT ID: 35609714
IF: 2.2 (D1)

2. LIST OF PUBLICATION NOT RELATED TO THE THESIS

I. Impaired regulation of PMCA activity by defective CFTR expression promotes epithelial cell damage in alcoholic pancreatitis and hepatitis
Madácsy Tamara, Varga Árpád, Papp Noémi, Tél Bálint, Pallagi Petra, Szabó Viktória, **Kiss Aletta**, Fanczal Júlia, Rakonczay Zoltan, Tiszlavicz László, Rázga Zsolt, Hohwieler Meike, Kleger Alexander, Gray Mike, Hegyi Péter, Maléth József
CELLULAR AND MOLECULAR LIFE SCIENCES **(D1)**
IF: 9.207

II. Thiopurines impair the apical plasma membrane expression of CFTR in pancreatic ductal cells via RAC1 inhibition
Tél Bálint, Papp Noémi, Varga Árpád, Szabó Viktória, Görög Marietta, Susánszki Petra, Crul Tim, **Kiss Aletta**, Sendstad Ingrid H, Bagyánszki Mária, Bódi Nikolett, Hegyi Péter, Maléth József, Pallagi Petra
CELLULAR AND MOLECULAR LIFE SCIENCES **(D1)**
IF: 9.234

III. Orai1 calcium channel inhibition prevents progression of chronic pancreatitis
Szabó Viktória, Csákány-Papp Noémi, Görög Marietta, Madacsy Tamara, Varga Árpád, **Kiss Aletta**, Tel Balint, Jójárt Boldizsár, Crul Tim, Dudás Krisztina, Bagyánszki Mária, Bódi Nikolett, Ayaydin Ferhan, Jee Shyam, Tiszlavicz Laszlo, Stauderman Kenneth A., Hebbar Sudarshan, Pallagi Petra, Maléth József
JCI Insight **(D1)**
IF: 9.484

IV. Human pancreatic ductal organoids with controlled polarity provide a novel ex vivo tool to study epithelial cell physiology
Árpád Varga, Tamara Madácsy, Marietta Görög, **Aletta Kiss**, Petra Susánszki, Viktória Szabó, Boldizsar Jojart, Krisztina Dudás, Gyula Jr. Farkas, Edit Szederkényi, György Lázár, Attila Farkas, Ferhan Ayaydin, Petra Pallagi, József Maléth
CELLULAR AND MOLECULAR LIFE SCIENCES **(D1)**
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3. INTRODUCTION

3.1. Epithelial ion and fluid secretion

Secretory epithelial cells line the luminal surfaces and ductal networks of multiple organs, including the airways, pancreas, liver, salivary and sweat glands. Their primary function is the vectorial transport of ions, predominantly chloride (Cl^-) and bicarbonate (HCO_3^-), coupled to water movement into the lumen. This process maintains mucosal hydration, supports mucociliary clearance, digestive enzyme function, and thermoregulation, and preserves the ionic composition of secreted fluids. Defective epithelial secretion underlies a broad spectrum of human diseases, ranging from the life-threatening manifestations of cystic fibrosis (CF) to chronic pancreatitis, autoimmune exocrinopathies and various secretory diarrheal conditions. The multi-organ impact of epithelial transport defects illustrates how proper baseline activity of secretory epithelia represents an essential prerequisite for human health and survival.

3.2.**The central role of CFTR in epithelial ion transport**

Among the transport proteins governing transepithelial secretion, the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel plays a central role. CFTR is a Cl^-/HCO_3^- channel localized mainly on the apical membrane, where it represents a rate-limiting step for fluid secretion. Activation of CFTR drives Cl^- and HCO_3^- efflux into the lumen, leading to sodium (Na^+) movement via paracellular pathways and osmotic water flow, thus generating fluid secretion. CFTR function is essential for maintaining the hydration, viscosity, and pH of epithelial secretions across multiple organs. Loss-of-function mutations in CFTR cause CF, characterized by dehydrated, acidic mucus in the respiratory and gastrointestinal tracts, pancreatic ductal obstruction, and NaCl-rich sweat. These manifestations highlight the systemic importance of CFTR-mediated transport in epithelial physiology.

3.3. **Molecular regulation of CFTR**

CFTR consists of two membrane-spanning domains (MSD1/2), two cytosolic nucleotide-binding domains (NBD1/2), and a regulatory (R) domain linking NBD1 to MSD2. Channel gating is tightly controlled by the R domain. In its unphosphorylated state, the R domain inhibits NBD dimerization and channel opening. Canonical CFTR activation is mediated by the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway. G protein-coupled

receptor (GPCR) stimulation activates adenylyl cyclases (ACs), increasing cAMP and activating PKA. PKA phosphorylates multiple serine residues in the R domain, releasing its autoinhibition and allowing NBD dimerization and opening of the channel, with up to a ~100-fold increase in open probability. Beyond phosphorylation-dependent activation, PKA can also regulate CFTR via direct binding, providing a reversible, phosphorylation-independent mechanism. CFTR function further depends on its spatial organization within macromolecular complexes anchored by A-kinase anchoring proteins (AKAPs) and Na^+/H^+ exchanger regulatory factor 1 (NHERF1) at the apical membrane. These scaffolds bring together CFTR, PKA, phosphatases, phosphodiesterases (PDEs), and cytoskeletal elements, enabling localized and temporally precise regulation. Inactivation involves GPCR desensitization, dephosphorylation, and local cAMP hydrolysis by PDEs such as PDE4.

3.4. The role of Ca^{2+} signalling in CFTR regulation

Although cAMP/PKA is the primary regulator of CFTR, calcium (Ca^{2+}) signalling represents a crucial complementary pathway. Ca^{2+} modulates CFTR via calmodulin binding, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), and tyrosine kinases, which can phosphorylate CFTR at distinct sites. Ca^{2+}-dependent regulation often acts through Acs, some isoforms (e.g., AC1, AC3, AC8) are activated by Ca^{2+}/calmodulin, while others (e.g., AC5, AC6) are inhibited by Ca^{2+}. The subcellular positioning of different AC isoforms creates cAMP microdomains that are shaped by local Ca^{2+} signals. In airway epithelia, AC1 colocalizes with CFTR at the apical membrane, supporting functional Ca^{2+}/cAMP crosstalk. However, the specific contributions of individual AC isoforms to CFTR regulation in different epithelial cell types remain incompletely understood. In pancreatic ductal epithelial cells, CFTR mediates both Cl^- and HCO_3^- transport, with HCO_3^- secretion playing an essential role in maintaining alkaline pancreatic juice. The regulation of this process involves complex interactions between CFTR, Cl^-/HCO_3^- exchangers (SLC26 family), and the WNK1/SPAK kinase pathway, all of which are modulated by Ca^{2+}-dependent mechanisms.

3.5. Store-operated and store-independent Ca^{2+} entry in epithelial cells

In non-excitable cells, including epithelial cells, store-operated calcium entry (SOCE) is a major pathway for Ca^{2+} influx. It is mediated by STIM proteins, which sense ER Ca^{2+} depletion, and ORAI channels, especially ORAI1, located in the plasma membrane. Upon ER Ca^{2+} store depletion, STIM1 oligomerizes and translocates to ER–PM junctions, where it activates ORAI1, leading to Ca^{2+} influx that refills ER stores and triggers downstream Ca^{2+}-dependent responses.

In airway epithelial cells, SOCE contributes to inflammatory signalling, ciliary beat regulation, and mucin secretion. However, accumulating evidence indicates that ORAI1 can also support store-independent calcium entry (SICE). A key player in SICE is the secretory pathway Ca^{2+}-ATPase isoform 2 (SPCA2), which, unlike the ubiquitously expressed SPCA1, can traffic to the plasma membrane and directly interact with ORAI1. This SPCA2-ORAI1 interaction activates Ca^{2+} influx independently of ER store depletion and STIM proteins and is uncoupled from the Ca^{2+}-ATPase activity of SPCA2. SPCA2-mediated SICE has been described in lactating mammary epithelium and breast cancer cells, where it supports high Ca^{2+} demand and contributes to the activation of the RAS-ERK signalling pathway. Despite the recognized importance of ORAI1-mediated Ca^{2+} entry in various cell types and the established role of Ca^{2+} in modulating CFTR activity, the potential contribution of ORAI1 to CFTR regulation in epithelial cells has not been systematically investigated. Given the colocalization of CFTR and Ca^{2+}-sensitive ACs at the apical membrane, and the emerging recognition of SICE mechanisms, it is plausible that ORAI1 channels contribute to the local Ca^{2+} microdomains that regulate CFTR function.

3.6. **Organoid models as tools for studying epithelial physiology**

Three-dimensional organoid cultures derived from tissue-specific stem cells recapitulate key structural and functional features of native epithelia. Pancreatic, airway, liver, and intestinal organoids maintain apical-basal polarity, tight junctions, and relevant differentiation profiles. For CFTR research, preserving apical localization and native protein–protein interactions are crucial. Organoids embedded in ECM and maintained in WNT/R-spondin/Noggin-containing media can be propagated long-term while retaining ductal markers and functional properties. Pancreatic ductal organoids express CFTR, KRT19, OCLN, and SOX9 and exhibit features of native ducts, including apical enrichment of mitochondria and brush border formation. Organoids provide a physiologically relevant platform to investigate CFTR regulation and epithelial ion transport, allowing functional assays (e.g. swelling, live-cell Ca^{2+} imaging, Cl^- flux) under conditions that preserve endogenous stoichiometry of signalling proteins and nanodomain organization.

4. AIMS

1. We aimed to characterize the store-independent activation of STIM1–ORAI1 by SPCA2 and to evaluate the physiological relevance of this signalling nanodomain in polarized secretory epithelial cells from multiple tissues, including the pancreas, airways, and liver.

2. We also aimed to determine how SPCA2-mediated Ca^{2+} influx regulates basal CFTR activity via Ca^{2+}-sensitive ACs and local cAMP signalling.

3. We aimed to standardize the experimental use of human organoid cultures as a model for epithelial fluid and ion secretion in primary, polarized epithelia.

5. MATERIALS AND METHODS

5.1. **Cell lines and animals**

HeLa, and HEK293 cells were cultured according to the provider's protocol and were used for transient transfection and subsequent measurements. L-WRN cell line (ATCC-CRL-3276) was utilized to produce conditioned medium required for organoid cultures. 8-12 weeks old and 20-25-gram weighted FVB/N mice were used to carry out ductal fragment isolation, fluid secretion measurements and organoid culture generation.

5.2. **Isolation of pancreatic ductal fragments and acinar cells**

Pancreatic ductal fragments were isolated as described previously (Maléth et al, 2015; Fanczal et al, 2020). Briefly, surgical removal of the pancreas was followed by enzymatic tissue digestion and stereomicroscope assisted microdissection. Following physical fragmentation of pancreatic tissue, enzymatic digestion supplemented by gentle centrifugation were applied to isolate acinar cells.

5.3.**Mouse and human organoid cultures**

Mouse pancreas, liver, and lung, as well as human pancreatic tissue, were minced and digested enzymatically at 37°C in a vertical shaker. After centrifugation and washing steps, cells were resuspended in Wash Medium, mixed with Matrigel, and plated as small domes (10 µl) in 24-well plates. After Matrigel solidification at 37°C, Feeding Medium was added and changed every second day. For passaging, domes were collected, digested with TrypLE™ Express, washed, and replated in fresh Matrigel. To generate apical-out human pancreatic organoids, Matrigel was enzymatically removed and organoids were transferred to suspension culture for ≥48 h, allowing polarity switching.

5.4. Constructs, transfection, site-directed mutagenesis and gene knockdown

Expression constructs for ORAI1, STIM1, SPCA2 and ACs were obtained from collaborators, ORIGENE and Addgene. Transient transfections were performed using Lipofectamine 2000. For site-directed mutagenesis Q5 high-fidelity, thermostable DNA polymerase was applied.

For gene knockdown in mouse ductal fragments, pre-validated siRNA or siGLOGreen transfection indicator were transfected with Lipofectamine 2000. A common concern with experiments derived from heterologous expression of multiple proteins is the altered endogenous stoichiometry of the proteins. To compensate for this, we performed a high number of experiments to buffer and reduce outliers, and we tried to confirm the crucial results in primary cells expressing endogenous proteins without overexpression.

5.5. End-point and qRT-PCR analysis

Total mRNA from mouse pancreatic ductal fragments was isolated by NucleoSpin RNA XS kit according to the manufacturer's instructions. mRNA concentration was measured by NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer, and cDNA was synthesized from 1 μg RNA. Gene expression changes after siRNA treatment were assessed by qRT-PCR and analyzed using the ΔΔCq method.

5.6. Gene expression analysis of mouse pancreatic ductal organoid cultures by RNA-Seq

RNA was extracted from the collected cell pellet after separation from Matrigel. RNA-sequencing was performed on an Illumina NextSeq 500 platform. Expression levels were determined according to transcript/million (TPM) values, with TPM <1 considered non-expressed. The TPM values of the human CFTR gene was published previously.

5.7. Immunofluorescent labelling for confocal microscopy

Organoids and sectioned ductal fragments were fixed in 4% PFA-PBS solution and followed by washing and antigen retrieval. Sections were then blocked with BSA solution and labelled with primary and secondary antibodies against the target proteins. Images were captured by a Zeiss LSM880 confocal microscope.

5.8. Western blot and Cell Surface Biotinylation

HeLa cells were transfected with ORAI1, STIM1, and SPCA2-carrying constructs. After 18h, cells were lysed in RIPA buffer with protease inhibitors. Proteins were separated by SDS-PAGE and transferred to membranes. Antibodies against GAPDH, HA, and relevant tags were used to detect expression levels. For cell surface protein measurements PierceTM Cell Surface Biotinylation and Isolation Kit were applied according to the attached protocol. Blot picture analysis and quantification were computed by ImageJ software.

5.9.Fluorescent microscopy

Fura-2-AM and MQAE dyes were used to monitor intracellular Ca^{2+} and Cl^-, respectively. Ratiometric Ca^{2+} imaging (F340/F380) was performed, and baseline Ca^{2+} levels and responses to pharmacological stimuli (e.g. CM5480, CPA, carbachol) were quantified. MQAE fluorescence changes were used to monitor CFTR-dependent Cl^- efflux. Bleaching and dye leak were corrected computationally using control recordings. Each ROI represented a cell or region, and summarized data were plotted as means.

5.10. *In vitro* and *in vivo* measurement of pancreatic fluid secretion

Isolated pancreatic ducts were attached to coverslips and perfused with HEPES- or HCO_3^--buffered solutions at 37°C. Changes in intraluminal volume were monitored by transmitted video microscopy with Olympus IX73 inverted microscope. For *in vivo* secretion, mice received CM5480 or vehicle before secretin injection. Pancreatic juice was collected for 30 min and secretion rates were normalized to body weight.

5.11. Direct Stochastic Optical Reconstruction Microscopy (dSTORM)

Samples labelled with immunofluorescent technique were immersed in glucose oxidase and catalase containing blinking buffer solution. dSTORM images were captured by Nanoimager S (Oxford Nanoimaging ONI Ltd.). Co-clusters were defined as clusters of two proteins within 300 nm. The proportion and size of co-clusters were quantified.

5.12. FLIM-FRET measurements

FLIM-FRET was used to assess nanoscale interactions between fluorescently tagged proteins. Donor lifetime was measured before and after acceptor bleaching. FRET efficiency was calculated for each cell based on the changes in the average donor lifetime ($E = 1 - \frac{\tau DA}{\tau D}$)

5.13. Statistical analysis

Data are presented as mean ± SEM. Normality was tested using Shapiro–Wilk test. Parametric (Unpaired t-test or one-way analysis of variance with Tukey's multiple comparisons test) and nonparametric (Mann-Whitney test and Kruskal-Wallis test) were used based on the normality of data distribution. $P<0.05$ was considered statistically significant. All statistical analyses were carried out by GraphPad Prism software (Version 8.3.1.).

6. RESULTS

6.1. **ORAI1-mediated extracellular Ca^{2+} entry is constitutively active in primary polarized epithelial cells**

RNA-Seq and PCR of mouse and human pancreatic organoids confirmed expression of ORAI1–3, STIM1/2, and regulatory proteins. Immunostaining showed ORAI1 enrichment at the apical membrane of pancreatic ductal cells. In isolated mouse ductal fragments, the selective ORAI1 inhibitor CM5480 decreased basal intracellular Ca^{2+} in the presence of extracellular Ca^{2+}, even without ER Ca^{2+} store depletion. Removal of extracellular Ca^{2+} similarly lowered $[Ca^{2+}]_i$, which was not further decreased by CM5480. Carbachol-evoked Ca^{2+} signals were diminished when CM5480 was applied during the plateau phase, suggesting that ORAI1 supports sustained Ca^{2+} elevations. ER Ca^{2+} measurements with D1ER confirmed that ER Ca^{2+} stores were not depleted under basal conditions, excluding store-operated activation as the main mechanism. siRNA knockdown of Orai1 or Stim1 reduced basal $[Ca^{2+}]_i$ and abolished the response to CM5480. CM5480-sensitive basal Ca^{2+} influx was observed in mouse lung and liver organoids and in human pancreatic organoids. These findings indicate that ORAI1 mediates a constitutive, store-independent Ca^{2+} entry that significantly contributes to basal $[Ca^{2+}]_i$ in primary secretory epithelial cells from multiple tissues.

6.2. **SPCA2 maintains constitutive ORAI1 activity in primary epithelial cells**

Whole-transcriptome analysis of mouse and human pancreatic organoids identified several potential interacting proteins of ORAI1, including Spca1, Spca2, and Septin7. Among these, SPCA2 emerged as a strong candidate for controlling SICE. siRNA-mediated knockdown of SPCA2 in mouse pancreatic ductal fragments lowered basal $[Ca^{2+}]_i$ and significantly reduced CM5480-sensitive constitutive Ca^{2+} influx. In contrast, knockdown of Spca1 or Septin7 had no significant effect, indicating a specific role for SPCA2. In HeLa cells expressing ORAI1 and SPCA2, confocal imaging revealed apical-polarized SPCA2 with a reticular ER-like pattern and ORAI1 puncta at the PM. dSTORM demonstrated partial colocalization of SPCA2 and ORAI1 clusters under resting conditions, which changed upon CPA-induced store depletion, suggesting dynamic reorganization of ER–PM contacts. FLIM-FRET analysis showed physical proximity between SPCA2 and ORAI1 in unstimulated cells, which was not increased further by CPA stimulation. In contrast, the presence of STIM1 was sufficient to remarkably increase the FRET between ORAI1 and SPCA2, which was again not increased further by CPA stimulation. As SPCA2 does not participate in SOCE, it is not unexpected that CPA stimulation

does not increase the SPCA2-ORAI1 interaction. Expression of ORAI1 and SPCA2 generated a constitutively active Ca^{2+} influx, which was not affected by the SPCA2 mutants. These results suggest that SPCA2 regulates the SICE via ORAI1 independently from SPCA2 Ca^{2+} influx in epithelial cells.

6.3. **SPCA2 increases the interaction between STIM1 and ORAI1**

To examine how SPCA2 affects STIM1–ORAI1 coupling, HeLa cells were co-transfected with STIM1, ORAI1, and SPCA2. In the absence of ER Ca^{2+} store depletion, STIM1 showed modest punctation and limited colocalization with ORAI1. Coexpression of SPCA2 markedly increased STIM1–ORAI1 cluster formation. dSTORM cluster analysis revealed that SPCA2 significantly enhanced the number and size of STIM1–ORAI1 co-clusters in resting conditions, and CPA further increased clustering. FLIM-FRET using STIM1-YFP and ORAI1-mCherry confirmed that SPCA2 elevated FRET efficiency, consistent with tighter STIM1–ORAI1 association. The constitutive Ca^{2+} influx and increased STIM1-ORAI1 clustering may be caused by enhanced Ca^{2+} leakage and reduced ER Ca^{2+} stores in SPCA2-expressing cells. To exclude the possibility that STIM1-ORAI1 clustering is caused by the leakage of the ER Ca^{2+} stores, we measured the ER Ca^{2+} store content in HeLa cells transfected with D1ER and treated with 10 μM CPA. These experiments showed no effect of SPCA2 on the ER Ca^{2+} content. Surface biotinylation and Western blotting showed that SPCA2 did not significantly increase total or surface ORAI1 expression, suggesting that its main role is to promote functional clustering rather than trafficking. Co-immunoprecipitation of HA-tagged ORAI1 pulled down both STIM1 and SPCA2, confirming that all three proteins form a biochemical complex. Taken together, these data support a model in which SPCA2 first acts on ORAI1 to promote its clustering, which then stabilizes STIM1 at ER–PM junctions, enabling constitutive SICE.

6.4. **ORAI1-mediated SICE regulates CFTR activity and fluid secretion in pancreatic ductal epithelial cells**

Given the central role of CFTR in epithelial secretion, we examined whether SPCA2–STIM1–ORAI1-mediated SICE influences CFTR activity. Immunostaining of mouse pancreatic organoids revealed CFTR and ORAI1 co-localization at the apical membrane. CFTR-mediated Cl^- efflux was monitored in isolated ductal fragments using MQAE. Removal of extracellular Cl^- from HCO_3^-/CO_2-buffered solution induced an increase in MQAE fluorescence, reflecting Cl^- efflux, which was abolished by the CFTR inhibitor CFTRinh-172. Inhibition of ORAI1 with CM5480 or chelation of intracellular Ca^{2+} with BAPTA-AM significantly reduced Cl^- efflux under basal conditions. Moreover, knockdown of Orai1 or Stim1 resulted in a marked

decrease in basal CFTR-mediated Cl^- efflux. On the other hand, the higher forskolin-stimulated maximal CFTR Cl^- efflux was not affected by the inhibition of ORAI1 with CM5480, suggesting that the stimulated secretion is independent of SICE. SPCA2 knockdown in ductal fragments reduced CFTR activity, whereas SPCA1 or SEPTIN7 knockdown had no effect, as in Ca^{2+} measurements. Basal HCO_3^- secretion and spontaneous swelling of ducts in HCO_3^-/CO_2 solution were impaired by CM5480, linking ORAI1 activity to both Cl^- and HCO_3^- transport and to basal fluid secretion. In vivo, CM5480-treated mice showed significantly reduced secretin-stimulated pancreatic fluid secretion compared to controls, supporting a physiological role for ORAI1-mediated SICE in ductal fluid secretion. Overall, these results demonstrate that SPCA2/STIM1/ORAI1-dependent SICE is a key determinant of basal CFTR activity and fluid secretion in pancreatic ductal cells.

6.5. **SICE via ORAI1 regulates CFTR activity in secretory epithelial cells**

To assess whether this regulatory mechanism is common across epithelia, we extended our studies to mouse airway and liver organoids and human pancreatic organoids. In all models, CFTR and ORAI1 showed apical colocalization by immunofluorescence staining. In unstimulated organoids, CM5480 markedly reduced CFTR-mediated Cl^- efflux measured by MQAE upon extracellular Cl^- removal. In airway organoids, inhibition of ORAI1 almost completely abolished basal CFTR-dependent Cl^- efflux. These findings indicate that ORAI1-mediated SICE is a general mechanism supporting basal CFTR activity in diverse secretory epithelia in both mice and humans.

6.6. SICE by ORAI1 regulates CFTR activity via Ca^{2+}-dependent ACs

To elucidate how SICE is transduced to CFTR, we analyzed AC expression and localization. RNA-Seq of pancreatic organoids identified expression of several AC isoforms, including AC1, 3, 6, 8, and 9. Because some ACs are Ca^{2+}-stimulated (AC1, AC3, AC8) while others are Ca^{2+}-inhibited (AC5, AC6), they are well positioned to mediate Ca^{2+}/cAMP crosstalk. dSTORM imaging of HeLa cells coexpressing CFTR and individual AC isoforms showed robust nanodomain colocalization of CFTR with AC1, AC3, and AC8, and weaker colocalization with AC6. These ACs also colocalized with ORAI1, and three-colour dSTORM confirmed that CFTR, ORAI1, and AC1/3/8 reside in the same PM nanodomains. In human apical-out pancreatic organoids, siRNA knockdown of AC1, AC3, or AC8 reduced basal CFTR activity, with AC8 knockdown producing the strongest effect. These data indicate that Ca^{2+}-activated ACs, particularly AC8, are crucial for maintaining basal CFTR function downstream of SICE.

Pharmacological inhibition of PKA using PKI (5–24) or KT5720 strongly diminished basal CFTR-mediated Cl^- efflux, demonstrating that PKA-dependent phosphorylation is required in this context. Together, these observations support a model in which ORAI1-mediated SICE activates Ca^{2+}-sensitive ACs (especially AC8) within apical nanodomains, generating local cAMP and PKA activity that sustain basal CFTR activity.

7. DISCUSSION

This study identifies a previously unrecognized regulatory pathway that maintains basal CFTR activity in polarized secretory epithelia via store-independent Ca^{2+} entry mediated by a SPCA2–STIM1–ORAI1 complex. We show that ORAI1 is constitutively active in resting epithelial cells, contributing significantly to basal $[Ca^{2+}]_i$, and that this activity depends on SPCA2 and STIM1 yet is independent of ER Ca^{2+} store depletion. Our data demonstrate that SPCA2 acts as a key organizer of SICE. It directly interacts with ORAI1, promotes ORAI1 clustering, and enhances STIM1–ORAI1 complex formation in the absence of ER depletion. SPCA2's effect does not require its Ca^{2+}-pump activity or changes in ER Ca^{2+} content, indicating a scaffolding role. SPCA2 expression is tissue-specific, which may explain why SICE is prominent in ductal epithelia but not in pancreatic acinar cells. We further show that SPCA2/STIM1/ORAI1-dependent SICE localizes to apical PM nanodomains where CFTR and Ca^{2+}-activated ACs (AC1, AC3, AC8) are co-localized. Within these nanodomains, constitutive Ca^{2+} influx through ORAI1 stimulates ACs, particularly AC8, leading to local cAMP production and PKA activation. This, in turn, maintains basal CFTR activity and fluid secretion. Notably, pharmacological inhibition or genetic silencing of ORAI1, STIM1, SPCA2, or ACs strongly impairs basal CFTR function and secretion, whereas forskolin-stimulated secretion remains intact. Thus, SICE defines a distinct regulatory layer specifically controlling basal, but not maximally stimulated CFTR activity. Our findings provide direct mechanistic support for previous indirect observations that basal CFTR currents depend on Ca^{2+} influx and membrane-bound AC activity. By combining super-resolution imaging, FLIM-FRET, functional assays, and organoid models, we characterized a compact signalling unit integrating Ca^{2+} entry, cAMP synthesis, and CFTR gating in native-like epithelia. Physiologically, basal CFTR activity is essential to maintain luminal hydration and mucociliary clearance in the airways, ductal patency and enzyme flushing in the pancreas, and bile and fluid composition in the hepatobiliary system. The SPCA2–STIM1–ORAI1–AC–CFTR nanodomain offers a mechanism by which epithelial cells generate stimulus-independent, steady-state ion and fluid secretion. This mechanism may be especially important under resting or low-stimulation conditions, when neurohormonal drive

is minimal yet a baseline of secretion must be preserved. Our data also suggest that different CFTR subpopulations and regulatory pathways underlie basal versus stimulated secretion. Forskolin activates CFTR independently of SICE through robust cAMP/PKA signalling, whereas basal CFTR activity depends on localized cAMP production by Ca^{2+}-sensitive ACs within lipid raft-like domains. This compartmentalization may contribute to finely tuned and context-dependent control of epithelial transport. The broader implications extend to disease. Dysregulation of ORAI1 or SPCA2 is implicated in cancer progression, and CFTR loss is linked to altered Wnt/β-catenin signalling and increased tumor risk in CF. The nanodomain described here couples these proteins and may thereby influence oncogenic pathways as well as epithelial homeostasis. Understanding how SPCA2 and ORAI1 are regulated in physiological versus pathological contexts may reveal novel therapeutic targets for CFTR-related diseases, inflammatory conditions, and malignancies. Finally, our work underscores the power of 3D organoid systems. Human pancreatic organoids retaining native polarity, tight junctions, and donor-specific traits offered a robust platform to interrogate Ca^{2+} dynamics and CFTR regulation in structurally intact epithelia. The ability to combine high-resolution imaging, targeted gene perturbation, and functional secretion assays in this setting was critical to uncovering the SICE–CFTR link.

In summary, we identified a protein nanodomain on the apical membrane composed of SPCA2/STIM1/ORAI1, CFTR, and Ca^{2+}-activated AC1, 3, and 8, highlighting distinct regulatory mechanisms for basal versus stimulated secretion. Our model proposes that constitutive Ca^{2+} influx maintains basal CFTR activity independently of neurohormonal stimulation that adjust the ion and fluid secretion to various stimuli. Importantly, this nanodomain may also impact cancer biology, as SPCA2 promotes breast cancer progression, loss of CFTR expression is linked to enhanced Wnt/β-catenin signalling and tumor risk in CF, and ORAI1 regulates proliferation and metastasis. Understanding ORAI1/SPCA2 interactions may therefore uncover novel therapeutic targets for cancer.

8. SUMMARY

The CFTR Cl^- channel is essential for transepithelial ion and fluid secretion in organs such as the pancreas, liver, and lungs. While the regulation of CFTR during stimulated secretion is well characterized, the mechanisms that maintain its basal activity are less clear. We identified a novel regulatory pathway in which store-independent Ca^{2+} entry (SICE) through an apical SPCA2–STIM1–ORAI1 complex sustains basal CFTR activity in secretory epithelial cells. We

demonstrate that ORAI1-mediated Ca^{2+} influx is constitutively active in primary polarized epithelial cells from pancreas, lung, and liver and significantly contributes to basal intracellular Ca^{2+} levels. This activity does not require ER Ca^{2+} store depletion but depends on STIM1 and SPCA2. SPCA2 interacts with ORAI1 and promotes its clustering and association with STIM1, forming a signalling nanodomain at the apical plasma membrane. Within this nanodomain, Ca^{2+} influx through ORAI1 activates Ca^{2+}-sensitive ACs (AC1, AC3, and particularly AC8), leading to local cAMP production and PKA activation. This, in turn, maintains basal CFTR-mediated Cl^- and HCO_3^- secretion. Pharmacological inhibition or genetic silencing of ORAI1, STIM1, SPCA2, or ACs markedly reduces basal CFTR activity and fluid secretion, whereas forskolin-induced maximal CFTR activation remains unaffected. Thus, SICE selectively controls basal, but not stimulated, CFTR function. Super-resolution dSTORM imaging and FLIM-FRET provide direct evidence that SPCA2, STIM1, ORAI1, ACs, and CFTR reside in tightly organized apical nanodomains. Human pancreatic organoids with preserved polarity were instrumental in validating these mechanisms in a physiologically relevant context. Overall, the thesis reveals a previously unrecognized mechanism by which epithelial tissues maintain basal secretion independently of external stimuli. The SPCA2–STIM1–ORAI1–AC–CFTR nanodomain represents a key hub integrating Ca^{2+} and cAMP signalling to sustain luminal hydration and epithelial homeostasis and may offer new targets for therapeutic intervention in diseases involving CFTR dysfunction or epithelial dysregulation.

9. SUMMARY OF NEW OBSERVATIONS

1. We revealed that in polarized secretory epithelial cells, CFTR activity is maintained under resting conditions by a constitutive, store-independent Ca^{2+} influx rather than by classical neurohormonal stimuli.
2. SPCA2 was identified as a central regulator that activates the STIM1–ORAI1 complex in the absence of ER Ca^{2+} depletion. This SPCA2-dependent SICE provides a continuous Ca^{2+} source required for maintaining basal epithelial ion and fluid secretion.
3. Pharmacological or genetic inhibition of STIM1 or ORAI1 reduced basal CFTR activity and resting fluid secretion, while stimulated (forskolin-induced) responses remained intact.
4. The results support a model in which SPCA2 continuously activates ORAI1-mediated Ca^{2+} influx that, via Ca^{2+}-activated ACs, generates local cAMP and PKA activity sustaining basal CFTR function. This mechanism operates independently of ER Ca^{2+}

depletion or neurohormonal stimulation and defines a distinct regulatory layer of epithelial homeostasis.

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