Regulation of ductal bicarbonate transport by intracellular chloride and its role in pancreatic secretion

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Directed by Professor Min Goo Lee

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ABSTRACT

Regulation of ductal bicarbonate transport by intracellular chloride and its role in pancreatic secretion

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The exocrine pancreas creates strong alkaline fluid. However, how the human pancreatic duct cells secrete copious amount of bicarbonate (HCO_3^-) has long been a puzzle for more than a century since it was realized. The present study demonstrates that intracellular Cl^- concentration ($[Cl^-]_i$)-sensitive mechanisms induces dynamic increase in the CFTR HCO_3^- permeability which play a pivotal role in the pancreatic HCO_3^- secretion. In the polarized PANC1 human pancreatic duct cells, CFTR activation rapidly decreased $[Cl^-]_i$ in response to the luminal Cl^- depletion. Interestingly, in human pancreatic tissues, CFTR-positive duct cells co-expressed WNK1, OSR1, and SPAK kinases, which are activated by low $[Cl^-]_i$. Notably, the WNK1-mediated OSR1 and SPAK activation by low $[Cl^-]_i$ strongly increased CFTR HCO_3^- permeability in the CFTR-transfected HEK 293T, PANC1, and guinea pig pancreatic duct cells making CFTR primarily a

HCO_3^- channel, which is essential for the secretion of pancreatic juice containing HCO_3^- at a concentration greater than 140 mM. In contrast, OSR1 and SPAK activation inhibited the CFTR-dependent Cl^-/HCO_3^- exchange activity, which may reabsorb HCO_3^- from the high HCO_3^--containing pancreatic juice. These results indicate that the $[Cl^-]_i$-sensitive activation of WNK1-OSR1/SPAK pathway is the molecular switch to generate HCO_3^--rich fluids in the human pancreatic ducts.

Keywords: CFTR, bicarbonate, pancreatic secretion, WNK1/OSR1/SPAK

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I. INTRODUCTION

In humans and many animals, pancreatic duct cells secrete bicarbonate (HCO_3^-) at a concentration more than five times higher than that found in plasma.[1-2] Importantly, HCO_3^- performs diverse functions in epithelial fluids including not only the pancreatic juice, but also airway surface fluid, and uterine fluid.[1,3-5] In case of pancreas, HCO_3^- secreted from duct cells neutralizes gastric acid and provides an optimum pH environment for digestive enzymes in the duodenum.[1] An overlooked function of HCO_3^- secretion is that HCO_3^- is a chaotropic ion that is important for the solubilization of macromolecules to prevent the aggregation of digestive enzymes and mucins.[1,3] In line with this notion, aberrant HCO_3^- secretion is associated with the obstructive types of pancreatic diseases such as cystic fibrosis (CF) and chronic pancreatitis.[6-8] In addition, recent evidence has

revealed that defects in HCO_3^- secretion are important pathophysiologic mechanisms of diseases in many other epithelia including the airways and genitourinary tract.[3-4] Therefore, pancreatic HCO_3^- secretion has attracted many people as a typical model to gain insight into the HCO_3^- transport mechanism in diverse epithelial cells.

However, how the pancreatic duct cells secrete copious amount of HCO_3^- has long been a puzzle for more than a century since it was realized.[9] Severe loss of pancreatic HCO_3^- secreting activity in the patients with cystic fibrosis,[7] which is caused by mutations in cystic fibrosis transmembrane conductance regulator (CFTR),[10] indicates that the CFTR gene product plays a critical role in HCO_3^- secretion. In the late 1980s, an early model for pancreatic HCO_3^- secretion suggested that the Cl^-/ HCO_3^- exchanger in the apical membrane mediates HCO_3^- secretion in cooperation with an apical Cl^- channel.[1-2] Subsequently, it was revealed that the CFTR protein functions as the apical Cl^- channel[11] and the activity of the apical Cl^-/ HCO_3^- exchanger is dependent on the expression of CFTR.[12-13] The idea that Cl^-/ HCO_3^- exchange mediates pancreatic HCO_3^- secretion has been strengthened by the finding that CFTR mutations associated with pancreatic insufficiency exhibit a severe defect in CFTR-dependent Cl^-/HCO_3^- exchange activity.[14] However, this model has been challenged because the classical 1:1 electroneutral Cl^-/HCO_3^- exchange is able to secrete only a maximum of 80 mM HCO_3^-, when intracellular concentrations of Cl^- ($[Cl^-]_i$) and HCO_3^- ($[HCO_3^-]_i$) are estimated to be equal (Figure 1).

Electrogenic HCO_3^- transporters can secrete higher concentrations of HCO_3^- than electroneutral transporters when the electrorepulsive force generated by the negative membrane potential is coupled to the efflux of HCO_3^-. Interestingly, recent studies have suggested that the apical Cl^-/ HCO_3^-

exchangers are actually electrogenic with distinct Cl^-: HCO_3^-stoichiometry.[15] The electrogenic Cl^-/ HCO_3^- exchangers, especially the exchanger with the stoichiometry of 1 Cl^-: 2 HCO_3^- can achieve a greater luminal HCO_3^- concentration.[2] Nevertheless, the transporter still cannot fully account for the HCO_3^--driven fluid secretion in pancreatic duct cells because the Cl^-/ HCO_3^- exchange is not able to attain 140 mM HCO_3^- in the lumen even with the 1:2 stoichiometry of Cl^-/ HCO_3^-.[2] More importantly, the Cl^-/ HCO_3^- exchanger always requires luminal Cl^- for HCO_3^- secretion. Therefore, the driving force for HCO_3^- secretion is greatly weakened when the luminal Cl^- concentration decreases in the later step of pancreatic secretion. However, it has been shown that a significant fraction of pancreatic HCO_3^- secretion is retained even in the absence of luminal Cl^-.[16-17] This implies that an unknown mechanism is likely to be responsible for ductal HCO_3^- secretion especially at the later stage when the HCO_3^- concentration, mediated by Cl^-/ HCO_3^- exchange, approaches equilibrium.

Present study firstly reports a novel mechanism whereby intracellular Cl^- concentration ($[Cl^-]_i$)-sensitive kinases play a critical role in pancreatic HCO_3^- secretion. Recently, two related kinase families, with-no-lysine (WNK) kinases and sterile 20 (STE20)-like kinases, have emerged as osmotic sensors that modulate diverse ion transporters.[18-19] In general, WNK kinases, including WNK1, are activated by osmotic stress such as a decrease in $[Cl^-]_i$, and subsequently phosphorylate and activate downstream STE20-like kinases, especially oxidative stress-responsive kinase 1 (OSR1) and STE20/SPS1-related proline/alanine-rich kinase (SPAK).[18] In pancreatic duct cells, CFTR activation greatly reduced $[Cl^-]_i$, and this in turn induced the activation of WNK1-OSR1/SPAK kinase cascade. Surprisingly, activation of the WNK1-OSR1/SPAK pathway resulted in a dramatic increase in CFTR

HCO_3^- permeability, making CFTR primarily a HCO_3^--selective channel. A HCO_3^- channel forms an electrodiffusive HCO_3^- efflux pathway that is essential for the generation of pancreatic juice containing HCO_3^- at a concentration greater than 140 mM. In contrast, OSR1 and SPAK activation inhibited the CFTR-dependent Cl^-/ HCO_3^- exchange activity, which prevents the duct cells from reabsorbing HCO_3^- from the high HCO_3^--containing pancreatic juice. These findings suggest that the dynamic regulation of CFTR and anion exchange activity, via the $[Cl^-]_i$-sensitive WNK1-OSR1/SPAK pathway, is the molecular switch that generates a HCO_3^--rich fluid in the human pancreatic duct.

II. MATERIALS AND METHODS

1. Plasmids and cell culture

The coding regions of hOSR1 and mSPAK were PCR-amplified using the plasmids purchased from Image Clones (http://image.hudsonalpha.org/) and subcloned into the pcDNA3.1(-) vector (Invitrogen, Carlsbad, CA) using the Xba I and BamH I restriction sites. Flag-tag was inserted at the C-terminus of each clone using BamH I and Afl II sites. The coding region of hSLC26A3 cDNA was PCR-amplified using the cDNA library of the T84 human colon cells and subcloned into the pCMV-Myc vector (Clontech, Palo Alto, CA) using Bgl II and Not I restriction sites. The hSLC26A3 cDNA sequence was verified by nucleotide sequencing, and was identical to a registered sequence (Genbank Accession NM_000111). The mammalian expressible plasmids for Myc-rWNK1[19], Myc-mWNK4[20], hCFTR[13], and mSlc26a6[21] were subcloned in PCMV vector (Clontech, Palo Alto, CA).The WNK1 siRNA, a mixture of four siRNAs against hWNK1, was purchased from Dharmacon, Lafayette, CO (Catalog # L-005362-00). Plasmids and siRNAs were transiently transfected into cells using Lipofectamine Plus and Lipofectamine 2000 reagents, respectively (Invitrogen, Carlsbad, CA). An average transfection rate was over 90% which was confirmed by transfection with a plasmid expressing green fluorescence protein or transfection with FITC-conjugated scrambled siRNA.

HEK293T and PANC1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-HG (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum and 100 U/ml penicillin and 0.1 mg/ml streptomycin. CFPAC1 cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. In experiments for $[Cl^-]_i$ measurements, PANC1 and CFPAC1 cells

were cultured on a permeable support to form monolayers. Briefly, PANC1 and CFPAC1 cells were removed from the culture flasks using trypsin/EDTA, and washed by centrifugation/re-suspension in a fresh medium. Aliquots of this suspension were plated (3×10^5 cells/cm^2) on permeable supports fabricated from Transwell-Clear Polyester membrane (0.4 μm pore diameter; Costar, Cambridge, MA), which had been coated with collagen (30 μg/ml) for 30 min. Membranes bearing cultured cells (culture area 0.3 cm2) were floated on the culture medium in Petri dishes and incubated for 4-5 days until the cells formed a functionally polarized monolayer.

2. Human pancreatic tissue samples. The experiments using human pancreatic tissue samples were approved by the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine, Seoul, Korea. Normal human pancreatic tissues were obtained from tumor margins. All subjects gave written informed consents and their clinical statuses are as follows.

3. Immunoblotting, immunoprecipitation, and immunohistochemistry. The antibody against phospho-T-loop was generated in rabbits using the peptide sequence in both OSR1 and SPAK (TRNKVRKpTFVGTP).[22] Antibodies against SPAK (#2281, Cell Signaling Technology, Danvers, MA), OSR1 (#3729, Cell Signaling Technology, Danvers, MA), WNK1 (ab53151, Abcam, Cambridge, MA), CFTR (M3A7, Upstate Biotechnology, Waltham, MA; 24-1, R&D Systems, Minneapolis, MN systems), Flag-epitope (F3165, Sigma, St. Louis, MO), Myc-epitope (sc-40, Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (sc-1616, Santa Cruz Biotechnology, Santa Cruz, CA) were obtained from commercial sources.

Immunoblotting, immunoprecipitation, and immunohistochemistry were performed using conventional methods as follows.[23] Briefly, cells were harvested with lysis buffer (20 mM HEPES pH 7.4, 200 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM NaVO4, and 1 mM β-glycerophosphate) containing a complete protease inhibitor mixture (Roche Applied Science, Mannheim, Germany). After lysis, cell debris was removed by centrifugation, and cleared lysates were mixed with the appropriate antibodies and incubated overnight at 4 °C. Immune complexes were collected by binding to protein G-beads for 2 hr at 4 °C and washed four times with lysis buffer prior to immunoblotting. Proteins were recovered in SDS sample buffers and samples were separated by the 4%-12% pre-cast poly-acrylamide gels. The separated proteins were transferred to nitrocellulose membranes, and the membranes were blocked by incubation for 1 h in a solution containing 5% non-fat dry milk in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20. The membranes were then incubated with the appropriate primary and secondary antibodies, and protein bands were detected with enhanced chemiluminescence solutions. For immunohistochemistry, human pancreatic tissues were embedded in OCT (Miles, Elkhart, IN), frozen in liquid N_2, and cut into 4-μm sections. The sections were fixed in 3.7% formaldehyde for 15 min and permeabilized with 0.1% Triton X-100 for 10 min at room temperature. Tissue sections were blocked with goat serum for 1 hr. Staining was performed with antibodies against CFTR (24-1), WNK1, OSR1, SPAK and fluorescence-labeled secondary antibodies. Images were obtained with a Zeiss LSM 510 confocal microscope.

4. Measurements of $[Cl^-]_i$ and Cl^-/HCO_3^- exchange activity.

The standard HCO_3^--buffered solution contained (in mM) 120 NaCl, 5

KCl, 5 HEPES, 1 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose, and 25 Na HCO_3^- (pH 7.4 with NaOH). Cl^--free solutions were prepared by replacing the Cl^- with gluconate. The high HCO_3^--containing solutions were prepared by replacing Cl^- with equimolar HCO_3^-. All HCO_3^--buffered solutions were continuously gassed with 95% O_2 and 5% CO_2 to maintain the pH of each solution. The osmolarity of all solutions was adjusted to 310 mOsm with the major salt prior to use.

Intracellular Cl^- concentrations were measured in the PANC1 and CFPAC1 monolayers using the Cl^--sensitive dye N-[ethoxycarbonylmethyl]-6-methoxy-quinolinium bromide (MQAE) and the double perfusion chamber. Briefly, after achieving confluency, the cells were loaded with MQAE by incubating in the HEPES-buffered solution A containing 10 mM of MQAE for 30 min at room temperature and for 40 min at 0 °C. The HEPES-buffered solution A contained (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose, and 10 HEPES (pH 7.4). A membrane bearing MQAE-loaded cells was mounted in a miniature Ussing chamber (AKI Institute, U. of Copenhagen, Denmark) attached to the stage of an inverted microscope. The membrane was located in between the two-half chambers, which separate the chamber into a luminal (upper) and a basolateral (lower) compartment. A transparent coverslip was placed at the bottom of the perfusion chamber, which allowed fluorescence measurements from the dye-loaded monolayers using objective lenses with a long working distance (greater than 2 mm). Separate luminal and basolateral perfusates were delivered to the chamber after warming (37 °C). Fluorescence emission at 450 nm in response to an excitation wavelength of 350 nm was measured using a recording setup equipped with the photomultiplier tube (Delta Ram; PTI Inc., Birmingham, NJ). At the end of each experiment, a calibration procedure was performed

using standard Cl^--containing solutions with 150 mM K^+, 5 μM nigericin and 10 μM tributyltin cyanide.

The Cl^-/ HCO_3^- exchange activity was measured in transiently transfected HEK 293T cells by recording pH_i in response to $[Cl^-]_o$ changes of the perfusate. In brief, cell-attached glass coverslips were washed once with the HEPES-buffered solution A and assembled to form the bottom of a perfusion chamber. Cells transfected with the gene of interest were targeted by co-transfecting plasmids expressing green fluorescence protein (1/10 of target gene in μg). After targeting, cells were loaded with the fluorescent pH probe BCECF by incubating for 10-min at room temperature in solution A containing 2.5 μM BCECF-AM. After dye loading, the cells were perfused with the standard HCO_3^--buffered solution and BCECF fluorescence was recorded at the excitation wavelengths of 490 and 440 nm at a resolution of 2/s using a recording setup (Delta Ram; PTI Inc.). The 490/440 ratios were calibrated intracellularly by perfusing the cells with solutions containing 145 mM KCl, 10 mM HEPES, and 5 μM nigericin with the pH adjusted to 6.2-7.6. Buffer capacity was calculated by measuring ΔpH_i in response to 5-40 mM NH_4Cl pulses. The total buffer capacity (βt) of HEK 293T cells was not significantly altered by transfection with plasmids used in this study. Therefore, the results of Cl^-/ HCO_3^- exchange activity were expressed as ΔpH/min, and this value was directly analyzed without compensating for βt.

5. Isolation of small intralobular ducts from guinea pig pancreas.

This study was approved by the Committee for the Care and Use of Laboratory Animals, Yonsei University College of Medicine. The guinea pig (180-210 g) was humanely killed by cervical dislocation according to the institutional and national guidelines. The pancreas was rapidly removed and

cut into small pieces in HEPES-buffered Dulbecco's modified Eagle's medium (HEPES-DMEM). Tissue was incubated in digestion solution containing collagenase type II (0.5 mg/ml; Serva, Heidelberg, Germany), bovine serum albumin (1.0 mg/ml; Sigma, St.Louis, MO) and trypsin inhibitor (0.1 mg/ml; Sigma, St.Louis, MO) for 30 min at 37°C. Small intralobular ducts proximal to the acini (≥ third branch from the main duct) were then isolated at room temperature by microdissection using stainless steel needles under a dissecting microscope at x 40 magnification. The isolated ducts were further digested in 50 U/ml elastase (sigma, St.Louis, MO) dissolved in HEPES-DMEM for 50 minutes at 37°C. The ducts were transferred to a microwell and teased apart using stainless steel needles in divalent cation-free buffer in order to dissociate into single cells. The isolated duct cells were kept in McCoy's 5A medium supplemented with 10% fetal bovine serum at 37^{o}C, 5% CO_2 before further experiments.

6. Patch clamp experiments

Whole-cell recordings were performed on CFTR transfected HEK 293T cells and pancreatic duct cells. Briefly, cells were transferred into the bath mounted on a stage with an inverted microscope (IX-70, Olympus, Osaka, Japan). The bath solution was perfused at 10 ml/min. The voltage and current clamp experiments were performed at room temperature (22-25°C). Patch pipettes with a free-tip resistance of about 2.5 MOhm were connected to the head stage of a patch-clamp amplifier (Axopatch-200B, Axon Instruments, Foster City, CA). pCLAMP software v.9.2 and Digidata-1322A (Axon Instruments) were used to acquire data and apply command pulses. Reference electrodes were connected to the bath via 3% (wt/vol) agar bridge containing 3M KCl solution. Liquid junction potentials were corrected with

an offset circuit prior to each experiment. For the HCO_3^- permeability test, individual data was corrected by measuring the offset potential shift induced by the replacement of HCO_3^- solution after each experiment. The conventional whole-cell clamp was achieved by rupturing the patch membrane after forming a gigaseal. Voltage and current traces were stored and analyzed using Clampfit v.10.2 and Origin v. 8.0 (Microcal Inc, Northampton, MA). Currents were sampled at 5 kHz. All data were low-pass filtered at 1 kHz.

The pipette solution contained (in mM) 138 N-methyl D-glucamine chloride (NMDG-Cl), 5 EGTA, 1 $MgCl_2$, 1 Mg-ATP, and 10 HEPES (pH 7.2). The low Cl^--containing pipette solutions were prepared by replacing Cl^- with equimolar glutamate. The bath solution contained 136 NMDG-Cl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Glucose, and 10 HEPES (pH 7.4). The high HCO_3^--containing bath solution was prepared by replacing NMDG-Cl with equimolar choline-HCO_3. The HCO_3^--containing solutions were continuously gassed with 95% O_2 and 5% CO_2. In experiments with guinea pig pancreatic duct cells, total anion concentration of pipette and bath solutions was adjusted to 150 mM by adding 10 mM of major salt (NMDG-Cl or choline- HCO_3).

The whole cell current reversal potential (E_{rev}) was measured either in current clamp mode or in voltage clamp experiments. Resting membrane potential (RMP) was recorded in zero-current clamp mode of the whole cell condition. To measure the current to voltage relationship (I-V curve) during zero-current clamp recording, clamp mode was shifted to the voltage clamp mode and the I-V curve was obtained by depolarizing ramp pulses from −100 to 100 mV (0.5 V/ms, inter-pulse holding potential; near the RMP). In all experiments, the currents generated by CFTR were confirmed by

following 3 characteristics: 1) activation of current by the treatment with cAMP (5 μM forskolin and 100 μM 3-isobutyl-1-methylxanthine [IBMX]), 2) a linear I-V relationship, and 3) inhibition of current by the treatment with CFTR inhibitor CFTRinh-172 (10 μM). The reversal potential (E_{rev}) was used to calculate the permeability ratio of HCO_3^- relative to that of Cl^- ($PHCO_3/PCl$) according to the Goldman-Hodgkin- Katz equation: $\Delta E_{rev} = Er(HCO_3^-) - Er(Cl^-) = (RT / zF) \ln [(PHCO_3 [HCO_3^-]_o + PCl [Cl^-]_o) / (PCl [Cl^-]_o)]$, where $[X]_i$ and $[X]_o$ represent intracellular and extracellular anion concentrations respectively, and R, T and F have their usual thermodynamic meanings.

7. Statistical Analysis

Data are presented as the means ± SEM. Statistical analysis was performed with Student's *t*-tests or with analysis of variance followed by Tukey's multiple comparison test, as appropriate. $P<0.05$ was considered statistically significant.

III. RESULTS

1. Decreased $[Cl^-]_i$ in pancreatic duct cells by CFTR activation

$[Cl^-]_i$ of pancreatic duct cells is one of the key parameters in the determination of luminal HCO_3^- concentration achieved by the apical HCO_3^- transporting proteins (Figure 1). Therefore, $[Cl^-]_i$ was measured in the stimulated duct cells. PANC1 human pancreatic duct cells expressing wild-type (WT) CFTR were cultured on a permeable support to form monolayers and $[Cl^-]_i$ was measured using a double perfusion chamber with separate luminal and basolateral perfusions (Figure 2A). When Cl^- in the luminal perfusate was replaced with HCO_3^- to mimic pancreatic secretion along the ductal tree, $[Cl^-]_i$ decreased. However, this was not reproduced when HCO_3^- was replaced on the basolateral side, indicating that functional Cl^- channels are expressed only at the apical membrane. It is well documented that cAMP signaling is the major pathway responsible for activating both CFTR and pancreatic secretion.[1,11] Interestingly, cAMP stimulation greatly enhanced the reduction in $[Cl^-]_i$ in response to luminal Cl^- depletion. In contrast, the cAMP effect was not observed when the same protocol was repeated in CFPAC1 cells expressing ΔF508 mutant CFTR (Figure 2B). Furthermore, $[Cl^-]_i$ reduction in the PANC1 cells in response to luminal Cl^- depletion and cAMP stimulation was completely blocked by CFTR inhibitor, CFTRinh-172. These results suggest that CFTR activation leads to a marked reduction in $[Cl^-]_i$ down to approximately 5 mM in duct cells during stimulated HCO_3^- secretion.

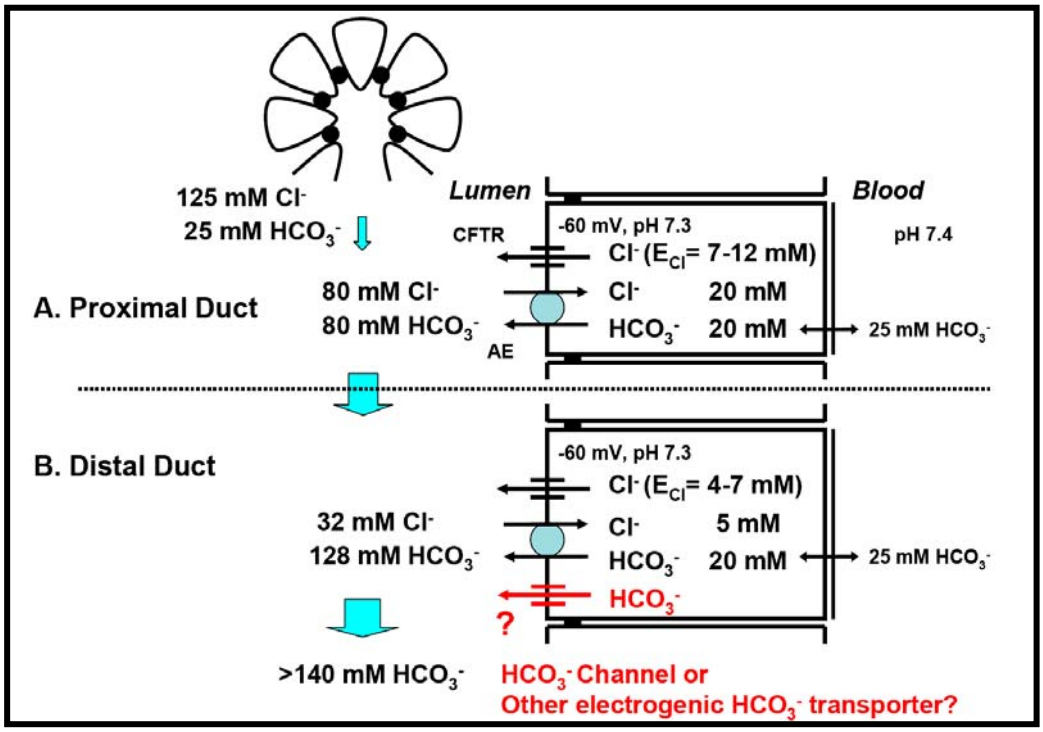

Figure 1. Possible mechanisms of HCO_3^- secretion in pancreatic duct cells. (A) An electroneutral Cl^-/HCO_3^- exchanger can secrete up to 80-mM of HCO_3^- when $[Cl^-]_i$ and $[HCO_3^-]_i$ are around same levels. (B) When $[Cl^-]_i$ decreases to a 5-mM level, the apical Cl^-/HCO_3^- exchanger can secrete HCO_3^- up to a 128-mM concentration. A HCO_3^- channel or other electrogenic mechanisms would be one of the strong candidates for the transporter responsible for high HCO_3^- secretion at the apical membrane of pancreatic duct cells. A HCO_3^- channel can secrete HCO_3^- up to a 200-mM concentration when cells maintain a -60mV membrane potential according to the Nernst equation.

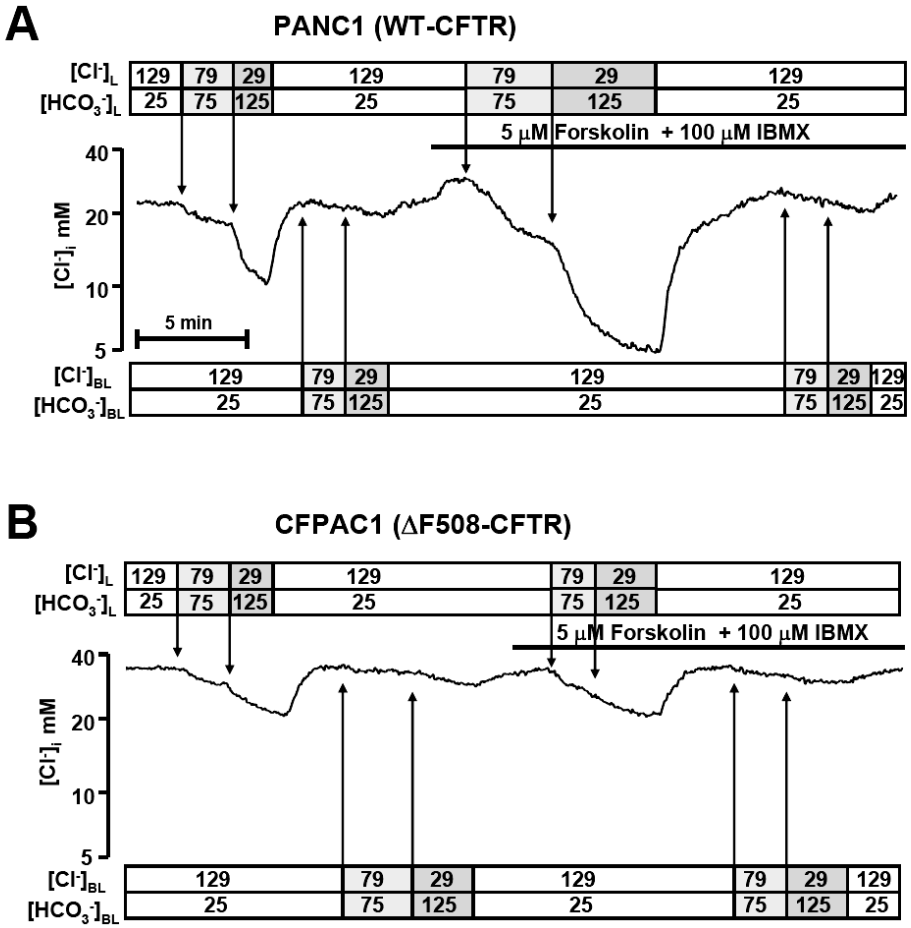

Figure 2. Measurements of $[Cl^-]_i$ in the monolayers of human pancreatic duct cells. (A) The PANC1 human pancreatic duct cells expressing WT-CFTR and (B) CFPAC1 cells bearing the ΔF508 mutant CFTR were cultured on permeable supports to form monolayers. The double perfusion chamber with separate luminal (L) and basolateral (BL) perfusions was used to measure $[Cl^-]_i$ in MQAE loaded cells. Activation of CFTR by cAMP stimulation (5 μM forskolin and 100 μM IBMX) augmented the luminal Cl^- permeability in PANC1 cells (A) but not in CFPAC1 cells (B).

2. WNK1, OSR1, SPAK expressed in human pancreas and their interaction with CFTR.

Since $[Cl^-]_i$ reduction induced via CFTR may possibly evoke pancreatic HCO_3^- secretion, the expression of WNK1, OSR1, and SPAK that are known to be activated by a decrease in $[Cl^-]_i$[18-19] was initially investigated in the human pancreas. Interestingly, immunoblotting and immunostaining in human pancreatic tissues revealed that WNK1, OSR1, and SPAK were abundantly expressed in the CFTR-positive pancreatic duct cells. In addition, these kinases highly co-localized with CFTR near the apical membrane of duct cells (Figure 3). To further identify the possibility of CFTR regulation by WNK and STE20-like kinases, immunoprecipitation between CFTR and these kinases was performed in HEK 293T cells. As shown in Figure 4, WNK1, OSR1, and SPAK were found to associate with CFTR in co-immunoprecipitation experiments. Collectively, these results strongly suggest that WNK1, OSR1, and SPAK may interact with and regulate CFTR in pancreatic duct cells.

3. $[Cl^-]_i$-sensitive activation of OSR1/SPAK mediated via WNK1 and CFTR.

A key step in OSR1 and SPAK activation by WNK1 is the phosphorylation at the T-loop, which can be detected by phospho-specific antibodies.[18] When HEK 293T cells were incubated in Cl^--free solutions for 40 min, phosphorylation of OSR1 and SPAK was greatly increased (Figures 5A and 5B). Application of hypotonic stress (165 mOsm) in addition to reduced extracellular Cl^- further activated OSR1 and SPAK as previously described.[22] Of interest, high HCO_3^- (> 125 mM) was also a potent activator of OSR1 and SPAK and was as effective as hypotonic stress at inducing T-

loop phosphorylation. Therefore, the high HCO_3^-/low Cl^--containing solutions was utilized to effectively activate OSR1 and SPAK in further experiments. It also has an advantage of mimicking conditions of the distal pancreatic juice. The importance of WNK1 in the low Cl^--induced phosphorylation of OSR1 and SPAK was verified by both gain-of-function and loss-of-function experiments. The overexpression of exogenous WNK1 enhanced OSR1 and SPAK phosphorylation, whereas knock-down of endogenous WNK1 greatly diminished the low Cl^- solution-induced phosphorylation (Figures 5C and 5D).

Next, the effects of CFTR on OSR1 and SPAK phosphorylation were examined. As shown in Figures 6A and 6B, CFTR activation greatly facilitated the low Cl^--induced phosphorylation of OSR1 and SPAK in HEK 293T cells, possibly due to a rapid decrease in $[Cl^-]_i$. In control cells, incubation with the low Cl^--containing solution for 40 min was required for phosphorylation. However, when CFTR was co-expressed and activated by cAMP, OSR1 and SPAK began to phosphorylate within 5 min of exposure to low Cl^- (Figures 6A and 6B). The low Cl^--induced activation of both kinases was rapidly reversed by replacing the solution with a standard 129-mM Cl^--containing solution (Figure 6C). Finally, the effect of CFTR on OSR1 and SPAK phosphorylation was investigated in human pancreatic duct cells. In accordance with the results in HEK 293T cells, incubation with low Cl^--containing solution induced endogenous OSR1 and SPAK phosphorylation in PANC1 cells expressing WT-CFTR but not in CFPAC1 cells expressing ΔF508-CFTR (Figure 6D). These results imply that OSR1 and SPAK are sensitive to changes in pancreatic fluid composition and can be activated during stimulated secretion in distal pancreatic ducts as luminal Cl^- decreases and HCO_3^- increases.

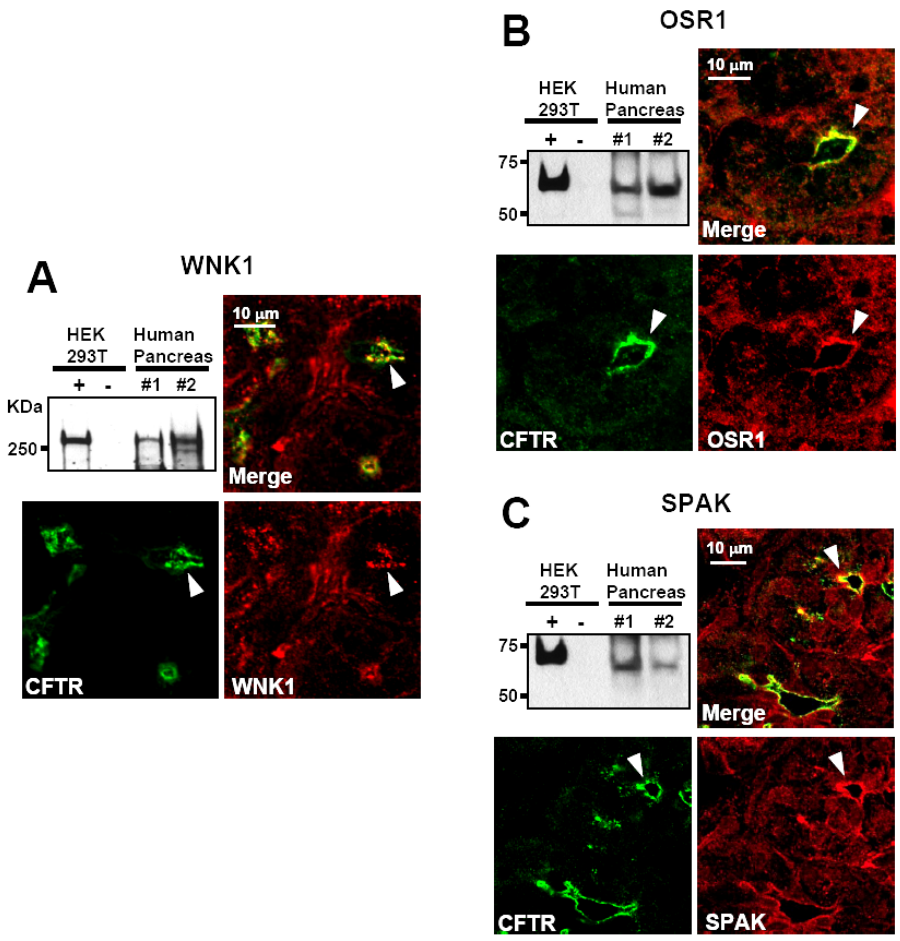

Figure 3. Expression of $[Cl^-]_i$-sensitive kinases in human pancreas. Expression of (A) WNK1, (B) OSR1, and (C) SPAK in human pancreatic tissues was analyzed by immunoblotting and immunohistochemistry. Protein samples from HEK 293T cells transfected with plasmids expressing Myc-WNK1, Flag-OSR1, or Flag-SPAK were used as positive (+) controls and those from mock-transfected cells were used as negative (-) controls for immunoblotting. In immunohistochemistry, CFTR-positive (green) pancreatic duct cells highly co-expressed WNK1, OSR1, and SPAK (red).

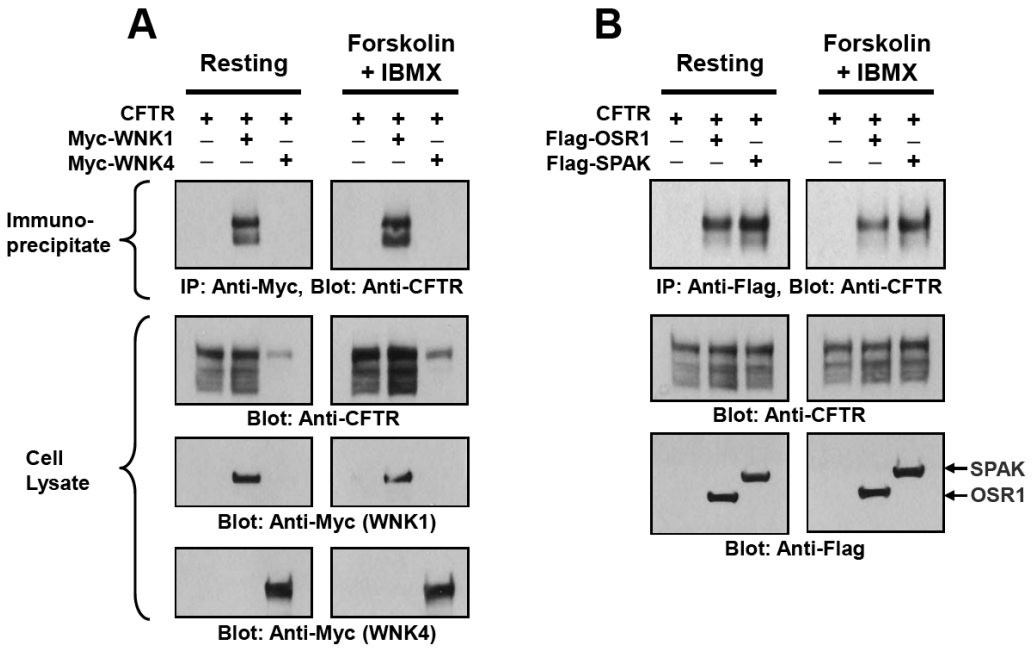

Figure 4. CFTR associates with WNK1-OSR1/SPAK kinases in a protein complex. Co-immunoprecipitation (IP) was performed in HEK 293T cells transiently transfected with plasmids expressing CFTR, Myc-WNK1 (A), Myc-WNK4 (A), Flag-OSR1 (B), and/or Flag-SPAK (B). In each right-hand panel of A and B, IPs were performed in cells incubated with 5 μM forskolin and 100 μM IBMX.

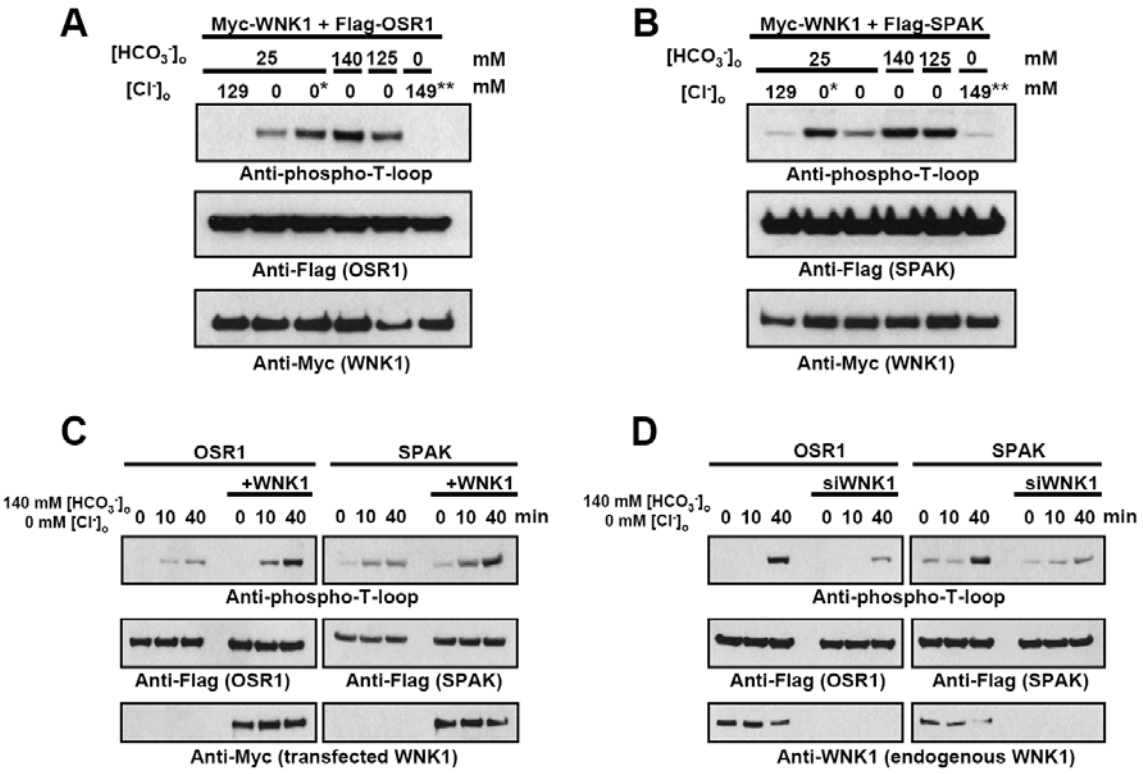

Figure 5. $[Cl^-]_i$-sensitive activation of OSR1/SPAK and the role of WNK1. The activation of OSR1 and SPAK was analyzed using antibodies against the phospho-T-loop. In panels A and B, HEK 293T cells transfected with indicated plasmids were incubated in solutions containing various concentrations of Cl^- and HCO_3^- for 40 min, and protein samples were collected. In some experiments, cells were incubated with hypotonic solution* (165 mOsm by reducing Na-gluconate concentrations) or bicarbonate-free, HEPES-buffered solution**. In panels C and D, the role of WNK1 in the $[Cl^-]_i$-sensitive phosphorylation of OSR1 and SPAK was analyzed. HEK 293T cells transfected with indicated plasmids were incubated with high HCO_3^-/low Cl^--containing solution for the indicated time. A batch of cells was transfected with the Myc-WNK1 plasmid (C) or hWNK1 siRNAs (D).

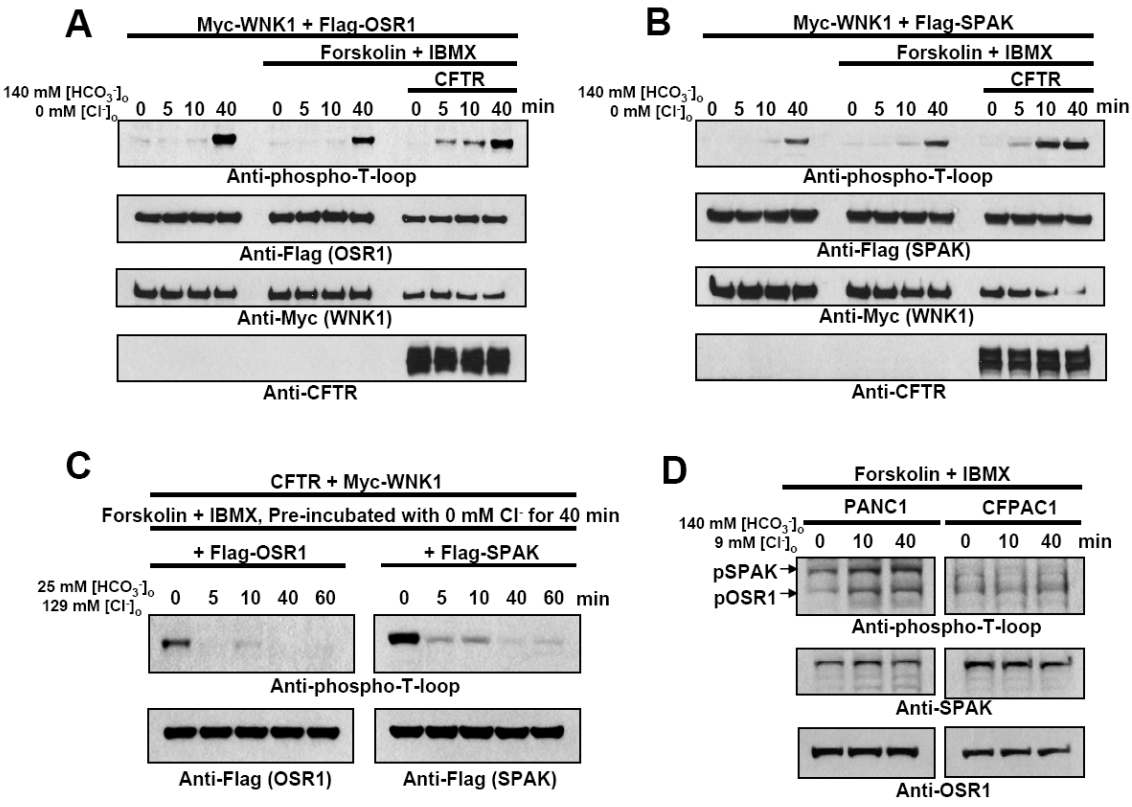

Figure 6. CFTR facilitates the $[Cl^-]_i$-sensitive activation of OSR1/SPAK. In panels A and B, HEK 293T cells were incubated in low Cl^- (0 mM)-containing solution for 40 min. Co-transfection with the plasmid expressing CFTR and cAMP stimulation induces the phosphorylation of OSR1 and SPAK in shorter time points. In panel C, HEK 293T cells expressing CFTR and indicated kinases were pre-incubated in the low Cl^- (0 mM)-containing solution for 40 min with cAMP stimulation, and then the solution was replaced to a standard 129-mM Cl^--containing solution for indicated time. In panel D, PANC1 cells expressing WT-CFTR and CFPAC1 cells bearing the ΔF508 mutant CFTR were incubated in low Cl^- (9 mM)-containing solution for up to 40 min with cAMP stimulation. Low Cl^--containing solution induces phosphorylation of OSR1 and SPAK in PANC1 cells but not in CFPAC1 cells.

4. Increase in CFTR HCO_3^- permeability via $[Cl^-]_i$-sensitive kinases.

To test the effect of OSR1 and SPAK activated by low $[Cl^-]_i$ on the HCO_3^--secreting transporters in the pancreatic duct cells, the effect of low $[Cl^-]_i$ on CFTR was measured, especially on CFTR HCO_3^--permeability. The activity of a hypothetical HCO_3^- channel has been considered one of the strong candidate mechanisms responsible for high HCO_3^- secretion into the lumen of pancreatic ducts.[2,24-25] Therefore, CFTR anion selectivity was assessed by replacing 140 mM extracellular Cl^- with 130 mM HCO_3^- and 10 mM Cl^- (Figure 7, A-D). The HCO_3^-/Cl^- permeability ratio (P_{HCO3}/P_{Cl}) was determined from the reversal potential (ΔE_{rev}) using the Goldman-Hodgkin-Katz equation. In all experiments, the currents generated by CFTR were confirmed by the following three characteristics: 1) activation of current by the treatment with cAMP (5 μM forskolin and 100 μM IBMX), 2) a linear I-V relationship, and 3) inhibition of current by the treatment with the CFTR inhibitor, CFTRinh-172.

In HEK 293T cells transfected with the CFTR plasmid only, replacing the bath solution with a 130 mM HCO_3^--containing solution induced a 24.5 ± 2.8 mV positive shift in ΔE_{rev} when the pipette contained a high concentration of Cl^- (140 mM), indicating that P_{HCO3}/P_{Cl} was 0.39 ± 0.05 (Figure 7A, left). The same protocol was repeated with a low Cl^--containing pipette solution. The Erev measurement with the 10 mM Cl^--containing pipette solution again showed a positive shift of ΔE_{rev} (18.4 ± 3.5 mV) by the high HCO_3^--containing bath solution, although HCO_3^- permeability was somewhat increased (P_{HCO3}/P_{Cl} = 0.53 ± 0.08, Figure 7A, right). Next, the above experiments were repeated in cells co-transfected with plasmids expressing WNK1 and SPAK. In the experiment with the 140 mM Cl^--containing pipette, replacing the bath solution with 130 mM HCO_3^- induced a small

increase in HCO_3^- permeability (ΔE_{rev} = 14.7 ± 2.0 mV, P_{HCO3}/P_{Cl} = 0.59 ± 0.07) compared with that of CFTR-only expressing cells (Figure 7B, left). Most surprisingly, WNK1 and SPAK activation by the low Cl^--containing pipette solution induced a remarkable increase in the CFTR HCO_3^- permeability. With 10 mM Cl^- in the pipette, replacing the bath to 130 mM HCO_3^- evoked a 7.6 ± 1.8 mV negative shift of ΔE_{rev}, indicating that HCO_3^- permeability is greater than that of Cl^- (P_{HCO3}/P_{Cl} = 1.50 ± 0.11, Figure 7B, right). Activation of OSR1 also increased CFTR HCO_3^- permeability when the same protocol was applied to cells expressing WNK1 and OSR1 (P_{HCO3}/P_{Cl} = 1.30 ± 0.10, n = 9, data not shown).

The increase of CFTR HCO_3^- permeability can be more clearly seen in the resting membrane potential (RMP) measurements (Figures 7C and 7D). In the CFTR-only expressing cells with 10 mM Cl^- in the pipette, replacing the bath with high HCO_3^- evoked a depolarization suggesting a decrease in anion influx (Figure 7C). In contrast, in the WNK1 and SPAK co-expressing cells, replacing the bath solution with 130 mM HCO_3^- evoked a 10-mV hyperpolarization, indicating an increase in anion influx (Figure 7D). These HCO_3^--mediated anion currents were inhibited by treatment with CFTRinh-172. All the above data imply that CFTR functions primarily as a HCO_3^- channel when WNK1 and SPAK are activated by low $[Cl^-]_i$.

5. Regulation of CFTR HCO_3^- permeability in physiologically relevant conditions

Most importantly, the low $[Cl^-]_i$-induced increase in CFTR HCO_3^- permeability was explored in guinea pig pancreatic duct cells. The ductal secretion of the guinea pig pancreas resembles that of humans which can accumulate over 140 mM HCO_3^- in pancreatic juice.[1] Single pancreatic duct

cells were freshly isolated from small intralobular and interlobular ducts that are the major sites of HCO_3^- secretion,[1] and were subjected to whole cell current measurement. In guinea pig pancreatic duct cells, cAMP stimulation evoked a CFTRinh-172-sensitive Cl^- current that mainly exhibits a linear I-V relationship (Figure 8). Importantly, decreasing $[Cl^-]_i$ greatly increased CFTR HCO_3^- permeability in guinea pig pancreatic duct cells. Parallel with the results from 293T cells, replacing the bath solution with a high HCO_3^--containing (146 mM) solution induced a depolarization (or a positive shift of the reversal potential) when the pipette contained high Cl^-, whereas the same high bath HCO_3^- induced a hyperpolarization (or negative shift of the reversal potential) when the pipette contained low Cl^- (Figures 8A-8D). On average, decreasing Cl^- from 150 mM to 4 mM in the pipette elevated P_{HCO3}/P_{Cl} from 0.24 ± 0.13 to 1.09 ± 0.29 (Figure 8E).

A similar phenomenon was also observed in PANC1 cells that endogenously express WT-CFTR, WNK1, and the two STE20-like kinases (Figure 9). Decreasing Cl- from 140 mM to 4 mM in the pipette boosted P_{HCO3}/P_{Cl} from 0.69 ± 0.03 to 2.42 ± 0.30 in PANC1 cells. Interestingly, this low $[Cl^-]_i$-induced increase in P_{HCO3}/P_{Cl} was abolished by transfection of siRNA directed against WNK1, indicating that WNK1 plays an essential role in this process.

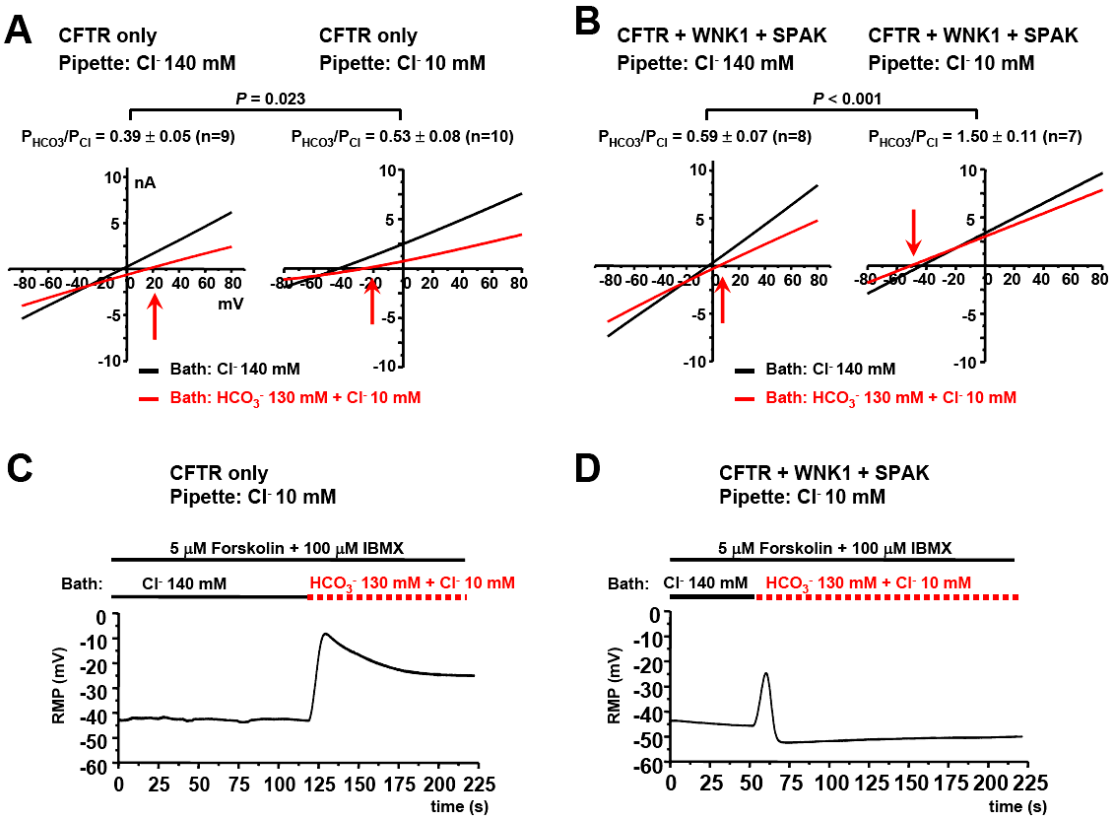

Figure 7. WNK1 and SPAK activation increases CFTR HCO_3^- permeability. Whole-cell recordings were performed to measure CFTR HCO_3^--permeability by replacing the bath solution with high HCO_3^--containing (130 mM) solution. The current to voltage relationship (I/V curve) was obtained by depolarizing ramp pulses from −100 to 100 mV. In panels A and B, the reversal potential (E_{rev}) was measured in HEK 293T cells transfected with plasmids expressing CFTR only (A), or co-transfected with plasmids expressing CFTR, WNK1, and SPAK (B) using pipettes containing two different concentrations of Cl^- (140 mM and 10 mM). In panels C and D, the resting membrane potential (RMP) was measured in HEK 293T cells transfected with plasmids expressing CFTR only (C), or co-transfected with plasmids expressing CFTR, WNK1, and SPAK (D) using pipettes containing 10 mM Cl^-.

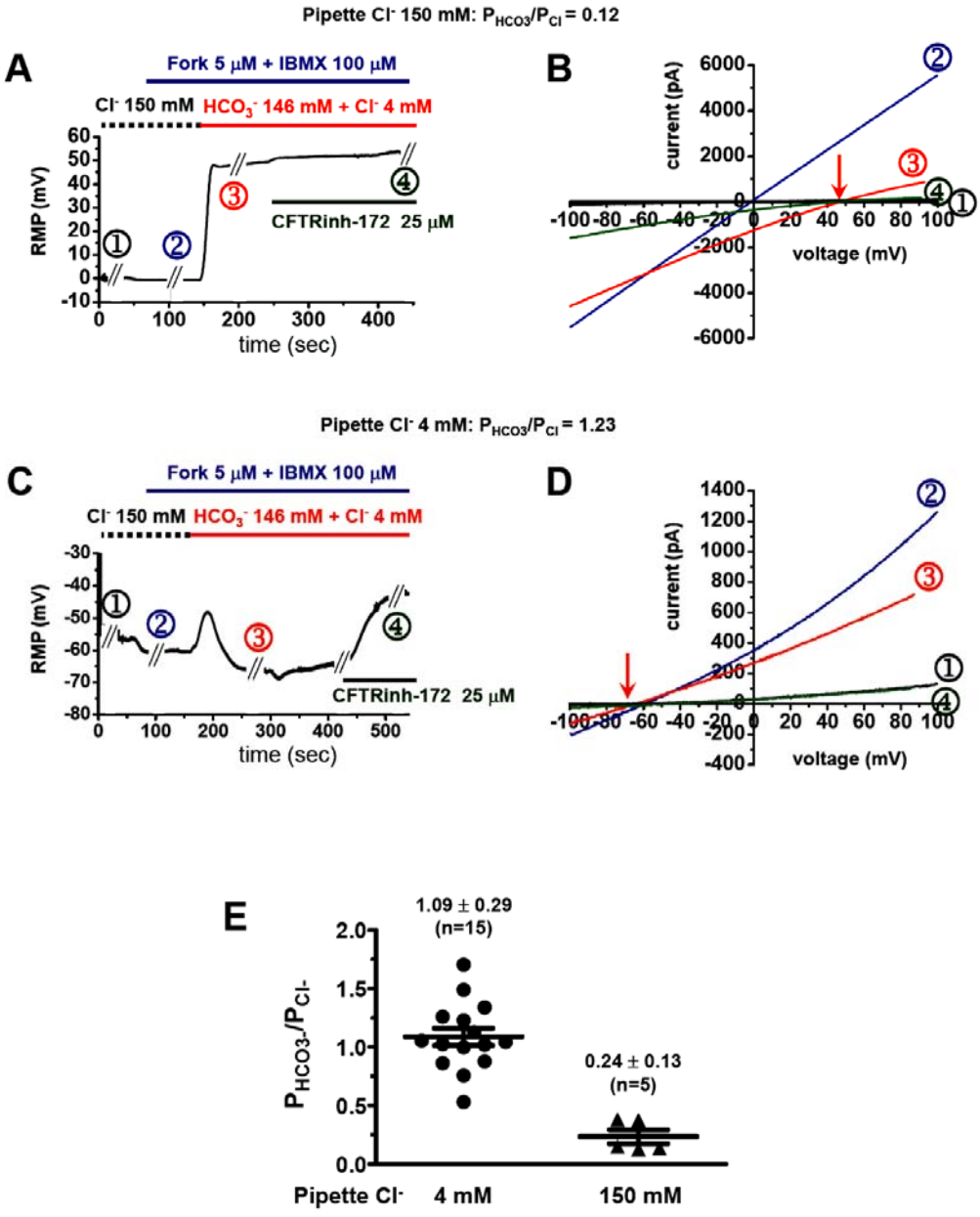
Pipette Cl- 150 mM: PHCO3/PCl = 0.12
A
Fork 5 μM + IBMX 100 μM
Cl- 150 mM HCO3- 146 mM + Cl- 4 mM
CFTRinh-172 25 μM
RMP (mV)
time (sec)
B
current (pA)
voltage (mV)
Pipette Cl- 4 mM: PHCO3/PCl = 1.23
C
Fork 5 μM + IBMX 100 μM
Cl- 150 mM HCO3- 146 mM + Cl- 4 mM
CFTRinh-172 25 μM
RMP (mV)
time (sec)
D
current (pA)
voltage (mV)
E
1.09 ± 0.29
(n=15)
0.24 ± 0.13
(n=5)
PHCO3-/PCl-
Pipette Cl- 4 mM 150 mM

Figure 8. Low $[Cl^-]_i$ increases CFTR HCO_3^- permeability in guinea pig pancreatic duct cells. Single pancreatic duct cells freshly isolated from small intralobular and interlobular ducts were subjected to whole cell recordings. During RMP measurement with zero-current clamp mode, I/V curve was obtained by applying ramp pulses from −100 to 100 mV after switching to the voltage clamp mode. Representative traces of RMP measurements using 150 mM and 4 mM Cl^--containing pipette solutions are shown in panels A and C, respectively, and resulting I/V curves are shown in panels B and D. From the basal current in guinea pig pancreatic duct cells (1), cAMP stimulation evoked an anion current that mainly exhibits a linear I/V relationship (2), is permeable to HCO_3^- (3), and is inhibited by CFTRinh-172 (4). Summarized results of P_{HCO3}/P_{Cl} are presented in panel E.

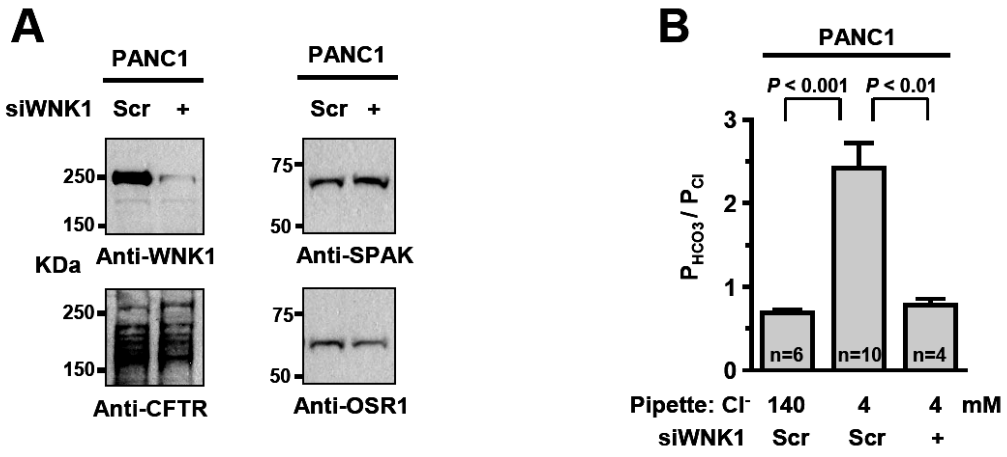

Figure 9. Low $[Cl^-]_i$ increases CFTR HCO_3^- permeability in PANC1 cells. (A) Endogenous CFTR (WT), WNK1, SPAK, and OSR1 in PANC1 cells were treated with siRNAs directed against WNK1. (B) Whole-cell recordings were performed to measure CFTR HCO_3^--permeability. Decreasing pipette Cl^- from 140 mM to 4 mM increased P_{HCO3}/P_{Cl} from 0.69 ± 0.03 to 2.42 ± 0.30. Importantly, this low $[Cl^-]_i$-induced increase in P_{HCO3}/P_{Cl} was abolished by the transfection with siRNA directed against WNK1 which indicates that WNK1 is essential in this process.

6. Inhibition of Cl^-/HCO_3^- exchange activities by the $[Cl^-]_i$-sensitive kinases.

The effects of OSR1 and SPAK activation on the CFTR-dependent Cl^-/HCO_3^- exchange were examined in HEK 293T cells. To activate OSR1 and SPAK, cells were pre-incubated with a Cl^--free solution for 40 min before measuring the $Cl^-_o/HCO_3^-_i$ exchange activity (Figure 11). As reported previously,[12-13] stimulation of CFTR with cAMP evoked a strong Cl^-/HCO_3^- exchange activity (Figure 11A). However, co-expression with OSR1 or SPAK greatly inhibited the CFTR-dependent Cl^-/ HCO_3^- exchange activity (Figures 11A and 11B). It has been previously suggested that activation of CFTR-dependent Cl^-/HCO_3^- exchange is mediated by interaction of CFTR and SLC26 transporters (Figure 10).[15,21] In accordance with this notion, activities of SLC26A3 (Figures 11C and 11D) and Slc26a6 (Figures 11E and 11F), two candidate transporters for the CFTR-dependent Cl^-/HCO_3^- exchanger in pancreatic duct cells,[15,21] were also inhibited by OSR1 and SPAK activation.

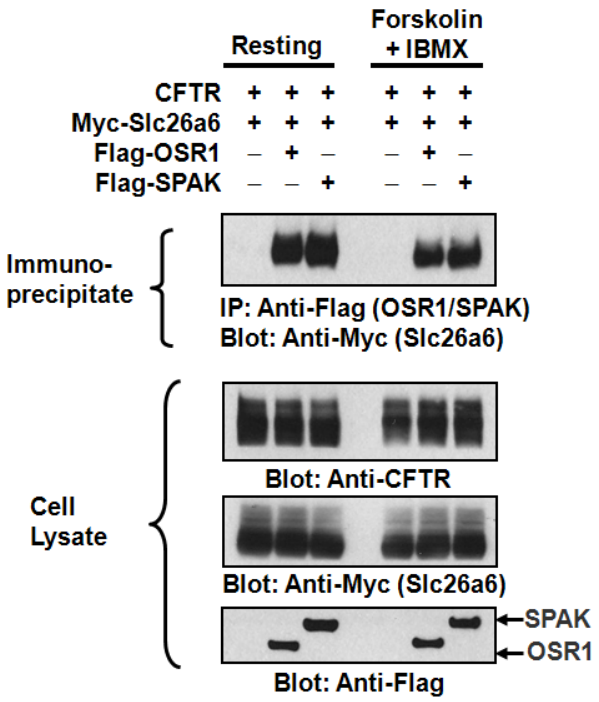

Figure 10. Association of CFTR/Slc26a6 with OSR1/SPAK kinases. HEK 293T cells were transiently transfected with plasmids for CFTR, Myc-Slc26a6, Flag-OSR1 and/or Flag-SPAK. OSR1 and SPAK associated with Slc26a6 in a protein complex. The interaction also took place when CFTR was stimulated with cAMP (5 mM Forskolin and 100 mM IBMX).

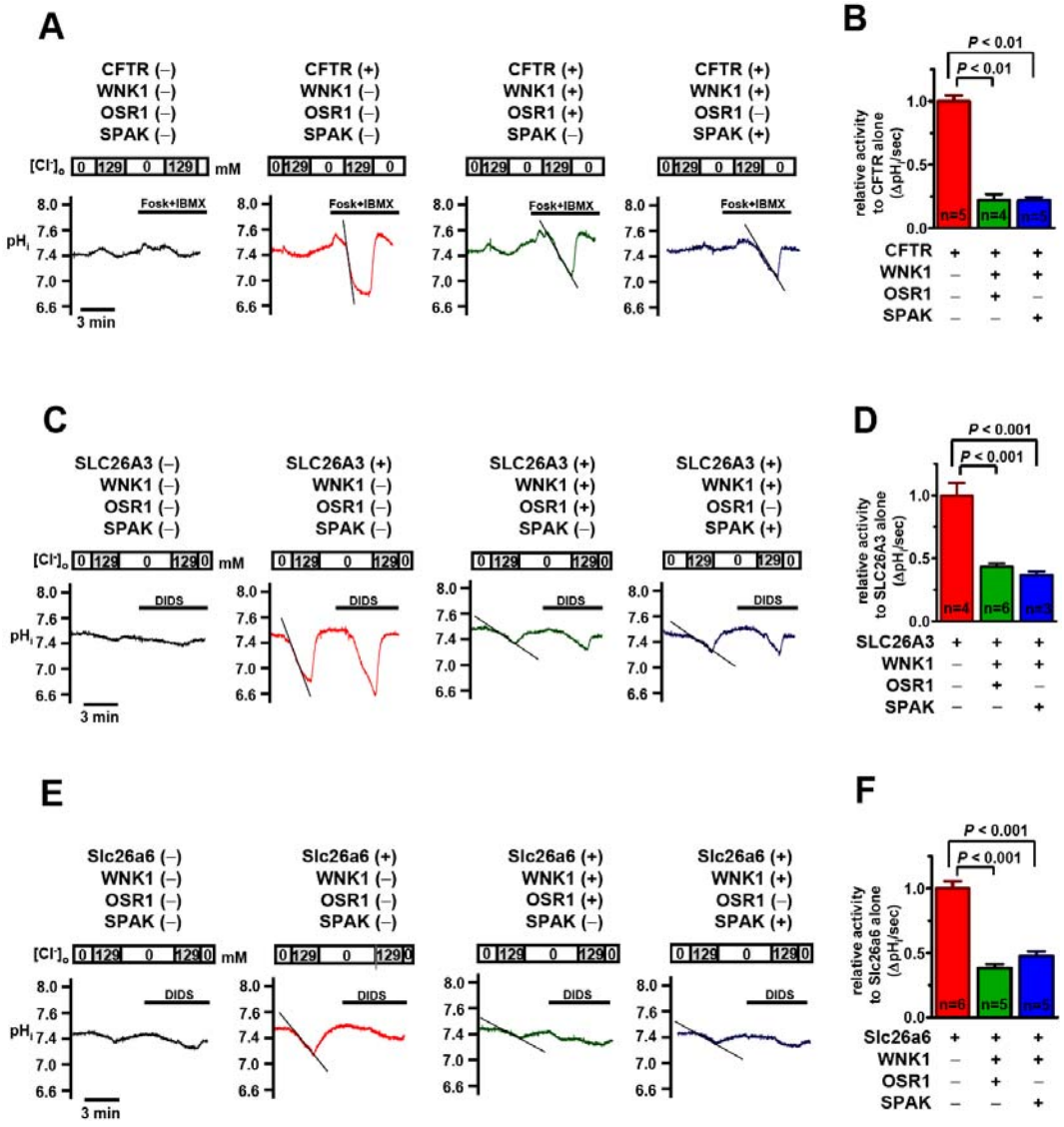
A
CFTR (–)
WNK1 (–)
OSR1 (–)
SPAK (–)
CFTR (+)
WNK1 (–)
OSR1 (–)
SPAK (–)
CFTR (+)
WNK1 (+)
OSR1 (+)
SPAK (–)
CFTR (+)
WNK1 (+)
OSR1 (–)
SPAK (+)
[Cl-]o 0 129 0 129 mM
Fosk+IBMX
pHi
3 min
B
P < 0.01
P < 0.01
relative activity to CFTR alone (ΔpHi/sec)
n=5
n=4
CFTR + + +
WNK1 – + +
OSR1 – + –
SPAK – – +
C
SLC26A3 (–)
WNK1 (–)
OSR1 (–)
SPAK (–)
SLC26A3 (+)
WNK1 (–)
OSR1 (–)
SPAK (–)
SLC26A3 (+)
WNK1 (+)
OSR1 (+)
SPAK (–)
SLC26A3 (+)
WNK1 (+)
OSR1 (–)
SPAK (+)
DIDS
D
P < 0.001
P < 0.001
relative activity to SLC26A3 alone (ΔpHi/sec)
n=4
n=6
SLC26A3 + + +
WNK1 – + +
OSR1 – + –
SPAK – – +
E
Slc26a6 (–)
WNK1 (–)
OSR1 (–)
SPAK (–)
Slc26a6 (+)
WNK1 (–)
OSR1 (–)
SPAK (–)
Slc26a6 (+)
WNK1 (+)
OSR1 (+)
SPAK (–)
Slc26a6 (+)
WNK1 (+)
OSR1 (–)
SPAK (+)
F
P < 0.001
P < 0.001
relative activity to Slc26a6 alone (ΔpHi/sec)
n=6
n=5
Slc26a6 + + +
WNK1 – + +
OSR1 – + –
SPAK – – +

Figure 11. Inhibition of Cl^-/HCO_3^- exchange activities by the $[Cl^-]_i$-sensitive kinases. HEK 293T cells transfected with indicated plasmids were preincubated in Cl^--free solution for 40 min to activate OSR1 and SPAK kinases. All solutions contained 25 mM HCO_3^- and were continuously gassed with 5% CO2. A and B, CFTR-dependent Cl^-/HCO_3^- exchange activities. Traces from cells transfected with indicated plasmids are presented in each panel. Summarized results are illustrated in panel B. Cells were stimulated with forskolin (Fosk, 5 μM) and IBMX (100 μM) to activate CFTR. C-F, SLC26A3- and Slc26a6-mediated Cl^-/ HCO_3^- exchange activities. Traces from cells transfected with indicated plasmids are presented in each panel. Summarized results are illustrated in panel D and F. Exposure to high Cl^--containing solution evoked $Cl^-_o/HCO_3^-_i$ exchange activity.

IV. DISCUSSION

The present study demonstrates that $[Cl^-]_i$-sensitive mechanisms play a pivotal role in the pancreatic HCO_3^- secretion by promoting a dynamic increase in the HCO_3^- permeability of CFTR anion channel and by simultaneously inhibiting the CFTR-dependent Cl^-/HCO_3^- exchange activity. A model for pancreatic HCO_3^- secretion based on the findings in this study is illustrated in Figure 12. In response to a meal, secretin hormone and vasoactive intestinal polypeptide released from the vagal nerve endings stimulate pancreatic duct cells and generate cAMP signals.[1] This initially activates HCO_3^- secretion mediated by the CFTR-dependent Cl^-/HCO_3^- exchange. The continuous increase in luminal HCO_3^-, and consequent reduction in luminal Cl^-, causes a decrease in $[Cl^-]_i$ via the apical CFTR Cl^- channel activity. The low $[Cl^-]_i$ in turn activates WNK1 and the downstream STE20-like kinases. Activation of OSR1 and SPAK exerts two critical effects on the apical ion transporting proteins. First, activation of OSR1 and SPAK generated an electrogenic pathway for HCO_3^- secretion via increasing the HCO_3^- permeability of CFTR, which is essential for the secretion of pancreatic juice containing HCO_3^- at a concentration greater than 140 mM. Second, OSR1 and SPAK activation inhibits apical Cl^-/HCO_3^- exchange activity, which may reabsorb HCO_3^- from the high HCO_3^--containing pancreatic juice.

Since it was proposed two decades ago that Cl^-/HCO_3^- exchange would be responsible for mediating HCO_3^- secretion at the apical membrane,[1-2] substantial effort has been devoted to identify the molecular nature and role of anion exchangers expressed in pancreatic duct cells. Despite a general consensus that the expression of CFTR is required for HCO_3^- secretion, its role was considered to mainly provide Cl^- for anion exchangers because

CFTR itself has a low capacity to transport HCO_3^-. Hence, SLC26A3 and SLC26A6 anion exchangers found in ductal cells, which are activated by CFTR, have been the focus as the key molecules to mediate pancreatic HCO_3^- secretion.[15,21] Although this mechanism is sufficient to account for the secretion of HCO_3^- up to ~80-100 mM, no theory has yet fully explained how human pancreatic ducts can accumulate over 140 mM HCO_3^- in pancreatic juice.

Electrogenic transporters including HCO_3^- channels are strong candidates for the transporter responsible for high HCO_3^- secretion.[1-2] Having a HCO_3^- selective channel at the apical membrane of a duct cell makes it theoretically possible to secrete up to 200 mM HCO_3^- if cells maintain a membrane potential of -60 mV (Figure 1). It has been previously suggested that CFTR may function as a HCO_3^- channel in pancreatic duct cells under some specific conditions.[17,25-28] However, P_{HCO3}/P_{Cl} of CFTR has been reported as 0.2-0.5 in pancreatic duct cells.[26,29] With this permeability ratio, the CFTR anion channel would secrete Cl^- much faster than HCO_3^-, thus CFTR would be unable to secrete sufficient HCO_3^- to account for high HCO_3^- levels. To date, nobody has reported a P_{HCO3}/P_{Cl} of CFTR greater than 1 in mammalian cells, nor have they provided a specific explanation for the modulation of CFTR HCO_3^- permeability. Present study demonstrates for the first time that CFTR can be more permeable to HCO_3^- than Cl^- in physiologically relevant conditions.

The inhibition of CFTR-dependent Cl^-/HCO_3^- exchange activities by $[Cl^-]_i$-sensitive kinases was a rather unexpected finding. Initially it was suspected that OSR1 and SPAK might activate the apical Cl^-/HCO_3^- exchange to promote HCO_3^- secretion. However, when the luminal HCO_3^- concentration is greater than 140 mM, continuous activation of apical Cl^-

/HCO_3^- exchange would actually absorb HCO_3^- from the lumen. Therefore, inhibition of the apical Cl^-/HCO_3^- exchange is required to prevent the reverse mode of Cl^-/HCO_3^- exchange activity and ultimately achieve HCO_3^- concentration above 140 mM in pancreatic juice. Interestingly, these findings correlate well with the previous mathematical models for high pancreatic HCO_3^- secretion.[2,29]

Recent evidence has revealed that defects in HCO_3^- secretion are important pathophysiologic mechanisms of diseases in many epithelia.[3-4] The major pathological finding observed in the patients with CF is the abnormally thick mucus that plugs airways, blocks gland ducts, and obstructs the lumina of hollow organs.[3] Bicarbonate is an important ingredient to maintain appropriate viscosity of mucin molecules by increasing their solubilization, hydration, and expansion in epithelial fluids.[1,3] It is important to mention that many conditions in the respiratory tract and intestinal lumen can induce hypo- or hyper-osmotic stress which will subsequently activate the WNK1 and STE20-like kinases. Therefore, HCO_3^- secretion regulated by the WNK1-OSR1/SPAK pathway and mediated by CFTR may play a role in epithelial defense against various noxious stimuli in the gastrointestinal, genitourinary, and respiratory systems. In addition, the fact that CFTR functions not only as a Cl^- channel but also a HCO_3^- channel will greatly influence the arena of CFTR research and drug development toward CF patients.

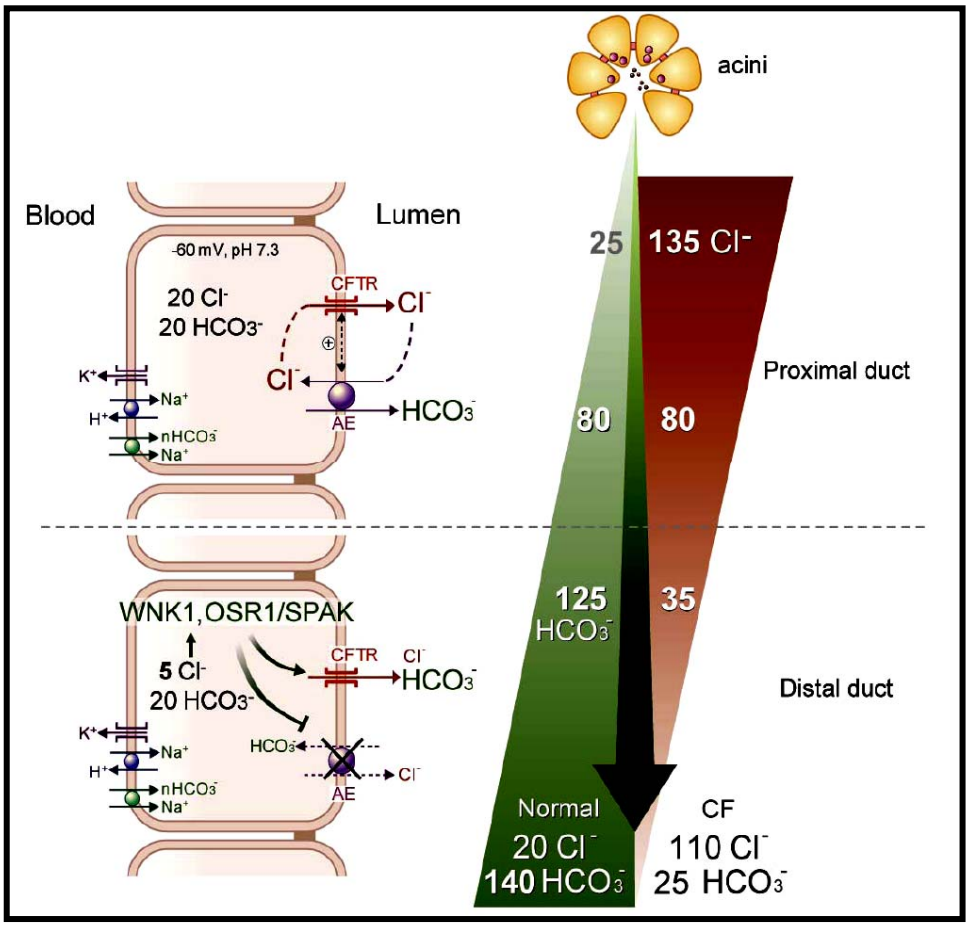

Figure 12. A model for pancreatic HCO_3^- secretion. cAMP signals initially activate the CFTR-dependent Cl^-/HCO_3^- exchange at the apical membrane of pancreatic duct cells. A gradual rise in luminal HCO_3^- and decline in Cl^- induces a decrease in $[Cl^-]_i$ due to the apical CFTR Cl^- channel activity. This in turn activates WNK1 and the downstream OSR1 and SPAK kinases. Activation of OSR1 and SPAK increases the CFTR HCO_3^- permeability, making CFTR primarily a HCO_3^- channel, and inhibits the activity of apical Cl^-/HCO_3^- exchange. These combinatorial functions maintain over 140 mM HCO_3^- concentration in the human pancreatic juice.

V. CONCLUSION

The mechanism by which human pancreatic duct cells secrete nearly isotonic HCO_3^- has long been a question for both physiologists and clinicians. Present study demonstrates for the first time, that the dynamic regulation of anion transporters in the pancreatic duct cells via the $[Cl^-]_i$-sensitive WNK1-OSR1/SPAK pathway play a pivotal role in the distal pancreatic HCO_3^- secretion. The low $[Cl^-]_i$ induced via CFTR markedly activated WNK1 and the downstream STE20-like kinases, OSR1 and SPAK. Activation of OSR1 and SPAK exerted critical effects on the apical ion transporting proteins in the pancreatic duct cells.

1. Activation of OSR1 and SPAK generated an electrogenic pathway for HCO_3^- secretion via increasing the HCO_3^- permeability of CFTR, which is essential for the secretion of pancreatic juice containing 140 mM HCO_3^- .

2. OSR1 and SPAK activation inhibited apical Cl^-/HCO_3^- exchangers in order to prevent the reabsorption of HCO_3^- from the high HCO_3^--containing pancreatic juice.

These novel molecular mechanisms provide insights into the physiology of exocrine pancreas and will initiate a new arena in the research of CFTR as a HCO_3^- channel. The findings in the present study will also greatly influence drug development toward wide range of diseases that are caused by abnormal epithelial HCO_3^- transport.

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국문 요약

세포 내 염소 이온에 의한 췌장관 중탄산 이온 수송 및 췌액 분비 조절

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박현우

췌장 관세포에서 140 mM의 중탄산 이온 (HCO_3^-) 분비는 소화기계의 정상적인 기능에 매우 중요한 역할을 하지만, 현재까지 췌장관에서 고농도의 중탄산 이온 분비와 관련된 분자적 기전은 불투명한 상황이다. 이에 대한 새로운 기전을 제시하고자 본 연구에서는 세포 내 염소 이온 농도 ($[Cl^-]_i$)의 감소가 췌장 관세포를 통한 중탄산 이온 분비를 매개하는 중요한 기전임을 밝히고자 하였다.

사람 췌장 조직에서 세포 내 염소 이온 농도 감소에 의해 활성화되는 것으로 알려진 WNK1, OSR1/SPAK kinase와 CFTR이 췌장 관세포의 내강 막 쪽에 같이 발현하고 있음을 확인하였다. 또한, PANC1 세포주에서 WNK1, OSR1/SPAK kinase는 CFTR의 활성화에 의해 감소한 세포 내 염소 이온 농도에 의해 급격히 인산화됨을 알 수 있었다. 따라서, 췌장 관세포에서 중탄산 이온

분비를 매개하는 이온 통로인 CFTR과 음이온 교환기가 활성화된 WNK1-OSR1/SPAK kinase에 의해 어떻게 조절되는지 알아보았다.

1. HEK 293T, PANC1, 그리고 기니피그 췌장 관세포에서 낮은 세포 내 염소 이온 농도에 의해 활성화된 WNK1-OSR1/SPAK kinase는 CFTR의 중탄산 이온 투과율을 급격히 증가시켰다. 이는 CFTR이 특정 조건에서 중탄산 이온 통로로써 작용할 수 있다는 것을 최초로 밝힌 것으로 140 mM의 중탄산 이온이 축적되는데 필수적인 역할을 한다.

2. 활성화된 WNK1-OSR1/SPAK kinase는 췌장관 세포에 존재하는 음이온 교환기인 SLC26A3와 Slc26a6를 억제시켰으며, 이는 고농도의 췌장관 중탄산 이온이 재흡수 되는 것을 방지함으로써 CFTR을 통해 축적된 중탄산 이온이 유지되도록 돕는다.

위의 결과를 통해, 낮은 세포 내 염소 이온 농도에 의해 활성화된 WNK1-OSR1/SPAK kinase가 췌장관 세포에서 고농도의 중탄산 이온을 분비하는 새로운 분자 기전임을 밝혔으며, 이는 상피세포에서 비정상적인 중탄산 이온 분비에 의해 유발되는 여러 호흡기계 및 소화기계 질환의 치료에 기여할 것으로 기대한다

핵심되는 말: 중탄산 이온, 췌장 관세포, CFTR, WNK1/OSR1/SPAK

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