

**THE EFFECTS OF Na^+/H^+ EXCHANGER REGULATORY
FACTOR 1 AND TRYPSIN ON PANCREATIC DUCTAL
BICARBONATE SECRETION**

Ph.D. Thesis

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Articles closely related to the subject of the thesis and cited in the thesis

- I. Pallagi P**, Venglovecz V, Rakonczay Z Jr, Borka K, Korompay A, Ózsvári B, Judák L, Sahin-Tóth M, Geisz A, Schnúr A, Maléth J, Takács T, Gray MA, Argent BE, Mayerle J, Lerch MM, Wittman T, Hegyi P. Trypsin reduces pancreatic ductal bicarbonate secretion by inhibiting CFTR Cl⁻ channels and luminal anion exchangers. *Gastroenterology* 141:2228–2239.e6 (2011). [IF: 11.675]
- II. Pallagi P***, Balla Zs*, Singh AK*, Dósa S, Iványi B, Kukor Z, Tóth A, Riederer B, Liu Y, Engelhardt R, Jármay K, Szabó A, Janovszky Á, Perides G, Venglovecz V, Maléth J, Wittmann T, Takács T, Gray MA, Gácser A, Hegyi P, Seidler U, Rakonczay Z Jr. The role of pancreatic ductal secretion in protecting against acute pancreatitis in mice. *Crit Care Med* (2013). Accepted. [IF₂₀₁₂: 6.124]. *The first three authors contributed equally.

Articles related to the subject of the thesis

- III.** Hegyi P, Venglovecz V, **Pallagi P**, Maléth J, Takács T, Rakonczay Z. Galanin - a potent inhibitor of pancreatic bicarbonate secretion - is involved in the induction and progression of cerulein-induced experimental acute pancreatitis. *Pancreas* 2011, 40(1):155-6.

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1.INTRODUCTION

The exocrine pancreas consists of two main types of cells: acinar and ductal cells. Acinar cells secrete an isotonic, NaCl-rich fluid containing a multitude of enzymes and precursor enzymes. The precursor enzymes are activated in the small intestine, initiated by conversion of trypsinogen to trypsin by the intestinal enzyme enteropeptidase. Trypsin then activates trypsinogen autocatalytically and also consequently activates other precursors. The major function of the pancreatic ductal cell is the secretion of an alkaline, HCO_3^- rich isotonic fluid, which serves two important functions: to flush digestive enzymes and toxic agents down the pancreatic ductal tree into the duodenum, and to help neutralize gastric acid.

1.1. Physiology of pancreatic ductal HCO_3^- and fluid secretion

The first step of HCO_3^- secretion is the accumulation of HCO_3^- inside the cell across the basolateral membrane of the duct cell by $\text{Na}^+/\text{HCO}_3^-$ co-transporters (NBC) and by the backward transport of protons via the Na^+/H^+ exchanger (NHE) and an H^+ -ATPase. HCO_3^- secretion across the apical membrane of pancreatic ductal epithelial cells (PDEC) is thought to be mediated by anion channels and transporters such as cystic fibrosis transmembrane conductance regulator (CFTR) and solute carrier family 26 (SLC26) anion exchangers.

CFTR, a plasma-membrane cAMP-activated Cl^- channel, is a member of the ATP binding cassette transporter superfamily. CFTR is expressed in numerous functionally diverse tissues, including the pancreas, kidney, intestine, sweat duct, heart and lung. In epithelial cells, CFTR mediates the secretion of Cl^- . In addition to its role as a secretory Cl^- channel, it also regulates several transport proteins, including the epithelial sodium channel, K^+ channels, ATP-release mechanisms, anion exchangers, sodium-bicarbonate transporters, and aquaporin water channels. CFTR has a PDZ-interacting domain, which mediates the binding of CFTR to several PDZ domain-containing proteins, including Na^+/H^+ exchanger regulatory factor isoform-1 (NHERF-1).

NHERF-1 is a scaffolding protein that is involved in the apical targeting and trafficking of several membrane proteins and anchors them to the cytoskeleton via ezrin. NHERF-1 also facilitates the association of multiprotein complexes via PDZ and ezrin, radixin, moesin-binding domains, a process that is essential for the adequate transport and function of

transporters, channels, and receptors. NHERF-1 is involved in numerous physiological processes, but the role of NHERF-1 in the pancreas has not yet been investigated, despite the fact that CFTR, a key regulator of epithelial functions, is controlled by this scaffolding protein.

SLC26 isoforms are members of a large, conserved family of anion exchangers, many of which display highly restricted and distinct tissue distribution. Several SLC26 isoforms function as $\text{Cl}^-/\text{HCO}_3^-$ exchangers. These include SLC26A3 (DRA), SLC26A6 (PAT1), SLC26A7 (PAT2), and SLC26A9 (PAT4). Out of these isoforms PAT1 and DRA were detected on the apical membrane of pancreatic ducts cells and play important role in the mechanism of pancreatic ductal HCO_3^- secretion

How SLC26 anion exchangers and CFTR act in concert to produce a high HCO_3^- secretion is controversial. One hypothesis is that HCO_3^- is secreted via the anion exchanger until the luminal concentration reaches about 70 mM, after which the additional HCO_3^- required to raise the luminal concentration to 140 mM is transported by CFTR. Another hypothesis is that all the HCO_3^- is secreted via the apical SLC26 anion exchangers and CFTR functions only to activate the exchangers and to provide the luminal Cl^- required for anion exchange to occur.

1.2. Pathophysiological role of pancreatic HCO_3^- secretion

Pancreatic ducts not only have prominent physiological, but also pathophysiological roles. Most studies of acute pancreatitis focus on the damage to acinar cells since they are assumed to be the primary target of multiple stressors affecting the pancreas. However, increasing evidence suggest that the ductal tree may have a crucial role in induction of the disease and is the primary target of stressors. Our hypothesis is that ductal secretion serves to defend the pancreas by washing out toxic agents such as bile acids, ethanol or activated trypsin. If this ductal defence mechanism is insufficient, ductal secretion will be inhibited and the harmful agents cannot leave the pancreas.

Trypsinogen is the most abundant digestive protease in the pancreas. Under physiological conditions, trypsinogen is synthesised and secreted by acinar cells, transferred to the duodenum via the pancreatic ducts and then activated by enteropeptidase in the small intestine. There is substantial evidence that early intra-acinar or luminal activation of

trypsinogen to trypsin is a key and common event in the development of acute and chronic pancreatitis. Therefore, it is crucially important to understand the effects of trypsin on PDEC.

Several studies have demonstrated that trypsin stimulates enzyme secretion from acinar cells via protease-activated receptor 2 (PAR-2), whereas the effect of trypsin on PDEC is somewhat controversial. Trypsin activates ion channels in dog PDEC and stimulates HCO_3^- secretion in the CAPAN-1 human pancreatic adenocarcinoma cell line. On the other hand, the protease dose-dependently inhibits HCO_3^- efflux from bovine PDEC. The effect of trypsin differs not only among species, but also with respect to the localization of PAR-2. When PAR-2 is localized to the basolateral membrane and activated by trypsin, the result is stimulation of HCO_3^- secretion. In contrast, when the receptor is localised to the luminal membrane, the effect is inhibition. Interestingly, there are no data available concerning the effects of trypsin on guinea pig PDEC which, in terms of HCO_3^- secretion, are an excellent model of human PDEC.

2. AIMS

The main aims of this work were to investigate the physiology and pathophysiology of HCO_3^- and fluid secretion of PDEC.

Our **specific aims** were:

1. To evaluate the role of NHERF-1 in pancreatic ductal localization of CFTR, and in HCO_3^- and fluid secretion.
2. To investigate the effects of trypsin on pancreatic ductal HCO_3^- secretion.

3. MATERIALS AND METHODS

3.1. Ethics

All experiments were conducted in compliance with the *Guide for the Care and Use of Laboratory Animals* (National Academies Press, Eight Edition, 2011), and were approved by Committees on investigations involving animals at the University of Szeged and at the Hannover Medical School and also by independent committees assembled by local authorities.

3.2. Maintenance of animals and genotyping of NHERF-1 mice

The mice and guinea pigs were housed in a standard animal care facility with a 12-h light/12-h dark cycle and were allowed free access to water and standard laboratory chow. NHERF-1-deficient mice were originally generated and described at Duke University Medical Center. NHERF-1 mutation was congenic for the FVB/N background for at least 15 generations. Genotyping was performed by PCR.

3.3. Isolation and culture of pancreatic ducts

Mice and guinea pigs were humanly killed by cervical dislocation, the pancreas was removed and intra-/interlobular pancreatic ducts were isolated by enzymatic digestion, microdissection, and then they were cultured overnight at 37 °C in a humidified atmosphere containing 5% CO₂. During overnight incubation, both ends of the isolated ducts seal and the ducts swell due to fluid secretion into the lumen.

3.4. Real-time reverse transcription polymerase chain reaction

3.4.1. mRNA expression of CFTR, PAT-1, DRA and NHERFs in mouse pancreatic ducts

Pancreatic ducts were homogenized by sonication in lysis-buffer and RNA was isolated with a NucleoSpin RNA XS Total RNA Isolation Kit. Reverse transcription was performed using Superscript III RT.

3.4.2. mRNA expression of PAR-2 in human pancreatic tissue

RNA was isolated from 30 human pancreata. Following reverse transcription, mRNA expression of PAR-2 and β -actin were determined by real-time PCR analysis.

3.5. Immunohistochemistry

3.5.1. Localization of NHERF-1 and CFTR proteins in wild-type and NHERF-1 knock-out mice

For NHERF-1 staining, paraformaldehyde-fixed, paraffin-embedded tissue sections (5 μ m) from mice of different genotypes were prepared on the same slide. For CFTR staining, pancreata were fixed in 2% paraformaldehyde in phosphate buffered saline (PBS). Fixed tissue was rinsed with PBS and transferred to 30% sucrose in PBS overnight. The tissue was embedded in tissue-freezing medium. Cryosectioning was done with a microtome cryostat at -20 °C and 10 μ m thick sections were collected on microscope slides. Immunohistochemistry

of the mouse pancreas was performed by using rabbit polyclonal antibodies against NHERF-1 and CFTR. Washing was followed by secondary antibody (Alexa Fluor 488-labelled goat anti-rabbit IgG) incubation. Slides were imaged on a confocal microscope.

3.5.2. Localization of PAR-2 protein in guinea pig, human and wild-type and PAR-2 knock-out mice

Pancreatic tissue from guinea pig, patients and wild-type (WT) and PAR-2 knock-out (KO) mice were investigated. Paraffin embedded, 3-4 μm thick sections were used for immunohistochemistry. The slides were incubated with the primary rabbit polyclonal antibody. Signal detection was achieved by ImPRESS reagent with secondary anti-rabbit IgG antibody. Diaminobenzidine was used to visualize immune complexes and nuclear counterstaining was performed with haematoxylin. The immunohistochemical reactions were digitalized with a Mirax MIDI slide scanner.

3.6. Western blot analysis

Western blot analysis was used to determine the specificity of the PAR-2 antibody. Proteins were extracted from fresh-frozen guinea pig (n=3) and human (n=3) pancreatic tissue stored at $-80\text{ }^{\circ}\text{C}$. Isolation was performed by using lysis buffer. Samples were homogenized, followed by centrifugation. Measurements of protein concentration were performed using Bradford-analysis. 30 μg of protein samples were loaded in each lane. Blots were incubated with polyclonal PAR-2 rabbit antibody and anti-GAPDH antibody at $4\text{ }^{\circ}\text{C}$ overnight. After washing with 0.1% TRIS, horseradish peroxidase-conjugated anti-rabbit antibody was applied at room temperature. Signals were visualized by enhanced chemiluminescent detection.

3.7. Microperfusion and measurement of intracellular pH, Ca^{2+} concentration

Intracellular pH (pH_i) and calcium concentration ($[\text{Ca}^{2+}]_i$) were estimated by microfluorimetry using the pH- and Ca^{2+} -sensitive fluorescent dyes BCECF-AM and FURA 2-AM, respectively.

3.8. Measurement of fluid secretion

In vitro

Fluid secretion into the closed luminal space of the cultured pancreatic ducts was analysed using a swelling method. Briefly, the ducts were transferred to a perfusion chamber. Bright-field images were acquired at 1 min intervals using a CCD camera. Digital images of

the ducts were analysed using Scion Image software to obtain values for the area corresponding to the luminal space in each image.

In vivo

Mice were anesthetized with 1.5 g/kg urethane by i.p. injection. The abdomen was opened, and the lumen of the common biliopancreatic duct was cannulated with a blunt-end 30-gauge needle. Then the proximal end of the common duct was occluded with a microvessel clip to prevent contamination with bile, and the pancreatic juice was collected in PE-10 tube for 30 min. Using an operating microscope, the jugular vein was cannulated for administration of secretin (0.75 CU/kg) and the pancreatic juice was collected for an additional 120 min.

3.9. Intravital video microscopy

The experiment was performed to assess the possible consequences of secretin treatment on the microcirculation of the pancreas in anaesthetized mice with urethane. Using an operating microscope, the right jugular vein was cannulated for i.v. administration of secretin and the fluorescence marker used for the intravital microscopic examination. The animals were placed in a supine position on a heating pad and a midline laparotomy performed. The majority of the intestines were exteriorized to gain good access to the pancreas which was carefully placed on a specially designed stage and covered with a microscopic cover slip. The microcirculation of the pancreas was visualized by intravital fluorescence microscopy using a single i.v. bolus of fluorescein isothiocyanate-labeled dextran. Video images of the microcirculatory network of the pancreatic tail were recorded at baseline and 20 min after the i.v. application of secretin.

3.10. Statistical analysis

Statistical analysis was performed by SigmaPlot (Systat Software Inc., Chicago, IL, USA). Data are presented as means \pm SEM. Both parametric (one- or two-way analysis of variance) and non-parametric (Kruskal-Wallis) tests were used based on the normality of data distribution (analyzed by the Shapiro-Wilk test). Post-hoc analysis (either Dunn's or Bonferroni's test) was performed according to the recommendations made by SigmaPlot. Statistical analysis of the immunohistochemical data was performed using the Mann-Whitney U test. Probability values of $P < 0.05$ were accepted as being significant.

4. RESULTS

4.1. Role of NHERF-1 in pancreatic ductal HCO_3^- and fluid secretion

4.1.1. mRNA expression of CFTR, DRA, PAT-1 and NHERFs in mouse pancreatic ducts

CFTR, DRA, PAT-1, NHERF-1 and NHERF-2, but not NHERF-3 mRNA were expressed in isolated pancreatic ducts of WT mice. Notably, quantitative RT-PCR indicated that NHERF-1 was expressed more abundantly than the other two CFTR-binding NHERFs (NHERF-2 and NHERF-3).

4.1.2. Apical NHERF-1 and CFTR localization in pancreatic ducts is reduced in NHERF-1-knock-out mice

NHERF-1 was highly expressed in the apical membrane of pancreatic duct cells, but only weakly expressed in some acinar cells of WT mice. No or weak staining was detected in NHERF-1-KO mice. The weak staining is non-specific and was not localized to the luminal membrane.

CFTR was expressed in both pancreatic acinar and ductal cells of WT and NHERF-1-KO mice. Compared to WT animals, apical CFTR staining in pancreatic ducts was markedly reduced and overall CFTR staining in the pancreas appeared more diffuse in the absence of NHERF-1.

4.1.3. Pancreatic ductal HCO_3^- secretion is decreased in NHERF-1-knock-out mice

To determine if mislocalization of CFTR affects pancreatic ductal function, we investigated HCO_3^- secretion in isolated ducts using three different, but complementary, methods that measure the rate at which HCO_3^- is secreted across the luminal membrane via $\text{Cl}^-/\text{HCO}_3^-$ exchangers and/or CFTR.

(1) Inhibitor stop. With this method the initial rate of pH_i acidification is measured after the basolateral membrane is exposed to H_2DIDS (0.2 mM) and amiloride (0.2 mM) which block HCO_3^- accumulation into the cell by the NBC and NHE. Using this approach the rate of transmembrane base flux [$J(B^-)$] was more than 4-fold lower in NHERF-1-KO compared to WT mice.

(2) Alkali load. Here the recovery of pH_i from an alkali load induced by exposure to 20 mM NH_4Cl in a $\text{HCO}_3^-/\text{CO}_2$ -containing solution reflects the rate of HCO_3^- secretion. The recovery from alkali load was about 2-fold lower in NHERF-1-KO vs. WT animals.

(3) Chloride removal. pH_i alkalinisation induced by removal of luminal Cl^- was significantly reduced in NHERF-1-KO compared to WT mice.

These data show that pancreatic ductal HCO_3^- secretion was significantly reduced in NHERF-1-KO compared to WT mice.

4.1.4. Fluid secretion is decreased in NHERF-1-knock-out mice

To investigate if fluid secretion was also compromised in KO mice, the rate of fluid secretion was measured. In the absence of secretagogue, we could not detect any significant changes in the volume of WT and NHERF-1-KO isolated pancreatic ducts. Stimulation of WT ducts with 5 μM forskolin caused dynamic swelling of the ducts as a result of fluid secretion into the closed luminal space. In contrast, ducts from NHERF-1-KO mice had a blunted response to forskolin.

We also examined the rate of pancreatic juice secretion *in vivo* in anesthetized mice. Under basal conditions, WT animals secreted pancreatic juice at a rate of 0.12 ± 0.02 $\mu\text{l}/\text{hour}/\text{g}$ body weight. In contrast, we could not detect any basal secretion in NHERF-1-KO animals. In response to secretin stimulation, we observed about 4-fold higher rates of pancreatic juice secretion in WT mice, values significantly higher than from NHERF-1-KO mice. These results demonstrate that pancreatic fluid secretion was significantly reduced in NHERF-1-KO compared to WT animals under both basal and secretin-stimulated conditions.

4.2. Role of trypsin in pancreatic ductal HCO_3^- secretion

4.2.1. Expression of PAR-2 in guinea pig and human pancreata

PAR-2 was highly expressed in the luminal membrane of small intra- and interlobular ducts, but was almost undetectable in the larger interlobular ducts. The localization of PAR-2 in the human pancreas was identical to that in the guinea pig gland. Measurements of relative optical density confirmed the significant differences between the expression of PAR-2 in small intra- and interlobular ducts and the larger interlobular ducts in both species.

4.2.2. Luminal administration of PAR-2 activating peptide and trypsin induces dose-dependent intracellular calcium signalling

Since PAR-2 expression was detected only on the luminal membrane of intralobular duct cells, we used the microperfusion technique to see whether these receptors can be activated by PAR-2 agonists. First, the experiments were performed at pH 7.4, in order to understand the effects of trypsin and PAR-2 under *quasi* physiological conditions. Luminal administration of PAR-2 activating peptide (PAR-2-AP) increased $[Ca^{2+}]_i$ in perfused pancreatic ducts. The $[Ca^{2+}]_i$ response was dose-dependent, and consisted of a peak which decayed in the continued presence of the agonist, possibly reflecting PAR-2 inactivation or depletion of intracellular Ca^{2+} stores. Pre-treatment of PDEC with 10 μ M PAR-2 antagonist (PAR-2-ANT) for 10 min completely blocked the effects of 10 μ M PAR-2-AP on $[Ca^{2+}]_i$. Removal of extracellular Ca^{2+} had no effect on the $[Ca^{2+}]_i$ rise evoked by luminal administration of 10 μ M PAR-2-AP; however, pre-loading ducts with the Ca^{2+} chelator BAPTA-AM at 40 μ M totally blocked the response.

Trypsin also induced a dose-dependent $[Ca^{2+}]_i$ elevation similar to that evoked by PAR-2-AP. 5 μ M (SBTI) trypsin inhibitor, 10 μ M PAR-2-ANT and 40 μ M BAPTA-AM totally blocked the rise in $[Ca^{2+}]_i$. These data show that trypsin activates PAR-2 on the luminal membrane of the duct cell which leads to release of Ca^{2+} from intracellular stores and an elevation of $[Ca^{2+}]_i$.

4.2.3. Luminal exposure to PAR-2-AP and trypsin evoke intracellular alkalosis in guinea pig PDEC

Luminal application of the CFTR inhibitor-172 (10 μ M) and the anion exchanger inhibitor H₂DIDS (500 μ M) induced an intracellular alkalization in PDEC. Both luminal PAR-2-AP and trypsin induced a dose-dependent elevation of pH_i . Pre-incubation of PDEC with either 10 μ M PAR-2-ANT or 5 μ M SBTI or 40 μ M BAPTA-AM for 30 min totally blocked the effect of trypsin on pH_i . Luminal Cl⁻-free conditions increased the pH_i of PDEC presumably by driving HCO₃⁻ influx on the apical anion exchangers. Note that luminal administration of trypsin further elevated pH_i in Cl⁻ free conditions and also in the presence of H₂DIDS and CFTR inhibitor-127. However, pre-treatment of ducts with a combination of H₂DIDS and CFTR inhibitor-172 markedly reduced the effect of trypsin on pH_i .

4.2.4. PAR-2 is down-regulated in patients suffering from chronic pancreatitis

It has been documented that there is activated trypsin in the pancreatic ductal lumen in chronic pancreatitis in human. If trypsin activity is elevated in the duct lumen, PAR-2 down-regulation should occur. Our data show a marked reduction in membranous PAR-2 protein level, but no significant changes in cytoplasmic PAR-2 protein in chronic pancreatitis. Furthermore, PAR-2 mRNA level was markedly reduced in chronic pancreatitis, suggesting that reduced PAR-2 mRNA transcription may cause PAR-2 down-regulation in chronic pancreatitis.

4.2.5. Luminal exposure to R122H mutant cationic trypsin induces elevation of intracellular calcium concentration and evokes alkalosis in PDEC

It has been demonstrated that mutations in cationic trypsinogen increase the risk of chronic pancreatitis, most likely because of the enhanced autoactivation exhibited by the mutant trypsinogens. Here we tested whether the most common mutation in cationic trypsin, R122H, affected the protease's ability to interact with PAR-2. 1 μM of R122H cationic trypsin caused comparable changes in pH_i and $[\text{Ca}^{2+}]_i$ to 0.4 μM wild type trypsin, suggesting that a trypsin-mediated inhibition of HCO_3^- secretion could play a role in the pathogenesis of hereditary as well as chronic pancreatitis.

4.2.6. Activation of PAR-2 is diminished in PAR-2 KO mice

Finally, we investigated the effects of both PAR-2-AP and trypsin on PDEC isolated from WT and PAR-2 KO mice. First we confirmed using immunohistochemistry that WT mice do, whereas PAR-2 KO mice do not express PAR-2 in their PEDC. Accordingly, our functional data clearly show that the pH_i and $[\text{Ca}^{2+}]_i$ responses to luminal administration of either trypsin or PAR-2-AP were markedly diminished in PAR-2 KO PDEC.

5. DISCUSSION

5.1. The effect of NHERF-1 on pancreatic ductal HCO_3^- secretion

We have demonstrated that NHERF-1 mRNA is highly expressed in mouse pancreatic ducts. Furthermore, the genetic deletion of NHERF-1 greatly reduced the translocation of CFTR to the luminal ductal cell membrane and also decreased both *in vitro* and *in vivo* pancreatic HCO_3^- and fluid secretion. Both basal and cAMP-stimulated (by forskolin or

secretin) secretion were affected in the transgenic animals, but this effect was not caused by alterations in pancreatic blood flow.

Localization of CFTR to the apical plasma membrane of epithelial cells is critical for vectorial transport of chloride in a variety of epithelia, including the airway, pancreas, intestine, and kidney. NHERF-1 has been shown to play an important role in the apical trafficking, targeting, membrane retention and activation of several membrane proteins such as CFTR. These effects on CFTR were shown to require PDZ domain interactions with NHERF-1 in several studies, although other similar studies failed to confirm these results. In accordance with our findings, CFTR mutations causing cystic fibrosis that impair the stability of the Cl^- channel in the plasma membrane also result in markedly reduced HCO_3^- and fluid secretion. Of course we cannot exclude the direct or indirect (e.g. via CFTR) effects of NHERF-1 deletion on other transporters involved in pancreatic HCO_3^- and fluid secretion, such as anion exchangers DRA and PAT-1. Of note, both DRA and PAT-1 are known to have PDZ domain binding motifs, and to bind to NHERF-1. In addition, activation of CFTR by SLC26 transporters was shown to be facilitated by PDZ ligands. Nevertheless, the reduced expression of CFTR in the apical membrane in NHERF-1 KO pancreatic ducts will likely decrease the activities of PAT-1 and DRA.

Several studies have shown that binding of CFTR to NHERF proteins may also be important for the regulation of CFTR activity. It has been demonstrated that NHERF-1 is required for full activation of transepithelial Cl^- and HCO_3^- secretion by cAMP- and cGMP-linked agonists in the duodenum and jejunum. A recent study has shown that CFTR activity is also dependent on NHERF-1 regulated cAMP compartmentalization and local protein kinase A activity in human airway epithelial cells. The particularly high expression of NHERF-1, as well as CFTR in pancreatic ducts, compared to other NHERFs and SLC26 anion transporters is quite different from the relative expression levels of these transporters and the NHERFs in the small intestine. These findings suggested to us that CFTR-NHERF-1 interaction may be crucial to pancreatic ductal secretion.

5.2. The effect of trypsin on pancreatic ductal HCO_3^- secretion

Until quite recently, the pathophysiological relevance of pancreatic ducts in acute pancreatitis has been neglected. However, there are important lines of evidence supporting the idea that pancreatic ducts play a role in the pathogenesis of pancreatitis: i) ductal fluid and HCO_3^- secretion are compromised in acute and chronic pancreatitis, ii) one of the main endpoints of chronic pancreatitis is the destruction of the ductal system, iii) mutations in

CFTR may increase the risk of pancreatitis, and iv) etiological factors for pancreatitis, such as bile acids or ethanol in high concentration, inhibit pancreatic ductal HCO_3^- secretion. Despite the above mentioned data, the role of PDEC in the development of pancreatitis has received relatively little attention.

There are important species differences regarding the localization of PAR-2 in pancreatic ducts and in the effect of PAR-2 activation on HCO_3^- secretion. For example, CAPAN-1 cells and dog PDEC express PAR-2 only on the basolateral membrane, whereas bovine PDEC express PAR-2 on the luminal membrane. Therefore, one of our aims was to determine which animal model best mimics human PAR-2 expression and thus would be the best for studying the effects of trypsin on PDEC function. Our results showed that in the human pancreas PAR-2 is localized to the luminal membrane of small proximal pancreatic ducts, which are probably the major site of HCO_3^- and fluid secretion. Since PAR-2 expression in the guinea pig pancreas was localized to the luminal membrane of duct cells, we performed our experiments on isolated guinea pig ducts.

First we characterized the effects of PAR-2 activation by trypsin and PAR-2-AP on PDEC. Previously, it has been shown that activation of the G-protein-coupled PAR-2 by proteinases requires proteolytic cleavage of the receptor, which is followed by an elevation of $[\text{Ca}^{2+}]_i$. As expected, luminal trypsin and PAR-2-AP caused a dose-dependent elevation of $[\text{Ca}^{2+}]_i$ in guinea pig ducts. Importantly, the trypsin inhibitor SBTI, PAR-2-ANT and the intracellular Ca^{2+} chelator BAPTA-AM all completely blocked the elevation of $[\text{Ca}^{2+}]_i$, whereas removal of extracellular Ca^{2+} had no effect. Next we characterized the effects of PAR-2 activation on pH_i . Luminal application of trypsin and PAR-2-AP both caused a dose-dependent intracellular alkalosis in PDEC. PAR-2 activation reduces HCO_3^- secretion in PDEC by inhibiting SLC26 anion exchangers and CFTR expressed on the apical membrane of the duct cell. In similarity with the $[\text{Ca}^{2+}]_i$ signals, the effect of PAR-2 activation on pH_i was blocked by SBTI, PAR-2-ANT and BAPTA-AM; the action of BAPTA-AM suggesting that the inhibition of HCO_3^- secretion follows from the rise in $[\text{Ca}^{2+}]_i$. Interestingly, an elevation of $[\text{Ca}^{2+}]_i$ is crucial for both stimulatory and inhibitory pathways that control HCO_3^- secretion by PDEC.

The pH of pancreatic juice [and therefore the luminal pH (pH_L) in the duct] can vary between approximately 6.8 and 8.0. It has recently been shown that protons co-released during exocytosis cause significant acidosis (up to 1 pH unit) in the lumen of the acini. However, it has clearly shown that the pH_L in pancreatic ducts is dependent on the rate of HCO_3^- secretion. pH_L can be elevated from 7.2 to 8.5 by stimulation with secretin or forskolin

and this effect was strictly dependent on the presence of HCO_3^- . Also, inhibition of ductal HCO_3^- secretion with H_2DIDS can decrease the pH_L to below 8.0. We showed that autoactivation of trypsinogen was relatively slow at pH 8.5, but decreasing the pH from 8.5 to 7 progressively stimulated autoactivation. These results suggest that under physiological conditions HCO_3^- secretion by PDEC is not only important for elevating the pH in the duodenum, but also for keeping pancreatic enzymes in an inactive state in the ductal system of the gland.

Receptor down-regulation is a phenomenon that occurs in the continued presence of an agonist and leads to a reduction in the cell's sensitivity to the agonist. In this study, we provide evidence that transcriptional down-regulation explains the reduced expression of PAR-2 seen in chronic pancreatitis.

In conclusion, we suggest for the first time that one of the physiological roles of HCO_3^- secretion by PDEC is to curtail trypsinogen autoactivation within the pancreatic ductal system. However, if trypsin is present in the duct lumen (as may occur during the early stages of pancreatitis due to leakage from acinar cells), PAR-2 on the duct cell will be activated leading to Ca^{2+} release from intracellular stores and a rise in $[\text{Ca}^{2+}]_i$. This causes inhibition of the luminal anion exchangers and CFTR reducing HCO_3^- secretion by the duct cell. The fall in HCO_3^- secretion will increase the transit time of zymogens down the duct tree and decrease pH_L , both of which will promote the autoactivation of trypsinogen. The trypsin so formed will further inhibit HCO_3^- transport leading to a vicious cycle generating further falls in pH_L and enhanced trypsinogen activation, which will favour development of the pancreatitis. Finally, the R122H mutant cationic trypsin also elevated $[\text{Ca}^{2+}]_i$ and pH_i in duct cells, suggesting that this mechanism may be particularly important in hereditary pancreatitis in which the mutant trypsinogens more readily autoactivate.

6. NEW FINDINGS

1. NHERF-1 mRNA is highly expressed in mouse pancreatic ducts.
2. Genetic deletion of NHERF-1 greatly reduces the translocation of CFTR to the luminal ductal cell membrane.
3. NHERF-1 is involved in both pancreatic ductal HCO_3^- and fluid secretion.

4. Both basal and cAMP-stimulated secretion is affected in the transgenic animals, but this effect is not caused by alterations in pancreatic blood flow.
5. In the human pancreas PAR-2 is localized to the luminal membrane of small proximal pancreatic ducts. PAR-2 expression in the guinea pig pancreas is localized to the luminal membrane of duct cells.
6. Luminal trypsin causes a dose-dependent elevation of $[Ca^{2+}]_i$ in guinea pig ducts via activation of PAR-2.
7. PAR-2 activation by trypsin reduces HCO_3^- secretion in PDEC by inhibiting SLC26 anion exchangers and CFTR expressed on the apical membrane of the duct cell.
8. Transcriptional down-regulation explains the reduced expression of PAR-2 seen in chronic pancreatitis.

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