Ion Channels in Pancreatic Duct Physiology

Viktoria Venglovecz,[1] Péter Hegyi,[2] Michael Gray[3]

[1]Department of Pharmacology and Pharmacotherapy, University of Szeged, Szeged, Hungary,
[2]Medical School, Institute for Translational Medicine, University of Pécs, Pécs, Hungary
[3]Biosciences Institute, Faculty of Medical Sciences, Newcastle University, Newcastle upon Tyne, UK.

1. Physiology of pancreatic ductal cells

The main function of pancreatic ductal cells is to secrete a HCO_3^- rich, isotonic fluid that washes out the inactive form of digestive enzymes from the ductal system, as well as provide pH conditions that are essential for normal pancreatic function. The rate of HCO_3^- secretion is influenced by several factors (such as secretory rate, species, or the location of the cell in the ductal tree) nevertheless upon stimulation, it can reach up to 140 mM, which means a significant concentration difference exists between the outside and inside of the ductal cell, that poses an extreme challenge to ductal cells. This high degree of HCO_3^- secretion is achieved through the coordinated action of ion transporters and channels, in which the Cl^-/HCO_3^- exchanger and the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel play a central role.

Ion transporters and channels on ductal cells are differentially expressed on the luminal and basolateral membranes, resulting in polarization of the ductal cell (Fig. 1) [1]. The major ion transporters on the basolateral membrane include the Na^+/H^+ exchanger (NHE), the Na^+/HCO_3^- cotransporter (NBC), the Na^+/K^+ ATPase and various types of K^+ channels [2]. The electroneutral, NHE1 isoform can be found on the basolateral membrane of ductal cells and acts as a proton extruder with a 1 Na^+:1 H^+ stoichiometry [3]. In addition to playing an important role in the regulation of intracellular pH, NHE1 promotes the formation of HCO_3^- from carbonic acid, by removing excess H^+ from the cell. The NHE3 isoform is located on the luminal membrane of ductal cells and, unlike NHE1, is involved in HCO_3^- salvage [4; 5]. HCO_3^- also enters the cell directly through NBC. The electrogenic NBC isoform, NBCe1B has been identified on the basolateral membrane of ductal cells, with 1 Na^+/2 HCO_3^- stoichiometry [6; 7]. Through this transporter, more HCO_3^- enters the cell than is formed during the dissociation of carbonic acid. The electroneutral form of NBC, the NBCn1 or NBC3, has been shown to be active on the luminal membrane of ductal cells, where it plays role in HCO_3^- salvage [8]. In addition, Na^+/K^+ ATPase, together with basolateral K^+ channels, maintains a negative

membrane potential, using the energy from the hydrolysis of ATP, which provides a driving force for anion secretion across the luminal membrane.

There are two major transporters on the luminal side of the ductal cells; the CFTR chloride channel and the Cl^-/HCO_3^- exchanger. Several hypotheses exist regarding the mechanisms by which the ductal cells are able to secrete up to five-times as much HCO_3^- as is present in the cytosol [9; 10]. The most generally accepted view is that HCO_3^- secretion occurs through both the Cl^-/HCO_3^- exchanger and the CFTR channel [11; 12]. Among the Cl^-/HCO_3^- exchangers, the Slc26a6 (PAT1) and the Slc26a3 (DRA) isoforms are present on pancreatic ductal cells [13-15]. PAT1 and DRA have different Cl^- : HCO_3^- stoichiometry (2:1 for DRA and 1:2 for PAT1) and are expressed in different parts of the ductal tree. In the initial stage of HCO_3^- secretion, HCO_3^- is secreted into the lumen through the Cl^-/HCO_3^- exchanger in exchange for Cl^- that is returned to the lumen via the CFTR Cl^- channel. As the concentration of HCO_3^- increases in the lumen and at the same time Cl^- decreases, the WNK1-OSR1/SPAK signalling pathway is activated, which increases the permeability of the CFTR channel to HCO_3^-, resulting in the formation of up to 140 mM HCO_3^- in the lumen [12].

2. Pathological aspects

Since fluid and electrolyte secretion by pancreatic ductal cells is a tightly regulated process it is not surprising that insufficient pancreatic ductal HCO_3^- and fluid secretion leads to the destruction of the gland, as classically observed in the inherited disease Cystic Fibrosis (CF) [16-18]. An important consequence of impaired HCO_3^- secretion is an acidic pancreatic juice (less than 6.5) that increases mucus viscosity, and decreases the solubility of secreted digestive enzymes, factors that predispose to the formation of mucin/protein plugs and eventually cysts within the ductal tree. A more acidic pH may also induce premature activation of digestive enzymes within the ductal tree, leading to the development of pancreatitis. Therefore, intensive research is underway to develop drug molecules capable of restoring the function of transporters, especially in CF.

2.1 Cystic fibrosis

CF is the most common, life-limiting, inherited disease in Caucasian populations (1 in 3,500 new-borns in Europe) [19; 20]. Over 2000 CF causing mutations have been identified in the *cftr* gene (http://www.genet.sickkids.on.ca/), although to date, only ~ 360 of these variants have been fully annotated (https://cftr2.org/). However, 70-90% of CF individuals harbour the F508del mutation on at least one allele [21], which results in misfolding and incorrect

processing of CFTR to the apical membrane. For those mutations that have been studied in detail, the genetic alteration leads to a variety of functional defects in the CFTR protein. These functional defects have been grouped into 6 classes [22]. Class 1-3 cause severe CFTR dysfunction, while Class 4-6 produce less severe effects on CFTR and, in general, the mutated protein retains some level of channel activity. In relation to pancreatic pathology, ~ 85 % of people with CF are born pancreatic insufficient (PI), which equates to a reduction in pancreatic function of more than 95 %. In these people, there is a very good correlation between pancreatic disease severity and the class of mutation [23-25] with 'severe' CF mutations, such as the most common Class 2 CF mutation, F508del, and the Class 3 gating mutant, G511D, strongly correlate with PI. For those with 'milder' mutations (some residual channel activity such as Class 4, R117H), pancreatic function is preserved (pancreatic sufficient, PS), albeit to differing levels. However, in general these PS individuals require less pancreatic enzyme replacement supplements, but can become PI with increasing age.

As described in the introduction, CFTR plays a fundamental role in pancreatic ductal $NaHCO_3$ and fluid secretion, where it regulates HCO_3^- secretion in two fundamentally different ways; firstly, as a direct exit pathway for HCO_3^- secretion and secondly as a regulator of SLC26A-mediated Cl^-/HCO_3^- exchange [1]. For the latter process, this involves physical interaction between CFTR and the SLC26A6 anion exchanger [14; 26-28], and loss of functional CFTR leads to loss of anion exchange activity. The exact mechanism underlying this complete loss of anion exchange activity is not fully understood, but studies from polarised cultures of CFPAC cells, a human CF pancreatic ductal cell line homozygous for F508del, showed that apical SLC26A Cl^-/HCO_3^-exchange activity was absent, despite evidence for mRNA expression. Importantly, viral-mediated CFTR transduction of the CFPAC cells restored anion exchange activity, suggesting that CFTR may be required for the trafficking of SLC26A6 to the apical membrane (169). Furthermore, it is interesting that the anion exchanger is activated by a number of CFTR mutants that lack Cl^- channel activity [27], and that this correlates with a good retention of pancreatic function in patients carrying those mutations [29]. Taken together, these results strongly suggest that a functional CFTR at the apical plasma membrane is a prerequisite for SLC26A6-mediated anion exchange, and that mild CFTR mutations are likely to preserve Cl^-/HCO_3^- exchange activity, although this needs formal demonstration.

Strategies for improving HCO_3^- secretion in the CF pancreas are limited because of the marked tissue destruction at birth in the majority of people with CF. However, the last decade has seen a major improvement in the treatment of the basic defect in CF, through the development of

small molecule CFTR modulators [30]. Clinically-approved drugs include the CFTR potentiator, Ivacaftor, which enhances CFTR open probability for a number of gating mutants, as well as CFTR correctors, which help restore processing CFTR trafficking mutants (including F508del-CFTR) to the apical membrane. These include the first generation corrector drug, Lumacaftor and as well more efficacious second-generation correctors, such as tezacaftor and elexacaftor. These drugs are either given alone, or in combination (double and triple combinations), depending on the functional defects(s), and have produced substantial improvements in lung function, number of hospitalisations and exacerbations, as well as BMI [31; 32]. Since lung dysfunction is the major cause of morbidity and mortality in people with CF, median survival rates are predicted to significantly improve. However, there are only a few studies that have directly assessed if these CFTR modulators also improve pancreatic function [33]. For example, pancreatic function measurements in young children with CF taking Ivacaftor over 24 weeks, showed a significant restoration of enzyme-secreting capacity (increased faecal elastase-1 levels), and by inference, pancreatic tissue regeneration, which is an extremely exciting finding [34], that warrants further research. A more recent, but limited study, also provided evidence that Ivacaftor restored some pancreatic function in an adult with CF (http://dx.doi.org/10.3390/)

2.2 Acute pancreatitis

Acute pancreatitis (AP) is a sudden inflammation of the pancreas, which in most cases is mild, but in approximately 20% of patients, a life-threatening, severe form can develop in which the mortality rate can reach up to 20-40% [35]. Large individual differences can be observed in the development and course of the disease, in which the disturbed balance of protective and damaging factors presumably plays a significant role [36]. Pancreatic ductal cells are considered as protective mechanism in the pancreas by the secretion of a HCO_3^--rich, isotonic fluid. The two main etiological factors in the development of AP are gallstone obstruction and excessive alcohol consumption. Both aetiologies associated with marked changes in HCO_3^- and fluid secretion based on *in vitro* intracellular pH and fluid transport studies from isolated microdissected ducts [37-42]. At low concentrations, both agents increased HCO_3^- secretion, a response that required CFTR and Cl^-/HCO_3^- exchange activity [41-43]. However, higher levels of these agents led to a severe inhibition of CFTR-dependent HCO_3^- secretion, which was due to profound mitochondrial damage and a consequent reduction in intracellular ATP levels [38; 40] (Table 1). These studies were the first to suggest that ductal HCO_3^- secretion could play a protective role against these noxious agents not hitherto thought of.

Bile acids

Under normal conditions, bile cannot enter the pancreatic ductal system; however, in the case of gallstone obstruction it may regurgitate into the pancreas through the common bile duct. Chenodeoxycholate (CDCA) is the most abundant hydrophobic, unconjugated bile acid in human bile. Using isolated guinea pig pancreatic ducts, it has been shown that luminal administration of CDCA at low concentrations (0.1 mM), significantly increases ductal HCO_3^- secretion, in which CDCA-induced intracellular Ca^{2+} oscillation play an important role [42]. The major source of CDCA-induced Ca^{2+} is the endoplasmic reticulum (ER), from which Ca^{2+} is released via an IP_3-mediated pathway. The released Ca^{2+} activates large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) on the luminal membrane of ductal cells, and opening of these channels hyperpolarizes the cell membrane, thereby enhancing the electrochemical driving force for anion secretion through the luminal membrane [41]. This stimulatory effect of CDCA has also been demonstrated in the CFPAC-1 cell line and has been shown to be highly dependent on CFTR expression.[43] In contrast, high concentration of this bile acid (1 mM) causes a toxic increase in Ca^{2+} and strongly inhibits HCO_3^- secretion [42]. The inhibitory effect of high concentrations of bile acids presumably results from the fact that at this concentration, CDCA damages mitochondria, resulting in ATP depletion and ultimately apoptosis [40; 42; 44; 45]. This dual effect of bile acid is thought to be important in the pathomechanism of AP. In the early stages of the disease, when bile acids reach ductal cells at low concentrations, the increased fluid secretion try to wash out the toxic bile acids from the ductal tree in order to avoid pancreatic damage. If the concentration of bile acids further increases, it energetically destabilizes the cell, inhibits the function of ion transporters and induces apoptosis. Bile acids reach the acini where they induce inflammatory processes. Interestingly, the toxic effect of bile acids highly depends on their hydrophobicity. The hydrophilic bile acid, ursodeoxycholic acid, the 7-alpha enantiomer of CDCA, is able to counteract the cell-damaging effects of high-dose of CDCA through stabilization of the mitochondrial membrane that raises the possibility of therapeutic use of this bile acid [46; 47].

Ethanol

EtOH also dose-dependently affect ductal HCO_3^- secretion. Low concentration of EtOH (0.3-30 mM) enhances both basal and secretin-stimulated ductal fluid secretion on intra-interlobular ducts isolated from guinea pigs, in which cAMP activation and Ca^{2+} release play a key role [48]. The stimulatory effect of low concentrations of EtOH, has also been demonstrated in the Capan-1 cell line and has been shown to be dependent on Ca^{2+} release from the ER via an IP_3-

mediated pathway [39]. In contrast, high concentrations of EtOH (100 mM) strongly inhibited the rate of HCO_3^- secretion and the activity of CFTR [39; 48]. The inhibitory effect of EtOH is presumably mediated by fatty acids (FAs) and fatty acid ethyl esters (FAEEs) formed during the non-oxidative metabolism of EtOH. Similar to the effect of bile acids, EtOH and its non-oxidative metabolites induce toxic Ca^{2+} signalling in ductal cells by completely depleting ER stores and promoting extracellular Ca^{2+} uptake into cells. Persistently elevated Ca^{2+} causes mitochondrial Ca^{2+} overload, resulting in decreased mitochondrial membrane potential and ATP production. Chelation of Ca^{2+} abolished the inhibitory effect of EtOH and fatty acid on HCO_3^- secretion, suggesting that the inhibitory effect of high dose of these agents is mediated by toxic intracellular Ca^{2+} [39]. Moreover, EtOH and its metabolites profoundly inhibit CFTR function on the ductal cells which can be prevented by the supplementation of intracellular ATP (ATP_i), indicating that the inhibitory effect of EtOH on CFTR is mediated by ATP_i depletion. Since CFTR works in close coordination with the Cl^-/HCO_3^- exchanger, improper CFTR function may contribute to decreased fluid and HCO_3^- secretion and thus to the pathogenesis of AP. Consequently, maintenance of ATP_i may represent a therapeutic option in the treatment of the disease [38; 39]. Decreased expression of CFTR has been also observed on the luminal membrane of human pancreatic ductal cells in alcohol-induced acute and chronic pancreatitis, and in response to FAs and FAEEs in which the accelerated turnover and decreased biosynthesis of the channel play role. The importance of CFTR in the alcohol-induced pancreatic damage has been further confirmed in CFTR knock out mice, where the absence of CFTR caused much more severe pancreatitis [39].

Trypsin

One of the most important role of ductal HCO_3^- secretion is to prevent intraductal activation of trypsinogen. Although there is no significant trypsin in the lumen under physiological conditions, it may leak from the acinar cells in the early stages of pancreatitis. By binding to PAR-2 receptors on the luminal membrane of ductal cells, trypsin or trypsin activating peptide (PAR-2-AP) increases $[Ca^{2+}]_i$ levels and inhibits Cl^-/HCO_3^- exchange and CFTR function, resulting in lower luminal pH [49]. Low luminal pH favours premature activation of trypsinogen, which will activate additional trypsinogens. This process leads to a vicious cycle in which more trypsin is formed, the more trypsinogen will be activated, resulting in even more inhibition of the activity of the luminal transporters [49]. The importance of PAR-2 activation in the pathomechanism of pancreatitis has been also demonstrated using PAR-2 knock out mice,

in which luminal administration of either trypsin or PAR-2-AP had significantly lower effect on both $[Ca^{2+}]_i$ and pH_i.

3. Therapeutic perspectives and clinical significance

The CFTR chloride channel is clearly the most investigated and most utilized ductal channel that has been targeted for therapy [50; 51]. In the first decades after the discovery of the CFTR gene, only symptomatic therapy was available. Difficulties over the years have been caused by the heterogeneity of CFTR gene mutations. Therefore, it is almost needless to say, that CF is typically a disease where personalized therapy needs to be considered. However, one approach that would be potentially be suitable for all people with CF, is gene therapy. The first randomized clinical trial, of a non-viral-based gene therapy for CF, was performed by Alton EWFW et al. They found that a 12-month-treatment by pGM169/G67A gene therapy formulation improved lung function among the CF patients [52]. Although the results were very promising, no further trials have taken place since the original observation, although pre-clinical development of a viral-mediated gene therapy treatment for CF is underway (https://www.cff.org/Trials/Pipeline/details/10160/Spirovant-Sciences). However, in the last decade the orally bioavailable correctors, potentiators and suppressors of CFTR gene mutations have become available for treatment [53].

CFTR-directed therapies may also be useful for the treatment of pancreatitis, since recent animal studies have suggested that strategies that help maintain levels of HCO_3^- secretion would limit the extent of pathology induced by bile and alcohol [39; 40; 54]. Furthermore, the effects of ethanol and ethanol metabolites on CFTR are consistent with reduced biogenesis, accelerated plasma membrane turnover, as well as channel inhibition [39]. Thus restoring cell surface expression and activity of CFTR may partly alleviate the ethanol-induced damage. This potentially could be through use of CFTR correctors (Lumacaftor, Tezacaftor), as well as potentiators (Ivacaftor) to improve channel activity. We have recently found that Ivacaftor (VX-770) and Lumacaftor (VX-809) restore the alcohol-induced CFTR expression defect in pancreatic ductal cells suggesting that Orkambi® may serve a therapeutic option in acute, recurrent or chronic pancreatitis [55]. Akshintala VS showed recently that CFTR modulators, alone or in combination, reduced the risk of recurrent acute pancreatitis within a 3-year-follow up therapy in adult CF patients [56]. A 24-year-old CF patient with R117H/7 T/F508del mutations with recurrent acute pancreatitis were reported no pancreatitis during ivacaftor therapy [57]. Carrion A et al. also found a reduced frequency and recurrence rate of pancreatitis in patients with CF during Ivacaftor therapy [58]. Both the basic research results and the

pancreatitis-free periods achieved in CF patients suggest that the drugs used for treating CF patients should be tested in randomized clinical trials in non-CF patients with recurrent pancreatitis as well.

Another possible way to compensate for defective CFTR would be to target alternative ion channels, such as the calcium-activated Cl channel, TMEM16A [59; 60], or transporters such as the SLC26 family members (A3, A6 or A9), or short-circuiting their regulation by CFTR and rebalancing exocrine homeostasis. It has been shown that variants (SNPs) in the SLC26A9 anion transporter influence disease severity in the CF lungs and intestinal tract, and therefore act as gene modifiers. Importantly, a recent study has suggested that SNPs in SLC26A9 also influence the degree of pancreatic insufficiency [61]. Furthermore, variants of SLC26A9 also influence the extent of CF-related diabetes, which may be due to beneficial effects of restoring ductal bicarbonate secretion on endocrine (islet) function in CF [62]. This opens up the possibility of targeting this anion transporter as a potential therapeutic target to slow the progression of exocrine dysfunction in CF. One important advantage of this 'alternate non-CFTR approach' is that it would benefit all CF patients regardless of genotype.

4. Summary

One of the most important functions of ductal cells is their ability to neutralize acidic pH within the pancreas and duodenum. This ability of ductal cells is due to the secretion of a HCO_3^- rich fluid, which results from the coordinated action of ductal ion channels and transporters. Impairment of transport processes can result in a decrease in both the volume and pH of pancreatic fluid, which can predispose to inflammatory processes and consequently the development of various diseases. In the case of CF, it is well known that the inadequate function of the CFTR channel underlies the disease, however, it is only recently that research has shed light on the pathological role of ductal cells in pancreatitis. Various etiological factors such as bile acids and EtOH are now known to impair ductal HCO_3^- and fluid secretion, which are likely to play an important role in initiating pancreatitis by creating an unfavorable pH environment. Consequently, drugs that enhance the function of ion transporters may be of great importance not only in CF therapy, but also in the treatment of pancreatitis.

5. Reference list

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6. Figure legends

Figure 1. Major iontransporters of pancreatic ductal cells. HCO_3^- enters the ductal cell directly via the basolateral Na^+/HCO_3^- cotransporter (NBC). In addition, carbonic anhydrase (CA) is involved in the intracellular accumulation of HCO_3^-, by catalyzing the formation of HCO_3^- and H^+ from carbonic acid. The resulting H^+ leaves the cell via the H^+ pump or Na^+/H^+ exchanger (NHE). HCO_3^- is secreted into the lumen through the Cl^-/HCO_3^- exchangers and the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel, respectively.

Table 1. The effects of bile, ethanol and trypsin on pancreatic ductal HCO_3^- secretion