The role of Ca^{2+} signaling in the physiology and pathophysiology of the pancreatic ductal cells

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Ph.D. Thesis

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LIST OF FULL PAPERS RELATED TO THE THESIS

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I. Pallagi P*, **Görög M***, Papp N, Madácsy T, Varga Á, Tim Crul, Szabó V, Molnár M, Dudás K, Grassalkovich A, Szederkényi E, Lázár Gy, Venglovecz V, Hegyi P, Maléth J. Bile acid- and ethanol-mediated activation of Orai1 damages pancreatic ductal secretion in acute pancreatitis.
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[IF_{2021}: 5.182]

*** Megosztott első szerzők**

II. Fanczal J, Pallagi P, **Görög M**, Diszházi G, Almássy J, Madácsy T, Varga Á, Csernay-Biró P, Katona X, Tóth E, Molnár R, Rakonczay Z Jr, Hegyi P, Maléth J. TRPM2-mediated extracellular Ca^{2+} entry promotes acinar cell necrosis in biliary acute pancreatitis.

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III. Molnár R, Madácsy T, Varga Á, Németh M, Katona X, **Görög M**, Molnár B, Fanczal J, Rakonczay Z Jr, Hegyi P, Pallagi P, Maléth J., Mouse pancreatic ductal organoid culture as a relevant model to study exocrine pancreatic ion secretion.

Lab Invest. 2020 Jan; doi: 10.1038/s41374-019-0300-3

[IF_{2018}: 5.662]

Number of full publications: 3 (1 first author)

Cummulative IF: 16.026

CONTENTS

LIST OF ABBREVIATIONS

AP: Acute pancreatitis

ATP: Adenosine triphosphate

BCECF-AM: 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester

BSA: Bovine serum albumine

cAMP: Cyclic adenosine monophosphate

CDC: Chenodeoxycholate

cDNA: Complementary deoxyribonucleic acid

$[Ca^{2+}]_i$: Intracellular Ca^{2+} concentration

CFTR: Cystic fibrosis transmembrane conductance regulator

Cl^-: Chloride ion

$[Cl^-]_i$: Intracellular Cl^- concentration

CO_2: Carbon dioxide

CPA: cyclopiazonic acid

DMEM: Dulbecco's Modified Eagle Medium

ER: Endoplasmic reticulum

Fura-2-am: Fura-2-acetoxymethyl ester

HBSS: Hanks' Balanced Salt solution

HCO_3^-: Bicarbonate ion

HEPES: 4-2-hydroxyethyl)-1-piperazineethanesulfonic acid

IP_3: Inositol 1,4,5- trisphosphate

IP_3R: Inositol 1,4,5- trisphosphate receptor

IRBIT: Inositol 1,4,5-trisphosphate receptor-binding protein

KO: Knock-out

MCU: Mitochondrial Ca^{2+} uniporter

MQAE: N-(Ethoxycarbonylmethyl)-6-Methoxyquinolinium Bromide 5

mRNA: Messenger ribonucleic acid

Na-TC: Na-taurocholate

NCX: Na^{+} / Ca^{2+} - exchanger

NHEs: Na^{+}/H^{+} exchangers

PA: Palmitic acidPBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PDEC: Pancreatic ductal epithelial cell

PFA: Paraformaldehyde

pH_i: Intracellular pH

PMCA: Plasma membrane Ca^{2+} ATP-ase

pNBC1s: Na^{+}/ HCO_3^{-} cotransporters

POA: Palmitoleic acid

ROS: Reactive oxygen species

SAM: Sterile α-motif

SERCA: Sarco/endoplasmic reticulum Ca^{2+} ATPase

SLC26: Solute carrier family 26

SOC: Store operated Ca^{2+} channel

SOCE: Store operated Ca^{2+} channel entry

TBS: Tris(hydroxymethyl)aminomethane buffered saline

TRPC: Transient receptor potential canonical

TRPM: Transient receptor potential melastatin

WNK/SPAK kinase: With-no-lysine/ SPS1-related proline/alanine-rich kinase

WT: Wild type

1. INTRODUCTION

1.1. The exocrine pancreas

The pancreas is a complex organ with exocrine and endocrine parts and located in the retroperitoneal region of the upper abdominal cavity. 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[1,2]. It has long been implied that the main function of pancreatic ductal epithelial cell (PDEC) is to provide a mechanical framework for acinar cells. In 1986, Barry Argent and colleagues developed a method that allowed the isolation of intact pancreatic ducts and PDECs[3]. Since then, it was possible to separately study the function of duct cells. Although the duct cells create 5% of the pancreas, a large proportion of the secreted pancreatic fluid is due to the ductal cells[4]. Nevertheless, the morphology of acinar cells is uniform, the structure of duct cells is much more various[5]. Epithelia are different as they are columnar in the large distal ducts and cuboidal in the proximal small ducts[6]. HCO_3^- secretion is said to occur primarily in the proximal part of the ducts[7]. The amount of daily secreted pancreatic fluid in humans is between 1 liter and 2,5 liter. The secretion depends on the body size and reduces with age[8]. The physiological function of alkaline secretion is in one hand to neutralise protons secreted by acinar cells, to flush digestive enzymes to the duodenum, and to neutralise the gastric acid entering the duodenum[9].

1.2. Physiology of pancreatic ductal HCO_3^- secretion

The secreted Cl^- by acinar cells is exchanged for HCO_3^- by the ductal cells to produce an alkaline fluid, which is essential for normal digestion[4,8,10]. The accumulation of HCO_3^- inside the ductal cell across basolateral membrane thought to be mediated by direct mechanism through Na^+/ HCO_3^- cotransporters (pNBC1) or indirectly via the passive diffusion of carbon dioxide (CO_2) across the cell membrane. CO_2 is converted to carbonic acid by the carbonic anhydrase enzyme and after hydrolyzed to HCO_3^- and H^+[11]. Backward transport of protons is mediated by Na^+/H^+ exchangers (NHEs) and an H^+-ATPase[7]. HCO_3^- secretion can occur through the apical membrane of PDECs via solute carrier family 26 (SLC26) anion exchangers and the cystic fibrosis transmembrane conductance regulator (CFTR)[10]. Although CFTR is predominantly a Cl^- channel, it can also handling HCO_3^- and other anions. Gray et al. have reported that CFTR is 3-5 times more selective for Cl^- than HCO_3^-[12,13].

HCO_3^- permeability of CFTR is regulated by With No-Lysine (WNK)/STE20/SPS1-associated proline/alanine-rich kinase (SPAK) kinase pathway, and sensitive to intracellular Cl^-[14,15]. The other key regulator factor in HCO_3^- secretion of CFTR is inositol 1,4,5- trisphosphate (IP_3) receptor (IP_3R)–binding protein released with IP_3 (IRBIT), which coordinates also basolateral HCO_3^- uptake and mediates synergy between Ca^{2+} and cyclic adenosine monophosphate (cAMP) signaling pathways[16]. It is demonstrated that luminal application of adenosine triphosphate (ATP) stimulated fluid and HCO_3^- secretion and raised $[Ca^{2+}]_i$[17]. Acetylcholine also plays a role in the regulation of ductal HCO_3^- secretion via elevation of $[Ca^{2+}]_i$[18,19]. Transporters and regulatory mechanism of pancreatic HCO_3^- secretion is summarized in Fig.1.

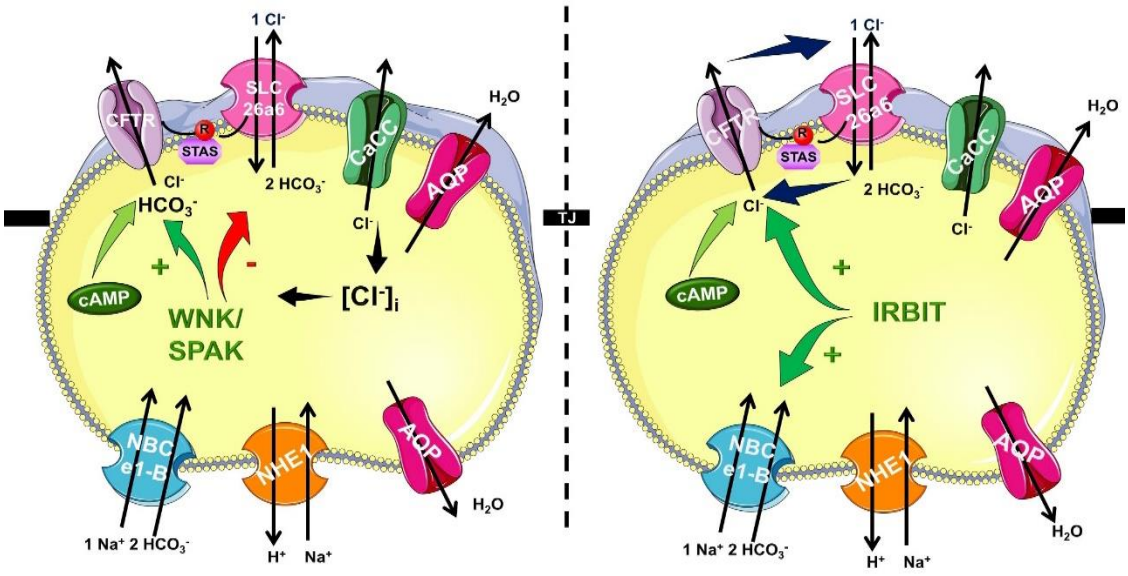


Figure 1. Mechanism of pancreatic ductal HCO_3^- secretion. Pancreatic ductal cells secrete HCO_3^- rich isotonic fluid as a result of a complex interplay by several transport proteins. HCO_3^- is accumulated across the basolateral membrane Na^+/HCO_3^- cotransporter NBCe1-B. Via the luminal membrane HCO_3^- is secreted by SLC26 Cl^-/ HCO_3^- exchangers and cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel. **Schematic depiction: Madácsy T. et al. Front Physiol. 2018 Dec 20;9:1585.**

1.3. Regulation of pancreatic ductal secretion by Ca^{2+} signaling

Complex signaling system is responsible for the physiological regulation of pancreatic ductal HCO_3^- secretion, in which intracellular Ca^{2+} play crucial roles, however uncontrolled Ca^{2+} release can lead to intracellular Ca^{2+} overload and toxicity, including mitochondrial damage and impaired ATP production[15]. There are two mechanisms which can maintain the

balance of the intracellular Ca^{2+} concentration: the Ca^{2+} entry into the cytosol via the plasma membrane ion channels and the Ca^{2+} clearance from the cytosol via the plasma membrane Ca^{2+} pump. If we would be able to block the entry or maintain the clearance, we could reduce the toxicity of sustained Ca^{2+} signals and thus cell damage.

1.3.1. Calcium release

During stimulation, phospholipase C β (PLCβ) is activated by agonist which binds to G protein coupled receptor. Phosphatidylinositol 4,5-bisphosphate (PIP_2) is hydrolized by activated PLCβ to inositol trisphosphate (IP_3) and diacylglycerol[20]. IP_3 receptors (IP_3R) are a Ca^{2+} release channels located in the endoplasmic reticulum (ER) membrane, which are activated by IP3 binding[21]. IP_3R2 and 3 are the major isoforms of IP_3Rs in exocrine pancreas[22,23] and the localization of IP_3R2 was demonstrated close to the apical pole of PDEC[24]. Besides channel opening and Ca^{2+} release, IP3 binding to their receptors also release IRBIT, which play crucial role in several regulatory process of PDEC[16,24,25]. Ca^{2+} influx via PM is induced by depletion of ER Ca^{2+} stores (calcium entry), which could contribute to the intracellular Ca^{2+} signals during longer stimulations (see below). Since a long sustained intracellular Ca^{2+} elevation can be toxic, it is necessary to extrude Ca^{2+} from the cytoplasm (calcium clearance).

1.3.2. Calcium entry

The release of endoplasmic reticulum (ER) Ca^{2+} stores by biologically active compounds (such as bile acids, or fatty acid ethyl esters) activates the influx of the extracellular Ca^{2+} via the Orai1 Ca^{2+} channel. This process is referred as store operated Ca^{2+} entry (SOCE). Although SOCE is part of the physiological Ca^{2+} signaling events in non-excitable cells, under pathological conditions it significantly contributes to the sustained intracellular Ca^{2+} overload. Regardless of different AP-inducing pathogenic factors, all cases are associated with sustained elevated intracellular Ca^{2+} concentration, which are a hallmark of AP pathogenesis[26]. On the other hand, independently from toxins increased intrapancreatic pressure due to ERCP can also lead to extracellular Ca^{2+} influx via the activation of the mechanoreceptor Piezo1 and TRPV4[27]. The other player of Ca^{2+} entry is transient receptor potential melastatin-2, which is sensitive to oxidative stress and can trigger the elevation of intracellular Ca^{2+} concentration.

Ca^{2+} entry is mediated by the store operated Ca^{2+} channels (SOC), mainly Orai1[28,29]. The Orai proteins have four putative transmembrane-spanning domains (TM1–4) (Figure 2)[30]. Orai2 and Orai3, human homologs of Orai1, also form Ca^{2+} -selective store-operated channels when co-expressed with Stim1[31]. Stim1 is a single-pass transmembrane protein located in the ER

membrane. The N terminus of Stim1 is located within the ER lumen[32]. During store operated Ca^{2+} entry an agonist binds to and activates IP_3 receptors (IP_3R) and causes the release of Ca^{2+} from ER, which leads to oligomerization and conformational switch of Stim1. Stim1 binds to Orai1 and trigger Ca^{2+} influx. Summarized, release of Ca^{2+} from ER induces the activation of the store-operated Ca^{2+} channels localized in the plasma membrane as showed by Figure 1[33,34].

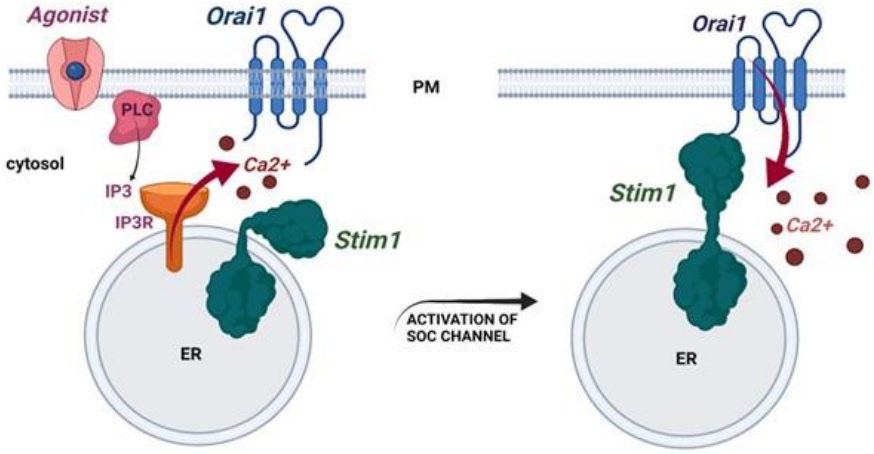


Figure 2. Store operated Ca^{2+} entry. Release of ER Ca^{2+} stores induce extracellular Ca^{2+} influx through the Orai1 Ca^{2+} channel. Following ER Ca^{2+} depletion, the Stim1 Ca^{2+} sensor protein is activated and translocates to ER-plasma membrane junctions to activate the Orai1 Ca^{2+} channel in the PM, through which extracellular Ca^{2+} flows into the cells.

Two different publications confirmed the role of selective Orai1 inhibition by GSK-7975A and CM-128 in the reduction of the severity of AP via decrease of bile acid-mediated extracellular Ca^{2+} influx and sustained Ca^{2+} overload in pancreatic acinar cells[35]. Moreover, CM-5480-mediated selective inhibition of Orai1 abolished myeloperoxidase activity and inflammatory cytokine expression in pancreatic and lung tissues and prevented oxidative burst in neutrophils [36]. Although the beneficial effect of selective Orai1 inhibition in AP is well-established, precisely how Orai1 inhibition affects pancreatic ductal secretion is unknown.

1.3.3. Calcium clearance

There are two ATP dependent mechanisms which are responsible for the calcium clearance: the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump, which returns Ca^{2+} from the cytosol to the endoplasmic reticulum (ER); and the plasma membrane Ca^{2+} ATP-ase (PMCA) pump, which moves Ca^{2+} from the cytosol to the extracellular space. SERCA has three water-filled pores which form cytosolic and luminal pathways. SERCA uses the energy produced by ATP hydrolysis to pump two Ca^{2+} ions from the cytosol to the ER, and two H^+ from the ER to

the cytosol[37,38]. PMCA is the major Ca^{2+} efflux pathway in non-excitable cells, such as pancreatic acinar cells, where the Na^{+}/Ca^{2+} - exchanger (NCX) is not expressed[39,40]. PMCA is encoded by four separate genes (PMCA1-4). PMCA1 and PMCA4 are ubiquitously expressed, PMCA2 and PMCA3 are expressed in β-cells[41,42].

1.4. The role of pancreatic ductal cells in acute pancreatitis

It is well know that impaired pancreatic ductal fluid and HCO_3^- secretion can lead to pancreatic damage and to the development of acute pancreatitis (AP). AP is one of the most common inflammatory disease of the gastrointestinal tract with high morbidity and mortality. The incidence of AP is increasing and has become the leading causes of acute hospitalization among non-malignant gastrointestinal diseases[43]. Many different risk factors can cause AP such as impacted gallstone, heavy alcohol consumption, hypertrigliceridaemia or through iatrogenic side effects of medical treatments like Asparaginase or endoscopic retrograde cholangiopancreaticography (ERCP) thus representing a major clinical challenge[44]. Primarily AP is a localized to and represents a sterile inflammation of the exocrine pancreas, which however can lead to local and systemic complications. Infected necrosis of the pancreatic tissue and surrounding visceral fat are the main local complications, whereas acute lung injury and renal dysfunction include the systemic complications which lead to multiorgan failure. Beside complications it has to be mentioned that nowdays there is no specific therapy to treat or prevent the progression. The severity of AP can vary among mild (no multiorgan failure), moderate (multiorgan failure is only temporal, < 48 h) to severe form (multiorgan failure is only permanent, > 48 h). The mortality rate of all AP types is ~ 3%, however in severe cases it can reach to 28-30%[44,45].

Although several factors are responsible for the development of AP, cell toxicity caused by sustained Ca^{2+} overload is a hallmark of the disease[46]. In pancreatic acinar cells, elevation of intracellular Ca^{2+} concentration can lead to premature trypsinogen activation[47], mitochondrial damage and cell necrosis[48]. Numerous publications demonstrated the contribution of several Ca^{2+} channels localized in PM in the cell damage during AP. Gerasimenko et al. showed the protective effect of the inhibition of extracellular Ca^{2+} entry via Orai1 in necrosis of acinar cell *in vitro*[49], and it was also confirmed that pancreatic oedema, inflammation and necrosis were significantly reduced by inhibition of Orai1 in experimental models of AP[35]. Additionally, Kim et al confirmed the role of an other Ca^{2+} channels (TRPC3) in development of AP. They found that deletion of TRPC3 significantly reduced sustained increase in cytosolic Ca^{2+} levels evoked by bile acid, prevented the pathologic inhibition of digestive enzyme secretion *in vitro* and

severity of cerulein-induced AP *in vivo*[50]. Ca^{2+} uptake into mitochondria is an important regulator of cytoplasmic Ca^{2+} signals, and play crucial roles in the spatial and temporal localization of the intracellular Ca^{2+} signals[51]. However, pathological elevation of mitochondrial Ca^{2+} concentration can trigger the opening of the mitochondrial membrane permeability pore (MPTP) across the inner and outer membranes of mitochondria, and the mitochondrial membranes will permeable to molecules and ions with molecular mass less than 1.5 kDa, including protons and water[52]. The $(\Delta\Psi)_m$ is disappeared by the changes of mitochondrial membrane permeability, mitochondria become swollen, their membranes ruptured and the energy production of the cells is decreased[52–54]. As mentioned above, SERCA and PMCA are ATP dependent Ca^{2+} pumps and therefore the drop of ATP production can further maintain the sustained Ca^{2+} rise[55] thereby generate vicious cycle which in turn triggers cell necrosis[56,57].

1.5. Pathophysiological Ca^{2+} signal generation in ductal cells

Recent publications confirmed the benefical role of physiological Ca^{2+} signaling in the regulation of PDEC. Howerver, several study showed that sustained intracellular [Ca^{2+}] elevation was induced by the most frequent pathogenic factors for acute pancreatitis, such as bile acids, non-oxidative ethanol metabolites and trypsin in pancreatic ductal cells, which inhibits the cellular functions[48,57,58]. These results suggest, that uncontrolled Ca^{2+} release can lead to sustained intracellular Ca^{2+} elevation which caused cellular damage during acute pancreatitis.

To confirm the role of Ca^{2+} signaling in the regulation of pancreatic HCO_3^- secretion, dose-dependent dual effects of non-conjugated bile acid chenodeoxycholate (CDC) were demonstrated by Venglovecz et al, which can depend on the type of Ca^{2+} signal[58]. Repetitive, short-lasting Ca^{2+} oscillations evoked by low concentrations (100 μM) of CDC stimulate HCO_3^- secretion from the luminal membrane of PDEC. Different type of inhibitors, such as IP_3R inhibitor caffeine, xestospongin C or the PLC inhibitor U73122 abolished the oscillations. If the cells were preincubated with intracellular Ca^{2+} chelator BAPTA-AM, the Ca^{2+} signals and the stimulatory effect of 100 μM CDC on HCO_3^- secretion were also abolished. In contrast, administration of high concentrations (1 mM) CDC induced a toxic sustained Ca^{2+} elevation and caused mitochondrial damage resulting the depletion of ATP production[59] which changes might explain the inhibition acid/base transporters including the basolateral NHE, NBCe1-B and the luminal CBE[58]. In contrast to the effect of low concentration of CDC, BAPTA-AM preincubation could not prevent the formation of Ca^{2+} signal, mitochondrial damage and the

inhibitory effect of CDC on the HCO_3^- secretion (Figure 3). This finding suggest that bile acid has a direct, Ca^{2+}-independent effect to mitochondria. This toxic effect might be explained by the protonophoric-like effect of the high concentration of CDC that can affect the mitochondrial ATP production.

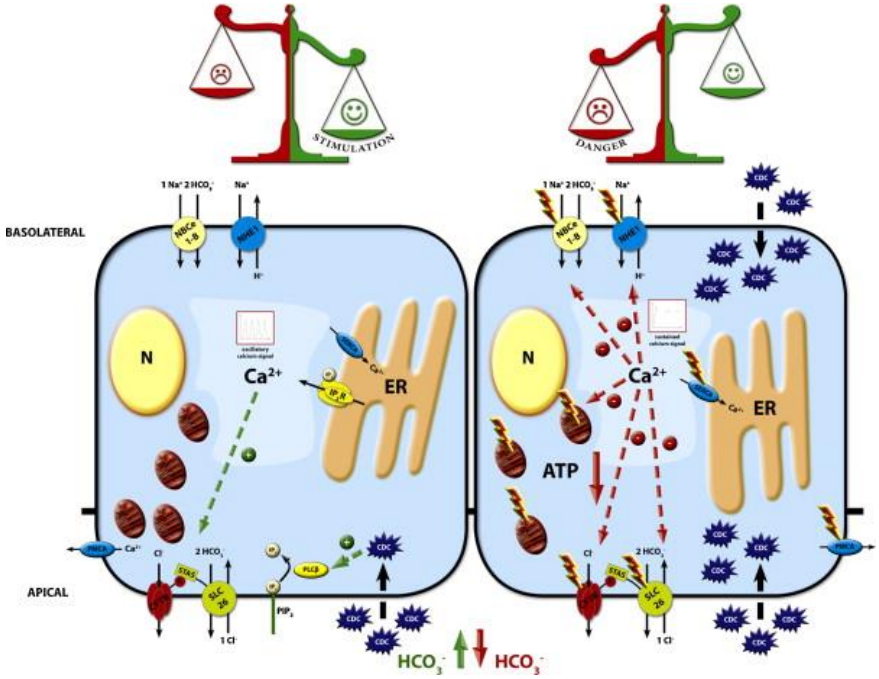


Figure 3. The effects of bile acids on PDEC. Low concentration of CDC induced repetitive Ca^{2+} oscillations and stimulated HCO_3^- secretion from the luminal membrane of PDEC. In contrast, high concentration of CDC induced toxic sustained Ca^{2+} elevation and severe morphological damage of the with a consequent $[ATP]_i$ depletion. These changes inhibited acid/base transporters including the basolateral NHE, NBCe1-B and the luminal SLC26 Cl^-/HCO_3^- exchanger. **Schematic depiction: Maléth J and Hegyi P. Cell Calcium 55 (2014) 337-345.**

Excessive ethanol consumption is the other leading cause of AP. Similarly to bile acids, ethanol and non-oxidative ethanol metabolites can evoke similar intracellular changes in PDEC and also has a dual effects on pancreatic ductal HCO_3^- secretion. Administration of low concentration (1 mM) of ethanol increase the elevation of $[Ca^{2+}]_i$, and secretin-stimulated fluid secretion in guinea pig pancreatic[60]. If 100 mM ethanol was used by Yamamoto et al weak inhibition of stimulated fluid secretion was observed. BAPTA-AM preincubation abolished the stimulatory effect of low concentration of ethanol which suggest that it was mediated by the $[Ca^{2+}]_i$ elevation. In our experiments administration of low concentration ethanol increase HCO_3^- secretion via stimulation of phospholipase C and IP_3-mediated Ca^{2+} release from the endoplasmic reticulum (ER) in CAPAN-1 cells[61]. On the other hand, high concentration of ethanol and non-oxidative ethanol metabolites (POA) evoke sustained intracellular Ca^{2+} elevation by releasing Ca^{2+} from the ER via IP3 and ryanodine receptor activation. Additionally, depleted ATP level was observed, which cause cell necrosis and decrease HCO_3^- secretion in PDEC via inhibition of the apical Cl^-/ HCO_3^- exchanger and CFTR (Figure 4). BAPTA-AM

preincubation completely abolished the inhibitory effects of ethanol and POA, suggesting that the inhibition was mediated by the sustained $[Ca^{2+}]_i$ elevation[61].

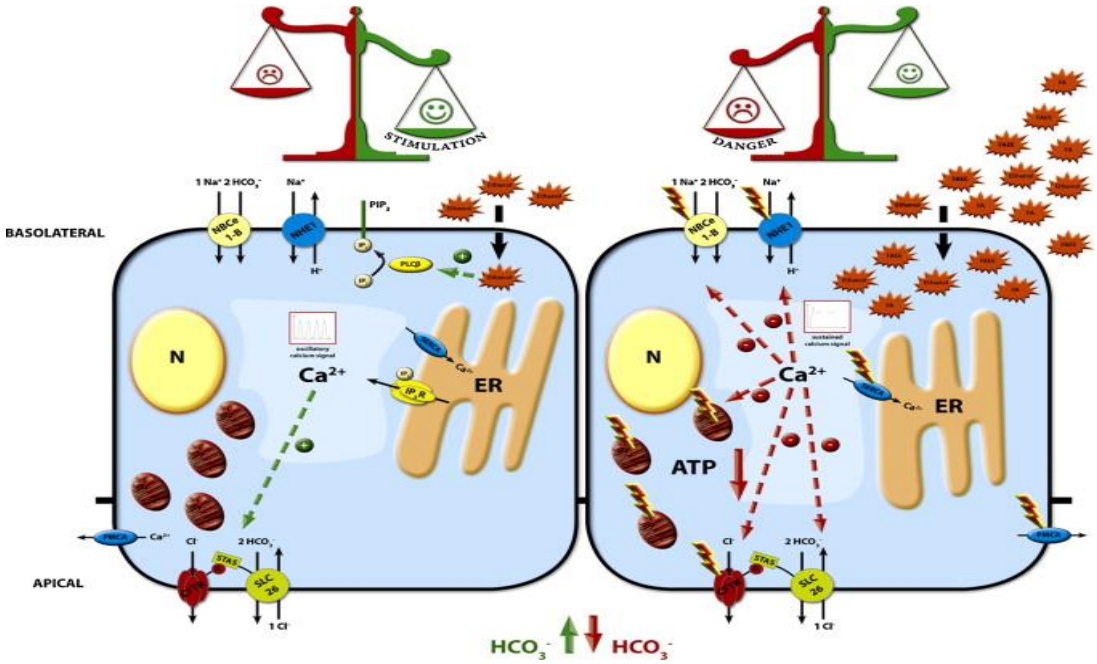


Figure 4. The effects of ethanol and ethanol metabolites in PDEC. Low concentration of ethanol stimulated the HCO_3^- secretion in PDEC via Ca^{2+} release from the ER. In contrast, high concentration of ethanol and fatty acids inhibited acid/base transporters including the basolateral NHE, NBCe1-B and the luminal CFTR, and SLC26 Cl^-/HCO_3^- exchanger. via toxic sustained Ca^{2+} overload and impaired $[ATP]_i$ production. **Schematic depiction: Maléth J and Hegyi P. Cell Calcium 55 (2014) 337-345.**

Early protease activation in the pancreas is a hallmark of the pathogenesis of acute pancreatitis. In our previous work, the effects of trypsin on the pancreatic ductal epithelia was investigated and showed that luminal administration of trypsin or PAR2 antagonist peptide inhibit the luminal Cl^-/ HCO_3^- exchanger and CFTR via elevation of intracellular Ca^{2+} concentration[62]. Moreover, the inhibitory effect of trypsin was abolished by BAPTA-AM preincubation, similarly to the inhibitory effects of ethanol and POA[62].

1.6. The role of reactive oxygen species in Ca^{2+} signaling

Beside cAMP and Ca^{2+}, reactive oxygen species (ROS) is also an important component of the signal transduction in cells and oxidant and antioxidant molecules provide the balance their generation. ROS are produced during mitochondrial respiration derived from complexes I and III of the mitochondrial electron transport chain[63]. Numerous effects of ROS in signalling pathways has been demonstrated in the recent years[64]. To confirm this essential regulatory role in intracellular signalling Booth et al showed the presence of H_2O_2 nanodomain on ER-

mitochondrial interface, which is induced by cytoplasmic Ca^{2+} spikes and is a positive regulator of Ca^{2+} oscillations. Such nanodomains can be considered as important elements of inter-organelle communication[65]. Criddle et al also demonstrated the effects of ROS production on the ion channels and pumps, which play fundamental role in the intracellular Ca^{2+} signalling[66]. However, disruption of lipid membranes, proteins and DNA induced by unbalanced generation of ROS are a crucial steps in the pathogenesis of diseases[67]. The role of ROS production in the pathogenesis of AP was confirmed by Criddle *et al.* who showed that menadion induced ROS production casued acinar cell apoptosis[68]. It has been also suggested that bile acids induces ROS generation in the mitochondria of the pancreatic acinar cells from mice and humans, which was dependent on the increase of mitochondrial Ca^{2+}[69].

1.7. Transient Receptor Potential Melastatin 2

Transient receptor potential melastatin-2 (TRPM2) is a member of Melastatin subfamily of TRP proteins[70] and has been identified to act as a cellular redox-sensor. TRP channels are nonselective cation channels that are activated by different chemical and physical stimuli. TRPM2 is also permeable for Ca^{2+} and play crucial role in different physiological and pathophysiological processes associated with redox signaling and oxidative stress[71]. TRPM2 is expressed in numeous different cell types and organs including pancreatic β cells[72], spleen, neurons[73], bone marrow cells[73,74] and immune cells such as T lymphocytes, macrophages and neutrophils[75–77]. Oxidative stress induced by ROS activate the channel and it can be also became active by free ADP-ribose in synergy with free intracellular Ca^{2+}. ADPR production induced by poly ADP-ribose polymerase enzyme (PARP) is a response to DNA damage evoked by oxidative stress and promotes apoptotic cell death. Oxidative stress could induce the production of free ADPR in the mitochondria therefore it is the another source of ADPR. TRPM2 has a crucial role in the development of inflammatory disorders[78]. Chemokine production were increased by Ca^{2+} influx via TRPM2 in monocytes, resulting to elevation of neutrophil infiltration in inflammatory bowel disease[76]. Furthermore, the role of TRPM2 has been established in the pathogenesis of irradiation-induced xerostomia. Liu et al. demonstrated that the elevated ROS production induced by irradiation activated TRPM2 and lead to extracellular Ca^{2+} overload in salivary glands, which damaged acinar cells and resulted loss of acinar cell function (saliva production) in the salivary glands[79]. Liu et al also reported that radiation treatment caused persistent salivary gland dysfunction via activation of intrinsic, TRPM2-dependent mitochondrial pathway of apoptosis, that resulting caspase-3-mediated cleavage of stromal interaction molecule 1 (STIM1), which then reduced store-operated Ca^{2+} entry[80]. It has

been also suggested, that TRPM2 is involved in diabetic stress-induced mitochondrial fragmentation in pancreatic β cells. Abuarab *et al.* demonstrated that ROS production is enhanced and TRPM2 is activated by high extracellular glucose concentrations, which caused permeabilization of lysosomal membranes and induced Zn^{2+}-mediated mitochondrial fission[81]. Several publications proved that TRPM2 could play key role in the regulation of insulin secretion and could represent a new target for diabetes therapy[72,82,83], however, the role of TRPM2 in other secretory function of the pancreas, such as fluid and HCO_3^- secretion of PDEC, has not yet been investigated.

2. AIMS

It is evident that Ca^{2+} signaling has important role in the regulation of pancreatic ductal physiology and pathophysiology, however several crucial questions are not answered yet. Thereofre during my Ph.D. studies I investigated the expression and function of two plasma membrane Ca^{2+} channels in pancreatic ductal cells.

Aim 1.

Inhibition of the Orai1 Ca^{2+} channel prevented the sustained elevation of the intracellular Ca^{2+} in pancreatic acinar cells and significantly decreased the severity of the experimental acute pancreatitis. However, the role of Orai1 channel in the physiological and pathophysiological function of the pancreatic ductal epithelial cells is currently unknown.

Specific aims:

1. To characterize the expression and function of Orai1 in pancreatic ductal cells.
2. To examine the role of Orai1 in bile acid and ethanol+PA induced ductal cell damage.
3. To investigate the role of Orai1 inhibition on pancreatic ductal function during AP.

Aim 2.

TRPM2 is a non-selective cation channel that mediates extracellular Ca^{2+} influx during oxidative stress in several cell types, however the presence and role of TRPM2 in pancreatic ductal cells is unknown.

Specific aims:

1. To examine the expression and functional activity of the TRPM2 channel in isolated pancreatic ductal fragments.
2. To characterize the role of TRPM2 in bile acid induced pancreatic ductal cell damage.

3. MATERIALS AND METHODS

3.1. Animals

2 months male FVB/N mice (20-25g) were used with adherence to the NIH guidelines and the EU directive 2010/63/EU for the protection of animals used for scientific purposes. The study was approved by the National Scientific Ethical Committee on Animal Experimentation under license number XXI. /1541 /2020.

TRPM2 knockout mice were generously provided by Yasuo Mori. The knockout mice were generated from a C57BL/6 background as described previously[76]. TRPM2 +/+ and TRPM2 −/− mice were bred from TRPM2+/− animals. Mice were genotyped using a standard polymerase chain reaction (PCR) assay[79]. 2 mounths male mice (20-25g) were used. The animals were kept at 22-24°C with a 12 h light–dark cycle. Mice were used with adherence to the NIH guidelines and the EU directive 2010/63/EU for the protection of animals used for scientific purposes. The study was approved by the National Scientific Ethical Committee on Animal Experimentation under licence number XXI. /2523/2018.

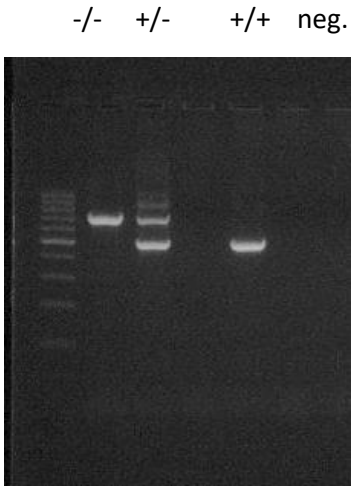


Figure 5. Agarose gel electrophoresis. The band at ~500 bp indicates the homozygous TRPM2 wild type (WT) (+/+), the band at ~700 bp indicates the TRPM2 knock out (KO) (-/-), the band at ~700 bp and ~500 bp shows the heterozygous (+/-) mice individuals.

3.2. Isolation of mouse pancreatic ductal fragments

Pancreatic ductal fragments were isolated as described earlier[58]. Briefly, following pentobarbital-induced terminal anesthesia, the pancreas was surgically removed, placed into cold Dulbecco's Modified Eagle Medium (DMEM/F12). The pancreas was injected with 100 U/ml collagenase, 0.1 mg/ml trypsin inhibitor, 1 mg/ml bovine serum albumin in DMEM/F12 and placed into a shaking water bath at 37ºC for 30 min. Small intra/interlobular ducts are then

isolated by microdissection using stainless steel needles under a stereomicroscope. The ducts were cultured in a 37°C incubator gased with 5% CO_2-95% air until the using.

3.3. Isolation of mouse pancreatic acinar cells

Pancreas was placed into ice-cold Hanks' Balanced Salt solution (HBSS). Next, the tissue was cut into small pieces and placed into 10 ml isolation solution (10 ml HBSS (Sigma; 8264), 200 U/ml of collagenase (Worthington; 5273), 10 mM (4-2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) (Sigma; 3375)). The tissue was incubated for 30 min at 37 ºC and it was vigorously shaken every 5 minutes during the incubation. Next, the pancreas was placed into 10 ml ice-cold buffered washing solution (10 ml HBSS (Sigma; 8264), 10 mM Hepes (Sigma; 3375), 0.5 ml Fetal Bovine Serum) and centrifuged at 700 rpm on 4°C for 2 min. This was repeated twice. At the end the pellet was resuspended in 1 ml HBSS solution. The acinar cells were kept in a 37°C incubator gased with 5% CO_2-95% air until the using.

3.4. Fluorescent microscopy

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), intracellular Cl^- concentration ($[Cl^-]_i$), or intracellular pH (pH_i) were measured as described earlier[84]. Isolated mouse pancreatic ductal fragments were attached to a poly-L-lysine-coated coverslip (24 mm diameter) as the base of a perfusion chamber. The ductal fragments were imaged using Olympus IX71 fluorescent microscope equipped with an MT-20 illumination system to asses pH with BCECF-acetoxymethyl ester (BCECF-AM) (1 μmol/L) 434/17 nm and 497/16 nm single-band bandpass filters for excitation and 537/26 nm single-band bandpass filters for emission, to Ca^{2+} with Fura-2-acetoxymethyl ester (Fura-2-AM) (2 μmol/L) 340/26 nm and 387/11 nm single-band bandpass filters for excitation and 510/84 nm single-band bandpass filters for emission, to Cl^- with N-(Ethoxycarbonylmethyl)-6-Methoxyquinolinium Bromide 5 (MQAE) (2 μmol/L) 340/26 nm single-band bandpass filters for excitation and 510/84 nm single-band bandpass filters for emission. The signal was captured by a Hamamatsu ORCA Flash 4.0 V3 CMOS camera through a 20X oil immersion objective (Olympus; NA: 0.8) with a temporal resolution of 1 sec. Ratiometric image analysis was performed by Olympus excellence software.

3.5. Immunofluorescence staining

Upon freezing in Shandon Cryomatrix, isolated pancreatic ducts were sectioned and stained as previously described[85]. Isolated mouse pancreatic ducts were labelled with Trypan Blue (Trypan Blue solution, Sigma, Cat.No. 93595-50ML) for 10 min then frozen -20°C in Shandon Cryomatrix (ThermoFisher Scientific, Cat. No.: 6769006) and sectioned 7μm thicks with

cryostat (Leica CM 1860 UV) at -20°C. Sections were fixed in 4% Paraformaldehyde-Phosphate-buffered saline (PFA-PBS) for 15 min then washed in 1x Tris buffered saline (TBS) for 3 x 5 minutes. Antigen retrieval was performed in Sodium Citrate Buffer - Tween20 buffer (0.001 M Sodium Citrate Buffer, pH 6.0 and 0.05% Tween20) on 92°C for 30 min. Sections were blocked with 0.1% goat serum and 10% bovine serum albumin (BSA)-TBS for 1 h. The sections were incubated with an anti-mouse ORAI1 antibody (Anti-ORAI1 (extracellular) Antibody, Alomone Labs, Cat. No ACC-062) or an ATTO-594 conjugated rabbit polyclonal primary antibody against TRPM2 (Anti - TRPM2 - ATTO-594; Alamone Labs; Cat. No: ACC-043-AR) in 1:200 dilution in 5xBSA-TBS overnight on 4°C. After washing three times with TBS the cells were labelled with Alexa Fluor 488 Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody (Invitrogen, Cat. No A11034, 1:400) for 2h at room temperature. Nuclei were visualized through staining with 1 μg/ml Hoechst33342 (ThermoFisher Scientific; Cat. No.: 62249) for 15 min and sections were mounted with Fluoromount (Sigma-Aldrich; Cat. No.: F4680). Images were captured with a Zeiss LSM880 confocal microscope using a 40X oil immersion objective (Zeiss, NA: 1.4).

3.6. Gene expression analysis and gene knockdown

Total messenger ribonucleic acid (mRNA) from acini and ductal fragments was purified with NucleoSpin RNA XS kit according to the manufacturer's instructions. One μg mRNA was used to synthetize complementary deoxyribonucleic acid (cDNA) with iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA; Cat. No.: 1708890). Conventional PCR amplification was performed with DreamTaq Hot Start DNA Polymerase and cDNA-specific Orai1 primers (forward: 5' CTTCGCCATGGTAGCGAT 3'; reverse: 5' TGTGGTGCAGGCACTAAAGA 3') for 35 cycles. For gene knockdown studies, isolated mouse ductal fragments were transfected with 50 nM pre-designed siRNA for Stim1 or siGLOGreen transfection indicator with Lipofectamine 2000 in feeding media for 24h.

3.7. In vivo acute pancreatitis models

In the cerulein-induced AP model, AP mice received 7 hourly injections of cerulein (50 μg/kg, i.p.) whereas control animals received injections containing physiological saline (i.p.) solution. One hour after the first cerulein injection, CM-5840 (20 mg/kg, i.p.) was administered. Twelve hours after the first cerulein injection, mice were sacrificed with pentobarbital (85 mg/kg, i.p.).

Biliary AP was induced by intraductal administration of 4% Na-taurocholate as previously described by Perides et al.[86]. Briefly, mice were anesthetized with a ketamine and xylazine

(respectively 125 mg/kg and 12.5 mg/kg, i.p.) cocktail followed by median laparotomy, the common biliopancreatic duct was cannulated across the duodenum with a 0.4 mm diameter needle connected to an infusion catheter, and the bile duct was occluded with a microvessel clip. Next, the mice received 2 µl/bwg of 4% Na-taurocholate (Na-TC) or physiological saline at a perfusion rate of 10 µL/min (TSE System GmbH). Following the infusion, the abdominal wall and skin were closed separately and the mice were placed on a heating pad until waking while buprenorphine (0.075 mg/kg, i.p.) was administered to relieve the pain. One hour after the 4% Na-TC infusion, the animals received CM-5480 (20 mg/kg, i.p.). Twenty-four hours after the operation, the mice were anesthetized with pentobarbital (85 mg/kg, i.p.) and sacrificed through exsanguination through the heart.

The mouse model of acute alcohol-induced pancreatitis was originally developed by Huang et al[87]. Mice received 2 hourly injections of ethanol (1.35 g/kg, i.p) mixed with palmitoleic acid (150 mg/kg). To prevent ethanol-induced peritoneal irritation, 200 µl physiological saline was injected before the ethanol/POA treatment. One hour after the first and directly before the second ethanol /POA injection, CM-5480 (20 mg/kg, i.p.) was administered. Control mice received 200 µl physiological saline (i.p.) instead of ethanol/POA. Twenty-four hours after the first ethanol/POA treatment, the mice were sacrificed under pentobarbital (85 mg/kg, i.p.) anaesthesia.

For all experimental models histological parameters were monitored to estimate the severity of induced pancreatitis. For histological scoring, pancreata were quickly removed, cleaned from fat and lymph nodes, and stored at 4°C in 4% formaldehyde. Paraffin-embedded pancreas samples were sliced in 4 µm thick sections and stained with haematoxylin–eosin. To estimate severity of induced pancreatitis, edema, inflammatory cell infiltration, and necrosis of the samples were scored by three independent investigators blinded to the protocol (0–5 points for edema, leukocyte infiltration and necrosis for the total histological score, or % of total area for 5). Averages of the obtained scores are included in the manuscript.

3.8. In vivo measurement of pancreatic fluid secretion

In all experimental pancreatitis models, pancreatic fluid was collected in vivo directly before sacrifice. Mice were anesthetized with ketamine/xylazine cocktail (respectively 125 mg/kg and 12.5 mg/kg, i.p.) and placed on a heated pad to maintain body temperature. The operation was performed as described in case of 4% Na-TC-induced AP. Following stimulation with secretin (0.75 Clinical Unit/kg, i.p.) for 30 min, the pancreatic juice was collected and the secretory rate was calculated as µl/body weight g for 1 h.

3.9. Statistics

Statistical analysis was performed with Graphpad Prism software. All data are expressed as means ± SEM. Both parametric (one-way analysis of variance) and nonparametric (Mann Whitney test and Kruskal-Wallis test – used for analysis of the acinar cell survival assay) tests were used based on the normality of data distribution. A p value below 0.05 was considered statistically significant.

4. RESULTS

4.1. Orai1 is expressed on the apical plasma membrane of pancreatic ductal epithelia

First, we analyzed the expression of Orai1 in mouse primary pancreatic ductal epithelial cells. End-point PCR analysis of acini and ductal fragments showed that the Orai1 gene is expressed both in pancreatic acinar and ductal cells **(Figure 6.A.)**. When immunofluorescent labelling of Orai1 was performed on ductal cells, the confocal images showed that Orai1 channels were expressed on the apical membrane of the pancreatic ductal cells and in the surrounding cells also. **(Figure 6.B.)**.

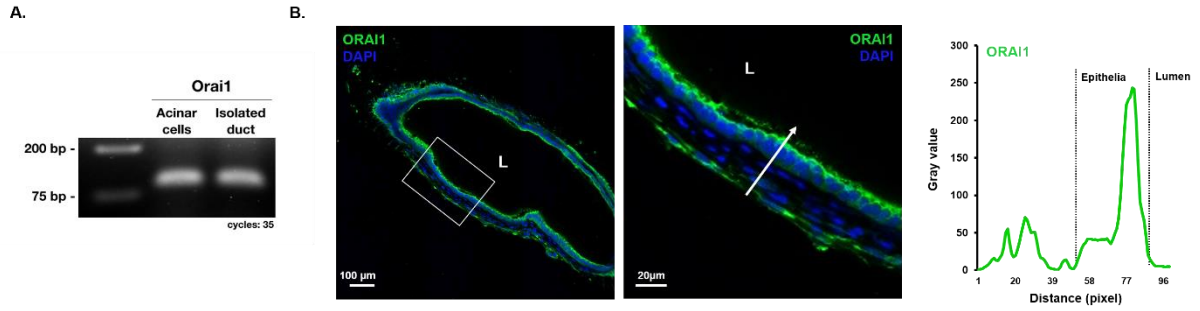


Figure 6. Expression of Orai1 in murine pancreatic ductal epithelial cells A. Agarose gel image proves active Orai1 gene expression in isolated mouse acinar cells and ductal fragments. **B.** Representative confocal image and exported line profile analysis of an isolated mouse ductal fragment demonstrates the dominantly apical localization of Orai1 in polarized epithelial cells. L: lumen.

Next, to demonstrate Orai1 functionality in isolated mouse pancreatic ductal cells, SERCA pump was inhibited with 25µM cyclopiazonic acid (CPA) in Ca^{2+} -free medium to deplete ER Ca^{2+} stores. Under these conditions, Ca^{2+} influx into the cytosol was induced by re-addition of extracellular Ca^{2+}, which was significant inhibited by perfusing Orai1 inhibitors at a concentration of 10µM. **(Figure 7.A.)**. Although the maximal inhibition did not increase when using higher CM-5480 concentrations resulting in a similar decrease of the plateau phase (45.15±3.41% at 10 µM CM-5480 vs. 52.38±2.45% at 30 µM CM-5480, respectively), it was achieved significantly faster at 30 µM CM-5480 **(Figure 7. B.)**.

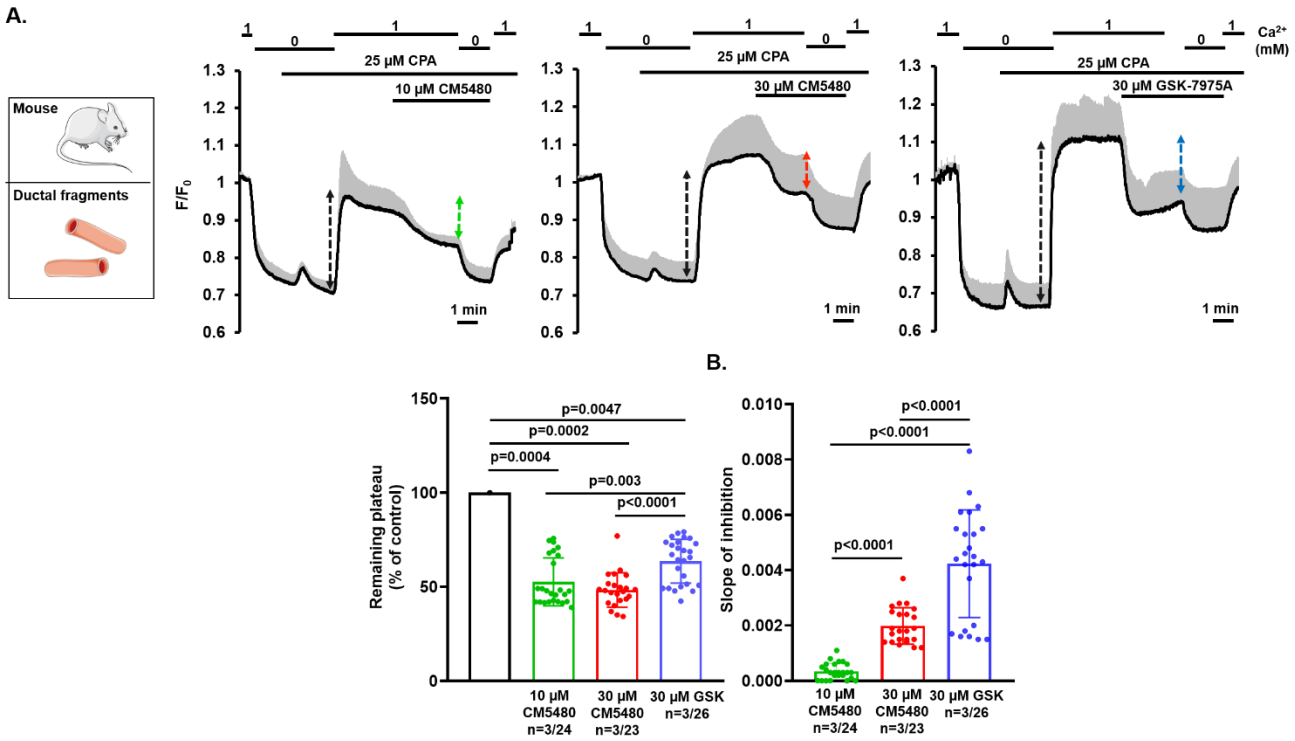


Figure 7. Functional activity of Orai1 in murine pancreatic ductal epithelial cells A. Isolated pancreatic ducts were perfused with standard HEPES solution, which was changed to Ca^{2+}-free HEPES solution. After Ca^{2+} store depletion via 25 μM cyclopiazonic acid and re-addition of 1 mM extracellular Ca^{2+}, mouse pancreatic ductal fragments were challenged with 10 or 30 μM CM5480 or 30 μM GSK-7975A specific Orai1 inhibitor resulting in a reduced Ca^{2+} influx (average traces). The degree of inhibitory effect of 30 μM GSK-7975A was significantly higher, than 10 or 30 μM CM5480, however there was no significant different between 10 and 30 μM CM5480. **B.** The slope of inhibition significantly increased upon administration of 30 μM CM5480 or GSK-7975A compared to 10 μM, and GSK-7975A was more effective than 30 μM CM5480.

As the plateau phase of the Ca^{2+} signal under the applied conditions is a mixture of Ca^{2+} influx and efflux, which may affect the characterization of the Orai1 mediated Ca^{2+} entry, we also applied another protocol. Addition of CM-5480 before the re-addition of extracellular Ca^{2+} significantly decreased the extracellular Ca^{2+} influx **(Figure 8.)**. Of note, despite the inhibition of the Orai1 channels, a significant proportion of the extracellular Ca^{2+} influx remained active in every case suggesting that other Ca^{2+} influx channels may contribute to the extracellular Ca^{2+} influx in ductal cells.

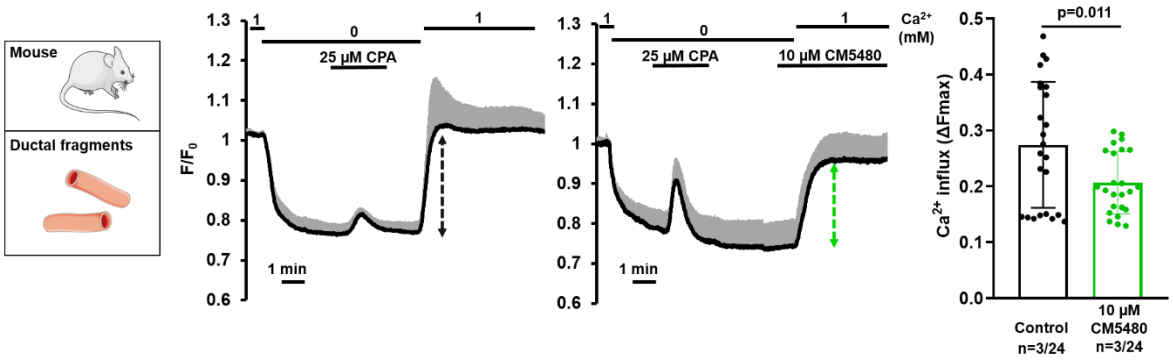


Figure 8. Functional activity of Orai1 in murine pancreatic ductal epithelial cells. Average traces demonstrates that CM5480 used prior to Ca^{2+} re-addition, significantly reduced extracellular Ca^{2+} influx in mouse pancreatic ductal fragments.

4.2. The inhibition of Orai1 abolishes toxin-induced extracellular Ca^{2+} influx in the pancreatic ductal epithelia

Next, we examined the effect of Orai1 channel inhibition on bile acid- or ethanol+ palmitic acid (PA)-induced intracellular Ca^{2+} elevation. 250 µM CDC induced sustained elevation of intracellular Ca^{2+} concentration in isolated mouse pancreatic ductal fragments. Reaching a stable plateau, the ductal fragments were challenged with 10 µM CM-5480, which reduced the extracellular CDC -induced Ca^{2+} influx significantly. 100 mM ethanol and 200 µM PA also induced intracellular Ca^{2+} elevation in ductal fragments. However, as the plateau phase of the evoked Ca^{2+} signal was not clearly separated from the peak in case of ethanol-, and PA treatment, CM-5480 was applied simultaneously with the ethanol and PA treatment. Similar to CDC, the extracellular ethanol-, and PA-induced Ca^{2+} influx significantly decreased for CM-5480 administration. **(Figure 9. A-B.)**. These results suggest the potential of CM-5480 to prevent Ca^{2+}-overload-mediated functional and morphological damage of ductal cells associated with biliary- or ethanol-induced AP.

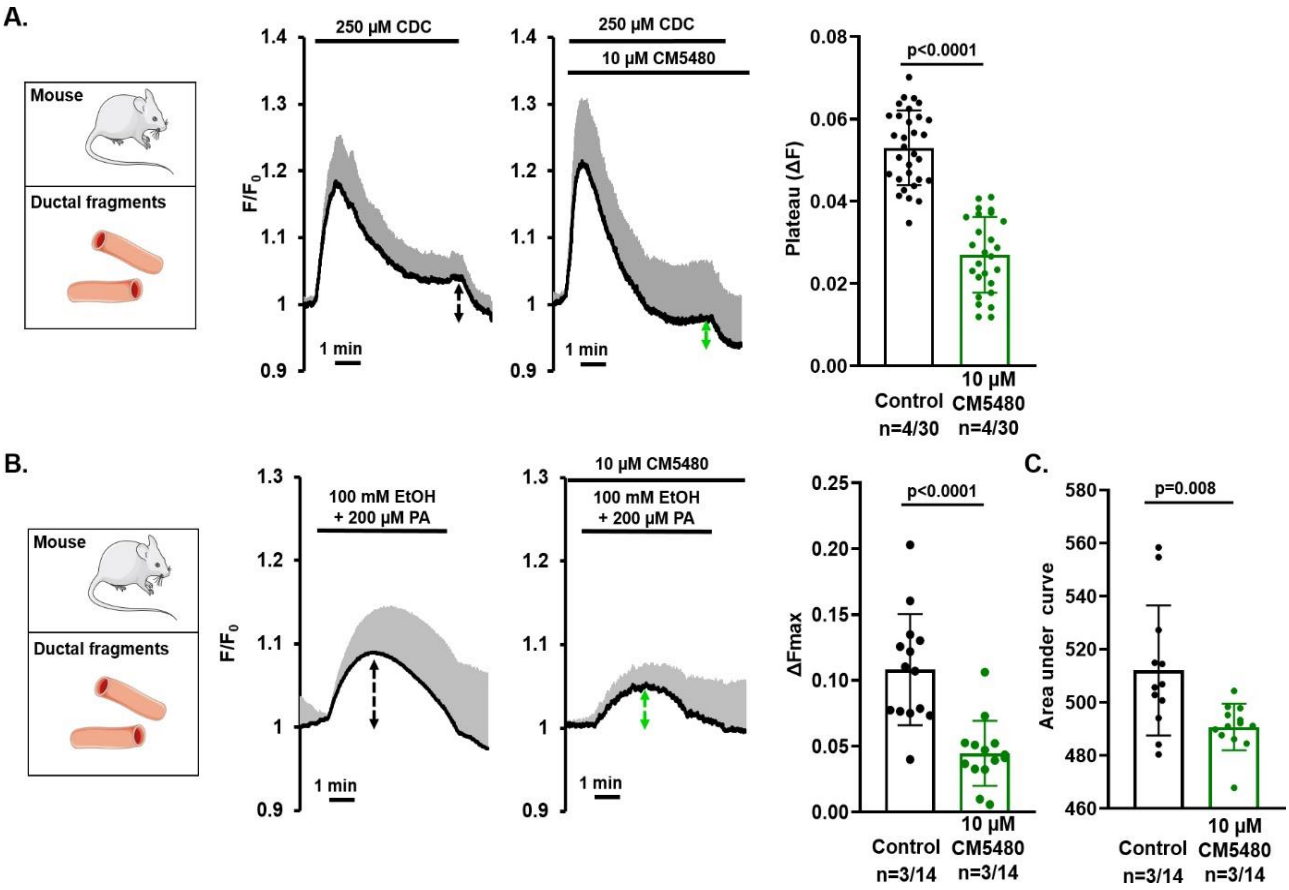


Figure 9. Pharmacological inhibition of Orai1 leads to reduced extracellular Ca^{2+} influx induced by bile acid or ethanol and fatty acid in pancreatic ductal epithelia. Isolated mouse pancreatic ductal fragments were challenged with 250 µM CDC or with a combination of 100 mM ethanol and 200 µM PA in standard HEPES buffered solution. **A.** Average traces and the bar charts demonstrate that the inhibition of Orai1 by 10 µM CM5480 significantly decreased the plateau phase of the CDC-induced intracellular Ca^{2+} signal. **B-C.** Orai1 inhibition also reduced the intracellular Ca^{2+} elevation and value of area under curve triggered by the combination of 100 mM ethanol and 200 µM PA.

4.3. Inhibition of Orai1 prevents bile acid- and ethanol-induced decrease of HCO_3^- secretion and CFTR function in pancreatic ductal epithelia

HCO_3^- secretion –the primary function of pancreatic ductal epithelia– is significantly impaired by bile acid- and ethanol-mediated sustained Ca^{2+} elevation and mitochondrial damage [88,89]. To assess the potential protective effect of Orai1 inhibition on ductal HCO_3^- secretion, we treated isolated mouse ductal fragments with CDC or ethanol/PA in the presence or absence of CM5480 and compared HCO_3^- efflux across the apical membrane [89]. The isolated mouse pancreatic ducts were perfused with 20 mM NH_4Cl in HCO_3^-/CO_2-buffered solution to measured HCO_3^- secretion across the apical membrane of pancreatic ductal cells. NH_4Cl containing solution triggered a rapid alkalization caused by the passive NH_3 uptake of the cells,

which was followed by a slow recovery of the alkaline pH - due to the SLC26 Cl^-/HCO_3^- exchanger - and CFTR-mediated HCO_3^- [88] efflux (i.e. secretion) from the ductal epithelia to resting pH_i. We found that 250 µM bile acid and 100 mM ethanol+200 µM PA significantly inhibited bicarbonate secretion, which was significantly increased by 10 µM CM-5480 **(Figure 10.A.)**. To calculate the base flux [$J(B^-)$] values (calculated as $\Delta pH/\Delta t$ [88]), the initial recovery rates were measured over the first 30 s. **(Figure 10.B.)**.

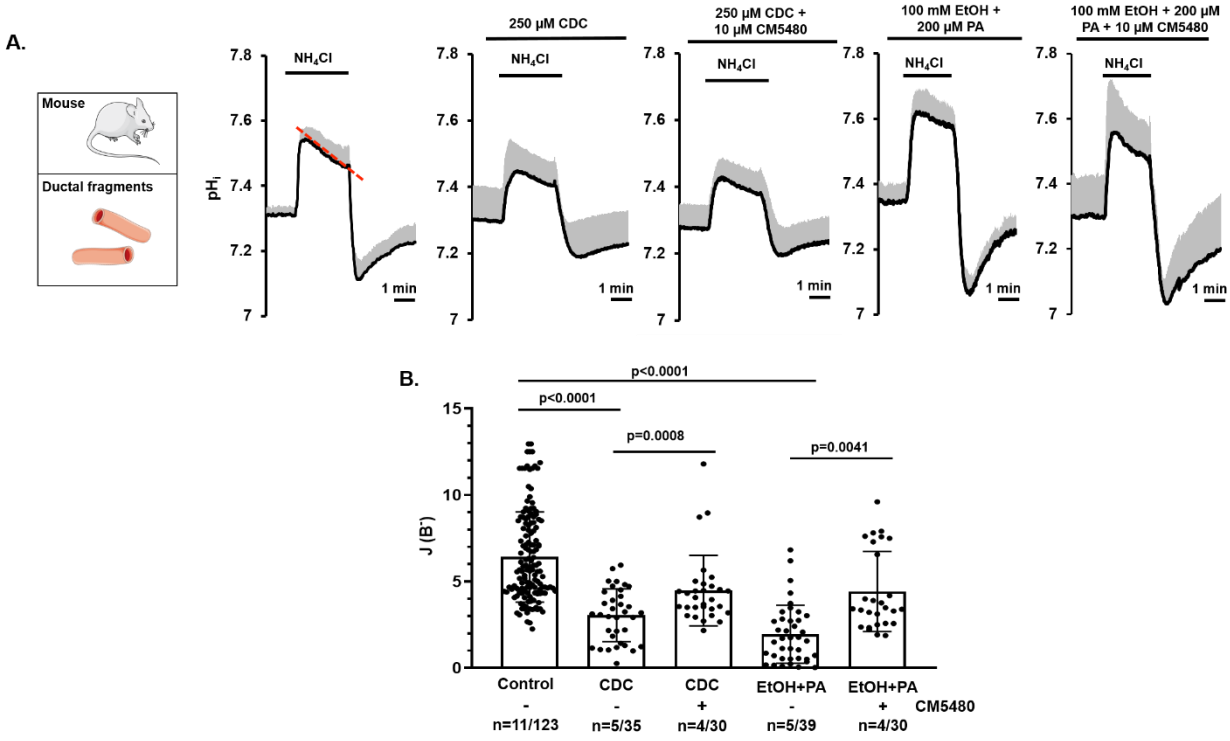


Figure 10. The inhibition of Orai1 prevents bile acid- and ethanol-induced decrease of apical HCO_3^- secretion in pancreatic ductal epithelia. A. Mouse pancreatic ducts were perfused with 250 µM CDC or 100 mM ethanol and 200 µM PA in HCO_3^-/CO_2-buffered extracellular solution and intracellular alkalization was achieved by 20 mM NH_4Cl administration in the absence or presence of Orai1 inhibitor CM5480. Both pathogenic conditions significantly reduced the base flux, which was offset using the Orai1 inhibitor. **B.** Bar charts of the calculated base fluxes of HCO_3^-. CM5480 significantly increased HCO_3^- secretion in the presence of CDC, ethanol+PA. Statistical analysis was performed by Tukey's multiple comparisons test.

Intracellular Cl^- concentration was measured also by microfluorimetry and an intracellular Cl^- concentration sensitive fluorescent indicator MQAE was used. The fluorescent signal emitted by MQAE is inversely correlated with intracellular Cl^-. When we removed extracellular Cl^- from the HCO_3^-/CO_2- buffered solution, intracellular Cl^- decreased due to Cl^- efflux through the CFTR channel. 250 µM CDC and 100 mM ethanol + 200 µM PA

significantly reduced Cl^- efflux. Treatment of these ductal cells with CM-5480 the Cl^- efflux significantly increased **(Figure 11. A-B.)**. These results suggest that CM-5480 treatment has the potential to prevent the AP-induced inhibition of the pancreatic ductal HCO_3^- secretion and CFTR activity.

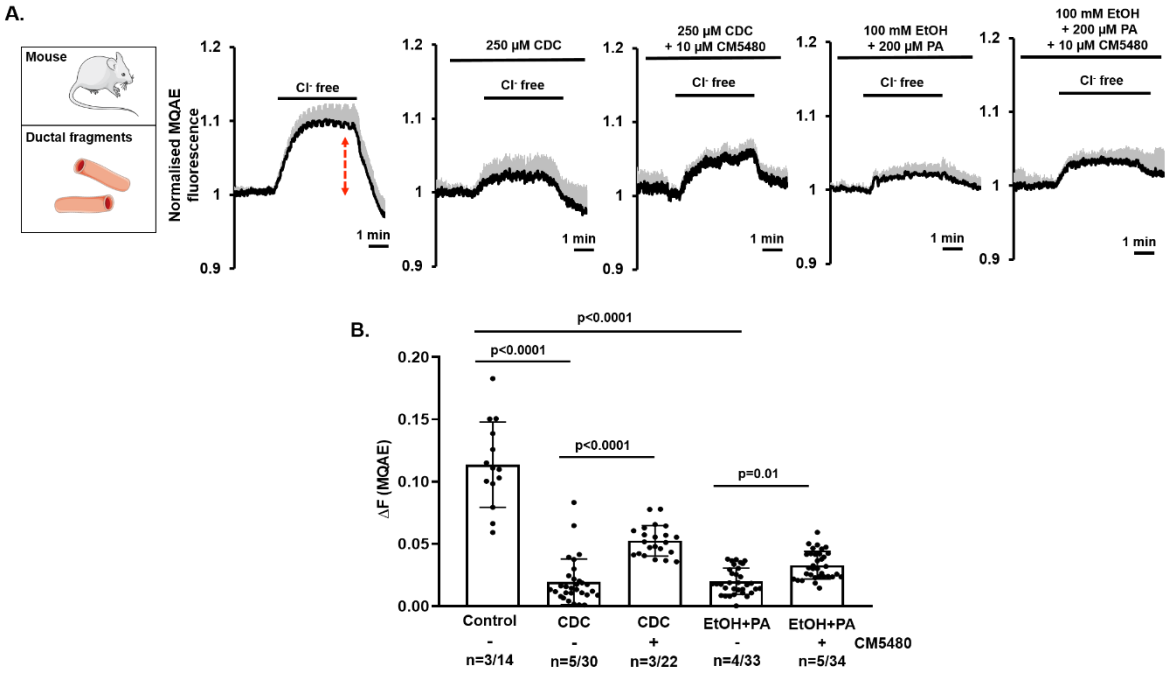


Figure 11. The inhibition of Orai1 prevents bile acid- and ethanol-induced decrease of CFTR function in pancreatic ductal epithelia. A. Average traces of intracellular Cl^- levels reflecting CFTR activity in mouse isolated ductal fragment using Cl^- sensitive fluorescent dye (MQAE). Removal of extracellular Cl^- induced a decrease in intracellular Cl^- levels (reflected by an increase in fluorescent intensity) due to CFTR activity. Cl^- removal from the extracellular solutions in the presence of 250 µM CDC or 100 mM ethanol and 200 µM PA inhibited CFTR activity which was improved by CM5480. **B.** Bar charts of the calculated Cl^- efflux. CM5480 significantly increased Cl^- efflux in the presence of CDC, ethanol+PA. Statistical analysis was performed by Tukey's multiple comparisons test.

4.4. Bile acid- and alcohol-induced Orai1-mediated extracellular Ca^{2+} entry depends on Stim1 activation

Exposure of ductal cells to bile acids and ethanol release Ca^{2+} from intracellular stores –most prominently from the ER [89,90]– which induces a conformational change of Stim1 triggering Orai1-mediated Ca^{2+} influx. Isolated ductal cells were treated with specific siRNA to knock down Stim1 expression and in order to assess the involvement of Stim1 in bile acid- and alcohol-induced ductal cell functional impairment. Treatment of pancreatic ductal cells with siStim1 did not alter CFTR-mediated HCO_3^-- or Cl^- secretion.

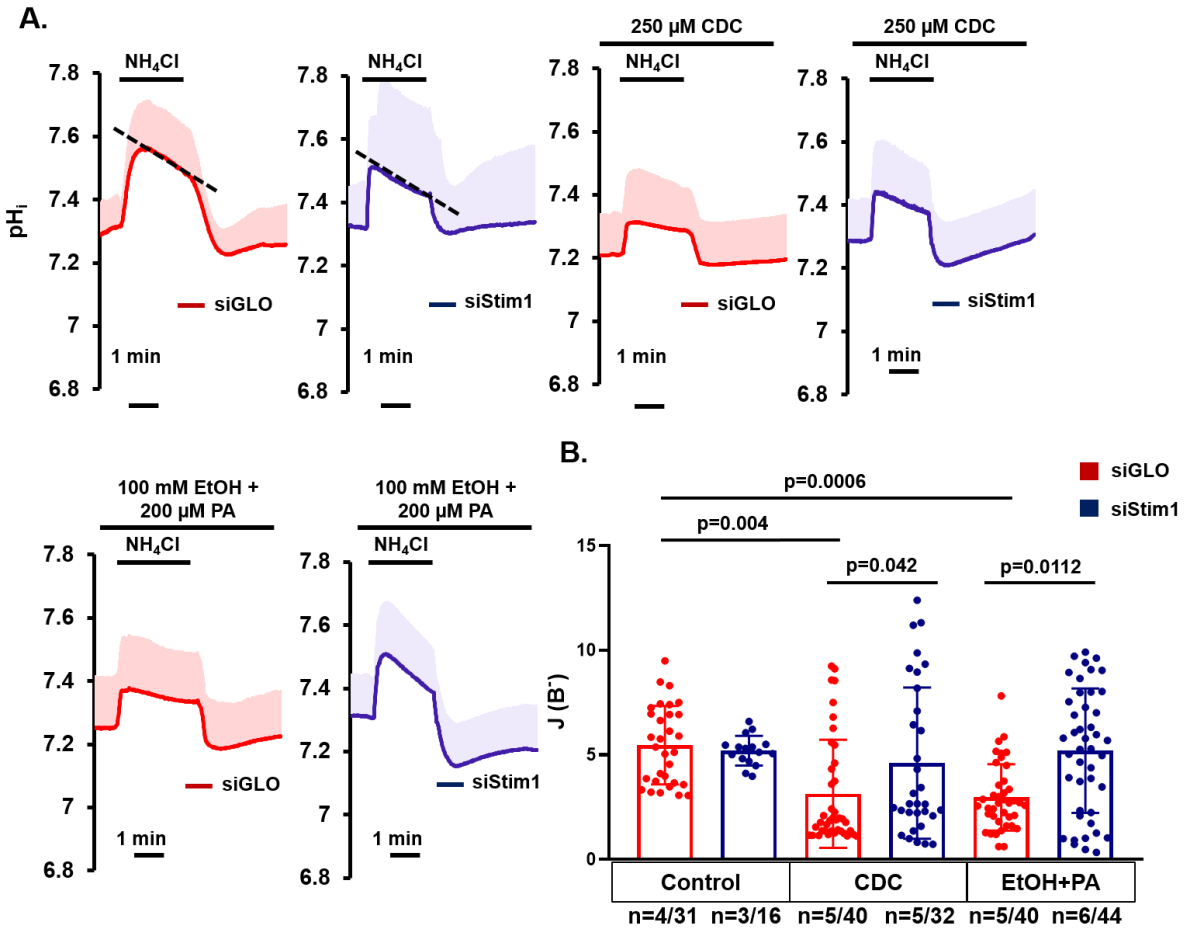


Figure 12. siStim1 treatment prevents bile acid- and ethanol-induced decrease of apical HCO_3^- secretion in pancreatic ductal epithelia. A. Average traces and bar charts demonstrate the effects of 250 µM CDC or 100 mM ethanol and 200 µM PA on control (red traces) and siStim1-treated (blue traces) pancreatic ductal fragments. Pancreatic ducts were challenged with 20 mM NH_4Cl in HCO_3^-/CO_2 buffered extracellular solution and the apical Cl^-/HCO_3^- exchange activity was determined. B. Bar charts of the calculated base fluxes of HCO_3^- show that administration of CDC or ethanol+PA significantly impaired pancreatic ductal HCO_3^- secretion in the siGLO-Green-treated control ducts. siStim1 treatment prevented the inhibition of HCO_3^- secretion. Statistical analysis was performed by Tukey's multiple comparisons test.

Whereas CDC or ethanol/PA significantly impaired HCO_3^- secretion **(Figure 12. A-B.)** or CFTR activity **(Figure 13. A-B.)** in siGLO-Green-treated pancreatic ductal cells, this was prevented in siStim1-treated cells. These results suggest that bile acids and ethanol induce Orai1-mediated Ca^{2+} influx in a Stim1-dependent manner.

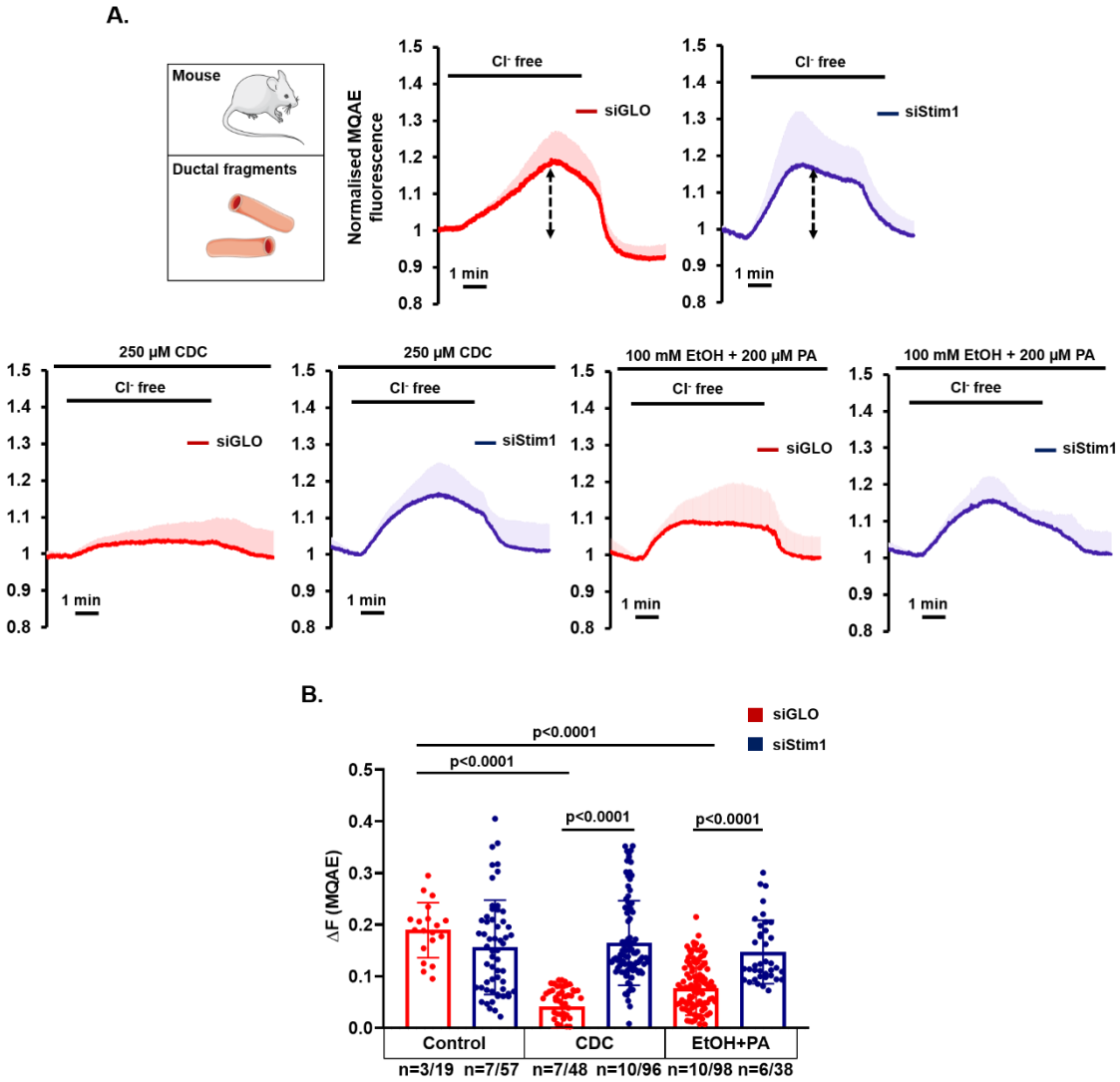


Figure 13. siStim1 treatment prevents bile acid- and ethanol-induced decrease of CFTR function in pancreatic ductal epithelia. Average traces and bar charts demonstrate the effects of 250 µM CDC or 100 mM ethanol and 200 µM PA on control (red traces) and siStim1-treated (blue traces) pancreatic ductal fragments. **A.** Extracellular Cl^- was removed to measure CFTR activity in control and treated ductal fragments. **B.** Bar charts of the calculated maximal fluorescent intensity changes of MQAE show that administration of CDC or ethanol+PA significantly impaired CFTR-mediated Cl^- secretion in the siGLO-Green-treated control ducts. siStim1 treatment prevented the inhibition of Cl^- secretion. Statistical analysis was performed by Tukey's multiple comparisons test.

4.5. Inhibition of Orai1 preserves pancreatic ductal secretion in vivo during acute pancreatitis

We wanted to analyze AP-mediated changes of fluid secretion in vivo. Upon establishment of experimental AP, secretin-induced fluid secretion was measured in vivo in anesthetized mice. In all three AP groups the in vivo fluid secretion was significantly decreased (i.e. cerulein-, Na-

TC-, and ethanol/POA-treated animals). CM-5480 treatment alone did not affect secretin-stimulated pancreatic secretion. In fact, in both cerulein- and ethanol/POA-treated animals, CM5480 significantly improved in vivo fluid secretion to levels comparable to the untreated – healthy– control group **(Figure 14. A., C.)**. Moreover, in the Na-TC-treated group, CM5480 resulted in an almost twofold increased fluid secretion compared to the Na-TC group; however, the difference failed to reach statistical significance **(Figure 14. B.)**. To confirm these results, we studied HCO_3^- secretion through fluorescence microscopy, using the alkali load technique on isolated pancreatic ductal fragments from CM-5480- and cerulein-treated mice. Cerulein significantly decreased HCO_3^- secretion in the ductal fragments, this secretory activity was preserved in ductal fragments derived of cerulein-induced AP mice receiving CM-5480 **(Figure 14. D.)**. Importantly, these results confirmed that inhibition of Orai1 preserves the ductal ion and fluid secretion both in vitro and in vivo in different forms of AP.

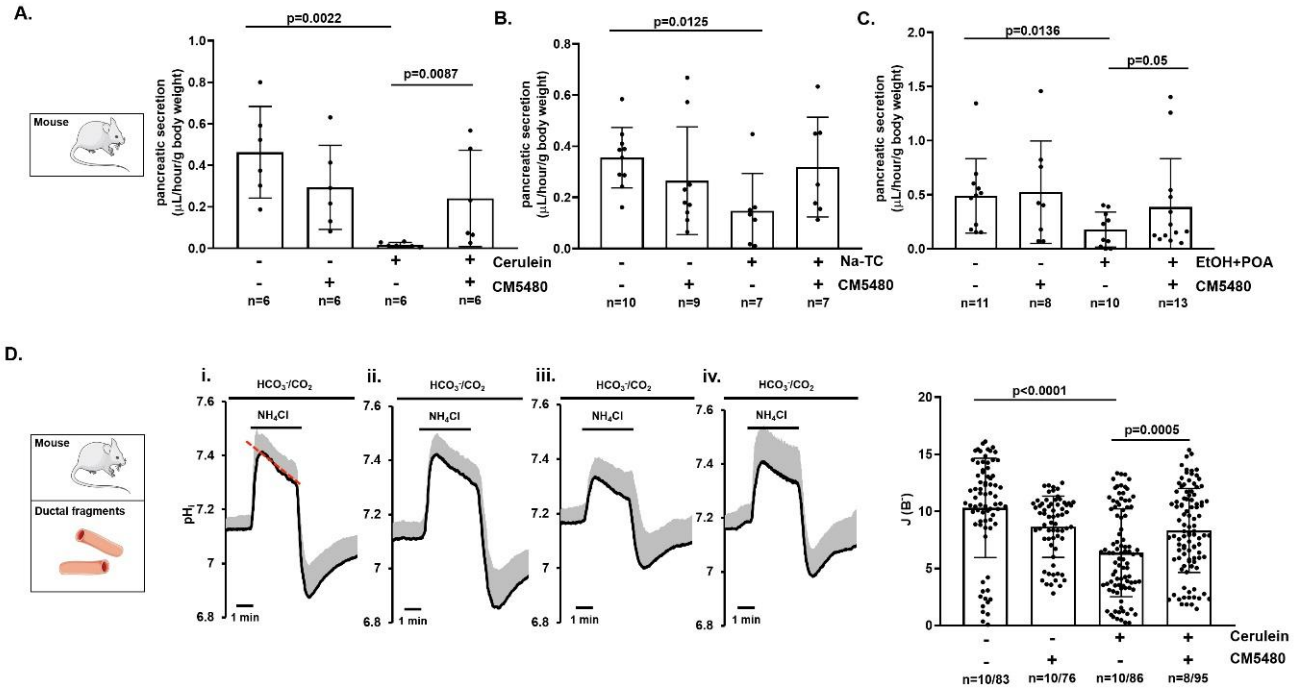


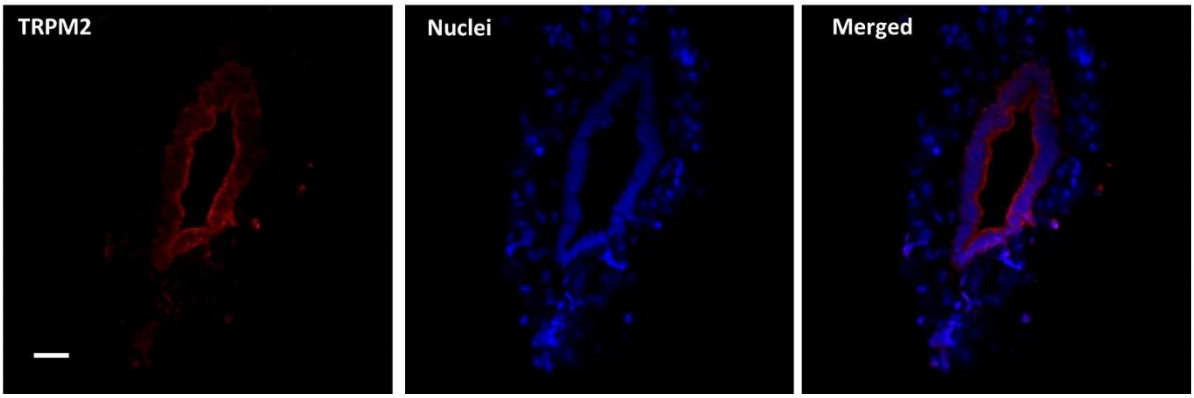
Figure 14. Orai1 inhibition by CM5480 prevents pancreatic ductal secretion during acute pancreatitis. Acute pancreatitis (AP) was induced in mice as described above. Pancreatic juice was collected for 30 min *in vivo* under secretin-stimulated (0.75 CU/kg i.p.) conditions from anesthetized mice after induction of AP. **A.** Summary bar charts show that cerulein administration significantly reduced the volume of pancreatic juice, which was preserved by CM5480 treatment. **B.** CM5480 administration increased the reduced *in vivo* fluid secretion caused by Na-taurocholate, however the difference was not significant. **C.** The volume of pancreatic juice was significantly lower after ethanol+POA treatment; however, Orai1 inhibition significantly improved it. **D**. Averages traces and bar charts demonstrate the *in vitro* HCO_3^- secretion of pancreatic ductal fragments isolated from mouse pancreas after the

induction of AP with cerulein. Pancreatic ducts were perfused with HCO_3^-/CO_2 buffered extracellular solution and intracellular alkalization was achieved by 20 mM NH_4Cl administration. Comparison of recovery from alkalosis shows that ductal HCO_3^- secretion was significantly reduced in the cerulein-treated group, which was restored by CM5480 treatment. Statistical analysis was performed by Tukey's multiple comparisons test.

4.6. Functional TRPM2 channels are present in pancreatic ductal cells

After the characterization the role of Orai1 in the pancreatic ductal secretion, we wanted to investigate the expression and function of an other Ca^{2+} channel, TRPM2, which play role in the Ca^{2+} entry process. Immunofluorescent labelling of TRPM2 showed an atypical expression on isolated ducts (**Figures 15. A.**). We have found that H_2O_2-induced oxidative stress activated TRPM2. 1 mM H_2O_2 induced an increased $[Ca^{2+}]_i$ in TRPM2 WT pancreatic ductal cells, than in TRPM2 KO ductal cells (0.30 ± 0.06 vs 0.10 ± 0.013, respectively). In these cells, Ca^{2+} elevation was significantly lower in Ca^{2+} free conditions. This suggests that the sustained elevation of $[Ca^{2+}]_i$ in response to H_2O_2 was largely due to TRPM2-channel-mediated influx of extracellular Ca^{2+} (**Figure 15. B.**). Because HCO_3^- secretion is the primary function of the ductal epithelia, the HCO_3^- efflux across the apical membrane was compared between WT and TRPM2 KO ducts using fluorescence pH_i measurements. With this assay, no difference in the activities of the apical and basolateral proteins was found between WT and TRPM2 KO ducts (**Figures 15C–D**). CDC markedly inhibited the HCO_3^- secretion both in TRPM2 KO and WT ductal cells, the genetic knockout of TRPM2 had no protective effect, suggesting that bile acids affect ductal cells via a TRPM2-independent mechanism (**Figures 15C–D**).

A.



B.

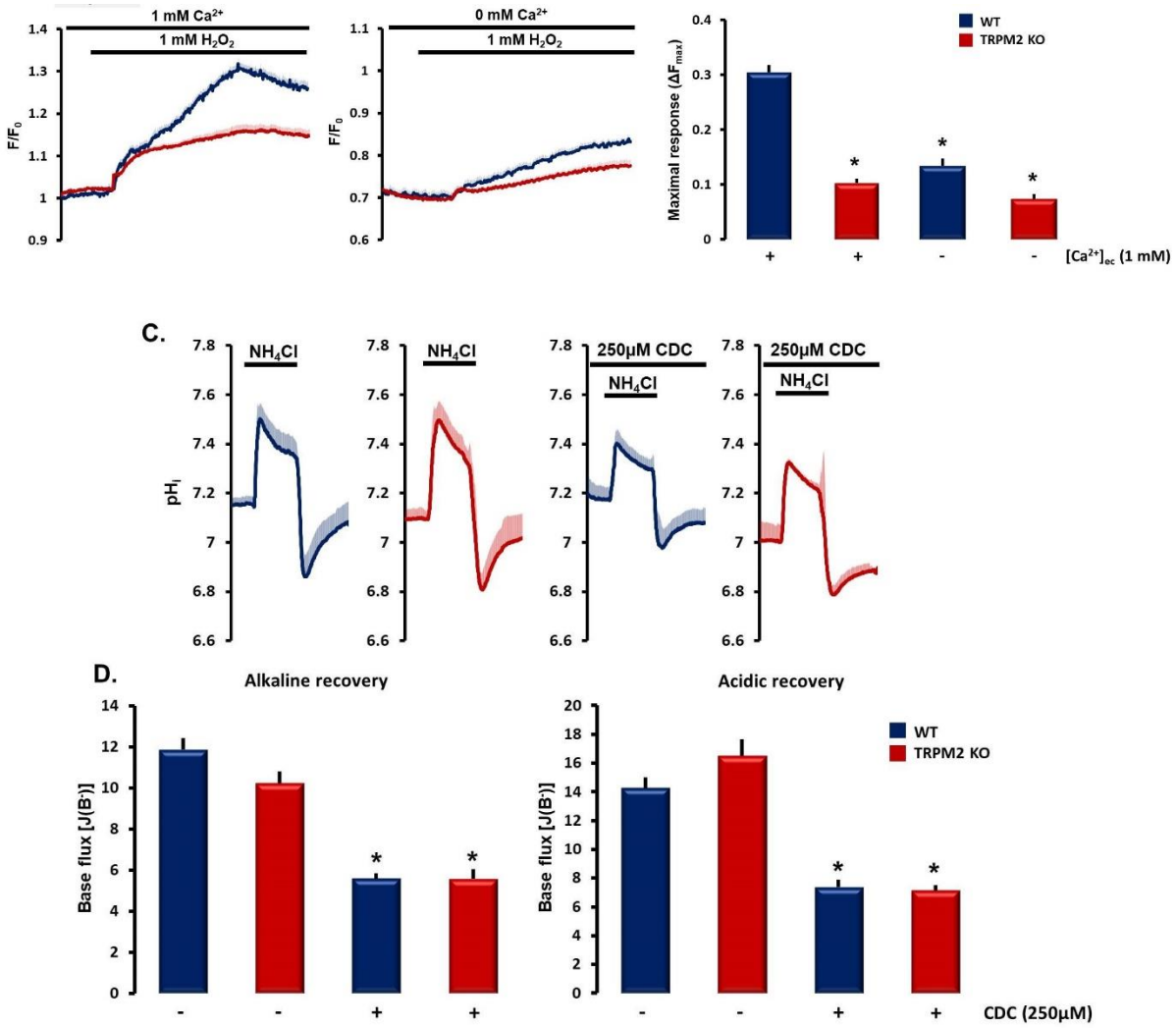


Figure 15A. Atypical expression of TRPM2 in mouse pancreatic ducts. **B.** TRPM2 KO ductal cells significantly inhibited the Ca^{2+} influx administration of H_2O_2. In TRPM2 WT and KO ductal cells the Ca^{2+} influx significantly decreased in Ca^{2+} free conditions administration of H_2O_2. **C.** Pancreatic ducts were alkalized with the administration of 20mM NH_4Cl. **D.** TRPM2 KO ductal cells had no effect on the HCO_3^- secretion but CDC significantly inhibited the HCO_3^- secretion both in TRPM2 KO and WT ductal cells.

5. DISCUSSION

Sustained elevation of intracellular Ca^{2+} is a hallmark in the development of AP-mediated cellular injury[26,46]. Although selective Orai1 inhibitors –limiting the uncontrolled extracellular Ca^{2+} influx– prevented acinar cell damage and decreased the severity of AP in multiple animal models[35,91], precisely how Orai1 inhibition affects the pancreatic ductal cell functions is currently unknown. During my work we first demonstrated that Orai1 resides in the apical membrane of the pancreatic ductal cells where it mediates extracellular Ca^{2+} influx upon ER Ca^{2+} store depletion. Next, we provided evidence that bile acid- and ethanol-mediated SOCE-activation contribute to sustained intracellular Ca^{2+} elevations leading to damaged ductal secretion and cell death. Finally, prevention of intracellular Ca^{2+} overload with selective Orai1 inhibitors preserved pancreatic ductal ion and fluid secretion and maintained exocrine pancreatic secretion during AP.

Expression of Orai1 in exocrine pancreas acinar cells was previously described by two independent groups. Lur et al. demonstrated Stim1-translocation and Orai1-activation in the lateral and basal plasma membrane[92], whereas Hong et al. reported a more pronounced Orai1 expression in the apical membrane[93]. In our experiments, Orai1 expression was observed on the apical membrane of ductal cells. Although the significance of this polarized expression pattern is currently unknown, it may be of importance in reuptake of intraluminal Ca^{2+} secreted by the acinar cells during digestive enzyme secretion. Interestingly, CM-5480-mediated functional inhibition of Orai1 did not completely abolish the ER store depletion-induced extracellular Ca^{2+} influx. In fact, in the current study, we achieved a maximal inhibition of around 50% –both in case of 10 μM and 30 μM CM-5480– suggesting that additional PM-residing Ca^{2+} channels contribute to SOCE in ductal cells. Interestingly, genetic deletion of the TRPC3 Ca^{2+} channel resulted in a 50% reduction of receptor-stimulated SOCE in pancreatic acinar cells and prevented bile acid- and ethanol metabolite-induced sustained Ca^{2+} elevation and intracellular trypsin activation. These beneficial effects ultimately resulted in reduced cerulein-induced AP severity *in vivo*[50]. Similar results were achieved with the specific TRPC3 inhibitor Pyr3[94]. However, the contribution of TRPC3 to SOCE in pancreatic ductal cells is currently unknown.

To achieve strict control and tune the activity of each other, Ca^{2+}- and cAMP/PKA signaling – a well-known key regulator of CFTR activity and HCO_3^- secretion– interact at multiple levels to facilitate maximal response[95]. On the other hand, the most common biologically active

molecules inducing AP –including bile acids, non-oxidative ethanol metabolites, and trypsin– induce toxic, sustained intracellular Ca^{2+} elevation in the exocrine pancreas[48,96]. Previous data indicated that the non-conjugated bile acid CDC dose-dependently impairs pancreatic HCO_3^- secretion[58] via sustained Ca^{2+} elevation and subsequent mitochondrial damage in pancreatic ductal cells[59] and isolated pancreatic acinar cells[97].

Considering the detrimental effect of heavy ethanol consumption - in combination with non-oxidative ethanol metabolites (such as fatty acid ethyl esters (FAEE)) - on acinar and ductal cells[15,98], our group previously demonstrated ethanol / POA-mediated impaired activity of the apical SLC26 Cl^-/HCO_3^- exchanger and CFTR Cl^- channel together with decreased HCO_3^- secretion in ductal cells[61]. Mechanistically, ethanol and POA induced a sustained Ca^{2+} elevation through IP_3- and ryanodine receptor-mediated Ca^{2+} release from the ER combined with extracellular Ca^{2+} influx; a mechanism which was also described in pancreatic acinar cells[48,99,100]. The involvement of Orai1 in the development of AP was first highlighted by Gerasimenko et al. by demonstrating that Orai1 inhibition decreased acinar cell necrosis in vitro[49]. In fact, selective GSK-7975A-mediated inhibition of Orai1 inhibited SOCE in a concentration-dependent manner and reduced the sustained Ca^{2+} elevation, trypsin activation, and acinar necrosis upon FAEE exposure. Others found that GSK-7975A and CM_128 – developed by CalciMedica– markedly impaired bile acid-induced extracellular Ca^{2+} influx and sustained Ca^{2+} overload in pancreatic acinar cells and significantly decreased pancreatic edema, inflammation, and necrosis in experimental models of AP[35]. By using CM-5480 – another selective Orai1 channel blocker by CalciMedica currently in clinical trials– Waldron et al. showed that inhibition of SOCE prevented trypsinogen activation, acinar cell death, NF-κB and NFAT activation, and inflammatory responses in multiple *in vitro* and *in vivo* models[91]. Other reports described cerulein-mediated interaction between Stim1 and Orai1, subsequent activation of SOCE, and calcineurin-mediated activation of NFAT and transcription factor EB promoting transcription of chemokine and autophagy-associated genes[101]. In our study, inhibition of Orai1 in pancreatic ductal cells significantly decreased the bile acids and ethanol / PA-mediated extracellular Ca^{2+} influx in pancreatic ductal fragments and organoids. The CM-5480-mediated inhibition of Orai1 was sufficient to significantly improve the *in vitro* HCO_3^- secretion and CFTR activity in pancreatic ductal cells. As specific siRNA knockdown of the ER Ca^{2+} sensor protein Stim1 reproduced the effect of selective pharmacologic Orai1 inhibition, CDC- and ethanol/PA-induced activation of Orai1 seems to be Stim1 dependent.

The importance of pancreatic ductal fluid and HCO_3^- secretion in the physiological function of the exocrine pancreas is supported by several independent studies. Di Magno et al. demonstrated that CFTR knockout mice –in which the exocrine pancreatic secretion was impaired– develop more severe cerulean-induced AP which is accompanied with increased pancreatic edema, neutrophil infiltration, and expression of inflammatory mediators[102]. In addition, our group showed that genetic deletion of Na^+/H^+ exchanger regulatory factor-1 (NHERF-1) –a scaffolding protein that anchors CFTR to the apical PM– reduces pancreatic fluid and HCO_3^- secretion in mice[103]. Compared to wild type littermates, NHERF-1 KO mice developed more severe experimental AP upon cerulein hyperstimulation or bile acid infusion to the main pancreatic duct. In addition, the alcohol-induced impairment of CFTR function and expression resulted in increased severity of experimental AP. In the current study, we confirmed previous reports by indicating that in vivo CM-5480-mediated inhibition of Orai1 in mice markedly decreases the severity of AP in three different model systems with independent pathogenic triggers. Importantly, in all three AP models, we demonstrated improved secretin-stimulated in vivo pancreatic fluid secretion in CM-5480-treated animals. Notably, at the tested dose, CM-5480 had no effects on the secretin-stimulated in vivo pancreatic fluid secretion itself in the control groups.

Restoration of pancreatic fluid secretion could have significant beneficial impact on the disease outcome. In a healthy pancreas, the digestive enzymes produced by the acini are washed out by HCO_3^--rich fluid into the duodenum where it neutralizes the local pH. Previously, our group demonstrated pH-dependent autoactivation of trypsinogen together with elevated trypsinogen activity in acidic environment indicating the primordial role of HCO_3^- to prevent early autoactivation of trypsinogen[62]. In addition, Zeng et al. reported that pharmacological correction of CFTR expression and activity rescues pancreatic acinar cell function and reduces autoimmune pancreatitis-induced inflammation, further highlighting the importance of proper ductal function in the disease outcome[104]. Recently, the role of Saraf[105] –an Orai1 channel regulator protein– was reported in AP[106]. In contrast to constant expression levels of Stim1 and Orai1, expression levels of Saraf decreased during AP in both mice and human. In addition, whereas Saraf knockout mice developed more severe AP accompanied by increased Ca^{2+} influx in acinar cells, its overexpression reduced acinar Ca^{2+} influx and decreased AP severity.

Very recently, a phase 2, open-label, dose-response clinical study evaluated the safety of Auxora –a selective Orai1 inhibitor drug developed by CalciMedica– in patients with AP, SIRS, and hypoxemia[107]. In this clinical study, the patients received low- or high-dose Auxora plus

standard of care (SOC). Overall, no differences in the number of serious adverse events with Auxora compared to SOC alone were reported. Of patients with moderate AP receiving low-dose Auxora, 36.5% improved to mild AP. Very interestingly, patients receiving Auxora better tolerated solid foods, had less persistent SIRS, and had a reduced hospitalization rate compared to SOC. It is tempting to speculate that the increased tolerance towards solid food may be explained by improved exocrine pancreatic secretion as observed in our current study. Based on these results, further clinical studies are needed to clarify the utility of Orai1 inhibition in AP patients.

As mentioned in the introduction, the Ca^{2+} entry is also mediated by the TRPM2 channel, a ROS-sensitive channel. To study this we characterized of TRPM2 expression in mouse pancreatic ducts, which was on the luminal membrane. The expression of TRPM2 has been demonstrated previously in different cell types, including inflammatory cells[108], myocytes[109] and epithelial cells[110], to our knowledge, we demonstrated first the expression of TRPM2 in the exocrine pancreas. When isolated TRPM2 WT ductal cells were challenged with H_2O_2 to increase ROS significantly increased the $[Ca^{2+}]_i$, which was due to the TRPM2-channel-mediated influx. Redox signals have been demonstrated to sensitise TRPM2 in other cell types. Those increased intracellular Ca^{2+} concentration at physiological body temperature, which plays an important role in the regulation of macrophage functions[111]. Perides et al. demonstrated that activation of the G-protein-coupled cell surface bile acid receptor (Gpbar1 or TGR5) at the pancreatic acinar cells leads to sustained Ca^{2+} elevation and intracellular activation of digestive enzymes[112]. On the other hand, CDC dose-dependently increased the intracellular Ca^{2+} level and inhibited HCO_3^- secretion in pancreatic ductal cells[58]. In our experiments, CDC also decreased the HCO_3^- secretion both in TRPM2 KO and TRPM2 WT ductal cells, but genetic deletion of TRPM2 had no protective effect, suggesting that bile acids affect ductal cells via a TRPM2-independent mechanism.

Taken together, we show that genetic deletion of TRPM2 protects pancreatic ductal cells from sustained intracellular Ca^{2+} overload triggered by H_2O_2. We also report that inhibition of Orai1 protects pancreatic ductal cells from sustained intracellular Ca^{2+} overload triggered by bile acids and ethanol in combination with non-oxidative ethanol metabolites. Importantly, this protection seems to be sufficient to maintain crucial ductal functions –such as fluid and HCO_3^- secretion– both in vitro and in vivo during AP. Taking into account that Auxora is currently in Phase 2b clinical trials to treat severe AP, our current results can further contribute to the development of specific pharmacological treatments for AP.

6. SUMMARY

Regardless of its etiology, sustained intracellular Ca^{2+} overload is a well-known hallmark of acute pancreatitis (AP). Toxic Ca^{2+} elevations in the pancreatic ductal cells damage the mitochondrial function and impair ductal ion and fluid secretion which –under physiological conditions– maintains the alkaline intra-ductal pH and washes out the protein-rich fluid secreted by acinar cells. While prevention of ductal cell injury decreases severity of acute pancreatitis, there is however no specific drug target identified yet in the ductal cells. Although Orai1 –a store operated Ca^{2+} influx channel– is known to contribute to sustained Ca^{2+} overload in acinar cells, details concerning its expression and function in ductal cells are currently lacking. In this study, we demonstrate that functionally active Orai1 channels reside in the apical plasma membrane of pancreatic ductal cells. Next, selective CM-5480-mediated Orai1 inhibition impaired Stim1-dependent extracellular Ca^{2+} influx evoked by bile acids or ethanol combined with non-oxidative ethanol metabolites. Furthermore, prevention of sustained extracellular Ca^{2+} influx protected ductal cell secretory function in vitro and decreased pancreatic ductal cell death. Orai1-inhibition maintained exocrine pancreatic secretion in in vivo AP models. Finally, H_2O_2-induced oxidative stress elevate the intracellular Ca^{2+} concentration in TRPM2 WT ductal fragments, not in KO ductal fragments so it is a TRPM2-channel-mediated influx. In conclusion, our results indicate that Orai1 inhibition prevents acute pancreatitis-related ductal cell function impairment and holds the potential of improving disease outcome.

New observations

- This is the first study demonstrating the expression of Orai1 in the apical membrane of pancreatic ductal epithelial cells
- We described the functional activity of Orai1 in isolated mouse pancreatic ductal cells
- The inhibition of Orai1 abolishes bile acid or ethanol+palmitic acid-induced extracellular Ca^{2+} influx in the pancreatic ductal epithelia thereby prevent the Ca^{2+}-overload-mediated functional and morphological damage of ductal cells
- Inhibition of Orai1 prevents bile acid- and ethanol-induced decrease of HCO_3^- secretion and CFTR function in pancreatic ductal epithelia
- Our results suggest that bile acids and ethanol induce Orai1-mediated Ca^{2+} influx in a Stim1-dependent manner.
- Inhibition of Orai1 preserves the ductal ion and fluid secretion both *in vitro* and *in vivo* in different forms of AP
- This is the first study demonstrating the expression and function of TRPM2 in pancreatic ductal epithelial cells
- Sustained elevation of $[Ca^{2+}]_i$ in response to H_2O_2 was largely due to TRPM2-channel-mediated influx of extracellular Ca^{2+}
- CDC inhibited the HCO_3^- secretion both in TRPM2 KO and TRPM2 WT ductal cells, the genetic knockout of TRPM2 had no protective effect, suggesting that bile acids affect ductal cells via a TRPM2-independent mechanism.

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