The role of intracellular Ca^{2+} signaling in the exocrine pancreatic damage during acute pancreatitis

Ph.D. Thesis

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LIST OF FULL PAPERS RELATED TO THE THESIS

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

I. **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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[IF$_{2018}$: 4.95]

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PLoS one. 2106 Oct 24; doi: 1.01371/jurnal.pone.0165244
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Number of full publications:	**4 (1 first author)**
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1. Introduction

The exocrine pancreas plays a central role in the digestion of meal in the duodenum under physiological conditions. Whereas pathophysiological stimuli (like excessive ethanol intake, or biliary reflux) can provoke acute pancreatitis (AP), which is a frequent disorder with high mortality in severe cases. Most of the gland (~80%) consist of acinar cells, with about 4% of ductal cells, 4% of blood vessels and 8% of endocrine cells and cells of the extracellular matrix. One of the features of the exocrine pancreas is the ´tree like´ structure whereas the acinar cells located at the end of the ´branches´ organized in lobolus. The pancreatic acinar cells produce and secrete the digestive enzymes in response to meal intake. The other cell type pancreatic ductal epithelial cells secrete high amount of HCO_3^--rich alkaline fluid. The alkaline pancreatic fluid secretion washes out the digestive enzymes of the pancreatic ductal tree and neutralizes the acidic chyme entering the duodenum. Under physiological conditions the acinar cells are protected against premature and intracellular activation of zymogenes. When this protective mechanism fails pancreatic autodigestion is initiated and AP can develop. Numerous studies suggesting that extra - as well as intracellular Ca^{2+} play an important role in the initiation of pancreatic protease activation.

1.1. Intracellular Ca^{2+} signaling in pancreatic acinar cells

In the exocrine pancreas intracellular Ca^{2+} signaling plays a major role in the signal transduction during neural and hormonal stimulation of digestive enzyme and fluid secretion. The produced digestive enzymes are packed in zymogen granules in the apical pole of the acinar cells, which is released in response to secretagogue stimuli induced intracellular Ca^{2+} elevation.

1.2. Acute pancreatitis

Acute pancreatitis (AP) is one of the leading causes of acute hospitalization among non-malignant gastrointestinal diseases and thus having a significant clinical and economic burden. Major etiologic factors leading to the development of AP are impacted gallstones causing biliary AP and heavy alcohol consumption. Primarily AP is a localized to and represents a sterile inflammation of the exocrine pancreas, which however can lead to local and systemic complications. Local complications include infected necrosis of the pancreatic tissue and surrounding visceral fat, whereas systemic complications are dominated by acute lung injury and renal dysfunction leading

to multiorgan failure. The average mortality rate is ~ 3%, however in severe cases it can reach to 28-30%. Although the disease pathogenesis is multifactorial sustained intracellular Ca^{2+} overload caused cell toxicity is considered as a hallmark of the disease. Intracellular Ca^{2+} overload can lead to premature activation of trypsinogen, mitochondrial damage and cell necrosis in acinar cells bile acid induced acute pancreatitis. Biliary pancreatitis is one of the most common forms of AP, although the mechanism of the disease development is still a matter of debate. When bile acid reach the pancreatic exocrine cells, several observations suggest that bile acids can trigger multiple intracellular changes in pancreatic acinar and ductal cells contributing to the development of biliary acute pancreatitis. In acini bile acids provoke a sustained elevation of the intracellular Ca^{2+} due to IP_3R and ryanodine receptor activation. In addition, taurolithocholicacid 3-sulfate induced mitochondrial damage in acinar cells accompanied by diminished intracellular ATP production and loss of the mitochondrial membrane potential ($\Delta\Psi_m$).

1.3. Reactive oxygen species role in Ca^{2+} signaling

Reactive oxygen species (ROS) are essential component of the signal transduction in cells and their generation is balanced by oxidant and antioxidant molecules. ROS are produced during mitochondrial respiration derived from complexes I and III of the mitochondrial electron transport chain. In the recent years several effects of ROS in intracellular signalling has been described. As an example of this regulation of intracellular signalling Booth et al showed that the ER-mitochondrial interface hosts a nanodomain of H_2O_2, which was triggered by cytoplasmic Ca^{2+} elevations and is a positive regulator of Ca^{2+} oscillations. Such nanodomains can be considered as important elements of inter-organelle communication. ROS production also have many effects on the ion channels and pumps involved in the intracellular Ca^{2+} signalling, which was reviewed by Criddle et al. In contrast, unbalanced generation of ROS has been suggested as crucial step in the pathogenesis of diseases via disruption of lipid membranes, proteins and DNA. In the pathogenesis of AP ROS production was shown to determine pancreatic acinar cell fate as the ROS induced by menadione triggered apoptotic cell death. Booth et al showed that bile acids induce ROS generation in pancreatic acinar cells from mice and humans. They also demonstrated that generation of ROS in response to bile acids occurred within the mitochondria and was dependent on the increase of mitochondrial Ca^{2+}. In addition, during the pathogenesis of AP, ROS released by circulating neutrophils during in the inflammatory response might also contribute to the

development of cell damage and local and systemic complications of AP.

1.4. Transient Receptor Potential Melastatin 2

TRPM2 is a member of Melastatin subfamily of the TRP channels, which is one of the largest cation channel family. Like most of the TRP channels are permeable to Ca^{2+} TRPM2 is also a Ca^{2+} permeable cation channel, which is involved in different physiological and pathophysiological processes associated with redox signaling and oxidative stress. The expression of TRPM2 has been demonstrated in different cell types and organs including pancreatic β cells , spleen, neurons, bone marrow cells, cardiomyocytes and immune cells such as T lymphocytes, macrophages and neutrophils. The channel is activated under oxidative stress by ROS and can be activated by free intracellular ADP-ribose (ADPR) in synergy with free intracellular Ca^{2+} as well. ADPR is produced by the enzyme Poly (ADP-ribose) Polymerase (PARP) in response to oxidative stress induced DNA damage and promotes apoptotic cell death. Another possible source of ADPR are the mitochondria, where oxidative stress could induce the production of free ADPR. The most prominent role of TRPM2 has been established in the development of inflammatory disorders. In monocytes Ca^{2+} influx via TRPM2 was shown to increase chemokine production, leading to enhanced neutrophil infiltration in inflammatory bowel disease. The intracellular Ca^{2+} overload damaged the acinar cells and lead to the loss of acinar cell function (saliva production) in the salivary glands Numerous of studies describe the expression of TRPM2 in various epithelial cells, moreover, emphasize the central role of the channel in the pathogenesis of oxidative-stress-related diseases. Although very likely, the expression or function of TRPM2 in exocrine pancreatic cells has never been investigated.

1.5. 3D organoids as models of epithelial physiology

In the last decades several studies highlighted that 2-dimensional cell cultures have several limitations (including artificial cell surface interactions, limited membrane contacts, lack of cell polarity), which limit their potential research use. On the other hand, rodent models of human diseases show major differences from the human disease phenotype and outcome inhibiting the translation of preclinical findings to clinical benefit. In then recent years, 3 dimensional organoid cultures emerged as potential preclinical tools for disease modeling and drug screening. Several reports suggest that epithelial cells in OC maintain the original cellular diversity and organization

of the organ of origin. As cell-to-cell contacts dominate OCs due to the spheroid shaped architecture of the organoids, it seems to be more relevant representation of the original cell microenvironment in contrast to 2D cultures. In pancreatic research currently OCs are studied as relevant human models of tissue development and carcinogenesis. Due to the above described advantages of the OCs, they might be a better model in exocrine pancreatic research, however we have only limited information about the functional properties (like Ca^{2+} signaling) of OCs.

2. Aim of the Study

I. (Pulication No. 1.)

Aberrant Ca^{2+} signaling and increased ROS production can cause mitochondrial damage, intraacinar digestive enzyme activation and cell death. TRPM2 is a non-selective cation channel that plays a major role in oxidative stress induced cellular Ca^{2+} overload in different cell types, but its expression and function is not known in the exocrine pancreas.

Therefore, our aims were to:

- characterize the expression and function of TRPM2 in the exocrine pancreas;
- assess the role of TRPM2 in bile acid induced cell damage;
- investigate the role of TRPM2 in the determination of acinar cell fate;
- compare the severity of cerulean induced and biliary AP in wild type and TRPM2 knockout mice.

II. (Pulication No. 2.)

Organoid cultures seem to be a suitable model for in vitro tests; however, we have no information how Ca^{2+} signals are developed in the epithelial cells in organoids.

Therefore, our aim was to:

- compare the Ca^{2+} signaling in primary epithelial cells and organoids.

3. Materials and Methods

3.1 Animals and Ethics

TRPM2 knockout mice were generously provided by Yasuo Mori, Kyoto, Kyoto University, Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering. The knockout mice were generated on C57B1/6 background as described previously. TRPM2 +/+ and TRPM2-/- mice were breed from TRPM2 +/- animals and used for experiments between the age of 8-12 weeks. Genotyping was performed by polymerase chain reaction (PCR) and visualized by standard agarose gel electrophoresis. Mice were used in adherence to the NIH guideline 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./2523/2018.**

3.2. Isolation of pancreatic acinar cells

WT and TRPM2 KO mice were sacrificed using terminal anesthesia with 250 mg/bwkg sodium pentobarbital. Acinar cells were isolated from the pancreas with enzymatic digestion.

3.2.1. Isolation of mouse ductal pancreatic ductal fragments and establishment of pancreatic organoid culture

Isolation of pancreatic ducts were performed by microdissection. Mouse pancreatic organoid cultures were established using the previously published protocol by Boj et al.

1.3. Gene expression analysis

The combination of reverse-transcription (RT-PCR) and conventional PCR were used to investigate the gene expression of TRPM2. The cDNA specific primers (forward: ACGGGCAATATGGTGTGGAG; reverse: CACCTCCCCTTTCCTTCGTT). To validate the primers mouse brain lysate was used.

3.4. Immunofluorescent labeling

The acinar cells of WT and TRPM2 KO mice were isolated and attached to poly-l-lysine coated cover glass. After the fixation and blocking the sections they were incubated with an ATTO-594 conjugated rabbit polyclonal primary antibody against TRPM2 (Anti - TRPM2 - ATTO-594; Alamone Labs; Cat. No: ACC-043-AR) in 1:50 dilution overnight on 4°C. Nuclear labelling was performed with Hoechst33342 for 15 minutes. visualized with a ZEISS LSM 880 confocal

microscope equipped with at 40x water based immersion objective.

3.5 Electrophysiology

For electrophysiology recordings, pancreatic acinar cells were isolated from mouse pancreas as described previously, with slight modifications. Whole cell currents were acquired at room temperature using an Axopatch 200B amplifier and a Digidata 1322A digitiser (Axon Instruments) at a 50 kHz sampling rate and filtered online at 5 kHz using a low-pass Bessel filter. Data acquisition was performed using p Clamp 9 software package (Axon Instruments). Cation currents were recorded during 100 ms long test pulses at step potentials between −60 and +120 mV both under control conditions and during treatment.

3.6. Measurement of intracellular Ca^{2+} concentration in acinar cells, ductal fragments and organoids

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), was measured by loading cells with 5μM Fura-2-acetoxymethyl ester (FURA-2-AM) for 15 min in acinar cells and 30 minutes in pancreatic ductal fragments or organoids in the presence of 0.05% Pluronic F-127. Acini, pancreatic ductal cells and organoids were attached to a poly-L-lysine-coated coverslip (24 mm diameter) as the base of a perfusion chamber and were mounted on the stage of an Olympus IX71 inverted microscope and were perfused with different solutions at 37°C. Region of interests (ROIs) were determined by the xcellence softver (Olympus) and changes of $[Ca^{2+}]_i$, was determined by exciting the cells with an MT20 light source equipped with 340/11 and 380/11 nm excitation filters. Excitation and emission wavelengths were separated by a 400 nm beam splitter and the emitted light was passed through a 510/80 nm emission filter. The changes of $[Ca^{2+}]_i$ were calculated from the F340/F380 fluorescence ratio.

3.7. Mitochondrial membrane potential measurements

Changes of the mitochondrial membrane potential ($\Delta\Psi_m$) were followed by using tetramethylrhodamine - methyl ester (TMRM) which accumulates in the mitochondria depending on $\Delta\Psi_m$. Pancreatic acinar cells were incubated with 100nM TMRM (ThermoFisher Scientific Cat. No.: T668) in standard HEPES solution for 20 min at 37°C on a poly-l-lysine-coated cover glass

and were then perfused with solutions at 37°C. The perfusion solutions were also supplemented with 100nM TMRM to avoid dye leakage. Changes in TMRM fluorescence was monitored using a ZEISS LSM880 confocal microscope equipped with a 40x water immersion objective. The cells were exited with 543 nm and the emitted light was captured between 560nm and 650nm. ROIs were places on the mitochondria of pancreatic acinar cells. Fluorescence signals were normalized to initial fluorescence intensity (F_1/F_0) and were expressed as relative fluorescence.

3.8. Investigation of acinar cell fate

To quantify acinar cell death an apoptosis/necrosis detection kit was used according to the manufacturer´s instruction (Abcam Cat. No.: ab176750). Briefly, pancreatic acinar cells from wild-type (WT) and TRPM2 KO mice were isolated with modifications to improve overall cell survival (shorter tissue digestion and gentle centrifugation was applied) and incubated with 1 mM H_2O_2 or 250 µM CDC for 30 min. Cells were then centrifuged and washed twice with PBS. Cells were then resuspended in 200 µL of Assay Buffer and loaded with CytoCalcein 450, Nuclear Green and Apopxin Deep Red at room temperature for 30–60 min. The cells were placed on a Cellview cell culture slide (Greiner Bio-One cat. no.: 543979) for imaging. Images were captured using a Zeiss LSM880 confocal microscope with different channels and wavelengths according to each dye: CytoCalcein 450 (Ex/Em = 405/450 nm), Nuclear Green (Ex/Em = 490/520 nm) and Apopxin Deep Red (Ex/Em = 630/660 nm). For each condition, five images were captured, and the total number of cells was counted by two independent investigators. Cells with Nuclear Green staining were considered necrotic, with Apopxin Deep Red staining apoptotic, whereas double stained cells were considered necrotic.

3.9. In vivo acute pancreatitis models

Cerulein-induced acute pancreatitis was triggered by 10, hourly intraperitoneal injections of 50 µg/bwkg cerulein (control groups received physiological saline). Two hours after the last injection, mice were euthanized with 85 mg/kg pentobarbital. Biliary AP was triggered by the administration of 4% Na-taurocholate (TC) into the common bile duct as described previously by Perides et al. Mice were sacrificed 24 h later using pentobarbital (85 mg/kg i.p.). In both cases, blood samples were collected after terminal anesthesia through the inferior vena cava, and the pancreas were

removed immediately. Blood samples were placed on ice and then centrifuged at 2500 RCF for 15 min at 4°C. Serum samples were used to measure serum amylase activity. Absorbance of the samples was detected at 405 nm using a FLUOstar OPTIMA (BMG Labtech) microplate reader. Formaldehyde-fixed pancreas samples were embedded in paraffin, and 4 μm thick sections were cut and stained with hematoxylin–eosin. Histologic parameters such as oedema, inflammatory cell infiltration and necrosis were scored by three independent investigators blinded to the protocol. Averages of the scores were calculated and included to the manuscript. Total histological score was calculated by adding the individual scores together.

3.10. Statistics

Statistical analysis was performed by GraphPad Prism software. All data are expressed as means ± SD. Both parametric (one-way analysis of variance) and nonparametric (Mann Whitney test, Kruskal Wallis test - for analysis of the acinar cell survival assay) tests were used based on the normality of data distribution. A p value of less than 0.05 was accepted as statistically significant.

4.Results

4.1. TRPM2 channel is expressed in the exocrine pancreas

End-point PCR analysis of isolated acini confirmed that the TRPM2 gene was expressed in the exocrine pancreatic cells. The immunofluorescent labelling of TRPM2 was performed on isolated acinar clusters the confocal images showed that TRPM2 channels were expressed on the basolateral membrane of the pancreatic acinar cells.

4.2. Functional TRPM2 channels are present in pancreatic acinar

When isolated WT pancreatic acini were challenged with 1 mM H_2O_2 to increase ROS, a rapid and sustained increase of $[Ca^{2+}]_I$ was observed, which was significantly reduced in the TRPM2 knockout (KO) acini (0.41 ± 0.09 vs 0.17 ± 0.029, respectively). In cells treated in an extracellular Ca^{2+}-free medium, Ca^{2+} elevation was found to be significantly impaired, and no difference was detected between WT and TRPM2 KO cells. 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+}. In

addition, H_2O_2 activated a reversible cationic membrane current, with a relative linear I–V relationship as was reported previously for TRPM2.

4.3. TRPM2 contributes to bile-acid-induced extracellular Ca^{2+} influx in pancreatic acinar cells

Bile acids have an important pathogenetic role in the development of biliary pancreatitis and can cause the release of Ca^{2+} from intracellular stores and trigger extracellular Ca^{2+} influx. Therefore, to study the role of TRPM2 in this process, the intracellular Ca^{2+} elevation in response to bile acid treatment was examined in pancreatic acini of WT and TRPM2 KO mice. Administration of 250 μM CDC was found to trigger a rapid, sustained increase in $[Ca^{2+}]_I$, which was markedly impaired in the TRPM2 KO acinar cells (0.834 ± 0.02 vs 0.655 ± 0.04). These results highlight that TRPM2 plays an important role in bile-acid induced extracellular Ca^{2+} influx in pancreatic acinar cells.

4.4. Lack of TRPM2 decreases acinar cell necrosis during bile acid exposure

Pancreatic acinar cell fate determines the severity of AP. Because of this, it was also important to characterize the role of TRPM2 in acinar cell death. In the untreated control samples, ~85% of the acinar cells were viable in both the WT and TRPM2 KO samples, which is comparable to previously published results. Incubation of WT and TRPM2 KO acini with 1 mM H_2O_2 for 30 min remarkably decreased the number of viable cells, and necrotic cell death was significantly increased. A lack of TRPM2 was observed to protect acinar cells from oxidative stress- induced cell necrosis during H_2O_2 treatment (% of viable cells: 19.4 ± 0.4 in WT vs 49.1 ± 1.2 in TRPM2 KO). The rate of apoptosis was similar in TRPM2 KO and WT acini (% of apoptotic cells: 9.1 ± 4.3 in WT vs 10.8 ± 2.5 in TRPM2 KO), whereas necrosis was significantly impaired in TRPM2 KO acini (% of necrotic cells: 71.5 ± 4.2 in WT vs 40.1 ± 3.2 in TRPM2 KO). Similarly, incubation of acinar cells with 250 μM CDC for 30 min decreased the number of live cells in WT sample, however overall cell survival was remarkably improved by TRPM2 deletion (% of viable cells: 48.3 ± 0.9 in WT vs 74.1 ± 1.3 in TRPM2 KO). TRPM2 deletion significantly decreased both apoptotic and necrotic cell death in the CDC treated group (WT: 15.4 ± 2.5% vs KO: 8.5 ± 1.3% and WT: 36.3 ± 2.2% vs KO:17.4 ± 1.3%, respectively). Importantly, the lack of TRPM2 channels

resulted in a ~30% decrease in acinar cell death, suggesting that TRPM2 has an important contribution to acinar cell death during biliary AP.

4.5. Lack of TRPM2 does not prevent mitochondrial damage during bile acid exposure

We wanted to further characterize the intracellular mechanisms that play a role in TRPM2-channel mediated cell necrosis. As TRPM2 has been reported to induce mitochondrial damage, therefore we compared the mitochondrial membrane potential ($\Delta\psi m$) in WT and TRPM2 KO pancreatic acinar cells. Administration of 1 mM H_2O_2 related in a marked drop of $\Delta\psi m$ in WT cells. The decrease of $\Delta\psi m$ was significantly lower in TRPM2 KO cells, whereas removal of the extracellular Ca^{2+} impaired the loss of $\Delta\psi m$ in WT cells to the level of TRPM2 KO acini. This suggests that extracellular Ca^{2+} influx through TRPM2 plays a crucial role in the oxidative stress induced mitochondrial damage seen in pancreatic acinar cells. The decrease of $\Delta\psi m$ in response to 250 μM CDC was also compared. However, no difference was seen between WT and TRPM2 KO cells. This may be due to the Ca^{2+} -independent direct mitochondrial toxicity of bile acids. Previously, TRPM2 channels have been suggested to be key mediators of diabetic stress-induced mitochondrial fragmentation in endothelial cell. Notably, in pancreatic acinar cells, fragmentation of mitochondria was not observed in response to either H_2O_2 or bile acid treatment.

4.6. Lack of TRPM2 decreases the severity of experimental biliary pancreatitis

To determine the role of TRPM2 in the pathogenesis of AP, the disease severity of WT and TRPM2 KO animals was compared in two well-established experimental AP models. In the cerulein induced pancreatitis experimental model, there were no significant differences were detected between WT and TRPM2 KO mice. The control animals had normal pancreatic histology in both groups, whereas cerulein hyperstimulation caused extensive pancreatic damage. Despite this, no significant differences were observed between the histological parameters of WT and TRPM2 KO animals. The extent of interstitial oedema (3.14 ± 0.25 for WT vs 3.03 ± 0.34 for KO), leukocyte infiltration (2.74 ± 0.53 for WT vs 3.04 ± 0.23 for KO, $p = 0.08$) or necrosis(18.64 ± 3.16 for WT vs 21.32 ± 3.58 for KO) showed no significant difference in the cerulein-treated groups. More importantly, the role of TRPM2 channel in the pathogenesis of biliary AP was also examined. In this model, pancreatitis was induced by intraductal infusion of 4% Na-taurocholate (control

animals received physiological saline) as described previously. The infusion of 4% Na-taurocholate induced necrotizing pancreatitis in both WT and TRPM2 KO mice, accompanied by elevated histological and laboratory parameters. The extent of interstitial oedema (2.8 ± 0.16 for WT vs 2.7 ± 0.2 for KO) or leukocyte infiltration (3.3 ± 0.38 for WT vs 2.7 ± 0.29 for KO, p = 0.08) was not significantly different in the Na-taurocholate-treated groups. Notably, the extent of necrosis was significantly higher in the WT group in comparison to the TRPM2 KO animals (41.3% ± 7.13% for WT vs 26.4% ± 5.5% for KO). In accordance with these findings, serum amylase activities were also significantly higher in the Na-taurocholate-treated WT animals versus the TRPM2 KO group. This perfectly mimicked the *in vitro* results obtained in this study, further confirming the crucial role of the TRPM2 channel in the pathogenesis of biliary AP.

4.7. Intracellular Ca^{2+} signaling in pancreatic organoids

Organoid cultures emerged as novel 3D models of epithelial physiology and pathology, which have several advantages (see in the Introduction). Therefore

OCs might be a better model in exocrine pancreatic research; however, we have only limited information about the functional properties (like Ca^{2+} signaling) of OCs. To investigate this, we compared Ca^{2+} signaling in primary pancreatic ducts and in OCs. For the release of Ca^{2+} from the endoplasmic reticulum Ca^{2+} stores, we used two Ca^{2+} mobilizing agonists ATP and carbachol). We observed that both agonists induced peak-plateau type Ca^{2+} elevation in the tested concentrations. These signals showed no significant differences in the maximal response. We also compared the store operated Ca^{2+} entry caused by the ER store depletion. Applying this assay, we detected that the ER Ca^{2+} release induced by 25 μM cyclopiazonic acid (CPA) was significantly higher in isolated ducts, whereas the Ca^{2+} influx was significantly higher in OCs. These observations need further investigation to determine the biological relevance of this phenomena.

5. Discussion

5.1. TRPM2-mediated extracellular Ca^{2+} entry promotes acinar cell necrosis in biliary acute pancreatitis

Several publications suggested that bile acids can generate prolonged intracellular Ca^{2+} elevation,

increase ROS production and damage the mitochondria in pancreatic acini. These pathologic changes can trigger the development of AP, which is a severe inflammatory disease of the gastrointestinal tract that has no specific treatment. The role of TRPM2 emerged in the development of Ca^{2+}-dependent cell injury as a ROS-sensitive non-specific cation channel, however the possible role of TRPM2 in the pathogenesis of AP has yet to be investigated.

Though the expression of TRPM2 has been demonstrated previously in different cell types, including inflammatory cells, myocytes and epithelial cells, to our knowledge, this is the first report demonstrating the expression of TRPM2 in the exocrine pancreas. Using conventional PCR and immunolabelling techniques, the expression of TRPM2 in the basolateral membrane of acinar cells was confirmed. In addition, increased intracellular ROS was found to trigger TRPM2-mediated extracellular Ca^{2+} influx. Intracellular Ca^{2+} signalling is one of the major signalling pathways in the exocrine pancreas which regulates the secretion of digestive enzymes in acinar cells as well as ion and fluid secretion in ductal cells. Therefore, it might be possible that TRPM2-mediated Ca^{2+} entry could contribute to physiological signalling, though further studies are required in order to confirm this.

Disturbed intracellular Ca^{2+} homeostasis has been suggested by several studies to play a pivotal role in bile-acid-induced exocrine pancreatic cell damage. In pancreatic acini, bile acids trigger dose dependent intracellular Ca^{2+} elevation via the activation of IP_3 and ryanodine receptors. In our experiments, CDC increased the $[Ca^{2+}]_I$ both in acinar and ductal cells, but genetic deletion of TRPM2 decreased Ca^{2+} elevation only in acinar cells. The results of this study show that the TRPM2 channel has a ~22% contribution to the bile-acid-generated Ca^{2+} signal in acinar cells.

Intracellular Ca^{2+} overload can lead to premature activation of trypsinogen, mitochondrial damage and cell necrosis in acinar cells. In this study, a knockout of TRPM2 resulted in a significant protection of pancreatic acinar cells from H_2O_2 and bile-acid-induced necrosis. Importantly, this protection was also observed in TC-induced AP as the extent of necrosis was significantly lower in TRPM2 knockout mice compared to the WT littermates.

As bile acids inhibited cellular ATP production and decreased $\Delta\Psi_m$, we also compared the changes of $\Delta\Psi_m$ in response to bile acid treatment in TRPM2 KO and WT acinar cells. The genetic knockout of TRPM2 and removal of the extracellular Ca^{2+} markedly reduced the drop of $\Delta\psi_m$, suggesting that extracellular Ca^{2+} influx through TRPM2 plays a crucial role in oxidative-stress-

induced mitochondrial damage. Despite this, we did not detect this protective effect in bile-acid-treated cells a result which might be explained by the Ca^{2+}-independent direct mitochondrial toxicity of bile acids. Previously, we and others also reported that the toxic effects of bile acids cannot be completely abolished by the removal of intracellular Ca^{2+} elevation. These results suggest that bile acids can induce mitochondrial damage in several different ways independently form intracellular Ca^{2+} overload. On the other hand, independently from mitochondrial damage, other Ca^{2+}-dependent toxic effects of bile acids have been described, which could also contribute to acinar cell necrosis.

In our study general TRPM2 knockout mice were used, therefore other factors might contribute to the observed protective effect of TRPM2 deletion in acute biliary pancreatitis. It is well described that inflammatory cells contribute to the severity of acute pancreatitis. Taken together, to the best of our knowledge, this is the first report of the expression and pathological function of the TRPM2 channel in the exocrine pancreas. We demonstrated that pancreatic acinar cells express functionally active TRPM2, which can be activated by increased oxidative stress. Importantly, we also provided evidence that TRPM2 activity contributes to bile-acid-induced extracellular Ca^{2+} influx in acinar cells, which promotes acinar cell necrosis independently from mitochondrial damage and increases the severity of bile-acid-induced experimental pancreatitis. These results suggest that inhibition of TRPM2 might be a potential option for use in treating biliary pancreatitis.

5.2. Intracellular Ca^{2+} signaling in pancreatic organoids

Pancreatic exocrine, secretory processes are challenging to investigate on primary epithelial cells. In pancreatic research currently OCs are studied as relevant human models of tissue development and carcinogenesis. OCs have recently emerged as promising *ex vivo* models of tissue physiology and pathophysiology. OCs are 3D self-organizing, organ like *in vitro* grown cell cultures where the cell-to cell contact is maintained. Reports suggest that cells in OCs maintain tissue specific gene expression, cell morphology and function and may represent features of malignant diseases. Although organoids are used in an increased number of studies, we only have limited experimental data about their physiological relevance, especially in case of pancreatic OCs. To answer these questions, we provide side by side comparison of Ca^{2+} signaling of epithelial cells in primary mouse isolated pancreatic ducts and organoids. To release the Ca^{2+} from the ER we used two Ca^{2+}

mobilizing agonist ATP and carbachol, where both agonists induced a peak-plateau type Ca^{2+} elevation. There were no significant differences in the maximal response, but Ca^{2+} release was slightly lower in organoid than in the isolated ducts. The ER Ca^{2+} released by CPA was higher in isolated duct, whereas the Ca^{2+} influx was significantly higher in OCs. These observations need further investigation to get to know the biological relevance of this fact.

6. Summary of new observations

- This is the first study demonstrating the expression of TRPM2 in the exocrine pancreas
- We described that TRPM2 is localized in the plasma membrane of pancreatic acinar cells
- H_2O_2 activated TRPM2 in pancreatic acinar cells as demonstrated by the intracellular Ca^{2+} elevation and characteristic cationic currents, which was absent in TRPM2 knockout acini
- Bile acids activate TRPM2 mediated extracellular Ca^{2+} influx
- H_2O_2 and bile acid -induced necrotic cell death was markedly reduced in acinar cells from TRPM2 knockout mice suggesting a remarkable role of TRPM2 in acinar cell fate
- the severity of bile induced (but not cerulean induced) experimental AP was lower in TRPM2 knockout mice suggesting that TRPM2 may represent a new pharmaceutical drug target for the treatment of biliary pancreatitis
- the agonist induced intracellular Ca^{2+} signaling was similar in isolated pancreatic ducts and pancreatic organoids, although the ER Ca^{2+} release induced by CPA was higher in isolated ducts than in organoids, whereas the Ca^{2+} influx was significantly higher in organoids.

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