CLOSING REPORT

OTKA PD 115974: Assembly and physiological role of the endoplasmic reticulum/plasma membrane microdomain in polarized epithelial cell

Period: 2015.09.01-2017.12.31

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I. Introduction

Epithelial cells line the surfaces of the entire gastrointestinal tract and its accessory organs. Existing at the interface of the body and the outside world, they are normally exposed to constantly changing extracellular stimuli and must have the capacity to adapt appropriately. These cells determine fluid and volume homeostasis of the body by secreting, or absorbing ions and fluid and they secrete several biologically active molecules. On the other hand disturbed epithelial functions can lead to severe inflammatory, or malignant diseases. It is can be easily conceded that proper spatiotemporal regulation of the signalling processes is essential in the maintenance of the physiological conditions. To ensure adequate regulation, the signalling complexes show polarized distribution and were suggested to cluster into domains in different subcellular regions. However our understanding of these regulatory mechanisms are only at the very beginning.

Very special subcellular signalling "highways" are the endoplasmic reticulum (ER)/plasma membrane (PM) junctions that are conserved structures where the ER is closely (the distance is 20-30 nm) tethered to the PM forming contact sites. Since their discovery [1] ER/PM junctions have been described in a plethora of cell types and have been reported to ubiquitously play role in multiple cellular functions. Increasing number of studies showed that within the ER/PM junctions signalling complexes organise to microdomains (MDs) that make signal transduction more specific and effective [2]. Despite their central role, we have limited information on how these MDs are formed, regulated or how they interact with each other. One of the most widely studied signalling process in the ER/PM junction is the store operated Ca^{2+} entry (SOCE). The hallmark of SOCE is the translocation of the ER Ca^{2+} sensor stromal interaction molecule 1 (Stim1) to the ER/PM junctions upon ER Ca^{2+} depletion [3], where it clusters and activates the PM Ca^{2+} channel Orai1 [4] to promote extracellular Ca^{2+} entry. Although Ca^{2+} influx is necessary for physiological responses, excess cellular Ca^{2+} overload is associated with cellular and tissue pathologies, therefore Ca^{2+} influx channels are extensively regulated. One form of Orai1 channel regulation is the slow Ca^{2+} dependent inactivation (SCDI), which is mediated by an inhibitor protein called Saraf [5, 6]. In our recent study we showed that Saraf can access Stim1 only when the protein complex is localised in a specific MD within the ER/PM junction [7]. We proved that directing Stim1 to a PtdIns(4,5)P_2-poor PM MD resulted in full activation of Orai1 current upon Ca^{2+} depletion, but completely abolished the interaction of Stim1 with Saraf and thus SCDI. In contrast, when Stim1 was targeted to PtdIns(4,5)P_2-rich PM MDs, Stim1 fully activated Orai1 and both the Stim1-Saraf interaction and SCDI were maximal. Most notably we showed that upon Ca^{2+} store depletion Stim1 is first recruited to the PtdIns(4,5)P_2-poor MD and then rapidly translocates to the PtdIns(4,5)P_2-rich MD to initiate SCDI. Previously, regulatory mechanisms by PtdIns(4,5)P_2 has been attributed to changes in PM PtdIns(4,5)P_2 levels [8]. Based on our original findings we proposed for the first time that dynamic translocation

of protein complexes between PtdIns(4,5)P_2 MDs is a novel form of regulation that can be particularly important for efficient temporal and spatial regulation of cell signalling.

II. Aims of the project

Based on our previous observations in this project I aimed to employ *in vitro* and *in vivo* cutting-edge techniques to (II.A) identify the molecular components of the ER/PM microdomains, (II.B) to determine the functional relevance of the ER/PM microdomains and (II.C) to understand the physiological role of ER/PM microdomains in epithelial cell functions.

III. Materials and methods

a. Constructs. Several important constructs were already available from our earlier studies [7]. Primers were obtained from Integrated DNA Technologies. Mutations were generated using the QuickChange Lightning site-directed mutagenesis kit from Agilent Technologies.

b. Cell transfection with cDNA and siRNA. HEK293, Hela or Cos7 cells were cultured in Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum. For cDNA or siRNA transfection Lipofectamine 2000 (Invitrogen) was used in OptiMEM (Gibco) according to the manufacturers instructions. For gene knock down 20–40nM siRNA was added [7]. The medium was changed to serum-containing medium 4-6 h after adding the siRNA duplexes or cDNA to the cells.

c. RT-PCR. RNA was extracted from cultured cells, or from isolated rodent pancreatic cells using the TRIZOL reagent and the mRNA levels was determined by quantitative PCR as described [7, 9]. The isolated mRNA was reverse transcribed into cDNA using a cDNA synthesis kit. The primers for quantitative reverse transcriptase-PCR will be purchased from Applied Biosystems. The fold change in the transcript levels of the specified genes was calculated by normalizing the Ct values to GAPDH.

d. FRET measurements. HEK293 cells were plated at low confluence on glass bottom dishes (MatTek Corporation) and transfected with ECFP (donor) and EYFP (acceptor) tagged constructs, for 12–16 h using Lipofectamine 2000 (Invitrogen) at 37°C. FRET imaging was performed at 37°C using an Olympus IX71 inverted fluorescent microscope equipped with X60 water immersion objective (NA: 1.2). Images were acquired at 5 s intervals using the three-cube method for sensitized emission [10, 11]. To minimize photobleaching, low power was used. Image analysis was performed with Olympus excellence software. FRET was determined on a pixel-by-pixel basis using a two-step FRET efficiency calculation protocol [7, 12].

e. Confocal imaging. For confocal imaging, HEK293, Cos7, or Hela cells were plated at low confluence on glass bottom dishes and was transfected with the indicated constructs for 8–12 h. The images were captured at room temperature with 40X objective water immersion objective (NA: 1.3). Images were processed with Photoshop CS3 (Adobe).

f. Ca^{2+} measurement. Cells attached to coverslips were loaded with 5 μM Fura2-AM by 30 min incubation at 37°C in culture media. After dye washing, florescence was measured using the 340 and 380nm excitation wavelength using the Olympus excellence imaging system as detailed before [13]. The results were given as the 340/380 ratio.

g. Pancreatic acinar cell isolation. Mouse pancreatic acinar cells were isolated according to the method of Pallagi et al [14]. Briefly, mice were anaesthetized with 85 mg/kg pentobarbital by i.p. injection. The pancreas was quickly removed and injected with 5ml of extracellular solution containing collagenase type IV (105 U/ml) then subjected to three successive 20-min incubations in this solution with vigorous shaking at 37°C.

h. Isolation of pancreatic ductal fragments. Mice were sacrificed and intra/interlobular ducts were isolated by collagenase digestion and microdissection from the pancreas and cultured overnight as previously described [13].

i. Measurement of pancreatic fluid secretion i*n vivo*. In vivo pancreatic fluid secretion was assessed in anesthetized mice. The abdomen was be opened, and the lumen of the common biliopancreatic duct was cannulated with a blunt-end 31-gauge needle. Then the proximal end of the common duct was occluded with a microvessel clip to prevent contamination with bile, and the pancreatic juice was collected in PE-10 tube for 30 min.

j. Measurement of intracellular pH and HCO_3^- efflux. Isolated pancreatic ducts were incubated in standard HEPES solution and loaded with BCECF-AM (1.5μmol/L) for 30 min at 37°C. The measurements will be carried out as described previously [15-17].

k. Cerulein-Induced Pancreatitis. Mice were administered 1, 7, or 10 hourly i.p. injections of cerulein (50 μg/kg per injection). Control mice were given physiological saline (PS: 0.9% NaCl) solution i.p. instead of cerulean as described previously.

IV. Results

A. Identification of the molecular components of the ER/PM microdomains

Increasing number of experimental data suggest that signalling and effector (such as ion channels) complexes in subcellular regions, such as the ER/PM junctions, have a highly integrated localisation determining their activity and function. These subcellular regions - membrane contact sites - are formed and tethered by several molecules. In yeast three proteins were found essential for ER/PM junction formation (Tcb1, Scs2 Ist2) [18]. The mammalian homologues of these are E-syt1-3, VAMP-associated protein (VAP) a, b and one of the 10 known Anoctamines (ANO1-10) [19-21]. However we have no information how Anoctamins participate in the formation of ER/PM junctions in mammalian cells.

1. The identification of ANO8 as a novel ER/PM microdomain protein

To investigate the role of anoctamin 1-10 isoforms (TMEM16 proteins) in the assembly of the ER/PM microdomains we used siRNA screen of ANO1-10 isoforms in HEK293 cells and

measured the SCDI of Orai1 by patch clamp. Briefly Palty et al. showed that the slow Ca^{2+} dependent inactivation (SCDI) of the Orai1 Ca^{2+} channel is mediated by it's regulatory protein Saraf [5]. In our recent study we described that Saraf can access Stim1 and inactivate Orai1 only if the protein complex is localized to the PtdIns(4,5)P_2-rich microdomain [7]. Therefore the SCDI of Orai1 can be used as a readout of the microdomain localisation of the Stim1-Orai1 complex. We found that only the knock down of ANO8 decreased the Saraf mediated SCDI of Orai1 (pink trace), the knock down of other isoforms had no effect (data not shown). This result suggests that that this is the ANO isoform playing role in the ER/PM microdomain formation (Figure 1.).

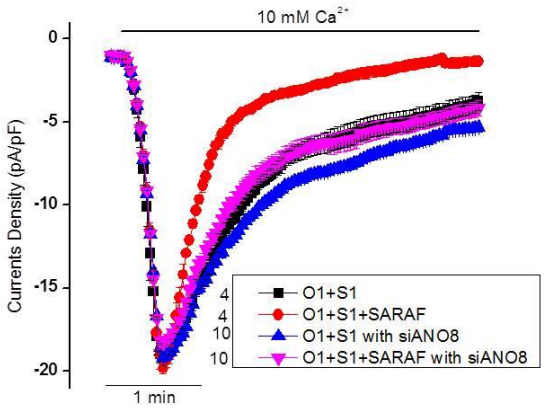

Figure 1. ANO8 knock down decreased the Saraf mediated SCDI of Orai1 Ca^{2+} channel in HEK293 cells. HEK293 cells were treated with scrambled or siANO8 and were transfected with Orai1, STIM1 and with or without Saraf and Orai1 current was measured dialyzing cells with pipette solution containing 1.5mM EGTA for 3 min in Ca^{2+}-free bath. CRAC current was initiated by superfusing with a solution containing 10mM Ca^{2+}. The currents were leak subtracted to facilitate demonstrating the SCDI. The numbers listed in parenthesis next to the traces indicates the number of experiments. All results are given as the mean±s.e.m.

2. Anoctamin 8 regulates the activity and inactivation of Orai1

Next we overexpressed ANO8 in HEK293 cells, which increased the Orai1 current amplitude (left panel) and the Saraf mediated SCDI of the channel (right panel, normalised current) (Figure 2.).

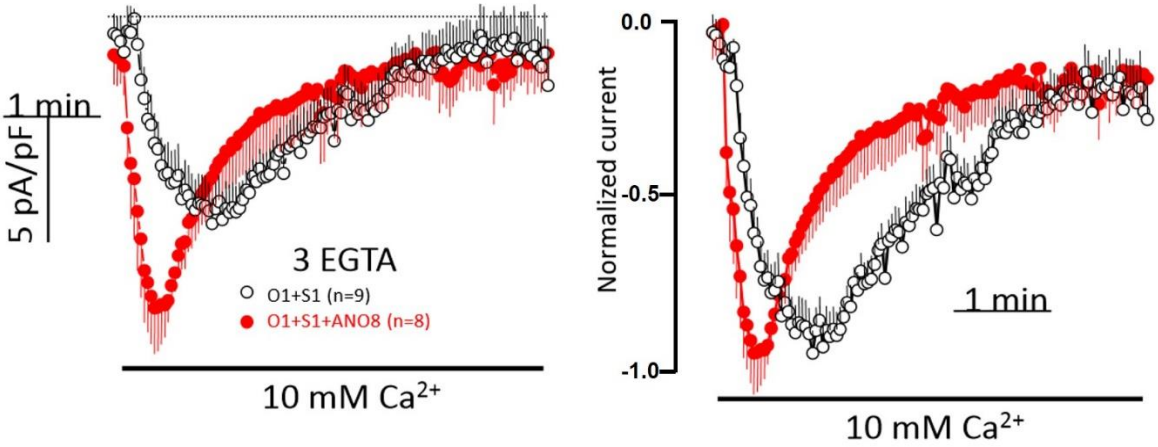

Figure 2. The overexpression of ANO8 increased the Orai1 current and the Saraf mediated SCDI of Orai1 in HEK293 cells. A. HEK cells were transfected with Orai1, Stim1 and with or without ANO8. Orai1 current was measured dialyzing cells with a pipette solution containing 3mM EGTA for 3 min in Ca^{2+}-free bath. CRAC current was initiated by perfusing with a solution containing 10mM Ca^{2+}. The

currents were leak subtracted. **B.** Normalised currents demonstrate the increased SCDI of the Orai1 channel in the presence of ANO8. The numbers listed in parenthesis next to the traces indicates the number of experiments. All results are given as the mean±s.e.m.

3. ANO8 interacts with Stim1 that can stabilise the Stim1-Saraf complex

In the next step we evaluated the interaction of ANO8 with Stim1 to get deeper understanding how ANO8 affects the Orai1 activity and inactivation. The intermolecular FRET measurements showed that Stim1 interacts with ANO8 under resting conditions, which is further augmented by the ER Ca^{2+} store depletion. We also wanted to see the effect of ANO8 on the interaction of Stim1 with Saraf. We found that te overexpression of ANO8 significantly increases the FRET between Stim1 and Saraf upon Ca^{2+} store depletion. ANO8 colocalizes with Stim1 and Orai1 upon ER store depletion and increased the number of Stim1 puncta. These results suggest that ANO8 is an important molecular component of the SOCE and its function is to stabilize the Stim1-Orai1 complex upon ER store depletion.

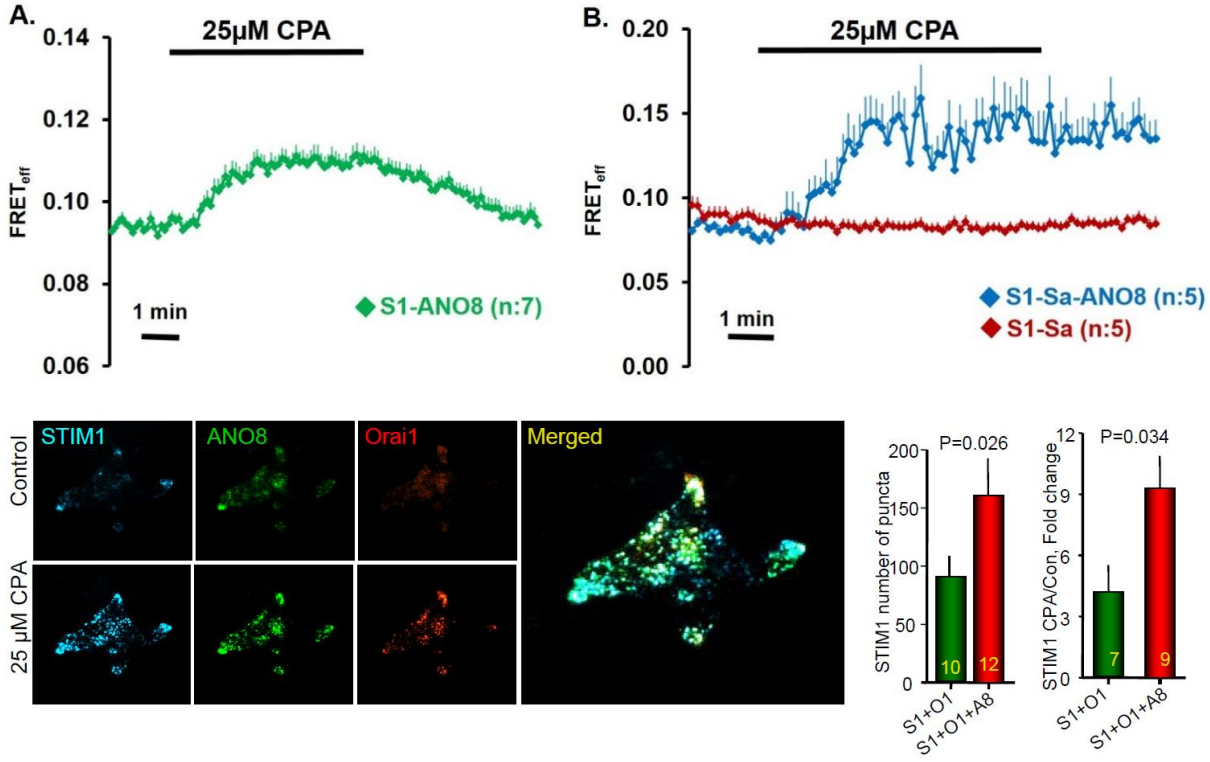

Figure 3. The interaction of ANO8 with Stim1. A. In intermolecular FRET we used Stim1-CFP as donor and ANO8-YFP as acceptor. ANO8 interacts with Stim1 in resting conditions, which is further increased during the depletion of the ER Ca^{2+} stores with cyclopiazonic acid (CPA). **B.** The overexpression of ANO8-HA increased the interaction of Stim1-CFP and Saraf-YFP upon Ca^{2+} store depletion. The numbers listed in parenthesis next to the traces indicates the number of experiments. **C.** Confocal images of Cos7 cells expressing Stim1, ANO8 and Orai1. As visible on the merged picture the depletion of the ER Ca^{2+} stores with 25μM CPA increased the colocalisation of the three proteins. In addition, the number of puncta formed by Stim1 is increased, if ANO8 is present. All results are given as the mean±s.e.m.

II.B. Determine the functional relevance of the ER/PM microdomains

As mentioned above Ca^{2+} and cAMP signalling are the hallmarks of epithelial signal transduction, however only limited information is available how they organize to MDs and how this localisation affect their activity and interactions with each other. It is described that Orai1 and the PM bound Ca^{2+} activated adenylyl-cyclase 8 (AC8) physically interact, which maximizes the efficiency of the response [22], whereas the activation of Stim1 recruits AC3 resulting in enhanced cAMP accumulation and PKA activation [23]. These suggest tight interrelated communication between the two signalling pathways. Although likely, the role of ER/PM junction MDs in this communication has not been investigated yet.

1. Orai1 and E-Syt1 increase the intracellular cAMP production

In our experiments we used Epac1 camp (developed and provided by Martin J. Lohse, DFG Research Center for Experimental Biomedicine, University of Würzburg) a FRET based intracellular cAMP sensor to assess the effect of Stim1, Orai1 and E-Syt1 on the cellular cAMP production. We found that the overexpression of Stim1 has no effect on the forskolin stimulated intracellular cAMP levels. On the other hand the overexpression of Orai1 enhanced the stimulated cAMP production (Figure 4.). This increase was significantly higher, when AC8 was present in the cells (Figure 5.). In addition, we showed that E-Syt1 significantly enhanced the stimulated cAMP production by AC8 (Figure 6.). This observation highlights a novel role of E-Syt1, since this ER/PM junction protein was not connected earlier to the intracellular cAMP production.

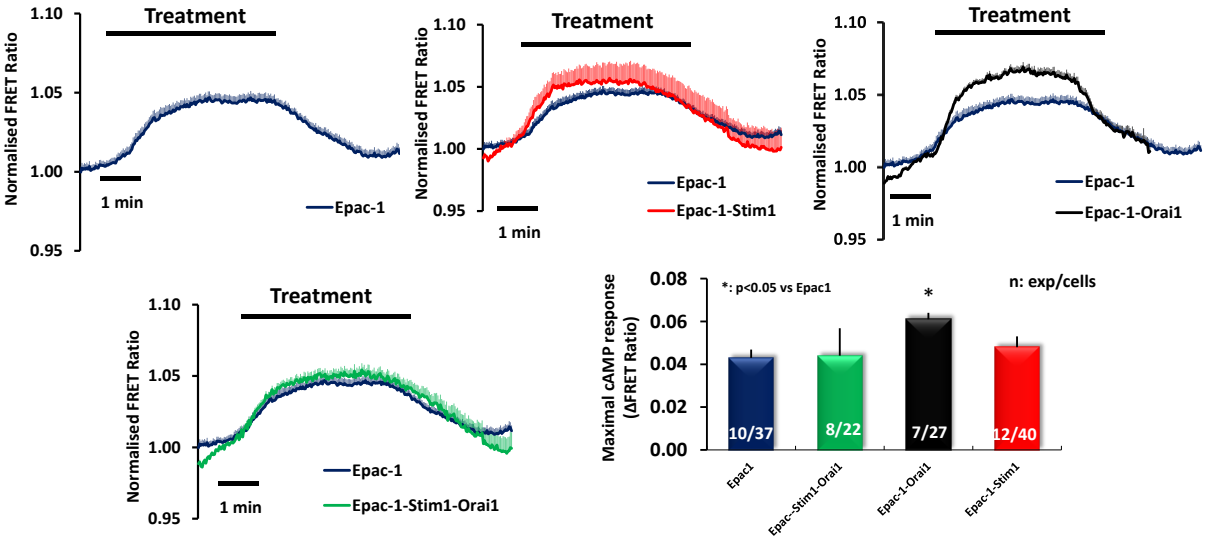

Figure 4. The effect of Stim1 and Orai1 on the cAMP production. The average traces of the cAMP measurements show the effect of Stim1 and Orai1 on the cAMP production. Cells were treated with 5µM forskolin that induced a reversible increase in the cAMP production. In Orai1 expressing cells the response to forskolin was significantly higher. The bar chart shows the maximal cAMP elevation upon stimulation. N= number of experiments/number of cells (highlighted on the bar charts). All results are given as the mean±s.e.m.

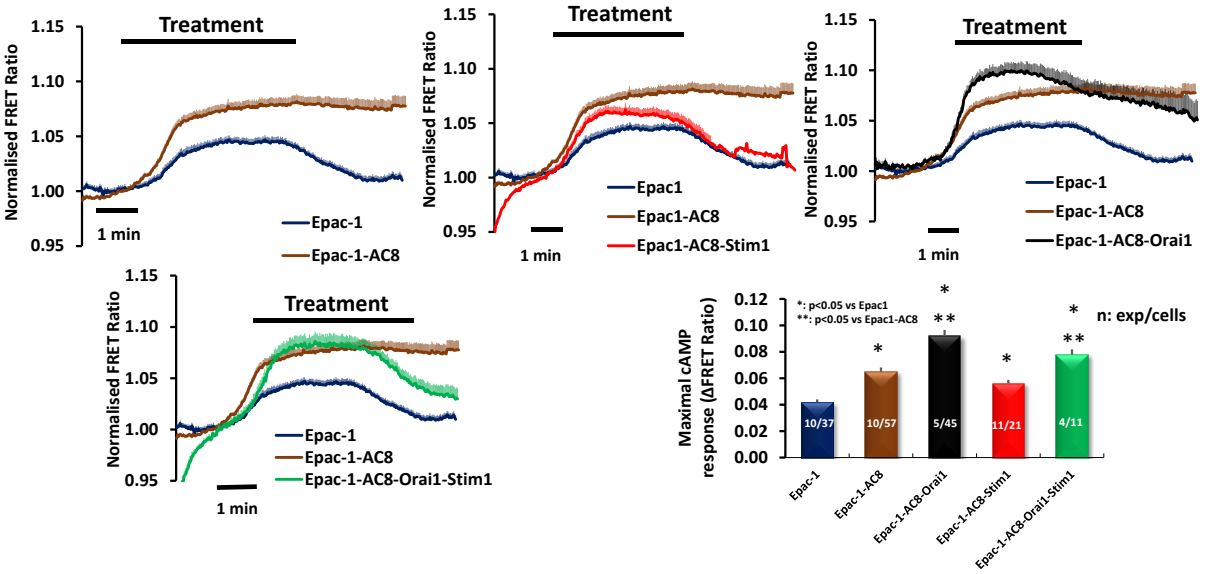

Figure 5. The effect of Stim1 and Orai1 on the cAMP production by AC8. The average traces of the cAMP measurements show the effect of Stim1 and Orai1 on the cAMP production by AC8. Cells were treated with 5µM forskolin that induced a reversible increase in the cAMP production. The expression of AC8 markedly increased the cAMP production. Orai1 expression induced a further increase in cAMP production in response to forskolin. The bar chart shows the maximal cAMP elevation upon stimulation. N= number of experiments/number of cells (highlighted on the bar charts). All results are given as the mean±s.e.m.

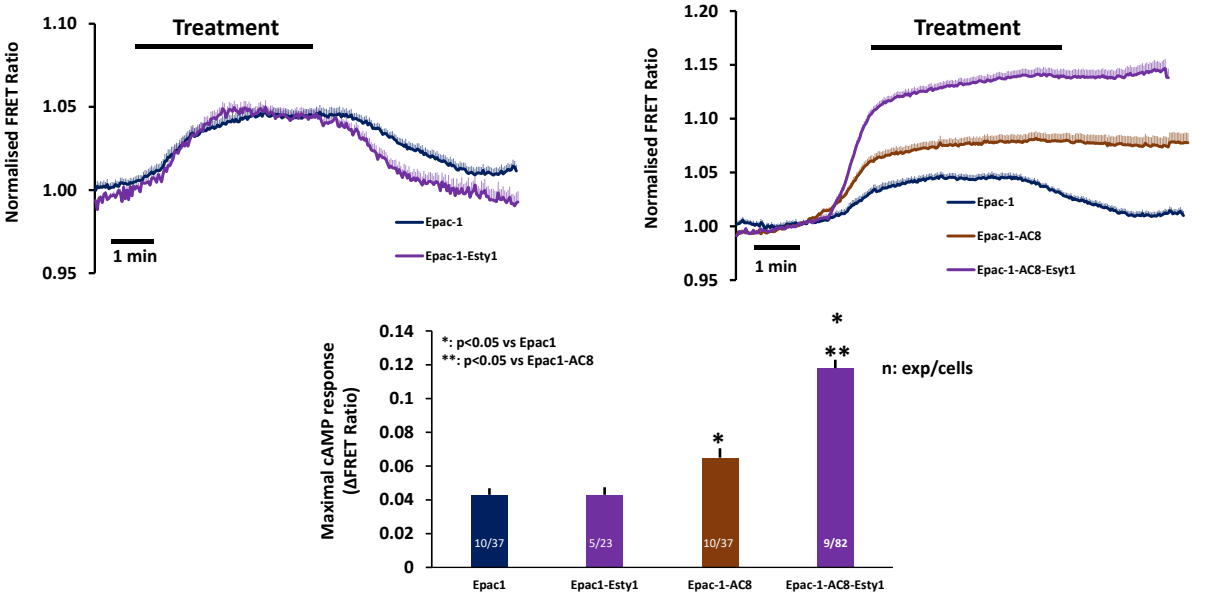

Figure 6. The effect of E-Syt1 on the cAMP production. The upper panels show the average traces of the cAMP measurements. Cells were treated with 5µM forskolin that induced a reversible increase in the cAMP production. In E-Syt1 expressing cells the response to forskolin was not altered, however if AC8 was overexpressed as well, we detected a marked increase in the stimulated cAMP production. The lower panel shows the maximal cAMP elevation upon stimulation. N= number of exp/number of cells. (highlighted on the bar charts). All results are given as the mean±s.e.m.

II.C. Understand the physiological role of ER/PM microdomains in epithelial cell functions

Crosstalk between Ca^{2+} and cAMP signaling plays pivotal role in the physiological functions of the exocrine pancreas[24, 25]. However the role of ER/PM microdomains in this synergism, or in any epithelial functions (such as fluid or ion secretion) has not been investigated before. An important aim of our project was to determine the localization of the proteins making the ER/PM microdomains in the polarized epithelial cells and study their role in the regulation of epithelial functions. In addition we attempted to develop a gene delivery method that allow us to knock down various microdomain proteins and study its effect on the pancreatic functions.

1. Adeno-associated virus mediated gene delivery in mice pancreatic cells

In these series of experiments we used adeno-associated virus 5 (AAV5) mediated delivery of green fluorescent protein (GFP) to investigate the efficiency of viral gene delivery to the pancreatic tissue. The main pancreatic duct of anesthetized C57BL/6 mice were cannulated and either empty, or GFP containing AAV5 vector was infused to the pancreas. The animals were sacrificed 6 weeks after the operation. The pancreatic inflammation (as a potential side effect) was investigated by histology. At this point quite unexpectedly we observed a massive inflammatory reaction in the mice pancreata, which almost completely destroyed the tissue. After several attempts to optimise our protocol (virus preparation, surgery) we switched to FVB/N mice, where the immune reaction was not observed. In the case of AAV5-GFP infusion we observed a marked expression of GFP in pancreatic acinar and ductal cells confirming that the approach works in our hands. In the future, we will use this approach to manipulate the gene expressions in the exocrine pancreas.

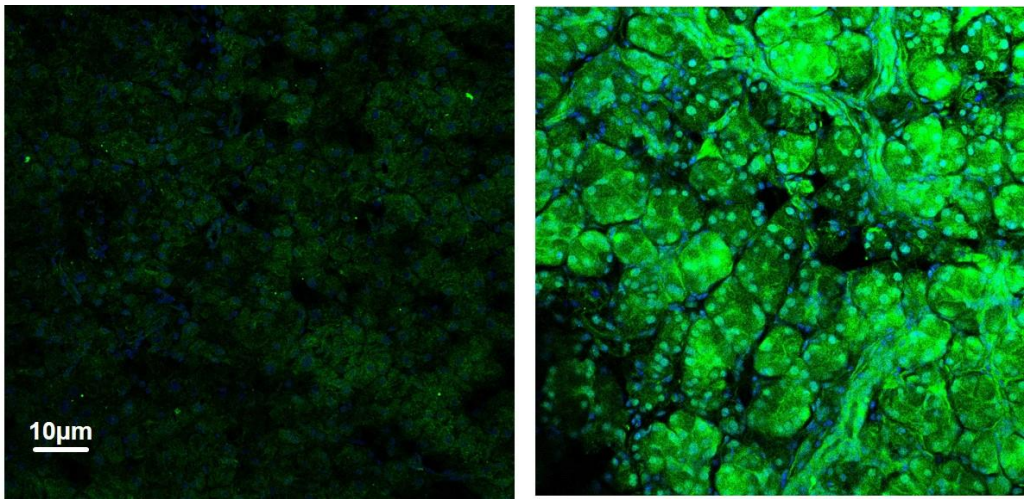

Figure 7. AAV5 mediated gene delivery into mice pancreatic tissue. The main pancreatic duct of anesthetized FVB/N mice were cannulated and either empty, or GFP containing AAV5 vector ($5*10^{10}$ particles; dissolved in 50µL PBS) was infused to the pancreas (infusion rate: 10µL/min). In the case of AAV5-GFP infusion (right panel) we observed a marked expression of GFP in the pancreatic tissue.

2. The role of Orai1 in the pancreatic ductal epithelial cells

To analyze the distribution and function of Orai1 in pancreatic ductal physiology and pathophysiology, we used isolated ductal fragments and *in vivo* model of acute pancreatitis. Our results demonstrated that Orai1 is expressed on the apical membrane of pancreatic ductal epithelial cells. The *in vitro* Ca^{2+} assays highlighted that Orai1 is an important contributor to the store operated Ca^{2+} entry in ductal cells. More importantly the inhibition of Orai1

significantly decreased the bile acid evoked Ca^{2+} influx in pancreatic ductal cells. Next we evaluated the effect of Orai1 inhibition in cerulean induced acute pancreatitis. Our results confirmed the previous report of Robert Sutton's group in Liverpool [26] and showed that the inhibition of Orai1 decreases the severity of acute pancreatitis. We also showed that in these animals the exocrine pancreatic secretion is significantly improved compared to the controls.

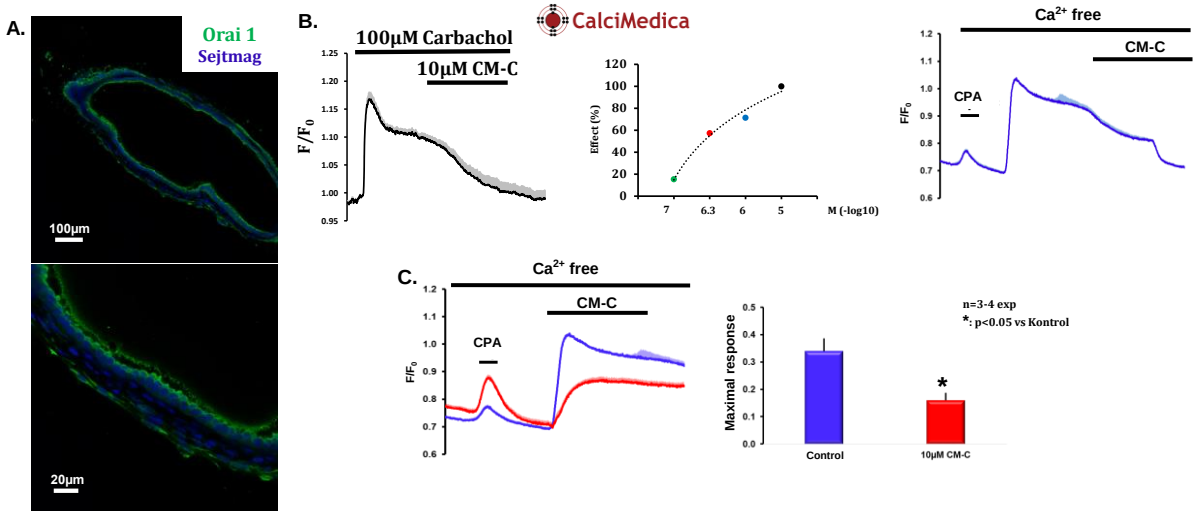

Figure 8. Expression and function of Orai1 in mice pancreatic ductal epithelial cells. A. Immunofluorescent staining of isolated mouse pancreatic ducts shows that Orai1 is expressed on the apical membrane of the primary ductal epithelial cells. **B.** CM-C, a selective Orai1 inhibitor provided by Calcimedica company, dose dependently inhibited the Ca^{2+} influx induced by 100µM Charbachol. **C.** Inhibition of Orai1 significantly decreases the extracellular Ca^{2+} influx in pancreatic ductal epithelial cells. A demonstrated on the bar chart, ~50% inhibition of the store operated Ca^{2+} entry can be achieved by the administration of 10 µM CM-C.

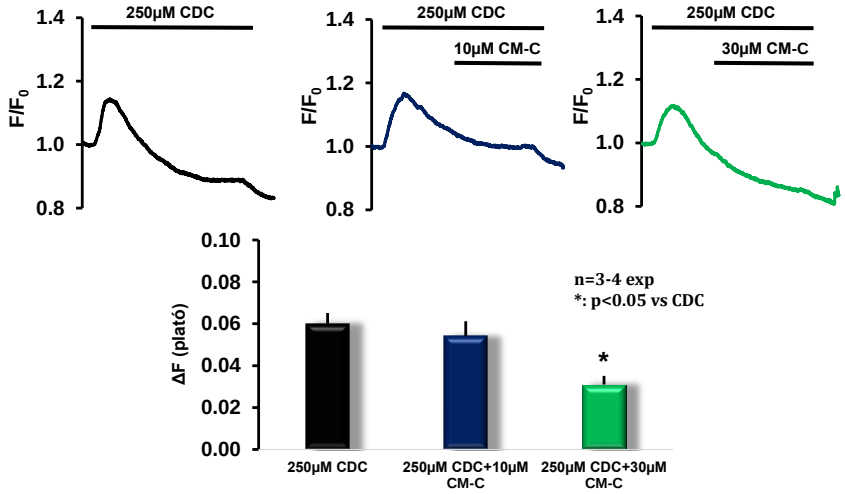

Figure 9. The inhibition of Orai1 decreases the bile acid induced extracellular Ca^{2+} influx. The upper sample traces show the effect of Orai1 inhibition on the extracellular Ca^{2+} influx induced by 250µM chenodeoxycholate (CDC). As summarised on the bar chart 30µM CM-C significantly decreased the extracellular Ca^{2+} influx.

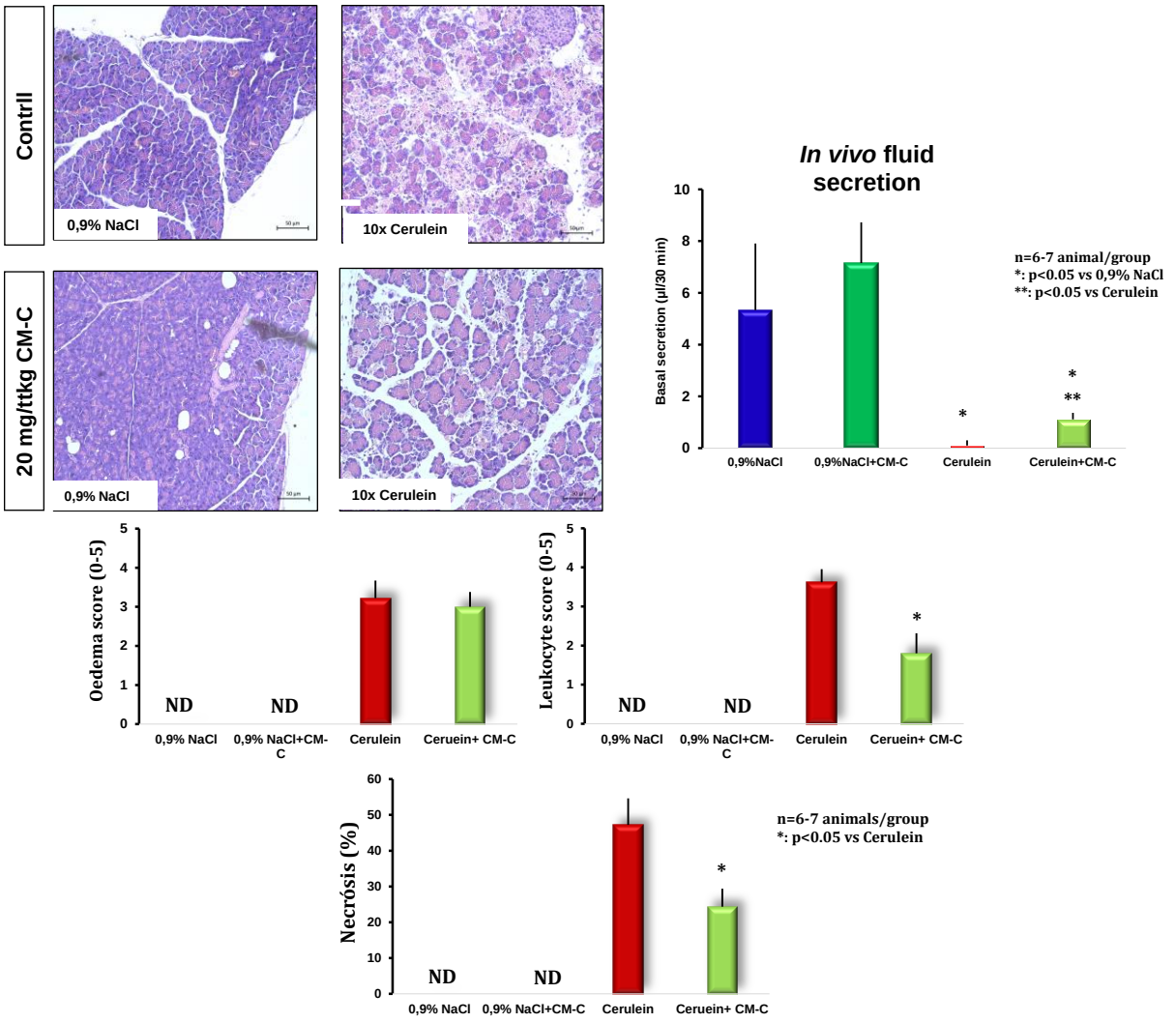

Figure 10. The inhibition of Orai1 decreases the severity of acute pancreatitis (AP) and restores exocrine fluid secretion. Sample histology pictures demonstrate that the severity of cerulean induced AP was significantly decreased by one 20mg/bwkg CM-C injection. Lower bar charts demonstrate that both the leukocyte infiltration and necrosis scores were significantly decreased by Orai1 inhibition. The right upper panel shows that the basal pancreatic fluid secretion is diminished during AP, which was significantly improved by the CM-C treatment. Notably, the fluid secretion is still significantly lower than the control values. Orai1 inhibition by itself had no effect on the fluid secretion.

CONCLUSIONS AND NOVEL OBSERVATIONS

In this research project our aim was to determine the composition and physiological role of ER/PM microdomains in polarized epithelial cells. In this complex work we successfully identified ANO8 as a novel ER/PM protein that promotes extracellular Ca^{2+} influx by stabilizing the Stim1-Orai1 complex. We also showed that proteins of the ER/PM junction have a significant role in the regulation of cAMP production by adenylyl cyclase 8. Importantly we established the role of Orai1 in the Ca^{2+} signalling and regulation of the secretory functions of pancreatic ductal epithelial cells.

Based on the results of the study we are preparing 3 manuscript that are expected to be submitted within 2-3 months. Due to my successful application for the HAS Momentum Grant I had to end the OTKA PD project on 2017 December 31 according to the regulations. It is important

to highlight that the OTKA grant support made possible to conduct several experiments that contributed to the successful application for the Momentum grant.

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