DIFFERENT ROLES OF CALCIUM SIGNALING IN THE GATROINTESTINAL TRACT

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Ph.D. Thesis

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Articles closely related to the subject of the thesis

I. Kemény LV*, Schnúr A*, Czepán M, Rakonczay Z Jr, Gál E, Lonovics J, Lázár G, Simonka Z, Venglovecz V, Maléth J, Judák L, Németh IB, Szabó K, Almássy J, Virág L, Geisz A, Tiszlavicz L, Yule DI, Wittmann T, Varró A, Hegyi P. Na+/Ca2+ exchangers regulate the migration and proliferation of human gastric myofibroblasts. *Am. J. Physiol. Gastrointest. Liver Physiol.* 305(8):G552-63. (2013) **[IF: 3.737]**

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II. József Maléth, Zsolt Balla, Balázs Kui, Anita Balázs, Máté Katona, Linda Judák, István Németh, Petra Pallagi, **Lajos V. Kemény**, Zoltán Rakonczay Jr., Viktória Venglovecz V., Imre Földesi, Zoltán Pető, Áron Somorácz, Katalin Borka, Doranda Perdomo, Gergely L.Lukacs, Mike A. Gray, Stefania Monterisi, Manuela Zaccolo, Matthias Sendler,Julia Mayerle, Jens-Peter Kühn, Markus M. Lerch, Miklós Sahin-Tóth, Péter Hegyi. Alcohol Disrupts Levels and Function of the Cystic Fibrosis Transmembrane Conductance Regulator to Promote Development of Pancreatitis, *Gastroenterology*, (2014), Accepted (GASTRO-D-14-00547R1) **[IF: 13.926]**

III. Kemény LV, Hegyi P, Rakonczay Z Jr, Borka K, Korompay A, Gray MA, Argent BE, Venglovecz V. Substance P inhibits pancreatic ductal bicarbonate secretion via neurokinin receptors 2 and 3 in the guinea pig exocrine pancreas. *Pancreas.* 40(5):793-5. (2011) **[IF: 2.386]**

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1. Introduction

1.1. Calcium signalling

Calcium signalling is an important second messenger that has role in signal transduction in response to extracellular signals and a regulatory role in several intracellular processes. Therefore, regulating intracellular calcium levels is still an important field of research, as there is still a lot to learn what mechanisms cells use to modulate intracellular calcium levels.

Different cell types express and use different sets of ion channels and transporters, according to their physiological role. For example intracellular calcium is crucial for cardiomyocites, smooth muscle cells and the interstitial cells of Cajal for contractions, while astrocytic calcium signalling is regulating vascular tones and functional hyperaemia. Besides its physiological role in several cells, toxic calcium signals have an important role in pathophysiological conditions as well. Our group has showed that ethanol and bile acids induce calcium signalling in pancreatic duct and colon cells, which might contribute to the pathogenesis of acute pancreatitis and to the development of diarrhoea.

However, there are other regulatory systems that do not utilize calcium signalling. Our group has shown that Substance P inhibits bicarbonate secretion of pancreatic ducts via a protein kinase C mediated pathway, thus regulating bicarbonate secretion in a calcium independent way.

Understanding more about the regulation of calcium homeostasis might offer a potential therapeutic benefit in certain diseases. Therefore, it is essential to learn more about the calcium homeostasis in different cell types of the gastrointestinal tract.

1.2. Gastric myofibroblasts and calcium signalling

Myofibroblasts are contractile, non-excitable, transitional cells between fibrocytes and smooth muscle cells. Their involvement in the regeneration of the gastric mucosa after ethanol-induced gastric damage has also been shown in rats. Besides their physiological roles, in pathophysiological conditions myofibroblasts contribute to chronic gastritis and to tumor development of gastric cancer too. Moreover, cancer-associated myofibroblasts reside in the tumor mass after the malignant transformation and promote the angio-neogenesis and metastasis formation of the malignant cells.

Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) is a signal important cellular processes, like motility and cell division, which determine the behavior of a cell in a particular environment. There are a number of pathways by which $[Ca^{2+}]_i$ is modulated according to the needs of cell function. Mainly, Ca^{2+} can enter the cell from the extracellular space into the cytosol via non-specific cation channels, voltage-sensitive Ca^{2+} channels and sodium-calcium exchangers (NCXs). It is known that NCX plays an important role in regulating the $[Ca^{2+}]_i$, in several cell types. NCX alters the migration of rat tendon fibroblasts, and migration and proliferation in human pulmonary artery smooth muscle cells.
As there is not enough information how human myofibroblasts regulate $[Ca^{2+}]_{i,}$ we set out to characterize the calcium homeostasis of human gastrointestinal myofibroblasts, focusing on human gastric myofibroblasts (HGMs) and on NCX, and to investigate the relationship between NCX and cell function, such as migration and proliferation.

1.3. Calcium signaling in pancreatic ductal epithelial cells (PDEC)

Calcium is an important signaling molecule in the fluid and HCO_3^- secretion of pancreatic ductal epithelium. Calcium regulates HCO_3^- secretion by promoting ATP production, but also activates calmodulin, which is involved in several cellular processes in PDEC. Although physiological Ca^{2+} signaling has many beneficial effects in PDEC, which help to promote HCO_3^- secretion, recent studies suggest that some of the pathogenic factors for acute pancreatitis (bile acids, trypsin) induce sustained elevation in $[Ca^{2+}]_i$. Therefore, it is essential to understand the mechanisms underlying the pathophysiological elevation of $[Ca^{2+}]_i$.
There is evidence, that not only acinar and stellate cells, but pancreatic ductal cells also play an important role in the pathogenesis of pancreatitis. Furthermore, we have shown that palmitoleic acid (POA), a known non-oxidative metabolite of ethanol, induces calcium elevation in PDEC cells. Additional experiments confirmed that ethanol and POA inhibits fluid secretion of PDEC cells via modulating $[Ca^{2+}]_i$, causing mitochondrial injury and altering the intracellular ATP levels. As sustained $[Ca^{2+}]_i$ elevation causes mitochondrial Ca^{2+} overload, which impairs mitochondrial membrane potential and ATP production, our goal was to identify the mechanisms that maintain the elevated $[Ca^{2+}]_I$ following POA treatment in PDEC.

1.4. Regulation of pancreatic bicarbonate secretion

The exocrine pancreas secretes 1-2L pancreatic juice a day. The pancreatic ductal epithelial cells (PDEC) contribute to the juice production by secreting a HCO_3^- rich fluid. The HCO_3^- rich alkaline pancreatic fluid, in response to meal, washes the digestive enzymes out of the pancreatic ductal tree and neutralizes the acidic chyme entering the duodenum. The regulation of pancreatic ductal secretion is a very complex system controlled by multiple stimulatory and inhibitory neural and hormonal mechanisms.

Although the stimulatory pathways of pancreatic juice secretion are well known, the inhibitory control of fluid and HCO_3^- secretion has not been fully characterized Previous work of the lab revealed that in the pancreas, SP directly inhibits basal and secretin-stimulated ductal fluid secretion. Substance P (SP) a well-known neuropeptide, which plays an essential role in numerous physiological processes. Recently, we have shown that the inhibitory effect of SP can be partially reversed by spantide, a nonselective neurokinin (NK) receptor antagonist in the guinea pig pancreas, indicating that NK receptors are involved in the inhibitory effect of SP. Furthermore, it has been shown that the inhibition of SP is calcium independent and mediated by protein kinase C, however it is not know which NK receptor subtype is involved in the regulation of ductal secretion. SP is known to exert its effects via 3 distinct mammalian NK receptors subtypes, NK1, NK2, and NK3. Therefore, we set out to characterize the role of NK receptor subtypes in the regulation of pancreatic ductal bicarbonate secretion.

2. Aims

The main aims of this work were to investigate the role of calcium in the physiology of HGMs and pathophysiology of PDEC and to further characterize the calcium independent neuroendocrine regulation of ion transporters in pancreatic ductal secretion. Our specific aims were:

1. To identify the ion transporter(s) involved in the regulation of the calcium homeostasis of HGMs and to investigate the role of the(se) ion transporter(s) in the migration and proliferation of HGMs.
2. To identify calium transport mechanisms involved in the POA induced sustained elevation of $[Ca^{2+}]_i$ in PDEC.

3. To investigate the expression of NK receptor subtypes in the guinea pig pancreas and to identify which subtype(s) mediate(s) the effect of SP on secretin-stimulated bicarbonate secretion.

3. Materials and Methods

3.1. Ethics

The study was approved by the Ethics Committee of the University of Szeged (Szeged, Hungary). All patients gave informed consent.

3.2. Patients, isolation and culture of myofibroblasts

Three specimens were obtained from patients undergoing gastric tumor resection surgery in the Department of Surgery, University of Szeged, Hungary, and oesophageal, duodenal and colonic myofibroblasts from two organ cadaver donors.

3.3. Isolation of pancreatic ducts from guinea pigs

4-8 week-old guinea pigs were sacrificed by cervical dislocation. Intra/interlobular ducts were isolated by enzymatic digestion and microdissection from the pancreas. Isolated ducts were cultured overnight.

3.4. Immunocytochemistry and immunhistochemistry

14 000 HGMs per chamber were seeded onto chamber slides and were allowed to recover overnight. After fixation and permeabilization, primary antibodies were added to the chambers and slides were incubated overnight in moist atmosphere at 4 °C. The following primary antibodies were used to identify phenotype of myofibroblasts: anti-α-SMA, anti-vimentin, anti-cytokeratin, anti-NCX1, NCX2, anti-NCX3,anti-ki67, anti-CD117, anti-DOG1 and anti-PDGFRα. Fluorescein isothiocyanate FITC-conjugated anti-guinea pig secondary antibody (1:400), Texas Red conjugated anti-mouse antibody (1:400), FITC-conjugated anti-goat and anti-mouse secondary antibodies (1:400) were used as secondary antibodies.

3.5. Immunohistochemistry of guinea pig pancreas

NK1, NK2 and NK3 receptor primary antibody antibodies were used for immunohistochemical staining that were carried out with HRP multimer based, biotin-free detection technique according to the protocol of automated Ventana

3.6. Measurement of intracellular Ca^{2+} and Na^{+} concentrations in HGMs

HGMs (5 000, 30 000 or 100 000 cells) were seeded onto a coverslip and were allowed to recover overnight. Then the coverslip was mounted on an inverted fluorescent light microscope. The cells were bathed in standard HEPES solution at 37 °C and were loaded with the Ca^{2+}-sensitive fluorescent dye FURA2-AM (5µM) for 60 min. For intracellular Na^{+} concentration ($[Na^{+}]_i$) measurements HGMs (100 000 cells) were handled the same way as for the Ca^{2+} measurements. The cells were bathed in standard HEPES solution at 37°C and loaded with the Na^{+}-sensitive fluorescent dye SBFI-AM (5µM) for 50 min. Changes in $[Ca^{2+}]_i$ and ($[Na^{+}]_i$) were measured using an imaging system (Cell R; Olympus).

3.7. Measurements of intracellular calcium levels in Capan-1cells

Capan-1 cells were were used for experiments between 20-60 passages. Cells were cultured according to the distributors' instruction. For intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) measurements 500.000 cells were seeded onto 24 mm-diameter cover glasses. Then Capan-1 cells were incubated in standard HEPES solution and loaded with Fura2-AM (2.5 µmol/L) for 30 min at 37°C. Cover glasses were then transferred to a perfusion chamber mounted on an IX71 inverted microscope.

3.8. Measurement of intracellular pH in isolated pancreatic ducts

Changes in intracellular pH (pHi) were measured by microfluorimetry using the pH-sensitive fluorescent dye 2',7'-bis (2-carboxyethyl),5 (and -6) carboxyfluorescein (BCECF After loading the ducts, pH_i was measured using a $Cell^R$ imaging system). Ducts were excited with light at wavelength of 490 and 440 nm, and the 490/440 fluorescence emission ratio was measured at 535 nm at a speed of one measurement per second.

3.9. Measurement of HCO_3^- secretion of pancreatic ducts

The rate of bicarbonate secretion was estimated by the alkali load technique. Briefly, HCO_3^- secretion is estimated by the rate of pH_i recovery from an alkaline load. Ducts were exposed

to 20 mM NH_4Cl in HCO_3^- buffered solution, which produces an increase in pH_i due to the influx of NH_3 across the membrane. Our group has demonstrated that recovery of pH_i under these conditions is correlated to HCO_3^- secretion of duct cells..

3.10. RT-PCR

RNA was isolated from HGM cultures using Qiagen RNeasy Mini Kit according to the manufacturer's instructions. 3µg of RNA was reverse transcribed to cDNA. 1 µl of cDNA was used as template for each PCR in a final volume of 25 µl, containing master mix, 10x Taq-buffer, 10 nM dNTPs per sample, 2.5 unit Taq-polymerase per sample, DEPC-treated water and 1 µM NCX primer set. The identities of the PCR products were confirmed by DNA sequencing.

3.11. Migration assay

HGMs (routinely 125 000 cells) were seeded onto six-well plates and were allowed to recover overnight in full media. On the following day, the HGM monolayer was gently scratched by a P2 tip in the middle of the well. Only wells containing even-sided and sharp-edged wounds were used for experiments. After gentle washing for three times with serum-free media, an inverted light microscope was used to measure and photograph wounds. Reagents were then added to the wells in serum-free media and HGMs were incubated in CO_2 incubator at 37 °C for 24 h. Migration was evaluated by counting the cells in the same area of the wound after 24 h.

3.12. Proliferation assay

HGMs (routinely 50 000 cells) were seeded onto cover glasses. After overnight recovery, they were synchronized by 30 hours of serum starvation, then 10 µM Click-iT 5-ethynyl-2-deoxyuridine (EdU) [Alexa Fluor 488 Imaging Kit, Invitrogen, Oregon, US] was added to the cells for overnight incubation with or without treatment. EdU incorporation was detected according to the manufacturer’s protocol.

3.13. Statistical analysis

Values are shown as means ± SEM. Statistical analyses were performed using non-parametric Kruskal-Wallis tests with post-hoc Wilcoxon tests for pairwise comparisons and Bonferroni

correction to test post-hoc significance within groups. Simple pairwise comparisons were tested with Student's t test. $P < 0.05$ was accepted as significant.

4. Results

4.1. Na^+/Ca^{2+} exchangers regulate the migration and proliferation of human gastric myofibroblasts

4.1.1. Immunocytochemical identification of cell cultures

To identify myofibroblasts from different gastrointestinal tissues (pancreas, oesophagus, stomach, duodenum, colon), cultured cells were subjected to immunocytochemical analysis using antibodies to vimentin and α-smooth-muscle actin (α-SMA) as specific markers of myofibroblasts. Cytokeratin and desmin antibodies were used for detecting epithelial and muscle cells, respectively. α-SMA and vimentin verified the presence of myofibroblasts, whereas desmin and cytokeratin negativity proved that no epithelial or smooth muscle cells were isolated. Purity of the myofibroblast cell cultures was 100 %.

4.1.2. Spontaneous Ca^{2+} oscillation in myofibroblast

Interestingly, depending on the source of isolation about 30-50 % of the cells showed spontaneous Ca^{2+} oscillations, but in pancreatic myofibroblasts no oscillation was observed. Not all cells show spontaneous oscillatory activity and oscillations were not synchronized in neighbouring cells. We tested different confluency levels (10-50-100 %) to see whether oscillation depends on the number of cells seeded onto the coverslip but we found no correlation. Cells showed stable but greatly variable oscillation pattern from cell to cell with different amplitudes and frequencies. As the proportion of oscillating cells (53.0±8.1%) was the highest in the gastric myofibroblasts, and they showed the highest oscillation frequency we decided to focus our further experiments on HGMs There were no significant differences between the samples isolated from different patients.

4.1.3. HGMs are positive for CD117 and PDGF-R, but negative for DOG1

When focusing on the α-SMA and vimentin positive HGMs, we performed a ki67 staining to determine if the proportion of oscillatory cells might correspond with cell cycle, ie. the S phase. We found that only 34±3.1 % of cells stained positive for ki67. As the maximum proportion of ki67 positive cells were always below 50 %, and in FURA-2AM experiments

occasionally 100 % of cells showed spontaneous oscillations. It can be concluded that oscillations are unlikely to be due to cell cycle, perhaps they are the result of cell division and migration together, which might explain the heterogeneity of Ca^{2+} oscillations.

As spontaneous intrinsic electrical activity is well-known in the interstitial cells of Cajal (ICC), we checked different ICC markers on HGMs. We found that HGMs stained positive for CD117, but negative for DOG1. As DOG1 is the most specific ICC marker and should correlate with CD117 staining, it can be concluded that CD117 is only a dedifferentiation marker in HGMs. PDGF-R, a stromal myofibroblast marker, was also positive for the HGMs.

4.1.4. Intracellular Ca^{2+} oscillations are dependent on the extracellular Na^{+} and Ca^{2+}

We used ion withdrawal techniques on non-oscillating and oscillating HGMs in order to identify the mechanism responsible for the oscillatory activity. Withdrawal of Ca^{2+} from the extracellular solution caused no effect on non-oscillatory cells, but a cease of Ca^{2+}-oscillation occurred in oscillatory cells. Re-addition of Ca^{2+} to the extracellular space made oscillatory cells to continue oscillation. Clearly, oscillation depends on extracellular Ca^{2+} indicating the role of a plasma membrane Ca^{2+}-channel/transporter. Na^{+}-removal also stopped oscillation and caused a relatively high-amplitude single calcium wave in oscillatory and non-oscillatory cells as well, in some cases with repeated waves with fade-out effect. These findings suggest a Na^{+}-dependent Ca^{2+}-transport mechanism in HGMs. Removal of both ions had no effect on non-oscillating cells but stopped oscillations in the other type. Oscillations returned immediately after re-addition of ions, which agrees with the former indication of a plasma membrane Na^{+}-dependent Ca^{2+}-exchange mechanism. Removal of K^{+} before removing Na^{+} had no effect on the Ca^{2+} signalling in non-oscillating cells and did not stop oscillations in oscillatory cells, suggesting that the oscillations were K^{+} independent. Removal of Mg^{2+} elevated the basal Ca^{2+} levels in HGMs, but in 16.8±3.6 % of the non-oscillating cells it caused Ca^{2+} oscillations. Furthermore, in oscillating HGMs Mg^{2+} removal had no effect on Ca^{2+} oscillations, suggesting that the oscillations are independent of Mg^{2+}.

4.1.5. Intracellular sodium also depends on extracellular sodium and calcium

Suspecting the presence of a Na^{+}-dependent Ca^{2+} exchange mechanism in HGMs, we determined to investigate the effects of ion withdrawal techniques on the intracellular Na^{+} ($[Na^{+}]_i$) levels using SBFI-AM dye. Withdrawal of the extracellular Ca^{2+} caused an increase

in the $[Na^+]_i$, meaning that not only extracellular Na^+ regulates the intracellular Ca^{2+} ($[Ca^{2+}]_i$), but Na^+ and Ca^{2+} regulate each other's entry to the cells. Removal of extracellular Na^+ caused a decrease in $[Na^+]_i$. Removal of both Na^+ and Ca^{2+} also decreased the $[Na^+]_i$, however the decrease was greater with the presence of extracellular Ca^{2+}. Not only the absolute change of the fluorescence, but the relative change to the baseline fluorescence was also significantly different in the presence of extracellular Ca^{2+}, providing another functional evidence for the presence of a plasma membrane NCX.

4.1.6. Effect of different Ca^{2+}-transport inhibitors on the intracellular Ca^{2+} and Na^+ levels of HGMs

Cells were constantly perfused with standard HEPES solution with or without reagents. We tested L-type Ca^{2+} channel blocker verapamil and NCX blockers CB-DMB and $NiCl_2$. The amiloride-derivative pan-NCX inhibitor CB-DMB in 1 µM concentration reversibly lowered $[Ca^{2+}]_i$, while stopping its administration recovered $[Ca^{2+}]_i$, but did not affect oscillation. However, it should be noted, that CB-DMB 1 µM concentration inhibited only 31.2% of HGMs, but higher concentrations inhibited all HGMs. The administration of 10 µM CB-DMB resulted in a decrease in $[Ca^{2+}]_i$ in all oscillatory and non-oscillatory cells. Also there was no significant difference in $[Ca^{2+}]_i$ decrease when non-oscillatory cells were treated with 1 µM or 10 µM concentrations of CB-DMB. 1 mM $NiCl_2$ – another potent pan-NCX inhibitor - slightly decreased $[Ca^{2+}]_i$ with a small overshoot after stopping its administration. 1 mM $NiCl_2$ stopped oscillation in oscillatory cells but it came back after stopping the administration. These findings also confirm that spontaneous oscillation is most likely maintained by NCX or at least it is involved in generating/maintaining oscillations.

10 µM and 100 µM verapamil had no effect on $[Ca^{2+}]_i$ suggesting that the oscillation is probably not voltage-mediated. Administering CB-DMB in 1 and 10 µM concentrations resulted in a decreased $[Na^+]_i$ level, but there was no significant difference between the two concentrations. The other NCX inhibitor $NiCl_2$ did not alter significantly the $[Na^+]_i$. These results suggest the importance of NCX not only in the regulation of $[Ca^{2+}]_i$, but also in the maintenance of the $[Na^+]_i$ in HGM cells.

4.1.7. mRNA and protein expression of NCX in HGMs

Based on the functional experiments we investigated the presence of the NCX isoforms at the mRNA and protein levels. Reverse transcription PCR confirmed the mRNA expression of all

three NCX isoforms on a transplant patient and on a cancer patient, which was confirmed by DNA sequencing. Immunocytochemistry revealed that NCX1, NCX2 and NCX3 are also present at the protein level in HGMs.

4.1.8. Motility decreased by NCX inhibitors

In scratch wound migration assays we utilized the inhibitors to examine their effects on the motility of HGMs. 1 µM CB-DMB and 1 mM $NiCl_2$ significantly decreased motility (by 28.4±3.9 % and 34.3±6.6 %) compared to the basal level. Stimulating migration with 100 ng/ml insulin-like growth factor II (IGF-II) caused a two-fold increase in motility (203.9±9.8 % vs. basal). 1 µM CB-DMB significantly inhibited stimulated migration (by 65.6±8.4 %), whereas 1 mM $NiCl_2$ treatment caused the strongest inhibition (98.9±10.9 vs. 203.9±9.8 %). We tried to use higher concentrations of CB-DMB to test dose-dependency but the cells detached above 1 µM after a few hours.

4.10. Proliferation is inhibited by blocking NCX

Next we tested whether NCX inhibitors have an impact on cell division. 100 ng/ml IGF-II was applied to stimulate proliferation of HGMs. We found that CB-DMB (1 µM) inhibited cell division (by 55.8±5.5 %), while 1 mM $NiCl_2$ caused a 78.3±5.9 % decrease in cell proliferation without IGF-II stimulation. 100 ng/ml IGF-II stimulation increased basal proliferation rate to nearly two-fold (191.3±10.1 %). 1 µM CB-DMB greatly inhibited cell division, but 1 mM $NiCl_2$ caused the strongest inhibition in the IGF-II stimulated HGMs.

4.2. POA increases intracellular calcium levels in PDEC

4.2.1. POA depletes ER Ca^{2+} stores of Capan-1 pancreatic ductal cells

The administration of 200µM POA evoked sustained $[Ca^{2+}]_i$ rise in Capan-1 cells. Removal of extracellular Ca^{2+} had no effect on the $\Delta Ratio_{max}$, suggesting that the initial rise in $[Ca^{2+}]_i$ is extracellular calcium independent. However, the plateau-phase of the signal was totally dependent on the presence of extracellular Ca^{2+} and blocked by gadolinium, suggesting the involvement of the store operated Ca^{2+} channels. To verify that 200µM POA completely depletes the ER Ca^{2+} stores, we administrated POA in Ca^{2+}-free media followed by the administration of 2µM thapsigargin (Tg). Under these conditions Tg was not able to induce further Ca^{2+} release. For control we administered Tg prior to POA administration, where

POA had no effect on $[Ca^{2+}]_i$. These data indicate that POA completely depletes the ER Ca^{2+} stores and induces extracellular Ca^{2+} influx.

4.2.2. POA decreases PMCA activity in Capan-1 cells

To further characterize the effects of POA on the extracellular Ca^{2+} influx, we performed the Tg-Ca^{2+} re-addition protocol. Tg treatment depleted ER Ca^{2+} and the re-addition of extracellular Ca^{2+} evoked store operated Ca^{2+} influx, where the steady state is maintained by the plasma membrane Ca^{2+}-ATPase (PMCA) activity. 200μM POA in Ca^{2+}-free extracellular solution mimicked the effect Tg (depleted the ER Ca^{2+} store and induced store-operated calcium entry (SOCE). However, after the SOCE mediated Ca^{2+} rise the decrease of $[Ca^{2+}]_i$ was markedly slower than in the case of Tg-treated cells and the plateau was reached on an elevated $[Ca^{2+}]_i$. These results suggest that POA not only depletes ER Ca^{2+} stores and induce extracellular Ca^{2+} influx, but also decreases PMCA activity, which can further contribute to the sustained Ca^{2+} elevation.

4.3. SP inhibits pancreatic ductal bicarbonate secretion via NK2 and NK3 receptors in guinea pig exocrine pancreas

4.3.1. NK2 and NK3 receptors are expressed on the lateral and luminal membranes of intra/interlobular ducts

Immunohistochemical staining revealed that all 3 NK receptors were expressed in pancreatic ductal cells; however, their localizations were different. Neurokinin 1 receptor was exclusively localized to the luminal membrane, whereas positive staining of NK2 and NK3 receptors were observed both on the lateral and luminal membranes of intra/interlobular ducts.

4.3.2. Inhibitory effect of SP on pancreatic ductal bicarbonate secretion is mediated by NK2 and NK3 receptors

First, we investigated the bicarbonate secretory rate of intact pancreatic ducts using the alkali load technique. Exposing ducts to 20 mmol/L ammonium chloride causes an increase in pH_i owing to the rapid influx of NH_3 into the cell. We have previously demonstrated that in a HCO_3^- / CO_2-buffered solution, the initial rate of pH_i recovery from alkalosis reflects the rate of HCO_3^- efflux across the apical membrane[2]. Administration of 10 nmol/L secretin

significantly increased basal secretion from 18.43 ± 0.96 to 47.72 ± 1.72 mmol/L base per minute, whereas application of 20 nmol/L SP completely blocked the stimulatory effect of secretin (23.84 ± 1.91 mmol/L base per minute). Basolateral administration of the NK1 antagonist (RP67580) at a concentration of 10 μmol/L, in the presence of secretin and SP did not influence the inhibitory effect of SP. However, the NK2 antagonist, MEN10376 (10 μmol/L) and the NK3 antagonist, SB218795 (10 μmol/L) significantly reversed the inhibitory effect of SP by 42.5 ± 2.1% and 68.1 ± 3.5%, respectively. Simultaneous administration of MEN10376 and SB218795 did not produce any further block of the effect of SP (63.6 ± 0.3%), indicating that other mechanisms may be involved.

5. Discussion

The role of the stromal microenvironment is essential in tumor development and angiogenesis. Regulating stromal cells might lead to therapeutic drugs that can modify tumor development and growth. Thereore, in our study we focused on the Ca^{2+} homeostasis, migration and proliferation of HGMs.

In our study, we showed that oesophageal, gastric, duodenal and colonic myofibroblasts display spontaneous Ca^{2+} oscillations, whereas pancreatic myofibroblasts do not..

Among the oscillatory myofibroblasts the HGMs displayed the highest number of oscillations with the highest frequency. These oscillations depended on the extracellular concentrations of Ca^{2+} and Na^{+}. As $[Na^{+}]_i$ did not vary in our experiments and the L-type Ca^{2+} channel blocker verapamil did not affect Ca^{2+} oscillations, the presence of any voltage mediated ion influx can be excluded.. The function of this phenomenon is unknown; it may be related to muscle cell origin or it may probably designate a different state in cell cycle. However, our results suggest that cell cyle could not be the main reason for oscillations. Perhaps they are the result of cell division and migration together, which might explain the heterogeneity of oscillations. Our results suggest that nerve innervations are not required for oscillations or contractions of at least 50% of HGMs. Therefore, these cells should be considered to have nerve independent spontaneous oscillatory activity. Administration of caused a single elevation in Ca^{2+} concentration, but did not switch on the oscillatory activity of the cells. However, bioactive molecules (matrix metalloproteinases, TGFβ, insulin-like growth factor binding protein-5, IGF-II progastrin, endothelin-1) were shown to modulate the proliferation and activation of stromal myofibroblasts. Therefore, it is more likely, that these cells are rather regulated by

themselves in an autonomous way and by bioactive molecules by their neighbouring cells (such as epithelial cells), thus contributing to inflammation and cancer progression.

With ion-withdrawal techniques we showed that an extracellular Na^+ and Ca^{2+} dependent transport mechanism plays an important role in regulating the cytosolic Ca^{2+} and Na^+ levels in HGMs, suggesting the presence of NCX. By K^+ withdrawal technique we showed that the Na^+ and Ca^{2+} dependent transport is independent of K^+, thus ruling out the involvement of Na^+/Ca^{2+}-K^+ exchangers in controlling the calcium homeostasis of HGMs. Our study indicates that extracellular Mg^{2+} might have a regulatory role in the Ca^{2+} oscillations, as the removal of Mg^{2+} causes Ca^{2+} oscillations in some non-oscillating cells. Probably that is due to the decreased intracellular Mg^{2+} concentration, which activates the NCX. Furthermore, NCX mRNA and protein were expressed in HGMs. The NCX inhibitor CB-DMB (which inhibits the forward and reverse mode of NCX) stopped the oscillations even after washout of the agent, highlighting the non-competitive irreversible effect of CB-DMB. $NiCl_2$, which is a competitive divalent anion for Ca^{2+} in eukaryotic cells, caused cease of oscillations for the period of administration, but oscillation returned after washout.

By controlling the intracellular Na^+ and Ca^{2+} levels in HGMs, NCX may regulate different important physiological processes. Therefore we investigated the effect of NCX inhibitors on the proliferation and migration rates of HGMs.

Migration was inhibited in HGMs under basal conditions following NCX inhibition by CB-DMB and nickel chloride. It is clear that migrating cells need intracellular free Ca^{2+} for actin polymerization, which is necessary for the formation of lamellipodia, suggesting that decreasing $[Ca^{2+}]_i$ might result in decreased motility. Furthermore, it has been shown that NCX1.1 associates with the F-actin cytoskeleton in NCX1.1 expressing Chinese hamster ovarian cells.

Basal proliferation rates were also impaired by either CB-DMB or $NiCl_2$ treatment. That may be a result of the above mentioned lack of free $[Ca^{2+}]_i$, which is necessary for signalling key events in cytoskeletal organization during S-phase or M-phase. Furthermore, without proper Ca^{2+} signaling, resting cells cannot get over the G_0 phase. In pulmonary artery smooth muscle cells, Ca^{2+} enters the cytosol after store depletion via the reverse-mode of NCX, and the NCX inhibitor KB-R7943 inhibited cell proliferation via disorganization of intracellular Ca^{2+} signalling.

IGF-II treatment greatly increased cell division in HGMs. However, administering NCX inhibitors abrogated this effect. In addition, $NiCl_2$ decreased both basal and IGF-II-stimulated

proliferation to surprisingly low levels and we believe that besides blocking NCX, this effect might be due to the inhibition of other enzymes as well. Thus we think that these effects of $NiCl_2$ in long-term (24h) proliferation experiments may contribute to the decreased proliferation rates of HGMs. Therefore such an enzyme inhibitor effect in migration experiments also cannot be excluded.

In conclusion, we showed for the first time that cultured HGMs display non-synchronized spontaneous monophasic Ca^{2+} oscillations, which depend on the extracellular Ca^{2+} and Na^{+}. In oscillatory and non-oscillatory HGMs $[Ca^{2+}]_i$ and $[Na^{+}]_i$ are dependent on extracellular Ca^{2+} and Na^{+} suggesting a role for NCX. We showed that NCX1, NCX2 and NCX3 mRNA and protein are present in HGMs and are involved in the migration and proliferation of HGMs. Functional experiments showed that NCX is necessary for proper basal and IGF-II-stimulated migration and particularly for proliferation of HGMs. As myofibroblasts are involved in pathophysiological conditions, such as chronic inflammation or tumor development, further investigations are needed to determine if modulating NCX function has a therapeutic effect on hyperproliferative gastric diseases.

Our lab has showed that ethanol in low concentration stimulates, whereas in high concentrations inhibits pancreatic ductal HCO_3^- secretion and decreases CFTR activity, thus contributing to the pathogenesis of pancreatitis. We further characterized the mechanism by which ethanol and its metabolites inhibit HCO_3^- secretion and CFTR activity. Low concentraction of ethanol (10mM) stimulated HCO_3^- secretion by IP_3R-dependent Ca^{2+} release from the ER. In contrast, high concentrations of ethanol and POA induced sustained $[Ca^{2+}]_i$ elevation mediated by both the IP_3R and RyR as well as extracellular Ca^{2+} influx.

Our results indicate that POA completely depletes the ER Ca^{2+} stores and induces extracellular Ca^{2+} influx, to sustain the elevated $[Ca^{2+}]_i$. Furthermore, POA not only depletes ER Ca^{2+}, but by decreasing PMCA activity, it decreases the removal of calcium from PDEC cells, contributing to the elevated $[Ca^{2+}]_i$. Notably, similar toxic Ca^{2+} elevation was found in pancreatic acinar cells, and in other cell types leading to premature protease activation and cell death. It is well documented that sustained $[Ca^{2+}]_i$ elevation causes mitochondrial Ca^{2+} overload, which impairs mitochondrial membrane potential and ATP production. Very recently ethanol was shown to sensitize pancreatic mitochondria to activate the mitochondrial permeability transition pore, leading to mitochondrial failure. Our lab has shown that, high concentrations of ethanol and POA also induced $[ATP]_i$ depletion and decreased mitochondrial membrane potential. The toxic effects of ethanol and POA were similar to

those of high concentration of bile acids. In conclusion, we found that the POA induced sustained elevation of $[Ca^{2+}]_i$ is a combined result of the depletion of ER stores and the inhibition of PMCA activity in PDEC. These observations could explain why ethanol consumption damages PDEC cell function, that might contribute to the pathogenesis of acute pancreatitis.

It is known that SP inhibits basal and secretin-stimulated ductal fluid secretion. This inhibitory effect may be physiologically important in limiting the hydrostatic pressure developed within the duct lumen during secretion and in turning off ductal secretion after a meal. Recently, we have shown that the inhibitory effect of SP can be partially reversed by spantide, a nonselective neurokinin (NK) receptor antagonist in the guinea pig pancreas, indicating that NK receptors are involved in the inhibitory effect of SP. Furthermore, it has been shown that the inhibition of SP is mediated by protein kinase C and independent of calcium signaling, however it is not know which NK receptor subtype is involved in the regulation of ductal secretion.

We characterized the localization of NK receptor subtypes in the guinea pig pancreas and showed that NK2 and NK3 receptors localize at the basal and lateral membrane of pancreatic duct cells, while NK1 receptors localize on the luminal side only. We provided evidence that SP inhibits bicarbonate secretion via both of the laterally expressed NK receptors, namely, NK2 and NK3, but not the luminal NK1. Our results, showing that all of the NK receptors are expressed in the luminal membrane of pancreatic ductal cells, open up new physiological questions, which need further investigations.

6. Summary

Ion transporters have a crucial role in modulating several cellular functions in the GI tract. Ion transporters modulating calcium level of cells have important roles is cellular signalling, thus they mediate proliferation, migration and survival of several cell types. As calcium might play different roles in different cell types, the main aims of this work were to further investigate the role of calcium ion transporters in the physiology of human gastric myofibroblasts (HGMs) and in the pathophysiology of PDEC and to further characterize the calcium independent neuroendocrine regulation of HCO_3^- transporters in pancreatic ductal secretion.

We showed for the first time that cultured HGMs display non-synchronized spontaneous monophasic Ca^{2+} oscillations, which depend on the extracellular Ca^{2+} and Na^{+}. We have identified NCX as a regulator of Ca^{2+} homeostasis of HGMs, and showed that NCX regulates migration and proliferation of HGMs. As myofibroblasts are involved in pathophysiological conditions, such as chronic inflammation or tumor development, further investigations are needed to determine if modulating NCX function has a therapeutic effect on hyperproliferative gastric diseases.

We found that palmitoleic acid (POA), a non-oxidative metabolite of ethanol, induced sustained elevation of intracellular calcium ($[Ca^{2+}]_i$). in PDEC. We have identified the mechanism of the sustained $[Ca^{2+}]_i$.elevation, which is a combined result of the depletion of endoplasmic reticulum stores and the inhibition of plasma membrane Ca^{2+}-ATPase activity in PDEC. As the prolonged increase in $[Ca^{2+}]_i$ can cause ATP depletion and mitochondrial injury to PDEC, these observations might explain why ethanol consumption might damage PDEC cell function, that might contribute to the pathogenesis of acute pancreatitis.

We showed that NK2 and NK3 receptors localize at the basal and lateral membrane of pancreatic duct cells in guinea pig pancreas, while NK1 receptors localize on the luminal side only. We provided evidence that SP inhibits bicarbonate secretion via both of the laterally expressed NK receptors, namely, NK2 and NK3, but not the luminal NK1. Our results, showing that all of the NK receptors are expressed in the luminal membrane of pancreatic ductal cells, open up new physiological questions, which need further investigations.

In conclusion, we have determined that NCX mediated calcium oscillation is required for the physiology of HGMs. Furthermore, we have concluded that POA can activate toxic calcium oscillations in PDEC that might contribute to the pathogenesis of ethanol induced pancreatitis. Moreover, we have identified that substance P inhibits bicarbonate secretion of pancreatic ducts via NK2 and NK3 receptors.

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