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Role of calcium dependent ion channels on secretory epithelial cell function

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I. Introduction

In my research, I investigated the role of different ion channels, their location in the plasma membrane and their functional relationships with intracellular Ca^{2+}-concentration $[Ca^{2+}]i$ on tear and pancreatic acinar cells isolated by enzymatic digestion. The primary tear secretion is a unidirectional water and ion transportation of the monolayer epithelial cells. This mechanism requires asymmetric apico-basal distribution of both the transporters and the intracellular Ca^{2+}-signal. Our main goal was to identify and localize Ca^{2+}-dependent ion channels in murine lacrimal acinar cells. Our results show a paxillin sensitive Ca^{2+}-dependent K^+-current, which mainly located apically and negligible in the basal region, and it is located in the same membrane compartment as the Ca^{2+}-dependent Cl^--current. All of this proposes that both K^+ and Cl^- are secreted into the acinar lumen, which explains the high luminal Cl^- concentration in the primary secretion (~141 mM), but contradicts the low K^+ concentration (<17 mM), suggesting that K^+ returns to the acinar cell from the primary tear secretion by some mechanism, that's why we hypothize the presence of a Na^+-K^+ pump in the apical part of the plasma membrane. The hypothesis was verified by immunostaining using a specific Na^+-K^+ pump antibody, and it was confirmed that the Na^+-K^+ pump located on both the apical and on the basal side of the lacrimal acinar cell. Based on our results, we propose a new tear secretion model in which paracellular Na^+-transport is complemented by a transcellular pathway driven by the Na^+-K^+ pump.

The intracellular Ca^{2+}-concentration of the pancreatic acinar cell plays a significant role in the secretion of the primary pancreatic juice, so we aimed to find previously unknown Ca^{2+}-dependent ion channels. Based on our QPCR test, the TRPM4 channel showed high expression in the gland, so in our experiments we examined the role of this channel in murine pancreatic acinar cells. Since the TRPM4 channel is a Ca^{2+}-activated non-selective monovalent cation channel, we hypothesized that after intracellular Ca^{2+}-concentration increases under the influence of a secretagogue stimulus, cations flowing into the cell through the TRPM4 channel depolarize the cell, thus reducing the driving force of Ca^{2+}. In our experiments, we demonstrated by patch-clamp that a Ca^{2+}-activated Na + current is present in murine pancreatic acinar cells, which can be inhibited by TRPM4 inhibitors (CBA, 9-Phenanthrol). Furthermore, under current-clamp conditions, we demonstrated that the Ca^{2+}-dependent Na^+-current causes an average ~17 mV depolarization of the cell

membrane, which could be inhibited by CBA. In order to test the effect of depolarization on Ca^{2+}-influx, $[Ca^{2+}]i$ measurements were performed, which showed that the slope of Ca^{2+}-influx is higher in TRPM4 knockout and CBA-treated acinar cells compared to control, while in TRPM4 knockout cells the amplitude of store operated Ca^{2+}-entry (SOCE) also increased. Based on our results, our hypothesis that TRPM4 activation reduces the driving force of Ca^{2+}-influx across the plasma membrane was confirmed.

II. Antecedents and objectives

II/1. Rethinking the tear secretion model

Tear secretion is essential for the proper functioning of the cornea and conjunctiva. If the amount or quality of tears produced decreases and wetting of the eye surface deteriorates, it can lead to dry eye syndrome (keratoconjuctivitis sicca). A significant proportion of the tear secretion is produced by the acinar cells of the lacrimal gland, so understanding the secretory mechanism of these cells is very important. Primary fluid secretion is unidirectional salt and water transport through monolayer secretory epithelial cells. The secretion model is based on the polarized location of ion transporters (K^+ and Cl^- channels). The electrochemical propulsion driving the Na^+-K^+-$2Cl^-$ cotransporter, the Na^+-H^+ and Cl^--HCO^{3-} exchangers is provided by the Na^+-K^+ pump. To the best of our knowledge, this transporters are located in the basolateral membrane. When $[Ca^{2+}]i$ is elevated by stimulation, calcium-activated chloride channels (CaCC) in the apical membrane open and Cl^- is secreted into the lumen, generating a transepithelial driving force that generates paracellular Na^+ transport. The Cl^- outflow shifts the resting membrane potential (~-45 mV) in a positive direction, and it become more positive than the Cl^- equilibrium potential (~-33 mV) (it cannot become more positive due to the Cl^- current alone, therefore the equilibrium is set around -30mV), so that the disappearing driving force of Cl^- would not allow the elongated Cl^- efflux. Luminally located Ca^{2+} -dependent K^+ channels play an important role in maintaining long-term Cl^- secretion, and the K^+ flowing out into the lumen through them helps to stabilize the membrane potential and maintain the luminal Cl^- driving force. However, there is a significant difference (17 mM vs 152 mM) in the secretion K^+ and Cl^- concentrations, and this difference suggests a K^+ reuptake mechanism in the luminal membrane of the acinar cell. The discrepancy is resolved either by the absence of a luminal Ca^{2+}-dependent K^+ channel (despite previous observations), or by the fact that some mechanism takes K^+ back into the cell very quickly. One of the goals of our research group was to supplement the missing mechanisms of the previous tear secretion model with a combination of full-cell configuration patch-clamp, Ca^{2+}-imaging and Ca^{2+}-uncaging methods, and by immunostaining.

II / 2. TRPM4 as a possible protection against calcium overload

Another goal of our research was to find potential mechanisms that could protect against Ca2+ overload. In 2002, based on their Ca2+ measurements on TRPM4 overexpressed HEK293 cells, Pierre Launay et al., suggested that TRPM4 may play a role in reducing Ca2+ driving force upon SOCE activation, and it was hypothesized that the TRPM4 and SOCE channels are located close to each other. In the pathogenesis of pancreatitis, the Ca2+ store of acinar cells is depleted to an extent that causes strong SOCE activation, so the TRPM4 channel has become the focus of our interest as a protective mechanism against potential Ca2+ overload. Because QPCR on murine pancreatic acinar cells showed as high expression level of TRPM4 channel encoding mRNA as the IP3 receptor channels, we began to develop our hypothesis. Our theory is that elevated [Ca2+]i activates the TRPM4 channel, Na+ flows into the cell, depolarizing it. Although the Na+ and K+ permeabilities of the channel are nearly identical, the propulsion conditions favor Na+ influx because on the resting membrane potential the driving force of Na+ is greater than that of K+ (the difference between the equilibrium potential of Na+ and the resting membrane potential is greater than in the case of of K+). If the membrane potential shifts to a more positive (but still negative) value due to Na+ influx, the driving force of SOCE activated by the temporary depletion of stores will also be smaller, and the influx of Ca2+ into the acinar cell would slow down in this case.

III. Methods

III/1. Isolation of acinar cells

All organ / tissue isolations complied with 2010/63 / EU and 40/2013. (II. 14.) and approved by both the Workplace Animal Welfare Committee of the University of Debrecen and the Animal Welfare Committee of the University of Rochester.

Isolation of acinar cells from murine lacrimal gland

The lacrimal acinar cells were separated by enzymatic digestion. Enzymes were dissolved in SMEM (ThermoFisher Scientific Inc., Waltham, MA). Following cervical dislocation of 3-4 months old mice, the lacrimal gland was immediately removed, finely chopped with surgical scissors, and digested in a 28 µg / ml trypsin solution for 8 min in a 37 °C water bath with shaking. The tissue was then washed with SMEM solution supplemented with trypsin inhibitor, followed by two additional digestion cycles (each ran 20 min in 10 ml digestion solution containing 0.18 Wünsch units / ml of Liberase TL (mixture of collagenase enzymes; Roche Diagnostics GmbH, Mannheim, Germany)). The nutrient solutions used were continuously bubbled with carbogen gas (95% O_2, 5% CO_2) to ensure a physiologically stable pH. In order for the tissue to disintegrate, it was pipetted several times through a cutted 200 µl pipette tip attached to a 10 ml serological pipette. The resulting cells were filtered through a 100 µm diameter nylon filter, washed, centrifuged (200xg, 3 min) and finally resuspended in BME medium.

Isolation of acinar cells from murine pancreas

Pancreatic acinar cells were separated by enzymatic digestion. Collagenase P (a mixture of collagenase enzymes; Roche Diagnostics GmbH, Mannheim, Germany) was dissolved in 100 U/ml in a 1:1 mixture of DMEM:HAM'S F12 (ThermoFisher Scientific Inc., Waltham, MA). After cervical dislocation of 3-4 months old mice (in CO_2 anesthesia), the pancreas was removed immediately, the tissue was injected with 2x1 ml of digestion solution, and then digested for 10 and 15 minutes in a 37 °C water bath with shaking in a solution containing 100U/ml Collagenase P, 0,1 mg/ml trypsin inhibitor and 2.5 mg/ml BSA (bovine serum albumine). The nutrient solutions used were continuously bubbled with carbogenic gas (95% O_2, 5% CO_2) to

ensure a physiologically stable pH. After digestion the tissue was blown out 4-6 times from a serological pipette, then filtered through a metal mesh filter (diameter: 60 mesh or 250 µm). The cells were then layered on a high concentration (400 mg/ml) BSA solution and washed by gentle manual centrifugation. The resulting pellet was washed in 2 ml of DMEM, centrifuged again carefully, and then the cell groups were again taken up in 2 ml of DMEM, kept at room temperature until used for Ca^{2+}-imaging measurements (maximum 2-3 hours).

In order to obtain individual cells from the acinar cell groups and to perform electrophysiological measurements on them, an additional digestion cycle was used, in which the cell groups were further digested in 100 U/ml Collagenase P-containing Ca^{2+} and Mg^{2+}-free PBS and finally 5 ml They were dispersed with a serological pipette.

III/2. Flash photolysis, electrophysiological and Ca^{2+} imaging measurements on lacrimal acinar cells

In the case of lacrimal acinar cells, a combined methodology was used in which the increase of intracellular Ca^{2+} concentration of acinar cells was induced by flashing UV light (using NP-EGTA), followed by Ca^{2+}-imaging method and in parallel by Ca^{2+}-activated K^+- or Cl^--currents were measured.

Total cell current was measured with an Axopatch 200A amplifier controlled by a Digidata 1322A analog-digital converter (Axon Instruments Inc., Foster City, CA, USA) under voltage-clamp conditions. Current was recorded by sampling at 50 kHz and was filtered at 5 kHz (low-pass Bessel filter), and data were recorded using pClamp 9 software (Axon Instruments). Pipettes with a resistance of 5-7 MΩ were prepared from borosilicate glass capillaries (Warner Instruments, Hamden, CT) with a horizontal puller. Continuous monitoring of acinar cell current was performed by the voltage-clamp method, K^+ current was measured at +40 mV, wjile Cl^- current was measured at -20 mV. The pipette solution (intracellular solution) had the following composition: 135 mM K^+-glutamate, 10 mM HEPES, 10 mM NP-EGTA, 2 mM $CaCl_2$ and 250 µM Fluo-4-K, pH 7.2. The potassium salt of Fluo-4 cannot cross the membrane due to its water-soluble properties, so it was added to the pipette solution. The composition of the external solutions was designed to examine the Ca^{2+}-dependent currents separately. K^+ current was measured in a low Cl^- containing solution having

the following composition: 135 mM Na^+ glutamate, 5 mM K^+ glutamate, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM HEPES, pH = 7.2. When measuring the Cl^- current, the K^+ current was inhibited with tetraethylammonium (TEA) so that the solution composition was as follows: 140 mM TEACl and 10 mM HEPES, pH 7.2.

Fluo-4 Ca^{2+}-sensitive dye was monitored with a monochromator (Polychrome IV) and a high-speed CCD camera (both devices manufactured by TILL Photonics). The images were given 4096 shades (16 bits) of gray leveling, which were subsequently provided with rainbow-scale coloring, with cold colors corresponding to low and warmer colors corresponding to higher [Ca^{2+}]. For flash photolysis, a 375 nm (UV) laser diode (maximum output 18 mW, Toptica) connected to an inverted microscope (nikon TE200) with an optical cable via a UV light capacitor (TILL Photonics) was used. UV light was focused on the stage with a 40x (NA = 1.3) magnification oil immersion objective (NIKON). The extent of the laser point at the half-maximum value was 0.7 μm in the x and y directions and 2.0 μm in the z direction, respectively. Whole cell current measurement, fluorescence imaging, and laser light flash time were synchronized by a Polychrome IV adapter and controlled by the Vision program (TILL Photonics). The Ca^{2+} buffer used for flash photolysis was nitrophenyl-EGTA (NP-EGTA), which binds Ca^{2+} photolabile, so that some of the Ca^{2+} bound by it can be released by UV flash. By directing the laser near the appropriate plasma membrane section, a Ca^{2+} signal limited to that area can be generated. It is important to have the right buffer-Ca^{2+} ratio so that the whole-cell Ca^{2+} signal, which we were able to achieve using 10 mM NP-EGTA and 2 mM Ca^{2+} in our measurement set-up, is not formed.

III/3. Electrophysiological and Ca^{2+}-imaging measurements on pancreatic acinar cells

Voltage-clamp

Whole cell patch-clamp measurements were performed at room temperature using an Axopatch 200B amplifier and a Digidata 1320A analog--digital converter (Molecular Devices, Sunnyvale, CA) under voltage-clamp conditions, and data were sampled at 50 kHz and obtained by filtration at 5 kHz (low-pass Bessel filter). Pipettes with a resistance of 5-7 MΩ were prepared from borosilicate glass capillaries (Warner Instruments, Hamden, CT) with a horizontal puller. Depending on the composition of the intra- and extracellular solutions, the current flowing through the TRPM4 or TMEM16a channel was measured. A voltage ramp protocol of -100 to +120 mM was used to measure the TRPM4 current, while a 1-second depolarization step protocol of -60 to +120 mV was used to measure the Cl^- current. Serial resistance compensation of at least 70% was used for each measurement.

In the case of TRPM4 current recording we used the following intracellular pipette solution: 144 mM Cs-glutamate, 1 mM $MgCl_2$, 100 µM EGTA, 48,6 µM $CaCl_2$ (100 nM ionized Ca^{2+}), 10 mM HEPES, 3 mM ATP, pH: 7.3. The composition of the extracellular solution: 140 mM Na-glutamate, 4 mM CsCl, 2 mM $MgCl_2$, 10 mM HEPES, pH: 7.4.

In the case of Ca^{2+} dependent Cl^- current recording we used the following intracellular pipette solution: 100 mM NMDG-Cl, 1 mM $MgCl_2$, 1.72 mM $CaCl_2$, 2 mM EGTA (1 µM ionized Ca^{2+}), 10 mM HEPES, pH= 7.2. The composition of the extracellular solution: 140 mM NMDG-Cl, 1 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES, pH= 7.3.

Current-clamp

The membrane potential of acinar cells was measured using current-clamp configuration of the patch-clamp method. In order to keep the intracellular environment as intact as possible and thus obtain the most evaluable voltage values, we chose the perforated patch-clamp method to perform our experiments. The essence of the method is to add a polyene macrolide antimicrobial agent (amphotericin B or nystatin) to the pipette solution, which goes into the patch under the pipette, forming pores through which monovalent ions (e.g. Na^+, K^+, Cl^-) can go across, but

divalent ions (eg Ca^{2+}) and uncharged molecules like glucose or greater cannot pass through. In our experiments, 300 µg/ml amphotericin B was added to the pipette solution, the pipette was filled with amphotericin B-free solution in the pipette tip and amphotericin B-containing solution behind it. In this way there is enough time for the high-resistance bond (Gigaseal) to form before amphotericin B would diffuse into the pipette tip. Between the cell and the pipette, amphotericin B is incorporated into the cell membrane within 5–10 min after Gigaseal formation, in which case perforation is indicated by slowly decreasing serial resistance and increasing cell capacity. In the case of perforated patch current recording we used the following intracellular pipette solution: 85 mM K-glutamate, 45 mM KCl, 15 mM NaCl, 2 mM $MgCl_2$, 100 µM EGTA, 48.6 µM $CaCl_2$, 10 mM HEPES, 300 µg/ml Amphotericin B, pH: 7.3. The extracellular solution was normal Tyrode, which had the following composition: 144 mM NaCl, 5.5 mM KCl, 2.5 mM $CaCl_2$,1.2 mM $MgCl_2$, 8.3 mM glucose, 5 mM Tris-HCl buffer, pH: 7.4.

Ca^{2+}-imaging

Pancreatic acinar cells were pipetted into a glass vessel (Ibidi) where they were allowed to adhere for 10 min. Cells were perfused with normal Tyrode's solution containing 144 mM NaCl, 5.5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM MgCl2, 8.3 mM glucose and 5 mM Tris-HCl buffer, pH 7.4. If Ca^{2+}-free normal Tyrode's solution was used in the measurement protocol, the solution contained 0.5 mM EGTA. For Ca^{2+}-imaging measurements, acinar cell groups were incubated in a 2 µM Fura-8-AM (AAT Bioquest) ratiometric Ca^{2+}-sensitive dye containing solution for 30 min at room temperature. During this time, the lipid-soluble acetoxymethyl ester (AM) form of the dye enters the cell and is degraded by esterases in the intracellular space, thus trapping the dye in the cell. If the cell is properly loaded with dye, then the cell has retained its healthy physiological properties. An advantageous feature of ratiometric dyes is the Ca^{2+} concentration determined by the ratio of the emission intensity values obtained at two different excitation wavelengths, so that the error due to different dye loading does not have to be taken into account. Fura-8 was excited at 360 and 405 nm at 1 Hz using an LED light source (FuraLED, Cairn Research Ltd, Faversham, UK) and the emitted light was passed through a 520 nm longpass filter and collected using a Qimaging Retiga R3 CCD camera. The imaging hardware setup was controlled by Micromanager software through an interface. Fluorescence values were determined using Image J software with Fiji plugins. Fluorescence ratios of emissions elicited by

excitations at 360 and 405 nm were calculated after background subtraction in single cells. Changes of ratios (ΔR) were determined for each cell, averaged and presented as mean ± SEM.

III/4. Immunocytochemistry

Immediately after isolation, acinar cell clumps were fixed in ice-cold methanol in suspension for 15 min. Antisera raised against the Na^+-K^+ ATP-ase (Abcam, Cambridge, UK, ref. #ab76020) and occludin (Novus, NBP1–87402) were added to acinar cell suspension in dilutions of 1:250 and 1:100, respectively. Subsequently, the cells were probed with Dylight 488 horse anti rabbit secondary antibody (ThermoFisher Scientific Inc., Waltham, MA) in the dilution of 1:2000 or 1:500. Cells were incubated with the antibodies at room temperature for 1 h and washed three times for 5 min by resuspending the cells in 3% BSA-PBS solution. After each wash, cells were collected by centrifugation. Primary antibodies were omitted in negative control experiments. Finally, acinar cell clumps were seeded on coverslips and examined using a Zeiss 510 Meta (Carl Zeiss Microscopy GmbH, Jena, Germany) confocal microscope equipped with a suite of diode and gas lasers.

III/5. Statistical analysis

Analysis was made in Origin 7.0 (Microcal Software, Northampton, MA, USA) or in Microsoft Excel. Data are presented as the average of cells obtained from at least three independent experiments and at least 3 animals. Averages are expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by using Student's t-test or one-way ANOVA with Bonferroni post test. Related samples were analyzed using repeated measure ANOVA and pairwise comparisons were carried out with Bonferroni corrected paired sample t-test. Differences were considered significant when p was less than 0.05.

The number of experiments (n) denotes the number of experimental repeats / total number of cells in the case of Ca^{2+} imaging and the number of cells in patch clamp measurements as indicated in the figure legends.

RNA isolation, RT, and quantitative real-time PCR

qPCR was performed on a Roche LightCycler 480 System (Roche) using the 5’ nuclease assay. Total RNA was isolated using TRIzol (Life Technologies Hungary Ltd), DNase treatment was performed according to the manufacturer’s protocol, and then 1 µg of total RNA was reverse-transcribed into complementary DNA using High-Capacity cDNA Kit from Life Technologies Hungary Ltd. PCR amplification was performed using the TaqMan Gene Expression Assays (assay IDs: Mm01175211_m1 for RYR1, Mm00465877_m1 for RYR2, Mm01328421_m1 for RYR3, Mm00439907_m1 for inositol 1,4,5-trisphosphate receptor (ITPR) type 1, Mm00444937_m1 for ITPR type 2, Mm01306070_m1 for ITPR type 3, Mm00613173_m1 for TRPM4, Mm01129032_m1 for TRPM5, and Mm00444690_m1 for TRPC3) and the TaqMan universal PCR master mix protocol (Applied Biosystems). As internal control, transcripts of the housekeeping gene (GAPDH; assay ID: Mm99999915_g1) were determined. The amount of the transcripts was normalized to the housekeeping gene using the ΔCT method.

V. Results

IV/1. Experiments on lacrimal acinar cells

IV/1.1 Spatially limited flash photolysis, Ca^{2+}-imaging and electrophysiological recording

In order to determine the localization of Ca^{2+}-activated K^+-channels in the plasma membrane, Ca^{2+} was transiently raised selectively in the apical or the basal membrane domain using flash-photolysis and the whole-cell current was measured simultaneously. The UV laser point (of ~0.7 μM FWHM) was positioned near the intracellular side of the apical or basal plasma membrane in a cell loaded with caged-Ca^{2+} and Fluo-4 through a patch pipette and exposed with the UV laser for 50 ms.

The intracellular solution used for flash fotolysis contained 10 mM NP-EGTA and 2 mM Ca^{2+}. The images were subsequently provided with rainbow-scale coloring, with cold colors corresponding to low, while increasingly warmer colors corresponding to higher [Ca^{2+}]. The flash was targeted to the apical or basal region, which is indicated by intense fluorescence signals. The images clearly show that in the field of photolysis, a large, short-lived, and locally extending Ca^{2+} signal can be generated under such conditions without significant propagation in the cell. When the laser dot was focused near the apical membrane region (the region was identified from the location of the granules), the mean increase in fluorescence was 5.26 ± 0.76 times greater than the baseline. The spatial extent of each measurement was analyzed on a line running from the apical membrane to the basal membrane of the cell. These studies show that the increase in the fluorescence peak occurred at an average distance of 0.65 ± 0.11 μm from the apical membrane and decreased exponentially with an average length constant of 3 ± 0.04 μm from the photolysis site, while the mean cell diameter was 22.8 ± 0.6 μm. The same evaluation was performed for cells exposed to flash photolysis focused on the basal area, which show a similar result: the normalized fluorescence intensity peak was 7.32 ± 1.44 from baseline, the distance from the basal membrane was 0.86 ± 0.3 μm and the length constant was 3.73 ± 0.12 μm. The mean diameter of these cells was 23.1 ± 2.1 μm. Based on these data, it can be concluded that the method is suitable for pole-selective elevation of [Ca^{2+}]i in acinar cells.

After setting up and validating the methodology, the aim of the first experiments was to investigate the presence of functional K^+ channels in the apical membrane. To investigate this, flash photolysis was combined with current cell current measurements of Ca^{2+}-dependent K^+ and Cl^--channels. The apical membrane was identified by the granular region and identified by the presence of the Ca^{2+}-dependent Cl^--current, since the Cl^--channels are found only in the apical membrane. Data were collected from small cell groups (2-3 cells). The middle cell of the selected cell group was attached to the patch pipette, which contained Fluo-4 Ca^{2+}-indicator dye and caged-Ca^{2+}. The K^+ current and Fluo-4 fluorescence were recorded in parallel as described in the Materials and Methods section. The UV laser beam was positioned near the apical plasma membrane. The laser was flashed 1.05 s after the start of electrical recording for 50 ms time duration. Our results clearly show that there is an instantaneous increase in $[Ca^{2+}]i$ on the apical side, which last for around 250 ms after the flash, while the fluorescence intensity did not change on the basal side. Our whole cell current measurements show a significant increase in K^+ current as a result of the local Ca^{2+} signal. The baseline of K^+ current was 0.5 nA, because the membrane potential was clamped at +40 mV, which partially opens voltage- and Ca^{2+}-dependent K^+-channels (BK channel, MaxiK) in a voltage-dependent manner. However, the BK channel inhibitor paxillin decreased the background current (baseline), and prevented the increase of the K^+ current due to Ca^{2+}-uncaging. Following the K^+ current measurement, the Na^+ and K^+ glutamate-based extracellular solution was replaced with a solution containing 140 mM TEA-Cl, which inhibited the K^+ current but provided enough Cl^- to record outward Cl^- currents. In this solution, a significant Cl^- current was measured by flash photolysis focused on the same spot, suggesting that the laser was precisely targeting the apical side. These observations were consistent for all 15 cells tested.

Subsequently, the K^+ and Cl^- currents of the basal membrane region were tested with the same experimental setup. Morphologically, the reticular structure and functionally the lack of Ca^{2+}-dependent Cl^- current helped to identify the membrane region. Although a slightly larger increase in $[Ca^{2+}]i$ was measured in these experiments, the K^+ current and the Cl^- current was not activated.

These data confirm that the BK channel in murine tear gland acinar cells is expressed in the apical region, in the same membrane region as the Ca^{2+}-dependent

Cl^- channel of the cell. This result is of great help in a more accurate understanding of the secretory mechanism of the lacrimal gland.

V/1.2 Immunocytochemistry

The apical location of K^+ channels suggests significant K^+ secretion during secretory activation of acinar cells, which raises the question of why tears may nevertheless have a relatively low K^+ content. Our hypothesis is that this discrepancy can be explained by the K^+ reuptake from the acinar cell lumen. In order to remain the membrane potential hyperpolarized during agonist stimulation, the function of the K^+ transporter responsible for reabsorption should be not depolarizing. For this reason, the Na^+-K^+ pump fits best into our model as a K^+ reuptake mechanism. To test this, Na^+-K^+ pumps were immunostained in freshly isolated acinar cell groups as described in the Materials and Methods section. It is noteworthy that the Na^+-K^+ pump is evenly distributed over the entire membrane surface, including the apical membrane region. The result of immunostaining confirms our hypothesis that the Na^+-K^+ pump can perform K^+ reuptake from acinar lumen through the apical membrane.

The functional significance of the apically located Na^+-K^+ pump is largely determined by the ratio of the apical membrane region to the total membrane surface. To estimate plasma membrane coverage by the apical membrane, the tight-junction protein, occludin was visualized by immunostaining. Cells were probed with antibody raised against occludin, and the primary antibody was probed with a secondary antibody DyLight488. Immunostaining showed a significant size of the apical membrane, corresponding to ∼30% of the total membrane section. Based on this results it is assumed the highly invaginated nature of the apical membrane and further suggests that the activity of apical Na^+-K^+ pumps may be functionally significant in determining the ionic composition of the primary tear fluid.

IV/2. Experiments on pancreatic acinus cells

IV/2.1 QPCR experiments

First, we performed reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) from murine pancreatic lysates using primer probes designed to recognize the two types of Ca^{2+}-dependent cation channels TRPM4 and TRPM5. In parallel, primer probes designed for the three isoforms of IP_3R, three isoforms of RyR, and TRPC3 channel, which served as internal control.. The household gene GAPDH was chosen as the reference gene. The IP_3R isoforms showed high, relatively uniform levels, while the RyR isoforms showed low levels, with RyR1 (“skeletal muscle type”) showing the highest levels of expression. These results are consistent with previous experience that IP_3R is significant, whereas RyR plays only a complementary role in pancreatic acinar cell Ca^{2+} signaling. The expression level of TRPM4 is comparable to that of IP_3 receptors and is about ten times higher than that of the TRPC3 channel, one of the Ca^{2+} channels responsible for the SOCE mechanism of acinar cells. However, the level of TRPM5 expression was below the detectable limit. Because of the high expression of TRPM5 in the endocrine pancreas, our negative result supports the fact that the Langerhans Island content of our pancreatic sample does not affect our data. On the other hand, it can be concluded that the expression level of TRPM4 is high in the pancreatic acinar cells.

IV/2.2 Electrophysiological measurements

Voltage-clamp (TRPM4, CaCC)

The following series of experiments aimed to verify the functional presence of TRPM4 using the full-cell configuration of the patch-clamp technique in voltage-clamp configuration. The composition of the intra- and extracellular solutions was designed to be specifically suitable for non-selective cation channel measurement. Accordingly, most of the Cl^- in the solutions was replaced with glutamate, thus avoiding the appearance of the Cl^- current, while the K^+ in the pipette solution was replaced with Cs^+, thus inhibiting the K^+ current. When running the ramp voltage protocol from -60 mV to +120 mV, an inward cation current is observed under control conditions, showing a slight voltage dependence in the positive voltage range. When the same cell was treated with CPA, the specific inhibitor of the SERCA pump, the current was significantly increased. The use of CPA is a commonly used method to increase $[Ca^{2+}]i$ because it inhibits the reuptake of Ca^{2+} by the SERCA pump into the ER, which compensates the resting leakage of Ca^{2+} from the ER and therefore causes an increase in $[Ca^{2+}]i$. Furthermore, the use of CPA over secretagogue activators for this purpose is advantageous because, in addition to increasing $[Ca^{2+}]i$, it also preserves the plasma PIP2 content, so that the PIP2 required for TRPM4 function remains until the end of the measurement, preventing a non-specific decrease in TRPM4 current. In addition, unlike other inhibitors, CPA can be washed out of the cell due to its water solubility, allowing for self-controlled experimental design. After the CPA induced TRPM4 current became constant, perfusion was started with a 100 µM solution of the widely used TRPM4 inhibitor, 9-Phenanthrol, which completely reduced the current. Unfortunately, in addition to the TRPM4 channel, 9-Phenanthrol also inhibits the Ca^{2+} dependent Cl^- channel, and since the currents of both channels are depolarizing, we can not use 9-Phenanthrol as a TRPM4 inhibitor to perform our experiments on pancreatic acinar cells (in physiological saline). In order not to obscure the depolarization caused by the TRPM4 current in another channel current, a more specific TRPM4 inhibitor is needed. We hope that a recently selected molecule, CBA, will be suitable for our further experiments. CBA at a concentration of 10 µM inhibited TRPM4 current with similar efficacy to 100 µM 9-Phenanthrol.

Cation current measurements were also performed on pancreatic acinar cells isolated from murines lacking the gene encoding the TRPM4 channel protein (TRPM4 knockout - TRPM4 KO) in the same experimental setup. Although the background current in these cells was higher than in the control, CPA did not increase it and was not sensitive to CBA. These results suggest that TRPM4 is also functionally expressed in pancreatic acinar cells in significant amounts.

Current-clamp

The effect of TRPM4 current on membrane potential was investigated by the perforated patch clamp method using the current clamp configuration. We measured under control conditions -44.38 ± 2.88 mV (n = 5) resting membrane potential, which began to increase slowly after CPA treatment until it reached -27.70 ± 2.99 mV (n = 5) equilibrium voltage value. Subsequently, the cells were perfused with a solution containing 10 μM CBA, as a result of which the membrane potential returned to a voltage value of -42.86 ± 1.65 mV (n = 5) close to rest.

Based on these experimental results, we hypothesize that the membrane potential of the pancreatic acinar cell is depolarized in a Ca^{2+}-dependent manner involving the TRPM4 channel. However, it is hypothesized that depolarization of the membrane potential reduces the electrochemical driving force of Ca^{2+}, thereby significantly reducing the influx of Ca^{2+} from the extracellular space.

IV/2.3 Ca^{2+}-imaging measurements

To verify that depolarization induced by TRPM4 current indeed reduces the driving force of Ca^{2+} influx, measurements of [Ca^{2+}] i on pancreatic acinar cell groups were performed by ratiometric fluorescence microscopy. Cells were stimulated by long-term treatment with low-concentration (10 pM) cerulein. Cerulein is a physiologically important secretory peptide with CCK-equivalent activity. Our experiments were performed in extracellular solution containing 2.5 mM Ca^{2+} and free of Ca^{2+} on both control and TRPM4 KO cells. Continuous treatment with 10 pM cerulein eliciated Ca^{2+} oscillation in all groups, suggesting that the cells retained their physiological responsiveness after isolation. When evaluating the curves, the Ca^{2+} spike arising between 500 and 600 seconds after cerulein treatment was examined. We chose this time window because, after this time, Ca^{2+} from the extracellular space is also expected to participate in the development of the Ca^{2+} signal. The mean

amplitude of the spikes measured in Ca^{2+}-containing and Ca^{2+}-free solution did not differ between the control cells (ΔR 500-600 s: 0.111 ± 0.008 and 0.091 ± 0.007), while it was significantly higher in the TRPM4 KO cells measured in Ca^{2+}-containing solution (ΔR 500-600 s: 0.087 ± 0.004 and 0.140 ± 0.011). Based on these data, there is a significant Ca^{2+} influx into TRPM4 KO cells after 500 seconds of cerulein treatment, which is not observed in control cells containing TRPM4 channel. In addition, it is important that the mean amplitude of the spikes measured in Ca^{2+}-free solution was essentially the same in control and TRPM4 KO cells, suggesting that by the end of treatment, the Ca^{2+} content of ER was similar in the two cell types. The area under the curve (AUC - area under the curve; Ca^{2+} spike between 500 and 600 seconds, which is proportional to the total Ca^{2+} level in the intracellular space) followed a rule similar to amplitude, but the difference was not statistically significant. Ca^{2+} spikes were repeated at similar frequency in control and TRPM4 KO acinus cells. In summary, the difference in the Ca^{2+} signal of the control and TRPM4 KO acinar cells in the Ca^{2+} containing extracellular solution suggests that the TRPM4 channel plays a significant role in the negative feedback regulation of Ca^{2+} influx.

To verify our theory, we designed further experiments in which we induced SOCE by significantly depleting the ER. The ER of pancreatic acinar cells was excreting for 10 min in a Ca^{2+} free extracellular solution containing 20 pM cerulein. Cerulein treatment caused rather sustained Ca^{2+} signals with fluctuations of gradually decreasing amplitudes. This behavior is an obvious sign of ER depletion and Ca^{2+} unloading due to the activity of PMCA. The extracellular solution was then replaced with a 2.5 mM Ca^{2+} solution, which resulted in a tonic increase in $[Ca^{2+}]i$ due to Ca^{2+} influx. When evaluating the curves, the fluorescence intensities measured in 2.5 mM Ca^{2+} were determined and it was found that the amplitude measured in TRPM4 KO cells (ΔR SOCE: 0.126 ± 0.004) was significantly higher compared to control acinar cells (ΔR SOCE: 0.012 ± 0.004).

For an even more specific study of SOCE, additional experiments were performed using CPA instead of cerulein to empty the ER. This experimental design provides an opportunity to avoid other factors that affect secretion of Ca^{2+} during secretagogue stimulation (e.g. PIP2 degradation). ER Ca^{2+} leakage was induced with a Ca^{2+} free extracellular solution containing 30 μM CPA. In the initial phase, as a result of treatment, $[Ca^{2+}]i$ increased and then slowly decreased, indicating that Ca^{2+} leak replenishment was abolished due to ER depletion and Ca^{2+} was pumped from the

intracellular space into the extracellular space. Ca^{2+} signals measured during ER depletion were not different in control, CBA-treated, and TRPM4 KO cells (0.18 ± 0.02, 0.22 ± 0.01, and 0.21 ± 0.03), which suggests that the ERs of the two cell types initially contained similar amounts of Ca^{2+}. After the fluorescence intensity returned to baseline, the Ca^{2+} free extracellular solution was replaced with a 2.5 mM Ca^{2+} containing solution, which caused a significant increase in $[Ca^{2+}]i$. Similar experiments were performed in the presence of CBA and on TRPM4 KO acinar cells. The administration of the TRPM4 channel inhibitor CBA was initiated only after complete ER depletion to avoid any undesirable side effect of CBA on ER depletion and Ca^{2+} content of the acinar cell. Evaluation of the fluorescence intensity ratios providing information on Ca^{2+} influx shows that the slope and amplitude of the fluorescence change were also higher in TRPM4 KO acinar cells compared to the control. In addition, the slope of the fluorescence signal increased significantly with CBA treatment, while no change in amplitude was observed (slope, CTRL: 0.76 ± 0.04; CBA: 1.06 ± 0.03; TRPM4 KO: 1.88 ± 0.1 AU (arbitrary unit); ΔRSOCE: CTRL: 0.065 ± 0.004; CBA: 0.071 ± 0.002; TRPM4 KO: 0.103 ± 0.005). These measurement results are consistent with the previous ones and further confirm our theory that TRPM4 regulates Ca^{2+} influx on murine pancreatic acinar cells by negative feedback mechanism.

V. Discussion

New tear secretion model

Based on our results, we concluded that the K^+ channels together with the Cl^- channels are located in the apical membrane, where the Na^+-K^+ pump density is the same as that of the basement membrane. Based on these, we created a new model of lacrimal gland secretion. Although the basic concept of the new model is very similar to the old model created by Wood and Mircheff in 1986, it describes the ionic movements across the membrane in much greater detail and accuracy, taking into account the new results. The BK channel plays an important role in the secretion of Na^+ and Cl^- rich fluid in secretory epithelial cells. Elevated $[Ca^{2+}]i$ for secretagogue stimulation activates CaCC channels, whereas Cl^- leaving the extracellular space would move the membrane potential towards its own equilibrium potential. Although depolarization further activates CaCCs, it reduces the driving force of Cl^- secretion. The BK channel plays a role in the fine regulation of Cl^- secretion, due to the fact that the BK channel is activated in parallel with CaCC for the Ca^{2+} signal, and the effluent Cl^- is also followed by the K^+ efflux, thus balancing the membrane potential. and ensuring continuous Cl^- driving force. The condition of said process is that the BK channel is located in the apical membrane, and this hypothesis was also confirmed in tear and pancreatic acinar cells using Ca^{2+} uncaging and immunofluorescent techniques. According to the old model, Na^+ transport into the cytoplasm is mediated through the Na^+-K^+-$2Cl^-$ and Na^+-H^+ cotransporters, and Na^+ circulates across the basal membrane via Na^+-K^+ ATPase. K^+ and Cl^- also enter the cell through the basolateral membrane via the Na^+-K^+-$2Cl^-$ and Cl^--HCO_3^- cotransporters. These ions leave the cell through the apical membrane into the lumen through Ca^{2+} dependent K^+ and Cl^- channels. According to the mechanism described above, K^+ secretion is primarily responsible for the formation of the osmotic gradient, thereby reduces the driving force of Na^+ through the epithelium and, consequently, lowers Na^+ secretion. However, the theory is not supported by the concentration ratio of ions in the tear fluid.

In our work, we confirmed the results of Trautmann and Marty that Ca^{2+} dependent K^+ and Cl^- channels are located in the apical membrane of the acinar cell of the lacrimal gland and hypothesized an apical K^+ reuptake mechanism that ensures low K^+ concentration in the acinar lumen. Our theory is supported by our

immunofluorescence recordings, which show an equal density distribution of the Na^+-K^+ pump in the apical and basolateral membrane sections. Our results support a significant role for K^+ reabsorption in tear secretion, which is consistent with several previous studies. Although, in order to judge the true functional significance of the Na^+-K^+ pump, we need to determine the ratio of the amount of apically and basally located transporters. Based on our immunofluorescence results, the ratio of the apical membrane section to the basolateral membrane region is 3: 7, suggesting that the apical membrane is large enough to cause significant K^+ reabsorption from the lumen through it.

A paper was recently published with the help of our research group in which our colleagues developed a mathematical model of the secretion mechanism of salivary gland acinus cells. This model suggested that fluid secretion was optimal when 40% of the total K^+ conductance and 30% of the pump activity was inserted into the apical membrane. Also, under these conditions it predicted that 27% of Na^+ was secreted through the transcellular pathway into the lumen, while without apical pumps, Na^+ used the paracellular pathway exclusively. We propose a similar overall mechanism in lacrimal glands.

The role of TRPM4 in the SOCE of pancreatic acinar cells and its pathological implications

We were the first to demonstrate the functional presence of TRPM4 on murine pancreatic acinar cells and to show that it inhibits Ca^{2+} influx by a negative feedback mechanism, thereby reducing the increase in $[Ca^{2+}]i$.

Abnormal, persistent elevations (Ca^{2+} overloads) of $[Ca^{2+}]i$ in pancreatic acinar cells are a critical, early pathological event that leads to premature intracellular zymogen activation, self-digestion, and ultimately acute pancreatitis. Because SOCE is essential for Ca^{2+} overload and mitigation of the severity of acute pancreatitis has been reported with the use of ORAI1 inhibitors, our data suggest that TRPM4 channel function may play a role in the prevention of pathological Ca^{2+} signaling. This hypothesis needs further testing by examining animal models of the disease. In the light of the results of animal experiments, the translational and therapeutic potential of the TRPM4 channel should be evaluated later.

A physiological function similar to that described by our group has been previously observed in the TRPM4 channel in T lymphocytes and mast cells. These suggest that silencing of the TRPM4 gene resulted in sustained $[Ca^{2+}]i$ elevations instead of Ca^{2+} oscillations in Jurkat T cells and increased IL-2 production, consistent with the idea that TRPM4 reduces Ca^{2+} influx into the cell by depolarizing the plasma membrane and reducing the electrochemical driving force of Ca^{2+}. Consistent with this, our results on acinar cells suggest that the TRPM4 current reduces Ca^{2+} influx by creating a lower Ca^{2+} driving force by depolarizing the membrane potential. However, the results of Park and colleagues raise another possibility. Accordingly, in HEK293T cells, TRPM4 has been shown to interact with TRPC3 (a Ca^{2+} channel activated by Ca^{2+} release), resulting in a decrease in TRPC3 channel activity. Because TRPC3 is highly expressed in pancreatic acinar cells, this also offers a possible explanation for our results. In addition, the allosteric inhibition of TRPC3 by TRPM4 may explain our finding that, contrary to expectations, CBA was unable to significantly increase the amplitude of SOCE. In this regard, it is hypothesized that the inhibitory interaction between TRPM4 and TRPC3 is not affected by CBA, so in the presence of CBA, TRPM3 remained in an inhibited state by TRPM4, which would explain that the amplitude of SOCE remained unchanged after CBA treatment.

The Ca^{2+} dependent cation current of pancreatic acinar cells also plays an important role in the regulation of transepithelial Cl^- secretion. Based on this, Kasai and Augustine developed a “push-pull” model of primary tear secretion. The model takes into account that the apico-basal propagation of the Ca^{2+} wave first activates apically localized Cl^- channels during secretagogue stimulation. Activation results in a Cl^- outflow because the membrane potential is more negative than the Cl^- equilibrium potential (“push” phase). Later, the Ca^{2+} wave reaching the basal plasma membrane activates the Ca^{2+} dependent cation channels (i.e., TRPM4) in this region, which depolarize the membrane over its Cl^- equilibrium potential, creating a driving force for Cl^- inflow (“pull” section). This alternating activation of the conductivities would result in unidirectional Cl^- and fluid flow in the epithelium. According to another hypothesis, Cl^- uptake is mediated by Na^+-K^+-$2Cl^-$ cotransporters located in the basal membrane. This model was strongly supported by a convincing study that Ca^{2+}-activated Cl^- channels are expressed exclusively in the apical plasma membrane. This new theory rejects the “push-pull” model and attributes no role to Ca^{2+}-dependent cation current in fluid secretion.

It is clear that the Cl^- current (via the recently identified TMEM16a channel) also acts as a major depolarizing current in pancreatic acinar cells, which raises the question of why acinar cells express ionic channels with the same role, which are responsible for ion currents that can be activated with the same ligand. The Ca^{2+} dependent cation current was previously hypothesized to play a role in Na^+ uptake across the basolateral membrane, providing a possible transcellular Na^+ transport pathway for Na^+. However, current measurements recorded at the equilibrium potential of Cl^- did not show an increased cation current as long as the level of $[Ca^{2+}]i$ increased only in the apical region of the cell, but if the value of $[Ca^{2+}]i$ increased in the entire intracellular space, it did. Although we were unable to demonstrate the expression of TRPM4 in pancreatic acinar cells by immunostaining in the absence of TRPM4 antibodies suitable for our immunofluorescence studies, the aforementioned previous study strongly suggests that Ca^{2+} activated cation channels are expressed only in the basal region of the plasma membrane. Kasai and Augustine also suggest that the apical membrane does not mediate significant cation currents, and consequently the only pathway of Na^+ secretion in the pancreatic acinar may be paracellular, and TRPM4 is unlikely to be involved in mediating transcellular Na^+ transport and thus transepithelial fluid secretion. Therefore, we concluded that TRPM4 functions as an

additional depolarizing current that is site-specifically located in order to negatively regulate Ca^{2+} entry through CRAC channels.

Based on our results, it was confirmed that the TRPM4 channel, when opened, inhibits the SOCE process with a negative feedback mechanism, in fact providing protection against Ca^{2+} overcharging. Secretagogue stimulation activates the IP3 pathway in the pancreatic acinar cell, causing Ca^{2+} release through both the IP3 channel and the RyR channel (Ca^{2+} induced Ca^{2+} release). Ca^{2+} released from the internal storage (SR) results in the depletion of SR, which causes store operated Ca^{2+} entry (SOCE). Activation of SOCE is directly triggered by a conformational change in the STIM1 protein. The maximal $[Ca^{2+}]i$ level is provided from the SR and extracellular space, which plays a key role in the exocytosis of zymogens and, in pathological cases, in their early activation. In parallel with the formation of SOCE, ascending $[Ca^{2+}]i$ activates the TRPM4 channels, Na^{+} flows into the cell, and depolarizes it. Due to depolarization, the driving force of Ca^{2+} influx is reduced, but the Ca^{2+} efflux by PMCA is maintained, thus shifting the Ca^{2+} movement towards the efflux, which provides protection against excessive Ca^{2+} overload.

VI. Summary

In the case of our experiments on lacrimal gland secretion, our goal was to develop a new model of tear secretion. The aim of the first series of experiments was to investigate the function of Ca^{2+}-dependent K^+-channels in the apical membrane of lacrimal acinar cells. Local uncaging was combined with whole-cell current recording and Ca^{2+}-activated K^+- and Cl^--current was measured. In the apical region, a local increase in $[Ca^{2+}]i$ resulted in a significant increase in K^+ current, and when BK channel inhibitor paxillin pretreatment was used, current activation was lacking.Next, the basal membrane was probed for K^+- and Cl^--currents by using the same approach. Although, in these experiments, the change in local $[Ca^{2+}]i$ was slightly higher, it failed to activate either K^+- or the Cl^-currents. These data strongly suggest that the primary K^+-channel in the murine lacrimal acinar cell is the BK channel, which co-localizes with the Ca^{2+}-activated Cl^--channels in the apical plasma membrane. Moreover, immunostaining shows that the area of the apical membrane is relatively large (~30% of the apparent membrane section circumference) and also contains Na^+-K^+ pumps. This result also suggests that apical Na^+-K^+ pump activity may be functionally significant in determining the ionic composition of the primary tear fluid. In summary, our new model suggests that K^+-secretion occurs on the apical membrane, but thereafter, K^+ is immediately taken up by apical Na^+-K^+ ATPase, which is associated with Na^+ secretion. Thus, this process redirects Na^+ secretion from the paracellular pathway in part to the transcellular pathway. In addition, the electronegative transport of the Na^+-K^+ pump helps to maintain the hyperpolarized membrane potential during stimulation, thus maintaining the electrochemical driving force of Cl^--secretion. Also, our model implies that K^+-reabsorption is flow-rate dependent. During stimulation (reflex or emotional lacrimation), when the high fluid secretion rate allows a shorter time for Na^+-K^+ exchange across the luminal membrane of the acinar cell, the K^+ content of the primary tear remains higher, while the Na^+ concentration is lower than in slower flow rate conditions.

Interesting results have also been obtained in the study of ion channels in pancreatic acinar cells. We found that TRPM4 ion channel mRNA is highly expressed in murine pancreatic acinar cells. The functional presence of TRPM4 under voltage-clamp conditions was also confirmed by the whole cell configuration of the patch-clamp technique. We found that 10 μM CBA inhibited the Ca^{2+}-dependent cation

current similarly to 100 μM 9-Phenanthrol, but did not affect the Cl^--current. As CBA is a specific TRPM4 channel blocker, these results strongly suggest that TRPM4 is functionally expressed in wild type PACs in significant amounts. We also showed the effect of TRPM4 current on membrane potential. We found that the plasma membrane of the pancreatic acinar cell is depolarized in a Ca^{2+}-dependent manner, in which the activation of the TRPM4 channel plays a significant role. In addition, our [Ca^{2+}] measurements show that 10 pM cerulein treatment induced significant Ca^{2+}-entry after 8 min in TRPM4 KO acinar cells, whereas not in control cells, indicating that TRPM4 is involved in the negative feedback regulation of the Ca^{2+}-entry. Moreover, when Ca^{2+}-entry was induced by 20 pM CCK treatment (SOCE), the magnitude of the Ca^{2+}-signal was significantly higher in TRPM4 KO cells compared to the control. To confirm these results, SOCE was examined even more specifically by excreting the Ca^{2+}-content of the ER in a receptor-independent manner with CPA to avoid the formation of possible experimental by-products. Analysis of the Ca^{2+}-signal showed that the slope and amplitude of signal formation were higher in TRPM4 KO pancreatic acinar cells compared to the control. Moreover, experiments were performed with the inhibitor of TRPM4 channel, which showed that CBA treatment significantly increased the elevation of the fluorescence signal rate.

Summarizing our results, it can be described that the TRPM4 current depolarizes pancreatic acinar cells in a Ca^{2+}-dependent manner and regulates Ca^{2+}-entry into the cell with negative feedback regulation under physiological conditions. Therefore, we conclude that TRPM4 functions as a complementary depolarizing current that is specifically localized in order to negatively regulate Ca^{2+}-entry near CRAC channels.

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Subject: PhD Publication List

Candidate: Gyula Diszházi
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Diszházi, G.**, Magyar, Z. É., Lisztes, E., Tóth-Molnár, E., Nánási, P. P., Vennekens, R., Tóth, I. B., Almássy, J.: TRPM4 links calcium signaling to membrane potential in pancreatic acinar cells. *J. Biol. Chem. 297* (3), 101015, 2021.
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