János Almássy: Final report for the NKFIH project # PD112199 entitled: Malignant hyperthermia susceptibility: a risk factor of pancreatitis?

In the current project, mouse pancreatic acinar cells were used in Ca^{2+} imaging experiments. Therefore, I started with optimizing the acinar cell isolation and the imaging parameters using confocal microscopy. During this early period, I encountered the following problem to solve: after cells were loaded using the standard protocol with Fluo-4 AM (2 μM, 30 minutes), as low as 1 mW laser intensity applied at 1 Hz, induced spontaneous, high amplitude, repetitive intracellular Ca^{2+} oscillations. Notably, this intensity represents the 1% of the maximal laser power. Eventually, the appropriate imaging conditions were established (0.5 mW laser power at 0.5 Hz) and used since then. Because this laser-related artefact severely affects imaging experiments and the information about it is limited to only few papers, I decided to describe this phenomenon in details, reveal the reasons and publish the result. The manuscript “Laser induced calcium oscillations in fluorescent calcium imaging” was submitted to General Physiology and Biophysics and requires minor revision to be accepted. This paper is a methodology paper that helps investigators use imaging tools properly.

The ultimate question of my research is whether malignant hyperthermia susceptibility (MHS) could be or could not be a genetic risk factor for pancreatitis. If (based on previous studies) we hypothesize that bile acids and alcohol ethyl esters cause acute pancreatitis by triggering high amplitude Ca^{2+} release events partially through ryanodine receptors (RyR) and that certain point mutations sensitize RyR to agonists; we reach the assumption that MHS might be a genetic risk factor for acute pancreatitis.

To test this idea, first we need to test whether bile acids and alcohol ethyl esters are RyR agonists. We found that taurocholic acid (TCA) triggered Ca^{2+} release in pancreatic acinar cells in Ca2+-free saline solution, which was significantly inhibited by the RyR1 antagonist dantrolene. Further, we showed that TCA enhanced RyR's ^{3}H-ryanodine binding and triggered robust Ca^{2+}-release from RyR-containing vesicles in the pathologically relevant concentration range. Single channel current experiments were also performed using purified RyR channels reconstituted into artificial lipid bilayers. Analysis of the currents demonstrated that 200 μM TCA induced a 5-fold increase in the channel's open probability (Kd=180 μM) and caused

a significant lengthening of the mean open time. TCA also suppressed Ca^{2+}-uptake rate and ATP-ase activity of SERCA pump-containing vesicles, but interestingly, failed to decrease the Ca^{2+} elimination rate in intact cells. Ca^{2+} imaging and Ca^{2+} release experiments were also performed using two other bile acids taurolithocholic acid sulphate (TLCS) and tauro-deoxycholate. These experiments yielded qualitatively the same results. But importantly, these bile acids were considerably more potent than TCA was.

Overall, our strongly suggest that bile acids open RyR by a direct allosteric mechanism, which contributes significantly to bile acid-induced pathologic Ca^{2+}-leak from the endoplasmic reticulum in pancreatic acinar cells.

These results were accepted for publication in March 2015 in the journal “Cell Calcium” (doi: 10.1016/j.ceca.2015.03.009.).

Interestingly, dantrolene failed to abolish TCA-evoked Ca^{2+} release from the SR vesicles, but the drug significantly delayed the Ca^{2+} release. As the molecular details of the mechanism of action of dantrolene is largely unknown, we believed that finding the reason for the delayed response could answer very important questions about the action of the drug. We found that ATP is responsible for the delay. We concluded that ATP is required for the inhibiting action of dantrolene, because after ATP is consumed up in the solution, dantrolene failed to inhibit the compensated leak from SR vesicles any longer. These results also imply that dantrolene is a weak drug, which cannot suppress more active channels. Very recently, Choi et al identified Mg^{2+} as the essential factor for dantrolene to act on RyR (Choi et al, doi: 10.1073/pnas.1619835114). Inspired by their work, we performed single channel current measurements showing that dantrolene inhibited channel activity by 36% in a Mg^{2+}-ATP containing recording medium. This is a very important result, because earlier, many groups (including us) failed to show the action of dantrolene in single channel experiments (probably due to the lack of “secret ingredients” in the recording medium). We summarized our results in a short manuscript and submitted it as a letter to the editor of the journal Proceedings of the National Academy of Sciences. It is currently under major revision.

Next, to answer the original question whether the acinar cells, harboring a malignant hyperthermia mutation are more susceptible to the stimulation with the parasympathomimetic drug carbachol, and whether they are more susceptible to the effect of bile acids and palmitoleic acid ethyl ester (POAEE), the effect of these

compounds were compared in pancreatic acinar cells from control and MHS mice, respectively. We found that 50, 100 and 250 nM carbachol or 1 mM TCA induced repetitive fluctuations of the intracellular [Ca^{2+}], but these responses were not significantly different in control and MHS acinar cells, respectively. These experiments were repeated using 5 mM TCA, which induced a robust, long lasting Ca^{2+} release in both cases, but the average amplitude and area under the curves were not statistically different.

As MHS RyR is a leaky channel, experiments were designed to assess the endoplasmic reticulum (ER) Ca^{2+} load and basal leak. Cells were treated with the SERCA pump inhibitor thapsigargin to empty the intracellular Ca^{2+} stores in Ca^{2+} free medium, then the slope and the amplitude of the fluorescence change was evaluated. Ca^{2+} release rate in MHS was steeper, but same size as in the control. These data indicate that the ER's Ca^{2+} content is maintained in MHS acinar cells, but the Ca^{2+} stores deplete more rapidly. This phenomenon raises the question whether the activity of store operated Ca^{2+} entry (SOCE) is different in the two groups. To answer this question, following store depletion cells were superfused with a medium supplemented with 5 mM Ca^{2+}. In MHS cells, significantly higher Ca^{2+} elevation was detected compared to control (209±18 vs. 136±16 AU), which indicates that the Ca^{2+} entry pathways are more active in MHS cells. Because SOCE is also significantly contributes to bile acid induced Ca^{2+} overload during the early stage of acute pancreatitis, the magnitude of Ca2+ entry was also investigated after TCA treatment. The amplitude of SOCE was 102±6,8 and 144.3±23.7, which are statistically not different.

Similar experiments were performed using POAEE. POAEE induced Ca^{2+}-release in both control and MHS pancreatic cells in Ca^{2+}-free bath solution, but no difference was observed between the two groups. POAEE was tested in Ca^{2+} release experiments and ATP-ase activity assays too, but no effect was observed. These results suggest that POAEE does not activate RyRs neither it alters SERCA activity. Therefore more experiments with this compound were not performed.

Next, I investigated whether Ca^{2+} release from permeabilized pancreatic acinar cells was different in malignant hyperthermia susceptible (MHS) mice compared to control. For this end, the permeabilized cell assay was established and optimized. Cells were loaded with the low affinity fluorescent Ca^{2+} indicator Fluo-5F AM for 40 minutes at room temperature. Fluorescence intensity of the emitted light (≥520 nm) was

monitored using a laser scanning confocal microscope. The cells were plated onto coverslips and washed with 0.05% saponin for ~3 minutes in order to permeabilize the plasma membrane. The cells were then superfused with intracellular-like medium to wash the dye out from the intracellular space. Washing was continued until the background fluorescence vanished and the Fluo-5F AM fluorescence of the reticular structure emerged. The endoplasmic reticulum was then loaded with Ca^{2+} using a Mg^{2+}-ATP and 1 μM Ca^{2+} containing solution. Ca^{2+} release was triggered with 700 μM taurocholic acid (TCA). TCA-induced Ca^{2+} -release rate was determined by fitting the release-curves with Boltzmann function. The slope of the curves recorded in MHS and control were not different (-0.00646 ± 0.000316 vs. -0.00647 ± 0.000298, n=136 and 144 cells). Apparently, the resting Ca^{2+} -leak was not different either in the two groups, but the maximal fluorescence of the curves (which is proportional to the ER Ca^{2+} load) was significantly higher in MHS cells (967 ± 26 and 1329 ± 34 AU). In order to answer the ultimate question of my project, whether malignant hyperthermia susceptibility is a risk factor of pancreatitis, a mouse pancreatitis model was generated. Mice were injected in every hour with 100 mg/kg body weight cerulein 2-6 times. The severity of pancreatic injury was assessed by measuring amylase activity of the plasma. However, even two cerulein-treatment induced significant elevation of plasma amylase activity, no difference was observed between control and MHS groups (3051 ± 87 and 2892 ± 426 U/l, n=4). In order to induce more severe pancreatic injury, similar experiment was performed with 6 consecutive injections, with very similar results. Some mice were treated with cerulein 7 times and were sacrificed 1 day later. Their pancreas was removed and after conventional histochemical preparations, pancreatic slices were stained with hematoxilin-eosin. The severity of the simptoms of panreatitis were evaluated using a scale of 0-5 or 0-100%, respectively. Tissue oedema scored 2.17 ± 0.19 in control vs. 2.42 ±0.1 MHS, lymphocyte infiltration was 2.33 ± 0.24 vs. 2.63 ± 0.21 and necrosis was 15.42 ± 3.05 vs. 15.42 ± 2.59%, suggesting that MHS do not increase the susceptibility of mice for pancreatic injury and pancreatitis.

As complimentary experiment, single channel current recordings on ryanodine receptors purified from control and MHS muscles were also performed, to determine the biophysical properties of the channels. Ryanodine receptors were purified using a multiple step isolation procedure by ultracentrifugation. The old isolation protocol for rabbit was slightly modified and optimized for lower amounts of mouse muscle tissue.

Ryanodine receptors were reconstituted in artificial lipid bilayers and the current was recorded under voltage clamp conditions. I found that open probability of MHS ryanodine receptors at maximally activating Ca^{2+} concentration (50 μM) was significantly higher compared to control (0.194 ± 0.061 and 0.357 ± 0.071, n= 11 and 15). However, channel activity at low (~20 nM) Ca^{2+} concentration was very similar in the two groups (0.028 ± 0.024 and 0.026 ± 0.01).

Alltogether, these results suggest that in the physiologically relevant concentration range of carbachol and relatively low concentrations of TCA, RyR1's deficiency is masked or compensated by other mechanisms. Our results also suggest that RyR1 might not have as significant pathological role as suggested earlier by other groups.

To show the distribution and the relative expression of RyR and the other Ca^{2+} release channel, IP_3R, immunofluorescent staining was performed on pancreatic acinar cells. Parotis acinar cells were used as negative control, because these cells do not express much RyRs. Unfortunately, our anti-RyR antibody did not work well, so we could not draw consequences about the distribution of the channels. In the same experiments, the plasma membrane of parotid acinar cells visualized using an anti-Na/K pump antibody. Surprisingly, we found that the antibody stained the whole circumference of the cell, indicating that the pumps are not only present in the basolateral, but the apical membrane too. This result is important for the field, because they explain the discrepancy between my previous results, showing that K channels are primarily localized in the apical membrane, and the fact that the primary saliva is low in K^+. Based on the immunofluorescent study, we created a new saliva secretion model, in which K^+ channels are localized mainly in the apical membrane. The secreted K is readily reabsorbed by the Na/K pump, whose activity contributes to Na+ secretion into the lumen. Based on a mathematical model, created by our collaboration partner, this active, transcellular mechanism partially (20%) substitutes for the paracellular Na^+ secreting pathway, while keeps the intraluminal K^+ concentration low. The results have been summarized in a manuscript, which was submitted to the journal Pflügers Archive European Journal of Physiology. Major revision is currently in progress.