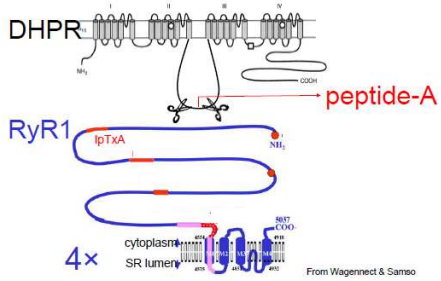
In the beginning of the project we have developed (modified) the purification procedure of the heavy sarcoplasmic reticulum vesicle (HSR) and of the skeletal type ryanodine receptor (RyR1) of the skeletal muscle of the pig. In the literature the isolation/purification procedure for rabbit can be found, but we were years ago the first to modify it for rats, and now we have worked it out for pigs. In course of the optimalization process the composition of the extraction solutions was varied systematically, the parameters of the sacharose gradient were used for separation. The KCl concentration of the actomyosin extracting medium was systematically varied, the lipid detergent ratio and concentration in the solubilization medium and the centrifugation times was also varied in order to obtain the best possible separation of the contaminating proteins (actomyosin, parvalbumin, actin etc.) and in order to obtain the highest possible RyR1 content of the HSR vesicles as well as of the solubilized receptor preparation. Due to these efforts we were able to obtain RyR1 concentration/content of the solubilized samples and incorporation ratios which are comparable to the values previously obtained for rabbit preparations according to the bilayer measurements, the channel parameters were also alike compared to the rabbit. These experiments took up quite more time, than originally planned – because of the difference of the pig skeletal muscle and because of experimental difficulties. For that reason part of the planned experiments for the first year has been completed in the second: the measurement of the effect of ATP on the channel parameters.

At the same time specific measurements planned – measurements required for the modeling of the ion conducting pore – were successfully completed. Within this subproject we have determined the influence of the calcium gradient on the specific conductance at negative and at positive holding potentials. Our experiments show that the change of the specific conductivity due to the change of the *trans* calcium concentration from 50 µM to 238 nM significantly differs at positive and at negative holding potentials. At negative potential the normalized specific conductance decreases by 3.7 % ± 1.6% while at positive potential it increases by 7.4% ± 4.3% upon change of *trans* calcium concentration. These data (n=6) normalized for the specific conductance determined at 50 µM *trans* calcium concentration. Throughout these experiments the *cis* calcium concentration were kept at 50 µM.



To study the electro-mechanical coupling (EC), a scorpion toxin: Maurocalcine (MCa) can be used, since its sequence mimics the active part of the 2-3 loop of the DHPR. Another series of measurements required for the modeling of the ion conducting pore and the interaction between the RyR1 and the MCa were also completed: the measurement of the potential generated by the current flowing through the channel (the “reversal potential”) due to calcium concentration gradient, in the presence of low MCa (5 nM) evoking about 50% of its maximal effect. These measurements were carried out using constant *cis* calcium concentration of 50 µM while varying the *trans* calcium concentration up to 15.4 mM in the presence of 5 nM MCa.

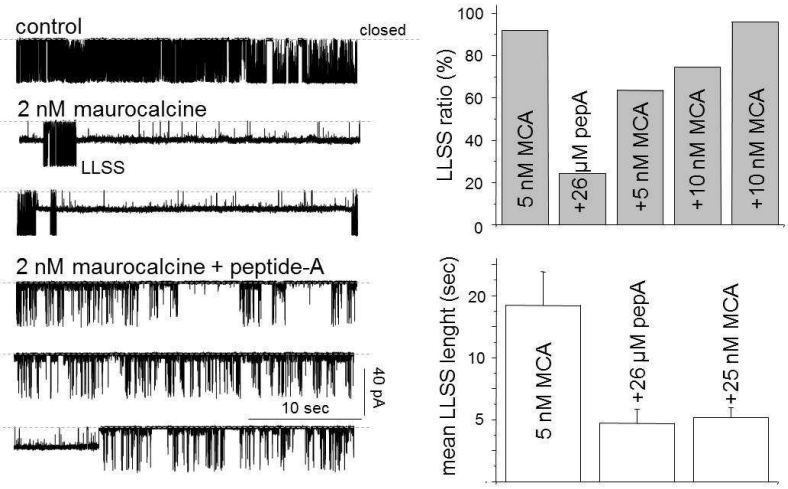
trans Ca	Nernst p	Meas. p.	Ratio
mM	mV	mV	meas./calc.
3.8	55	8.2	14.9%
7.7	64	12	18.8%
11.5	69	15	21.7%
15.4	72.8	17.3	23.8%

As the table shows, in the presence of MCa, the measured reversal potential is much lower than the calculated one: it is only 14.9% (at 3.8 mM *trans* calcium concentration) and 23.8% (at 15.4 mM *trans* calcium concentration) and the difference between the calculated and measured values decreases with the increase of the driving force.

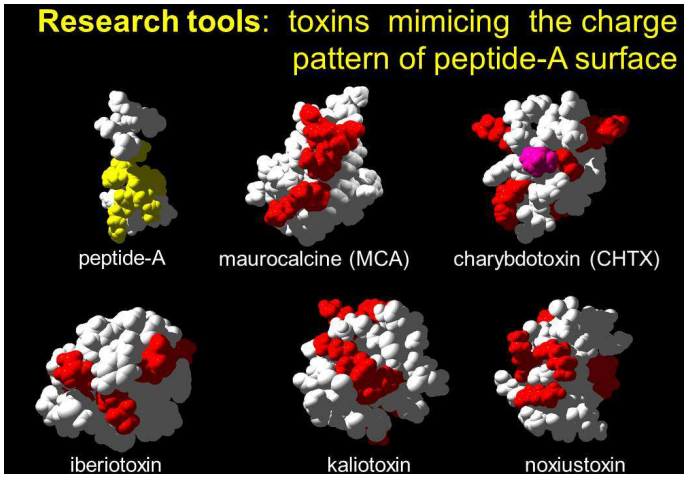
Since the very basic aim of the research is to understand, how and in which way the electromechanical coupling is altered in case of MH, we studied first the most important step of the EC coupling: the interaction of the dihydropyridine receptor (DHPR = voltage sensor) and the SR calcium release channel (RyR1). First – by competition experiments – we have shown that the binding site of the peptide A – which is the interacting part of the 2-3 loop of the DHPR) and of the MCa is the same so the effect of MCa successfully mimics the effect of the DHPR-RyR1 interaction.

	LLSS ratio	error
controll	0	-
+ 5 nM MCa	92.0%	2.5%
+ 26 µM peptide A	24.6%	3.2%
+ 5 nM MCa	63.6%	4.1%
+ 10 nM MCa	74.6%	3.1%
+ 10 nM MCa	96.2%	1.9%

MCA and Peptide-A competes for RyR

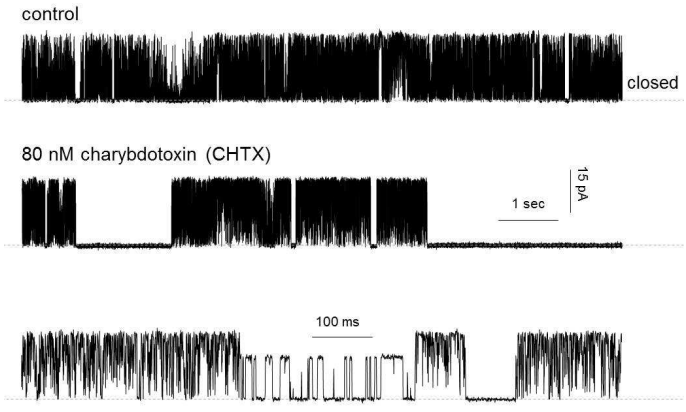


Going one step further, we have tested several beta scorpion toxins: such as Charibdotoxin (CHTx), Slotoxin (SLTx) and Iberiatoxin (IBTx). Two beta scorpion toxins, the SLTx and the IBTx have not influenced the channels parameters – even though they share a very similar structure – not even at 10 µM of toxin concentration.
The effect of CHTx is similar to the effect of MCa in a way, that the channel alternates between two substantially different gating modes: it has normal gating or it exhibits long lasting subconductance state (LLSS).

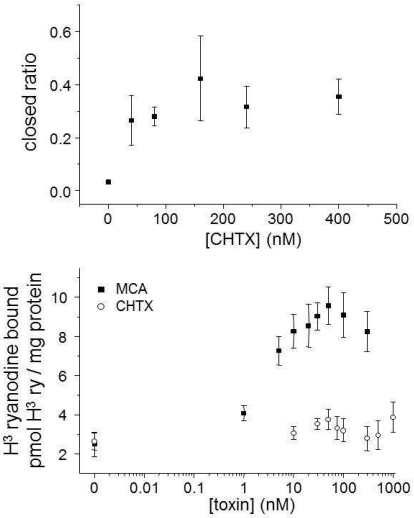


The conductance of the CHTx induced sub-conductance state is so low, that it could be called as closed state – in case of ions having larger or equal ionic diameter as the potassium ion. It has also been shown, that the binding site of the MCa and the CHTx is the same in contrast to the fact that they have quite different amino acid sequence. On the other hand hey have very similar spatial structure and almost identical surface charge distribution, so their identical binding site is not necessary a surprise. Recording the gating of the channel after the addition of 100 pM MCa, the characteristic MCa type LLSS phenomena shows up – intermittently with the normal gating – and after the addition of 100 pM CHTx (to the MCa modified RyR1), the LLSS gating shows the CHTx type LLSS pattern. These competition experiments indicate that the affinity of the CHTx is way long higher than that of the MCa – for the DHPR interacting site of the RyR1. This could be understood taking into account that the MCa and the CHTx are composed of similar motifs, but the CHTx is smaller in size which leads to a deeper position in the conducting pore than that of the MCa.

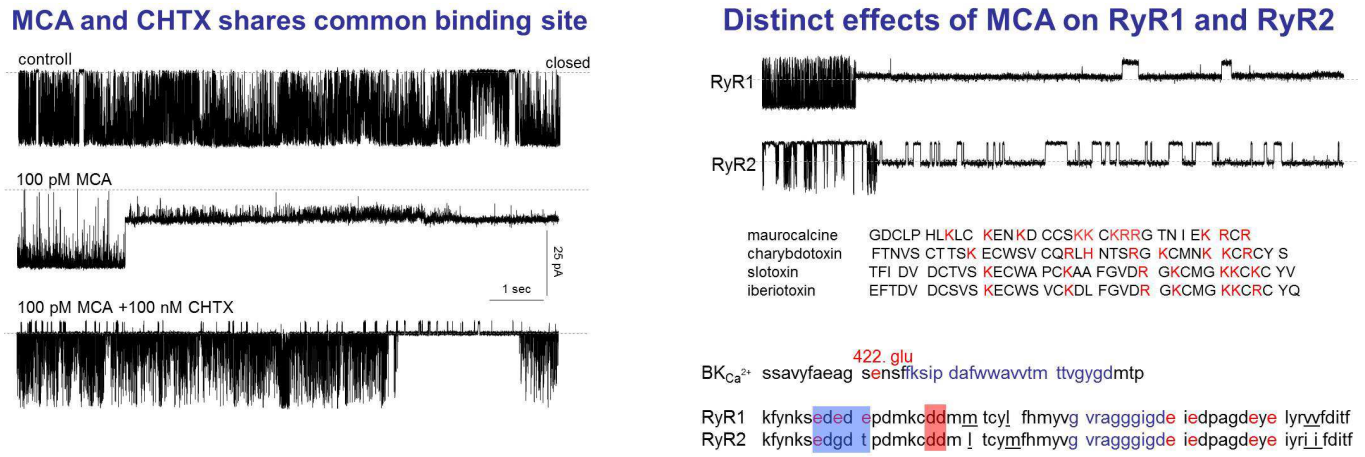
Effect of charybdotoxin on RyR



[CHTX] (nM)	Rel. Po	± S.E.
0	1	0
40	2.52	0.36
80	3.71	1.49
160	4.69	3.10
240	2.06	0.59
400	3.16	1.03
800	4.20	1.80



This idea is further supported by the fact, that the effect of these toxins are dependent on the holding potential as well as on the calcium concentration. These findings – beside their face values – are also important for the *in silico* reproduction of the parameters of the ion conducting pore, and the paper is in preparation for publishing.



We investigated of the effect of ATP on the RyR1 using wt pig RyR1 (Duroc strain) and the $^{arg}615^{cys}$ mutant (Pietrin strain) RyR1 channel. We have shown previously that the activity of the Ryanodine Receptor / Calcium release channel (RyR/CRC) is modulated by ATP. Increasing ATP concentration results in an increase of open probability of the RyR/CRC and the dose response curve of the phenomena is biphasic having two distinctive activatory processes. The amplitude ratio of the two steps are 1:3 suggesting strong cooperation between the ATP binding sites and independent but cooperative binding of the ATP on the RyR1 monomers. It was also shown by several laboratories that mutations leading to malignant hyperthermia (MH) also increases the calcium sensitivity of the channel.

Our aim was to test whether the ATP pharmacology is affected in MH and if this is the case in what way. Heavy SR vesicle was prepared as described previously using longissimus dorsi of a swine (Pietrin strain), which carries an MH causing homozygous $^{arg}615^{cys}$ mutation. Following CHAPS+lipid solubilization, the functional RyR1 tetramer - the channel complex - was incorporated into a lipid bilayer. The bathing medium contained symmetrical 250 mM KCl – 20mM PIPES – pH:7.4. Free (ionized) calcium concentration was established using Ca-EGTA calcium buffer, calculated by Fabiato's method. Under voltage clamp conditions the channel current was recorded and the channel parameters were determined such as open probability (P_o), mean open time and specific conductance. The ATP pharmacology of the RyR/CRC was determined using 50 µM Ca^{2+} free *trans* and 472 nM Ca^{2+} free *cis,* applying increasing Na_2ATP concentration on the *cis* side.
The wt pig RyR1 channel ATP pharmacology was essentially very similar to the pharmacology of the rat RyR1, showing two activation phase characterized by a half activatory ATP concentration ≈ 25 µM and ≈350µM with a relative $\Delta P_{o,1}$≈0.1 and $\Delta P_{o,1}$≈ 0.28 at the calcium concentrations. The mutant (pig) channel showed higher open probability compared to the wild type even in the absence of ATP. The mean open time was slightly higher in the mutant (0.46±0.12 ms), but not significantly different from the wt (0.37±0.1 ms). The ATP pharmacology of the mutant channel was different from the wt: the pronounced two phases disappeared from the ATP dependence of the open probability function. The increase of the open probability has two components: the mean open time increased significantly at and above 100 µM ATP concentrations, and the number of open events increased even more pronounced above 150 µM ATP. The majority of the P_o increase was attributed to the increase of the number of open events. All point histograms showed clearly two peaks without a trace of subconductance state: meaning that the synchrony of the four RyR1 monomers has not been changed due to the given mutation. (These data were presented as a lecture at the annual meeting of the Hungarian Physiologycal Society, while the abstract published in the Acta Physiologica (formerly Acta Physiologica Scandinavica: Sárközi S, Lukács B, Jóna I: Altered modulation of the skeletal type Ryanodine receptor/ calcium release channel by ATP in malignant hyperthermia).
In line of the characterization of the critical elements of DHPR-RyR1 interaction, we started a modeling studies of this interaction. First, we tested the competition of MCa and peptide A (which is the proposed interacting sequence of the DHPR). First 5 nM MCa was applied resulted a 92% LLSS ratio, then 26 µM peptide A, resulted an LLSS ratio of 26%, clearly shows that peptide A chased away the MCa from the common binding site. Increasing the MCa concentration to 10 nM resulted an increase of LLSS ratio to 62%, and again increasing the MCa concentration to 20 then 30 nM resulted an increase of LLSS ratio to 76% and 96% respectively. For these calculations the involvement of electrostatic shielding was studied testing the maurocalcine (MCa) - RyR1 interaction at different – symmetrical – KCl concentrations, such as 50 mM, 100 mM, 250 mM and 400 mM . These studies showed, that the RyR1-MCa interaction at 5 nM or 10 nM MCa does not altered by 50 mM nor by 100 mM KCl (LLSS ratio ≈ 90%). However 250 mM KCl almost completely suspended the RyR1-MCa interaction at 5 nM MCa concentration (LLSS ratio ≈ 8%). At 10 nM MCa concentration 250 mM KCl was ineffective (LLSS ratio ≈ 85%), but increasing the KCl concentration to 400 mM the RyR1-MCa interaction has been greatly reduced (LLSS ratio ≈ 12%). We tested whether these subconductance states are indeed calcium conducting states - by calcium release experiments carried out using

heavy SR vesicles actively loaded by calcium. Release of calcium was evoked by addition of MCa while the extra vesicular calcium concentration were monitored by AP III. These calcium release experiments showed that the MCa induced LLSS represents a conducting conformation. These finding implies, that Coulomb forces play substantial role in the RyR1-MCa interactions. A simple model has been proposed to describe the three-fold effect of MCa on the RyR1 gating. (These data were presented at the Biophysical Society Meeting 2013 February (*Biophysical Journal*: 102(3) pp. 305a & pp. 306a - 307a) and at the Gordon research Conference at Les Diablerets, Switzerland 03-08 June, 2012).

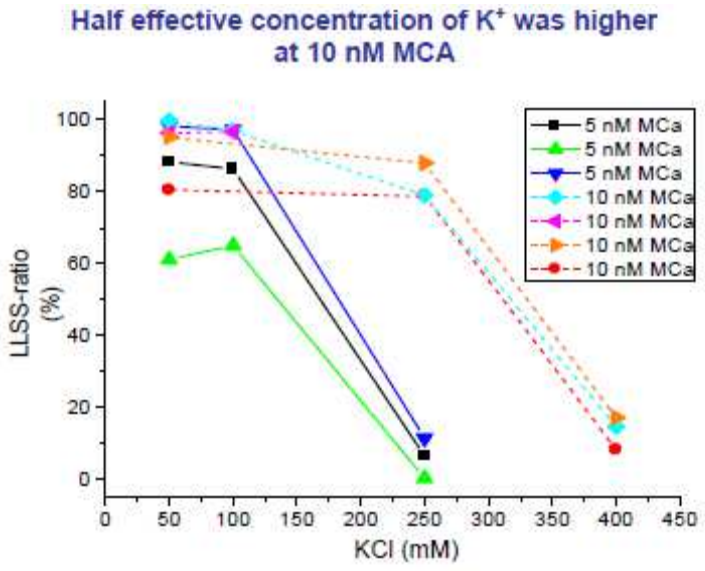
We continued to study the effect of the peptide A (the II-III loop interacting domain of the DHPR) mimicking maurocalcine (MCa), and we were interested, whether this interaction influenced or not by the ionic strength of the microenvironment. Electrostatic shielding might occur, if the interaction is charge motivated, and this reflects to the influence of the Coulomb interaction on the MCa-RyR interaction. The question was addressed by the measurement of the open probability of the RyR1 at low concentration of MCa using different concentration of KCl (i.e. different ionic strength). The applied KCl concentrations were 50mM, 100 mM, 250 mM and 400 mM. First we determined the LLSS (Long Lasting Subconductance State) ratio, which leflects to the time portion in wich the channel exists in the subconductance state. Applying 5 nM sMCa on the cis side of the channel the LLSS ratio was the following

[KCl]	LLSS [%]	SE
50	82,6	11,1
100	82,9	9,5
250	6,2	3,2
400	3,2	4,3

. Repeating the same experiments using 10 nM sMCa, the following data were obtained:

[KCl]	LLSS [%]	SE
50	92,9	4,3
100	96,6	0,2
250	82,0	3,0
400	13,5	2, 7

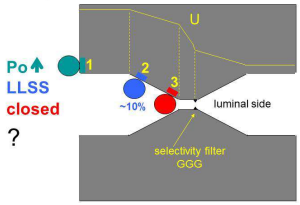
Using vesicular system it was shown, that in the presence of functional accessory protein (which is absent in the bilayer system) the influence of the ionic strength of the microenvironment is similar to bilayer system, so the interaction is genuine effect on the RyR1 itself. These data suggest, that the binding site for MCa is deeply int he pore, where the strength electric field is high, therefore the interaction substantially influenced by the ionic strength due to the shileding effect of the potassium ions, since at lower concentration of MCa 250 mM potassium was sufficient to gratly reduce the LLSS ration (i.e. the MCa – RyR1 interaction), while at higher (at 10 nM) MCa concentration simmilar effect could be achieved only at 400 mM KCl.



Ww carried out simlar experiments on the MH ($^{arg}615^{cys}$) mutant RyR1, buti n contrast to our previous hypothesis no difference was found, so the DHPR-RyR1 direct interaction is not influenced by the given mutation.

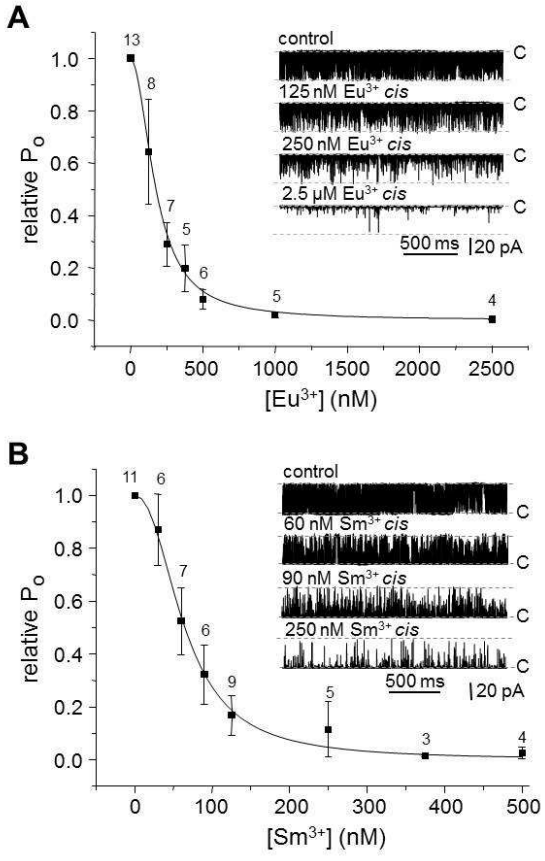
Possible mode of action

Data indicate electrostatic interaction: the smaller the positively charged surface area on the toxin, the weaker is the affinity to the binding site 2, and the easier is the binding site 3 to access, nevertheless toxins with even smaller charged surface area can not enter into the pore.

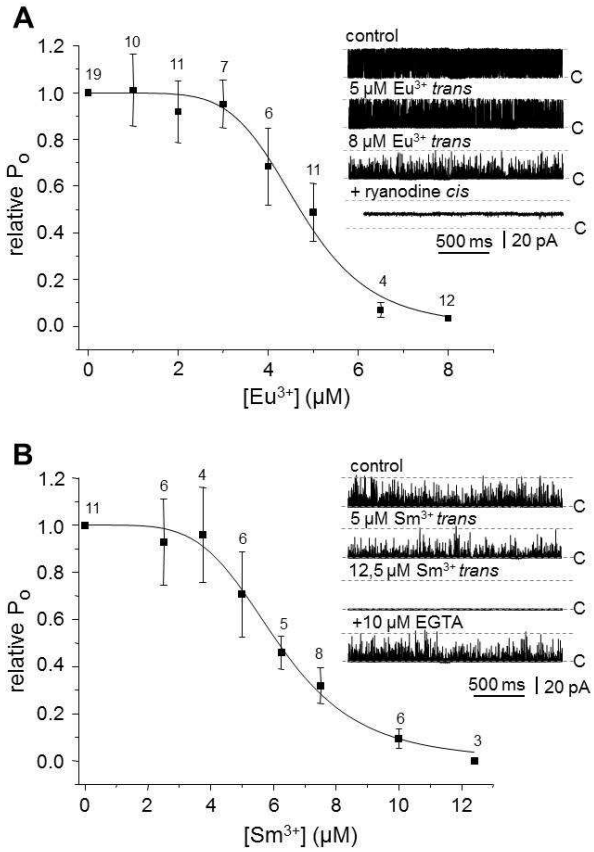


In the second half of the project we had to face with three substantial changes. First, the post doc – who was employed by the grant – Balázs Lukács – received an offer for a permanent position and consequently left the team (and the faculty). He was replaced by Nikolet Geyer a pre-doc who is presently submitting her PhD thesis in muscle physiology. In connection with this change, we successfully applied for this personal change and for an extension of the grant (with slight rearrangement between the different categories within the approved overall budget) until the end of this year. This extension was necessary to train the pre-doc to perform some techniques, with which the new pre-doc was not deeply experienced with. Secondly a

key member of the group – Sándor Sárközi – left the department due to teaching irregularities.
At the same time – due to financial difficulties and philosophy changes of the Agriculture Faculty – the Pietrin pig breeding stock population dismantled, so we were not able to get the muscles for the channel preparation anymore. We tried several other breeder companies, but – according to the genetic test – they were able to provide only heterozygous animals, and we had no influence on the timing of the samples either. Therefore finally we had to adapt ourselves to use mice models instead of pig models. This was possible, because there was a mice strain available for malignant hyperthermia, carrying the y522s mutation, developed at the University of Texas. After the necessary authorization we obtained these mice from our previous EU Research Network partner, from Ulm, and started to develop the necessary modification of the channel isolation protocol. This work also required some extra time.
Parallel with the above mentioned methodological development we continued the measurements aiming at the characterization of the channel pore. Using ryanodine binding assay we have determined the open probability of the channel as a function of Eu^{3+} concentration, which resulted a two- phase curve: first an activation (by about 40%) up to about 10 µM Eu^{3+}, followed by an inhibition which was almost complete at 30 µM. This proves, that the Europium ion acts as an agonist on both the activatory and the inhibitory binding sites as an agonist. This effect was verified by bilayer electrophysiology, where we showed, that – in extremely low calcium conditions – the cis side applied Europium dependent channel activity shows activation up to 500 nM (about 5 fold increase of Po at this concentration) but at higher concentration it causes an inactivation, showing that Eu ion is an agonist at the low affinity (inhibitory) binding site of the channel.
Repeating these single channel experiments applying Eu^{3+} on the trans (luminal) side showed, that – at medium cis calcium concentration – Eu^{3+} reduced the RyR's open probability in a dose dependent manner (IC50=4.7 µM). The action of Eu3+ was highly voltage-dependent with a preference to positive voltages, which drives Eu3+ into the vestibule. Eu3+ induced long closed events at positive membrane potentials by occluding the channel's pore, which could be reversed by the chelation of Eu3+ using EGTA. These data suggest that Eu3+ acts through two independent mechanisms on the luminal side of the channel, as it exerts two qualitatively different actions. Eu3+ inhibits RyR by binding possibly to the luminal Ca^{2+} -binding site, but at high concentrations it causes sudden quiet events by pore



Modulation of the RyR1 by *cis* lanthanides.



Modulation of the RyR1 by trans lanthanides.

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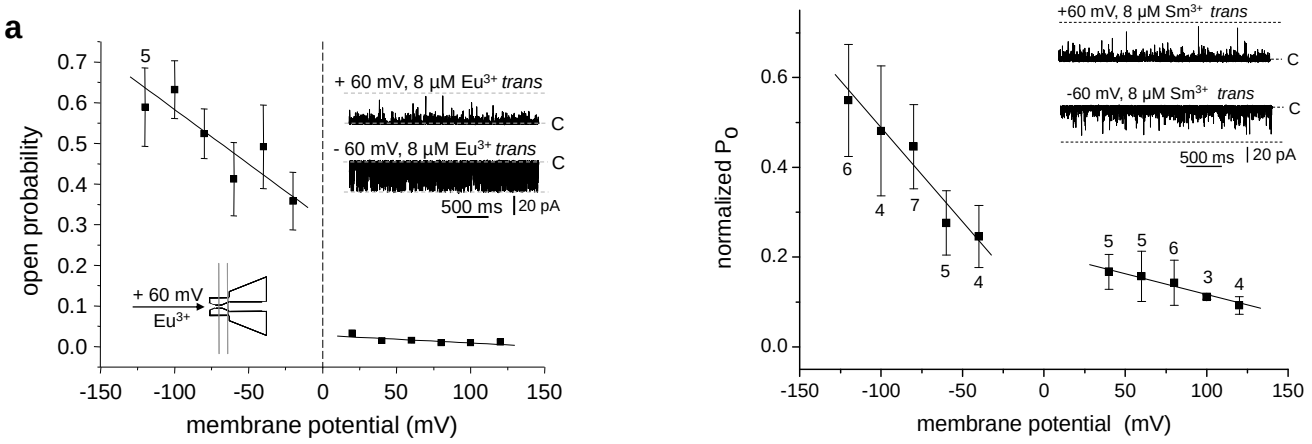

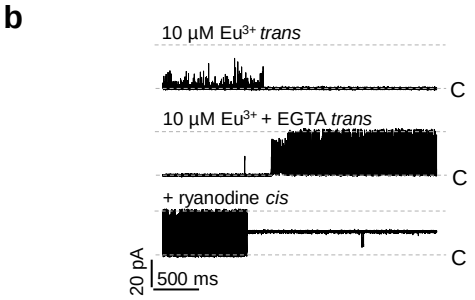

Potential dependence of the ionic interactions

The action of Eu3+ was highly voltage-dependent with a preference to positive voltages, which drives Eu3+ into the vestibule. Eu3+ induced long closed events at positive membrane potentials by occluding the channel's pore, which could be reversed by the chelation of Eu3+ using EGTA. These data suggest that Eu3+ acts through two independent mechanisms on the luminal side of the channel, as it exerts two qualitatively different actions. Eu3+ inhibits RyR by binding possibly to the luminal Ca^{2+} -binding site, but at high concentrations it causes sudden quiet events by pore occlusion.Investigating the activatory/inhibitory effect of Eu^{3+} applied on the cis side, we found an anomalous voltage dependence of the open probability (anomalous compared to the calcium, magnesium, potassium, ATP, etc – which show polarity and voltage independent open probability changes). As unexpected for a small cationic inhibitor, Eu3+ was significantly less potent in decreasing channel Po at negative voltages when it is driven into the pore. In contrast, inhibiting potency was higher at positive potentials, when pore occupancy of Eu^{3+} is less probable compared to negative voltages. Additionally, high concentrations of Eu^{3+} (≥1 µM) caused a classic voltage dependent block by occluding the pore, only at negative voltages. Together with our findings, these data suggests that Eu^{3+} acts as an agonist at both the activating and inhibiting Calcium-binding sites, the data (showing that the inhibition by Eu^{3+} dominates at positive potentials instead of negative), suggest that the activating but not the inhibiting Ca^{2+} -binding site is located in a strong electric field (i.e. in the RyR's vestibule).

To find further evidence that the Ca^{2+} -binding site might be located in the vestibule, we used the peptide-toxin maurocalcine (MCA) with RyR-blocking ability as a tool. MCA effect was studied at 50 µM Calcium. First we applied MCa, and after the development of the characteristic long-lasting subconductive state (it is a Calcium-dependent process and the sign of MCA pore-occupancy) calcium concentration was lowered to 100 nM. When MCA leaves the vestibule allowing normal gating, open probability is still high despite the fact that the calcium concentration was already low. This finding suggests that MCA renders RyR resistant to low calcium. Furthermore, when the polarity of the membrane potential was switched to relief RyR from MCA block, RyR also returned into a high Po mode, which gradually decreased with time. To explain our results, we constructed a model in which we propose that the activating Calcium-binding site might be located in the pore. MCA can be used as a lid to close Ca^{2+} in the vestibular space. With the lid on, RyR is resistant to calcium concentration reduction outside of the vestibule. When the lid is open, Ca^{2+} slowly leaves the vestibule and Po decreases. These data were presented at the Annual meeting of the American Biophysical Society, 2014 February by János Almássy.

At the present we are repeating these experiments using RyRs from the y522s MH mutant mice to investigate the effect of malignant hyperthermia on the channel gating and calcium binding properties. While waited for the mutant mice to get numerous which let us make isolation of the mutant channel, we successfully adopted of the RyR1 preparation procedures from rabbit to mice, and managed to get reasonable yield in case of mice. First we determined the single channel parameters of the control channels (with WT mice), using Sm^{3+}, which is one step closer to the calcium than the previously applied lanthanides, since the ionic radius of Sm^{3+} is 0.964 Å (while the calcium has 0.99, the Eu^{3+} 0.947 and the Gd^{3+} 0.938). By measuring the Sm^{3+} dependence of the open probability of RyR1 channels of the wild type mouse, we found, that the half inhibiting Sm^{3+} concentration is 64.3 ± 2 nM on the *cis* while 6.2 ± 0.1

μM on the *trans* side. The Hill coefficients – which refers to the number of binding site – were 2.2 ± 0.2 on the *cis*, while 4.7 ± 0.5 on the *trans* side. Comparing these data to the data obtained with other lantanides and with calcium, a space filling model can be constructed, showing, that – due to the diameters of these ions – there are four calcium binding site exist in the channel pore: one binding site for each monomer in this tetrameric channel. We concluded, that at the *cis* side of the channel pore due to the geometry of the pore and of the vestibule there are only two is calcium binding site can be occupied in case of Calcium, and of Samarium and of Europium but four in case of Gadolinium. In another words, the diameter of the channel pore (assuming circular shape since the geometry is unknown at the present) at the level of the modulatory calcium binding site falls between 3.79 Å and 4.29 Å. The fact, that Samarium and Europium shows different binding affinity (64.3 nM *versus* 167 nM) reflects probably to the differences in the geometrical microenvironment caused by the different disturbance of the binding site due to the slightly different surface charge distribution of this two lanthanides.

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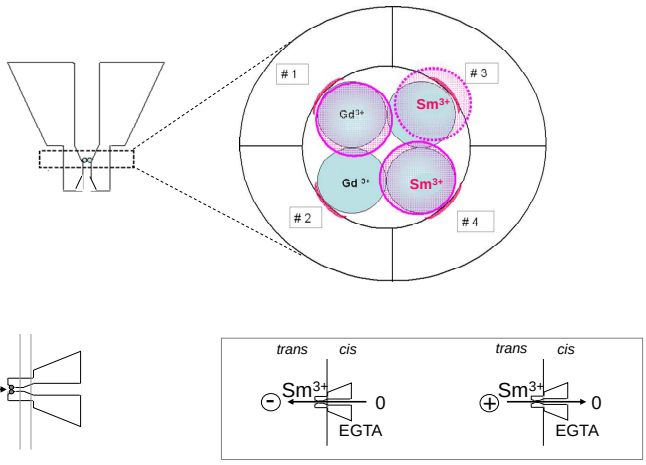
	Ca^{2+}		Sm^{3+}		Eu^{3+}		Gd^{3+}	
	cis	*cis*	*cis*	*trans*	*cis*	*trans*	*cis*	*trans*
IC50 (EC_{50})	(9.37± 1.3 μM)	287 ± 21 μM	64 ± 2 nM	6.2± 0.1 μM	167 ± 5 nM	4.7 ± 0.1 μM	5.65 ± 0.33 μM	5.47 ± 0.24 μM
N_{Hill}	1.18 ± 0.12	1.28 ± 0.11	2.3 ± 0.2	4.7 ± 0.5	2.0 ± 0.15	5.9 ± 0.9	4.7 ± 0.8	4.3 ± 0.6
Ionic radius	0.99 Å		0.964 Å		0.947 Å		0.938 Å	

Comparing these data to the data obtained with other lantanides and with calcium, a space filling model can be constructed, showing, that – due to the diameters of these ions – there are four calcium binding site exist in the channel pore: one binding site for each monomer in this tetrameric channel. We concluded, that at the *cis* side of the channel pore due to the geometry of the pore and of the vestibule there are only two is calcium binding site can be occupied in case of Calcium, and of Samarium and of Eu^{3+} but four in case of Gadolinium. In another words, the diameter of the channel pore (assuming circular shape since the geometry is unknown at the present) at the level of the modulatory calcium binding site falls between 3.79 Å and 4.29 Å. The fact, that Samarium and Eu^{3+} shows different binding affinity (64.3 nM *versus* 167 nM) reflects probably to the differences in the geometrical microenvironment caused by the different disturbance of the binding site due to the slightly different surface charge distribution of this two lanthanides.



The interpretation of the data of the binding sites, located at the *trans* side is more complicated. The fact, that *trans* side data shows that the number of binding site is very similar for all of the lanthanides suggest, that the trans side binding sites are in the luminal vestibule of the channel. This conclusion is further supported by the fact, that the hill coefficients suggest about four binding site per functional channel = one binding site per RyR1 molecule. The fact that these binding sites are the luminal modulatory site or an a non-modulatory binding site remains to be seen.