SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Ca^{2+}-dependent and pharmacological regulation of the skeletal muscle's ryanodine receptor

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BACKGROUND OF THE DOCTORAL THESIS

Precise regulation of intracellular calcium ion concentration ($[Ca^{2+}]_{IC}$) is essential to maintain proper cellular function. An important component of this regulation is the sarcoplasmic reticulum's (SR's) Ca^{2+} channel, the ryanodine receptor (RyR), which is involved in elevating $[Ca^{2+}]_{IC}$ in many cell types. The importance of RyR is shown by the fact that its genetic deficiency is incompatible with life and its point mutations result in abnormal channel function that can lead to cardiac arrhythmias, muscle weakness or even increased muscle tone and malignant hyperthermia syndrome (MHS). These diseases are collectively known as ryanopathies.

Our work aims to understand how molecular-level alterations in RyR function are linked to the skeletal muscle and heart symptoms, and how these abnormalities can be reversed by drug therapy.

My research focused on the most common disease; malignant hyperthermia syndrome (MHS), which is caused by point mutations in skeletal muscle-type RyR (RyR1). MHS is a lethal hypersensitivity reaction of mutant RyR1s to volatile anesthetic gases. It is thought that a common feature of ryanopathies is hypersensitivity of RyR to SR $[Ca^{2+}]$, but the controversy of the results on this hypothesis in MHS has required verification. The reason for the discrepancies is the difficulty of selective investigation of the Ca^{2+} binding sites facing the SR lumen, since Ca^{2+} can also affect the channel function by passing through the pore of the RyR to the cytoplasmic side ('feed-through'), making it difficult to isolate the role of luminal binding sites. To overcome this problem, another specific but impermeable ligand; the Eu^{3+}, was introduced in this study, which allowed side-selective analysis of the Ca^{2+}-binding sites of RyR, thus helping to elucidate details of the pathophysiology of the MHS.

The only available drug to treat MHS crisis is the non-depolarising muscle relaxant dantrolene. The clinical introduction of the dantrolene has dramatically reduced the number of deaths from MHS. Although it has been used since 1967, the exact mechanism of action is still unclear. Its specific binding to RyR1 has long been demonstrated, and the scientific uncertainty about its action is due to the long-standing observation that while dantrolene can inhibit Ca^{2+}-release in intact skeletal muscle fibres in a RyR1-specific manner, by clearing the channel protein and incorporating it into an artificial lipid membrane, sensitivity to dantrolene appears to be lost. In our

work, we have elucidated the cause of this insensitivity and shown that inhibition by dantrolene requires the combined presence of Mg^{2+} and ATP.

PROBLEM STATEMENT AND AIMS

Selective analysis of the luminal Ca^{2+}-binding site of RyR

RyR is a ligand-gated Ca^{2+} channel. The most important ligand of the RyR is the Ca^{2+}. RyR is regulated by Ca^{2+} from both the cytoplasmic side of the channel and from the SR lumen. Although there are many data in the literature on the regulation of RyR by luminal-side Ca^{2+}, the interpretation of the results is complicated by the fact that the divalent ions used to study luminal binding sites can exert their effects by binding to cytoplasmic Ca^{2+} binding sites through the channel pore. To rule this out, we used Eu^{3+} in our experiments, which has a high affinity to Ca^{2+}-binding sites but its unable to cross the channel.

In cardiac-type ryanopathies, Ca^{2+}-release occurs due to the luminal high Ca^{2+} sensitivity of the mutant RyR, so that at a given degree of SR Ca^{2+} load, the RyR opens in diastole. The released Ca^{2+} and NCX activity cause membrane depolarization and form an extrasystole. In the case of MHS, a similar molecular pathogenesis as in myocardium is assumed. RyR is also more active at rest in MHS, but it is currently unclear whether this abnormal activity is due to hypersensitivity of the luminal Ca^{2+}-binding sites on the SR side of RyR1 or the cytoplasmic Ca^{2+}-binding sites, which are reached by Ca^{2+} flowing through the pore, binding and thus activating the channel.

1. Our aim was to understand how the luminal Ca^{2+}-binding site regulates RyR1 and RyR2.
2. In the second group of experiments, we sought to answer the question whether store overload-induced Ca^{2+}-release (SOICR) mechanism underlies the increased channel activation in MHS, like in myocardial ryanopathies.
3. Using our functional data and in silico analysis, we aimed to identify RyR sequences that may contain the luminal Ca2+-binding site.

Mechanism of action of dantrolene

Dantrolene can directly or indirectly inhibit Ca^{2+} release via RyR1. Szentesi et al. have shown that the drug inhibits Ca^{2+}-release from intact skeletal muscle fibres as well as SR vesicles but unable to inhibit purified RyR1s in single-channel experiments. It was concluded that an important factor of the effect of dantrolene was lost during the RyR1 purification procedure. Thus, although dantrolene is the only muscle relaxant used to treat MH episodes and its binding site has been identified in the primary sequence of

the RyR1 protein, its exact mechanism of action is still unknown and therefore the missing factor(s) required to inhibit RyR must be identified to clarify this.

The topic on which my dissertation is based was motivated by a recent study that aimed to identify these unknown components. In this study, Choi et al. noticed that Mg^{2+} was missing from the measuring solution in previous single-channel experiments and hypothesized that this might be the missing factor required for the effect. They showed that 10 µM dantrolene requires at least 1 mM Mg^{2+} to significantly inhibit Ca^{2+} release in permeabilized muscle fibers. However, the authors did not provide single-channel measurements to confirm their results, so the direct evidence that dantrolene is mediated by Mg^{2+} on RyR1 remains unproven.

1. Therefore, the aim of the present work was to investigate purified RyR1s (devoid of accessory proteins) to determine whether direct binding of Mg^{2+} is indeed required for dantrolene sensitivity of the channel.
2. We also aimed to test the effect of the drug on Ca^{2+}-release from SR vesicles under different [Mg^{2+}] conditions.
3. We also wanted to investigate the role of ATP in the mechanism of the action of dantrolene using the methods mentioned above.

MATERIALS AND METHODS

Experimental animals

All experiments were in accordance with the Hungarian Animal Welfare Act, the European Union Directive 2010/63/EU and approved by the Animal Welfare Committee of the University of Debrecen (22/2012/DEMÁB). For our experiments we used skeletal muscle from adult rabbits (n=4), ventricular myocardium from adult beagle dogs (n=5) and skeletal muscle from control and transgenic C57Bl/6 mice. The transgenic mice carried the Y524S mutation in the RyR1 gene (n=20), which is corresponding to the human Y524S mutation, and wild-type littermates were used as controls (n=18).

Terminal cisterna vesicle fraction and purified ryanodine receptor preparation

We performed Ca^{2+}-release experiments from terminal cisterna vesicles (TCV) and single-channel current measurements on purified RyRs. In TCV both SERCA and RyR1 are present. TCV were prepared by differential centrifugation after homogenization of white muscle excised from rabbit/mouse or canine ventricular myocardium.

Ryanodine receptors were solubilized in a mixture of TCV suspension and a solution containing 1% detergent and 0.45% phospholipid. The sample was then layered on a linear (10-28%) sucrose gradient and purified by ultracentrifugation and the fractions containing the highest amounts of RyRs were used for single channel current measurements.

Ca^{2+}-release measurement

TCVs can be loaded with Ca^{2+} by their SERCA pumps and then Ca^{2+} can be released again by activation of RyR1. The Ca^{2+} concentration of the extravesicular solution can be monitored by Ca^{2+}-sensitive dye (antipyrilazo III) in spectrophotometric measurements at 710 nm. After loading the TCV, the compound of interest is added to the test solution and the dynamics of Ca^{2+}-release is measured after activation of the RyRs by the agonist. To investigate the effect of dantrolene, 10 μM dantrolene was added to the sample during the preincubation phase, followed by Ca^{2+} release using

the RyR agonist 4-chloromethacresol (4CMC, 400 μM) and the process was compared to that sample which was not treated with dantrolene.

Single-channel current measurement on a RyR in a planar lipid bilayer

The solubilized RyR1 molecules were incorporated into an artificial lipid bilayer and their current was measured under voltage-clamp conditions. The lipid bilayer is prepared from a mixture of phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine in a 5:4:1 mass ratio and dissolved in n-decane to a lipid concentration of 20 mg/ml. The artificial membrane is generated in a 200 μm diameter aperture of a cuvette designed for this purpose. The aperture separates two equal volume liquid compartments into which a solution of identical composition (250 mM KCl, 100 μM EGTA, 150 μM CaCl2, 10 mM HEPES, pH = 7,2) is added. One side is called the cis (cytoplasmic) side and the other side is called the trans (SR lumen-facing) side. After the membrane is prepared, intensive mixing and voltage steps of -300 to +300 mV are used to promote RyR incorporation, depending on the stability of the membrane. Since the solution on both sides of the bilayer had the same ionic composition, a voltage difference of +/- 60 mV was maintained by an Axopatch 200 amplifier to uphold the electrochemical gradient. The current signals were filtered at 1 kHz using an 8-pole low-pass filter and recorded at 3 kHz using pClamp 6.02 software after analog-to-digital conversion.

Statistical analysis of the data

The open probability (P_o) of RyRs was determined using the pClamp software package (Molecular Devices, Sunnyvale, CA). Statistical analysis was performed in Origin 7.0 (OriginLab, Northampton, MA) and Excel (Microsoft, Redmond, WA). Results are expressed as mean ± SE. Relative P_o data were calculated by normalizing each set of data points to their respective controls. Statistical significance of differences was assessed by one-way ANOVA. Tukey's hsd test was performed as a post hoc test. An independent two-sample t-test was used to compare individual data. A significant difference was considered to be $p<0.05$.

RESULTS

Regulation of RyR by luminal [Ca^{2+}]

The knowledge on the SR regulation of RyR2 is consistent in the literature, therefore we based the validation of our experiments with RyR1 on whether we could reproduce the results for RyR2 in our experimental setup. The effect of luminal Ca^{2+} was first tested with cytoplasmic [Ca^{2+}] (50 μM - RyR1 and 5 μM - RyR2) saturating the activating Ca^{2+} binding sites. By increasing luminal [Ca^{2+}], RyR1 was strongly inhibited, whereas the current on RyR2 was not affected. At low (100 nM) cytosolic [Ca^{2+}], increasing luminal [Ca^{2+}] activated RyR2 as expected, but still inhibited RyR1. Our results for RyR2 are in agreement with previously published data, and the RyR1 data are also in agreement with some previously published results.

These results raise the question of why the responses of RyR1 and RyR2 are different. One possible reason is that increasing luminal Ca^{2+} along its gradient can cross the pore of RyR1 and interacts with the cytosolic, inhibitory Ca^{2+}-binding site of the channel (the activating binding site is not available as it is saturated with 50 μM cytoplasmic Ca2+). If this is true, then it would be the lower affinity of the cytoplasmic inhibitory binding site for RyR2 that prevented RyR2 inhibition. This "feed-through inhibition" hypothesis is supported by the fact that the inhibitory effect of luminal Ca^{2+} on RyR1 was highly voltage-dependent, preferring negative membrane potentials that force luminal cations through the pore. Accordingly, increasing luminal [Ca^{2+}] should activate RyR1 when cytoplasmic binding sites are unsaturated ("feed-through activation"). Surprisingly, high luminal [Ca^{2+}] at 100 nM cytoplasmic Ca^{2+} did not enhance RyR1 activity at any negative membrane potential. This result indicates that under our experimental conditions, the feed-through Ca^{2+} current was not significant enough to affect cytoplasmic Ca^{2+}-binding sites, at least in RyR1. Thus, these data suggest that inhibition by luminal Ca^{2+} occurs directly through one or more luminal Ca^{2+}-binding sites. In this case, the extreme voltage dependence of the inhibition suggests that the binding site is in the channel pore where the voltage drop is expected.

A new tool for selective analysis of luminal Ca^{2+} binding sites

To specifically test the hypothesis that RyR1 carries a true inhibitory Ca^{2+}-binding site on the luminal side of the protein, and to avoid "backflow" effects, we had to choose a research tool to selectively probe the putative luminal binding site. Our research group has previously shown that Eu^{3+} is a specific and potent agonist of RyR1 cytoplasmic

Ca^{2+}-binding sites and is unable to cross the pore. Taking advantage of these properties, next we investigated the effect of Eu^{3+} in the following experiments to determine whether Eu^{3+} is an agonist of the luminal Ca^{2+}-binding site. First, we adjusted the cytoplasmic [Ca^{2+}] to 50 μM and added Eu^{3+} to the luminal side of RyR1. Luminal Eu^{3+} significantly inhibited RyR1 activity in a concentration-dependent manner with an IC_{50} of 4.7 μM. We also observed a qualitatively different state of RyR1 at membrane potentials that pushed Eu^{3+} inside the pore (opposite to physiological current direction, with a positive membrane potential), especially at high concentrations of the ion, as a prolonged closed state suddenly appeared upon Eu^{3+} treatment, suggesting that the ion blocked the channel pore. This effect was only observed at ≥5 μM Eu^{3+} and negative membrane potentials. The block was removed by chelation of Eu^{3+} with EGTA but was sometimes not reversible. The long latency (a few seconds) of EGTA action indicated that Eu^{3+} was strongly bound within the pore of RyR, causing a persistent physical blockage, as previously reported for L-type Ca^{2+} channels. The conductance of RyRs was ~800 pS under control conditions and did not change in response to Eu^{3+}. These data suggest that Eu^{3+} is unable to penetrate the channel therefore unable to reach the cytosolic binding sites. In addition, the analogy between the effects of Eu^{3+} and Ca^{2+} provides indirect evidence that Eu^{3+} is an agonist of luminal Ca^{2+}-binding sites.

In the following series of experiments, we wanted to learn more about the function of the putative luminal Ca^{2+}-binding sites of RyR1 and RyR2, and therefore investigated the effects of luminal Eu^{3+} at low (non-saturating) cytoplasmic [Ca^{2+}]. First, we adjusted the cytoplasmic [Ca^{2+}] to 100 nM and added Eu^{3+} to the luminal side of the channel. Luminal Eu^{3+} also inhibited RyR1 activity in this case. Since some researchers have reported that RyR is activated by luminal Ca^{2+} only when the channel is pretreated with caffeine, we also performed some experiments in the presence of 2 mM caffeine, but the inhibitory effect of Eu^{3+} was only more evident in this case.

In summary, no concentration and combination of cytoplasmic and luminal Ca^{2+} and Eu^{3+} (and caffeine) was able to activate RyR1, and Eu^{3+} inhibited the channel even more strongly at 100 nM cytoplasmic Ca^{2+}, when the P_o was significantly lower than at 50 μM Ca^{2+}.

We also performed experiments with RyR2 under similar conditions, which showed that the effect of Eu^{3+} was biphasic: it activated RyR2 at submicromolar concentrations but inhibited it at higher concentrations. These results clearly show that the regulation

of both channels by Ca^{2+} and Eu^{3+} is qualitatively similar, suggesting that both RyR1 and RyR2 harbour genuine luminal Ca^{2+}-binding sites, but these sites function in opposite ways in the two isoforms. In addition, the fact that the effect of Eu^{3+} was analogous to that of Ca^{2+} in both RyR1 and RyR2 provides evidence that Ca^{2+} and Eu^{3+} are qualitatively equivalent ligands for Ca^{2+}-binding sites, and thus these results validate our experimental approach.

The voltage dependent effect of luminal Ca^{2+} on RyR1 raises the question whether the effect of Eu^{3+} is similarly voltage dependent. The inhibitory effect of Eu^{3+} was strongly polarized, favoring negative voltages that drive Eu^{3+} into the channel pore. For positive membrane potentials, a decrease in driving force between +20 and +120 mV was accompanied by a progressively weakening inhibition. The effect of Eu^{3+} remained voltage-dependent even at low (100 nM) cytoplasmic [Ca^{2+}], as the inhibition was significantly stronger at -60 mV than at +60 mV. We also analyzed the voltage dependence of the activating effect of Eu^{3+} on RyR2 at 100 nM cytoplasmic Ca^{2+}, but the activation of RyR2 by Eu^{3+} was independent of membrane potential. Since RyR is impermeable to Eu^{3+}, the voltage-dependent inhibition of RyR1 could not be a consequence of feed-through the channel. These results therefore suggest that the Ca^{2+}-binding sites that are responsible for luminal side regulation of RyR1 and RyR2 must be in the channel pore, whereas the activating binding site of RyR2 must be located outside the electrical field of the channel pore.

Control of the Y524S MHS-RyR1 from the luminal side

Our results so far show that Ca^{2+} and Eu^{3+} inhibit RyR1 function do not support the previously proposed molecular mechanism of MHS, i.e., that a reduced SOICR threshold is responsible for the development of malignant hyperthermia seizures. Hypothetically, luminal Ca^{2+}-induced Ca^{2+} -release in the MHS skeletal muscle could also occur if the inhibitory potential of luminal Ca^{2+} is weaker in MHS-RyR1. This possibility was tested with mouse Y524S MHS-RyR1s. The experimental conditions were the same as before: first, cytoplasmic [Ca^{2+}] was adjusted to 50 µM (saturating) or 100 nM (non-saturating) Ca^{2+}, and then luminal [Ca^{2+}] was subsequently adjusted. Under control conditions, the P_o of MHS channels at 100 nM cytoplasmic Ca^{2+} was significantly higher than the P_o of wild-type (wt) channels, as expected from the MHS phenotype (P_o wt 100 nM=0.0031±0.001 (n=5), P_o MHS 100 nM=0.02±0.007 (n=13). The results were qualitatively consistent with those obtained with control RyR1s,

namely Y524S MHS-RyR1 was inhibited by luminal Ca^{2+} in the same way as wild type. It is important that even at 100 nM cytoplasmic Ca^{2+}, strong inhibition by luminal Ca^{2+} was observed, and above 5 mM Ca^{2+} slight activation was observed, indicating that self-activation by Ca^{2+} flux was only slightly present in our experiments (despite the higher sensitivity of MHS-RyR1 to cytoplasmic Ca^{2+}). For MHS-RyR1, inhibition by Eu^{3+} was similar to that of wild-type channels at both (50 μM and 100 nM) cytoplasmic [Ca^{2+}]. These data argue against previously published conclusions that increased sensitivity to SOICR is a relevant pathomechanism in MHS.

The mechanism of action of dantrolene

In the next topic I would like to present the results on the mechanism of action of dantrolene. This identification process has been hampered by a lack of knowledge about the mechanism of action of dantrolene, mainly due to conflicting data in the literature. Our research group has previously shown that the drug inhibits Ca^{2+}-release from intact skeletal muscle fibres and SR vesicles but does not inhibit the current of RyR1 channels. Therefore, we conclude that an important aspect of the effect of dantrolene is lost during the RyR purification process. To elucidate the mechanism of action of dantrolene, we sought to identify the missing factor(s) required for RyR1 inhibition.

Effect of dantrolene and magnesium on Ca^{2+}-release

We first performed Ca^{2+}-release experiments on SR terminal cisterna vesicles. The SR vesicles were first suspended in buffer in the presence of 1 mM Mg^{2+} and 0.5 mM ATP, then loaded with Ca^{2+} and, after the transmittance remained constant (spontaneous Ca^{2+}-leakage from the vesicle and Ca^{2+}-uptake by the SERCA pump were in equilibrium), Ca^{2+}-release was induced by RyR agonist (4CMC). The experiment was also repeated in the presence of 10 μM dantrolene and it was found that Ca^{2+}-release occurred with an average delay of 26 s. Next, the [Mg^{2+}] of the buffer was increased to 3 mM, which alone caused an average delay of 108 s under control conditions. However, we used 10 μM dantrolene with 3 mM Mg^{2+} it resulted in a delay (180 s) almost seven times longer than that observed with 1 mM Mg^{2+} and 10 μM dantrolene. The rate of Ca^{2+}-release in the presence of 3 mM Mg^{2+} was significantly lower than in the presence of 1 mM Mg^{2+} (2.04±0.08 vs. 0.9±0.07 nmol Ca^{2+}/s, n=3-4), whereas dantrolene only slightly reduced the rate of release (2.04±0.08 vs. 1.4 ±0.22 nmol

Ca^{2+}/s at 1 mM Mg^{2+} and 0.9±0.07 vs. 0.73±0.03 nmol Ca^{2+}/s at 3 mM Mg^{2+}). The 180 s delay caused by dantrolene in the presence of 3 mM Mg^{2+} indicates that the suppressive effects of dantrolene and Mg^{2+} on RyR1 potentiate each other.

ATP is also needed for the dantrolene effect

The fact that dantrolene caused Ca^{2+}-release delay instead of significant reduction in the rate of Ca^{2+}-release suggests that as long as ATP was present in the buffer, dantrolene was able to exert its inhibitory effect. It is thought that once SERCA had used up all ATP to compensate for Ca^{2+}-leakage from vesicles, dantrolene lost its inhibitory effect and Ca^{2+}-release began. To test this hypothesis, the effect of dantrolene was also tested at different ATP concentrations. Under control conditions, 0.5 mM ATP and 1 mM Mg^{2+} were used and an immediate Ca^{2+}-release was observed after the administration of 4CMC. Dantrolene significantly delayed the Ca^{2+}-release induced by 4CMC. Repeating the same experiment at a higher ATP concentration (1.5 mM), dantrolene caused an average delay of 158 s longer. However, increasing the ATP concentration alone did not cause delay in the Ca^{2+}-release.

Similar experiments (0.5 mM ATP and 1 mM Mg^{2+}) were performed on SR vesicles isolated from canine myocardium, but we failed to reproduce the results obtained on skeletal muscle SR vesicles, which is consistent with dantrolene being a selective inhibitor of RyR1.

Testing the dantrolene effect in Mg^{2+} and ATP-containing solution by single-channel current measurements

One of the main reasons for the uncertainty surrounding the mechanism of action of dantrolene was that it had previously been shown to be ineffective in single-channel current measurements on RyRs incorporated into artificial lipid bilayers. Therefore, the main question of our study was whether Mg^{2+} is the missing factor required for dantrolene action in these measurements and whether it can make purified RyRs dantrolene-sensitive. The channels were incorporated into the lipid bilayer in the presence of 50 µM Ca^{2+} (control), and 3 mM Mg^{2+} was added to the cytoplasmic side of the same channel a few minutes later, which significantly reduced the channel's P_o, since Mg^{2+} is a competitive antagonist of the activating Ca^{2+}-binding site. Then we added 1 mM ATP to the same compartment of the bilayer chamber, which increased RyR activity. After reaching steady-state, 10 mM dantrolene was also added to the space corresponding to the cytoplasmic side of RyR. The results of our current analysis

showed that dantrolene reduced the P_o of MgATP-treated channels by 48±5% (P_o=0.0177±0.025 vs. 0.0123±0.021, n=12). In four of our measurements, we further increased [ATP] from 1 mM to 2 mM after dantrolene treatment. Surprisingly, this further reduced P_o - rather than increasing channel current with agonist ATP - to 17%±8% of control (0.0177±0.025 vs. 0.0018±0.003, n=4, effect: -0.71;), consistent with the therapeutic effect of the drug. Our results therefore suggest that ATP enhances the effect of dantrolene. However, when Mg^{2+} was omitted from our measurements, dantrolene remained ineffective in the presence of 2 mM ATP (91%±3% of control, Po=0.38±0.35 vs. 0.35±0.33, n=10, effect%: -0.08;), suggesting that ATP is a necessary but not an exclusive condition for dantrolene action.

DISCUSSION

The role of Ca^{2+}-stores in the regulation of sarcoplasmic reticulum Ca^{2+}-channels in physiological and pathological conditions

Our group compared for the first time in the literature the regulation of luminal Ca^{2+} of the two RyR isoforms under the same conditions, whereby Ca^{2+}-leakage to the cytoplasmic side was avoided by using a specific agonist of luminal Ca^{2+}-binding sites; the Eu^{3+}. We demonstrated that (1) RyR2 is activated by luminal Ca^{2+} while RyR1 is inhibited, (2) these results were reproducible using Eu^{3+}, (3) under our experimental conditions the effect of feed-through regulation is below the limit of detection, (4) the inhibitory effect of Eu^{3+} on RyR1 is strongly voltage-dependent, whereas its activating effect on RyR2 is voltage-independent, and (5) the luminal regulation of the Y524S MHS-RyR1 channel is not different from the wild-type. The fact that the depolarization of the membrane potential attenuates the inhibition, and that the inhibition is stronger at negative voltages can be explained by the Eu^{3+} moving in the electric field towards its binding site.

In ryanopathies, impairment of Ca^{2+}-release regulation results in uncontrolled Ca^{2+}-release (SOICR), which can lead to cardiac arrhythmias such as heart failure or CPVT, or cause MHS in skeletal muscle. In the latter pathological state, halothane induces a malignant hyperthermia crisis by activating a sensitive mutant RyR1. Recently, Chen's group has proposed an alternative pathomechanism in which halothane triggers Ca^{2+}-release in MHS by lowering the threshold of SOICR. Unfortunately, since it is not possible to induce SOICR in skeletal muscle fibres, all their intracellular [Ca^{2+}] data were obtained from measurements in a heterologous expression system. Chen et al. were able to induce SOICR from 12 different MHS-RyR1-overexpressing HEK cells (including the Y523S rabbit RyR1, identical to mouse Y524S) in extracellular solutions containing high Ca^{2+}, but only when the cells were pretreated with 2 mM caffeine, which is known to sensitize RyR1 to cytoplasmic Ca^{2+}. Although bilayer experiments are lacking from the mentioned study, the authors have previously shown that porcine R615C-RyR1 is activated by millimolar luminal [Ca^{2+}] in lipid bilayer experiments. In contrast, our results in my dissertation show that both wild-type and Y524S MHS-RyR1 are inhibited by both Ca^{2+} and Eu^{3+} under similar experimental conditions, thus disproving their hypothesis. This discrepancy may be since our RyRs were generated from heterozygous mice, so our preparation contains RyR tetramers combining wild-

type and Y524S MHS-RyR1 monomers. The MHS pathomechanism proposed by Chen and his group, i.e. increased SOICR susceptibility, is argued against by the fact that in our experimental setup RyRs are inhibited by luminal Ca^{2+}, but heterozygous mice still show a strong MHS phenotype. The results of Chen et al. can be explained by feed-through activation of MHS-RyRs. Taken together, we conclude that low SOICR threshold is not a relevant mechanism in MHS, and thus the pathomechanisms of CPVT and MHS are not identical. In susceptible individuals, halothane activates the channel as an RyR agonist via direct allosteric binding sites. Furthermore, the pathological process is accelerated by the fact that the cytoplasmic Ca^{2+}-binding sites of mutant RyR are more sensitive and inhibition by Mg^{2+} is weaker, which makes CICR stronger.

Investigation of the Eu^{3+} binding site with *in silico* methods

Briefly summarizing our structural analysis and functional data, we believe that RyR2 carries two luminal Ca^{2+}-binding sites: an activating site outside the electric field and an inhibitory site located in the pore. In RyR2, the activating site dominates over the function of the inhibitory site, the latter being of negligible importance. In RyR1, the activating binding site is absent, and the affinity of the inhibitory site is high enough to help significantly suppress RyR1 activity in resting muscle.

RyR1 inhibition by dantrolene requires ATP and Mg^{2+}

Dantrolene is the only life-saving agent in MHS, so increasing the therapeutic efficacy of the drug and creating the conditions for an ideal drug effect is an important challenge.

The motivation for this topic was the desire to resolve the controversy about the mechanism of action of dantrolene, which arose from its ability to suppress Ca^{2+} -release on intact skeletal muscle fibres but lost its effect on artificial lipid bilayer-embedded ryanodine receptors.

Our work was directly stimulated by the publication of Choi and colleagues, in which they showed that Mg^{2+} is an essential cofactor for dantrolene action in $[Ca^{2+}]_{IC}$ measurements on skeletal muscle fibers. Indeed, in previous studies with dantrolene by our group, the use of Mg^{2+} was avoided because it led to inhibition of RyR1 to a degree that did not allow detection of further inhibition.

To clarify the discrepancies, we set out to perform single-channel current measurements on RyR1s for the above-mentioned studies. Our key finding is that we

have demonstrated for the first time at the single-channel level that dantrolene requires direct binding of Mg^{2+} to RyRs, with no other contributing protein required for the effect. Furthermore, we have shown that P_o further decreases with increasing [ATP], suggesting that ATP potentiates the effect of dantrolene. These single-channel data correlated with our results from Ca^{2+}-release measurements. In summary, ATP is required for drug action in addition to Mg^{2+}.

Our functional experimental data, as well as data from molecular modeling, suggest two possible molecular mechanisms for dantrolene action: 1) dantrolene binding requires a specific allosterically modified state mediated by Mg^{2+} and ATP, or that, 2) dantrolene binding allosterically increases the affinity of RyR1 for Mg^{2+}. Our *in silico* analysis supports the latter possibility, as the distances between the relative domains are similar in structures defined in the presence of caffeine, ATP and Ca^{2+} (open) and in the EGTA-induced (closed) conformational state. This structural information indicates that the dantrolene binding site is not subject to structural rearrangements during gating; therefore, it is likely to be always available - regardless of whether Mg^{2+} occupies the Ca^{2+}-binding sites - suggesting that dantrolene likely acts by allosterically increasing the affinity of Mg^{2+} for RyR.

Choi et al. and Cannon et al. also propose that in the presence of dantrolene, RyR1 is stabilized in a conformational state that causes it to increase its affinity for Mg^{2+}. They conclude that during MH episodes, increased metabolism due to a hydrolyzing MgATP complex, decreased [ATP] and consequently increased free [Mg^{2+}] may be the mechanism behind the drug effect of dantrolene. Furthermore, their data also suggest that dantrolene loses its efficacy at low [ATP] and excessively high [Mg^{2+}]. This implies that there may be an optimal time window for drug administration during MH crises and highlights the importance of immediate treatment.

SUMMARY

Ryanodine receptors (RyR) are ligand-gated Ca^{2+} channels of the sarcoplasmic reticulum (SR). Their main ligand is the Ca^{2+}, which regulates channel function acting from both the cytosolic and luminal side of the RyR. Impaired regulation by Ca^{2+} leads to cardiac and skeletal muscle diseases. For instance, Ca^{2+} overload-induced Ca^{2+} release (SOICR) is associated with cardiac arrhythmias and malignant hyperthermia (MH). Data published on the luminal-Ca^{2+} regulation of RyR is difficult to interpret due to the fact that the divalent ions used to study luminal Ca^{2+}-binding sites can also bind to cytoplasmic side binding sites as they flow through the channel pore. To overcome this problem, we used Eu^{3+} instead of Ca^{2+}, which can specifically bind to Ca^{2+}-binding sites but is not conducted by the channel. To investigate the mechanism at the molecular level, we performed single-channel current measurements using both skeletal muscle- and cardiac-type RyRs (RyR1 and RyR2). These measurements showed that increasing [Ca^{2+}] on the luminal side of RyR2 increased channel open probability (P_o), while RyR1 was inhibited. These results were reproduced by using Eu^{3+}. Luminal regulation of RyR1 carrying a mutation (Y524S) associated with MH was not different from wild type. Inhibition of RyR1 by Eu^{3+} was voltage dependent, whereas activation of RyR2 was not. These results suggest that the inhibitory luminal Ca^{2+}-binding site of RyR1 is located in the membrane's electrical field, i.e., in the pore of the channel, whereas the luminal activation site of RyR2 is outside of this region. Based on these functional data and *in silico* analysis we predicted Ca^{2+}-binding site sequences and suggest that RyR2 carries two luminal Ca^{2+}-binding sites: an activation site in the S1-S2 loop and an inhibitory site in the pore. The activating site dominates over the function of the inhibitory site, thus its role is not relevant. In contrast, in RyR1 the activating binding site is absent, and the affinity of the inhibitory site is high enough to significantly suppress RyR1 activity in relaxed muscle, where the average [Ca^{2+}] of SR falls within the range tested in this study. During Ca^{2+}-release, when SR [Ca^{2+}] decreases, RyR1 is gradually freed from inhibition, which compensates for the decreasing driving force.

In the second part of my dissertation, I sought to answer the question about the mechanism of action of dantrolene. Dantrolene is a RyR1 inhibitor which is as a non-depolarizing muscle relaxant in MH. Although it is known that dantrolene binds to the RyR protein, its mechanism of action is unknown, mainly due to the controversial results that dantrolene inhibits Ca^{2+}-release from intact fibres and SR vesicles but is unable to inhibit purified RyRs. It is therefore concluded that an important factor of the effect of dantrolene is lost during the RyR purification process. Recently, it has been shown that Mg^{2+} is essential for RyR inhibition by dantrolene in Ca^{2+}-release experiments in skeletal muscle fibers. Our experiments aimed to confirm these results at the single-channel level. 10 μM dantrolene added along with 3 mM Mg^{2+} and 1 mM ATP significantly reduced the P_o of RyR, and the channel P_o was further reduced to ~20% of control when [ATP] was increased to 2 mM. Our results show that Mg^{2+} is required for the action of dantrolene and suggest that ATP is also needed.

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Doctoral School: Doctoral School of Molecular Medicine
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List of publications related to the dissertation

1. **Magyar, Z. É.**, Bauer, J., Bauerová-Hlinková, V., Jóna, I., Gaburjakova, J., Gaburjakova, M., Almássy, J.: Eu3+ detects two functionally distinct luminal Ca2+ binding sites in ryanodine receptors.
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2. Diszházi, G.*, **Magyar, Z. É.***, Mótyán, J. A., Csernoch, L., Jóna, I., Nánási, P. P., Almássy, J.: Dantrolene Requires Mg2+ and ATP To Inhibit the Ryanodine Receptor.
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   * These authors contributed equally this work.
   IF: 3.664

List of other publications

3. Szentandrássy, N., **Magyar, Z. É.**, Gyöngyösiné Hevesi, J., Bányász, T., Nánási, P. P., Almássy, J.: Therapeutic Approaches of Ryanodine Receptor-Associated Heart Diseases.
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