Ph.D. THESIS

STUDY OF SKELETAL TYPE CALCIUM RELEASE CHANNEL (RyR1)

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1. Introduction

1.1 Contraction-relaxation cycle

At the beginning of electromechanical coupling the action potential propagates along the surface membrane and enter into the invaginations of membrane called T(transverse)-tubules, where it activates voltage sensors adjacent to terminal cistern of sarcoplasmic reticulum (SR). The L-type calcium channel (DHPR) which has a capability of binding dihydropyridine with high affinity is the voltage sensor. Under physiological conditions the DHPR works as voltage sensor and it has no calcium conductance. The action potential modifies the conformation of DHPR and the interaction between the II-III loops of four DHPRs and one SR calcium channel results in the opening of the latter one. This calcium channel which is also called ryanodine receptor (RyR) is located close to the T-tubiles, in the wall of the terminal cistern of the SR.

In the intracellular space the calcium diffuses to the calcium binding molecules primarily to the troponin C. After calcium binding to troponin C the troponin I – actin interaction fails making the tropomiosin to change its position on the surface of the actin. In this way miosin binding sites become free and miosin can start its spatial movement of the filaments (sliding theory) and finally in the shortening of the sarcomers (muscle contraction). Reuptake of calcium to SR is carried out by active transport. The myoplasmic calcium concentration is elevated until calcium has been transported back to the lumen of longitudinal SR by Ca^{2+} pump (SR CaATPase) located on the membrane of SR.

1.2. Skeletal type calcium release channel (RyR1)

Ryanodine receptor is high conductance (520-600 pS) and low selectivity calcium channel which consists of four polypeptides molecules (4x560 kDa) forming a homotetramer. The RyR1 (skeletal type) isoform of RyR is mainly present in skeletal muscle but it was also detected in the brain (Purkinje neurons) and in smooth muscle cells. The channel has a large extramembrane domain which was identified as a „foot" structure at cytoplasmic side. In the case of humans in this cytoplasmic ending numerous inheritable mutations have been identified. In the disease of malignant hyperthermia different mutations have been shown (Arg163Cys, Gly248Arg,

Tyr522Ser, Gly2433Arg, Gly341Arg, Arg614Cys etc.) to be responsible altered function of RyR.

1.3. DHPR, domain A, $IpTx_a$

In skeletal muscle the most important peptide regulator of the RyR is DHPR locating in T–tubule and consisting of five subunits (α_1, α_2, β, γ, δ). It has been shown that domain A – the cytoplasmic loop linking domains II and III of the α1 subunit (671. Thr – 690. Leu) – modifies the activity of RyR. It is suggested that a small sized basic cluster (681 RKRRK 685) is responsible for domain A – RyR interaction.

$IpTx_a$ – isolated from scorpion venom Pandius imperator – presents a strong sequence and three–dimensional structure identity with domain A. $IpTx_a$ and domain A are able to bind to the RyR1. Both of them potentiates the [^{3}H]ryanodine binding and induces Ca^{2+} release from SR vesicles. In the presence of domain A the $IpTx_a$ – induced [^{3}H]ryanodine binding decreased in concentration dependent manner. These data suggests that there is competition between $IpTx_a$ and domain A for binding site(s).

1.4. The maurocalcine

Maurocalcine (MCa) is a 33 amino acid residues peptide which was isolated from the venom of a scorpion (Maurus palmatus). Its folding is stabilized by three disulfide bridges. The position of basic amino acid residues suggests that there is a cluster of positively charged residues on the surface of toxin. MCa presents 82% amino acid sequence identity with $IpTx_a$. Maurocalcine and $IpTx_a$ activate the RyR1 in nanomolar concentration range. Maurocalcine shares sequence identity with domain A but the structure of domain A (α-helix at the end of N-terminal) playing role in DHPR – RyR interaction differs from the structure of MCa and $IpTx_a$. In skeletal muscle maurocalcine binds mainly to the RyR1. It was shown that MCa induced Ca^{2+} release from SR vesicles in concentration dependent manner (EC_{50}=17.5 nM). MCa-induced Ca^{2+} release was inhibited by ryanodine (500 μM) and ruthenium red (5 μM). Maurocalcine was found to potentiate [^{3}H]ryanodine binding in concentration dependent manner (EC_{50}=1.2 nM).

We have previously revealed that in the presence of MCa purified RyR1 channels show long-lasting openings/subcoductance states characterized by a conductance equivalent to 60% of the full conductance. We identified the critical role of ^{24}Arg. In the absence of ^{24}Arg (mutation) the effect of MCa failed. Other team of our department revealed that in developping skeletal muscle cell the addition of MCa into the external medium induced a calcium transient arising from the direct activatiory effect of the toxin on the skeletal muscle type ryanodine receptor. The depolarization-induced calcium release itself was not affected by the presence of the toxin.

Our collaboration partner suggested that MCa crosses biological membrane and penetrates into different cell types by passive way like cell penetration peptides (CPP). Maurocalcine allows the penetration of the macro protein complex (for example: biotinylate derivative of MCa coupled to fluorescent streptavidin indicator) so it may be used as vector for delivery of different molecules into the cytoplasm. These data revealed that MCa may use as a carriers of various molecule or drug of therapeutic, diagnostic and technological value.

1.5. Gadolinium

Gadolinium belongs to the „rare earths" elements, a series which comprises the elements with atomic number 57 through 71. The ionic radii of the lanthanide ions decrease through the period (lanthanide contraction). The lanthanides have three positive charges and the ionic radii of ions are similar to calcium. Because of their ferromagnetic and transport (they can cross blood vessels) properties gadolinium compounds are used as radio contrast agents to enhance images in medical magnetic resonance imaging (MRI). Earlier studies suggested that lanthanide ions have several physiological effects on different cells and on calcium binding and transporting protein. In skeletal muscle gadolinium was able to displace calcium from sarcoplasmic reticulum in micro molar range. Studies revealed that lanthanide ions inhibited muscle contraction depending on their ion radius. Among lanthanides the gadolinium had the strongest inhibitory effect. In skeletal muscle the inhibitory effect of Gd^{3+} on SR Ca-CaATPase, due to the binding to the Ca-binding site, was also described by Jona and Martonosi.

Objectives

Earlier studies revealed that scorpion toxin called maurocalcine – shows high sequence identity with RyR-modulator domain A – modifies the skeletal type calcium release channel (RyR1). ^{24}Arg and basic cluster of surface of MCa may play role in toxin-RyR interaction. Using single channel experiments on purified RyR1 we wanted to clarify whether MCa and its mutants modify the gating and pharmacology of RyR1. We wanted to determine the effect of MCa on the activity of SR Ca^{2+}-pump involved strongly in calcium homeostasis using coupled enzyme assay technique.

The lanthanide ions and mainly gadolinium are able to bind to different calcium binding peptide and modify the calcium homeostasis of the cell. It was revealed that gadolinium blocks N-, T- and L-type calcium channels and inhibits the activity of SR Ca-pump. At the second part of our project we studied the effect of Gd^{3+} on RyR1 principally the location of its binding site(s) using single channel experiments, [^{3}H]ryanodine-binding and calcium efflux measurements.

2. Materials and methods

2.1. Isolation of heavy SR vesicles from rabbit skeletal muscle

Heavy SR vesicles were isolated from *musculus longissimus dorsi* of rabbits. Several protease inhibitors were included in the solutions to prevent proteolysis during the isolations. Following a homogenization step (4ºC) first crude microsomes were collected by centrifugation at 40,000 *g*, 30 min. After removing the actomyosin contamination, by dissolving it in 600 mM KCl, microsome fraction was collected at 109,000 *g*, 30 min. The pellet was resuspended and loaded onto a 20–45% linear sucrose gradient. HSR vesicles were collected from the 36–38% region of the continuous sucrose gradient, pelleted by centrifugation at 86000 *g*, 16 hours. HSR vesicles were resuspended and collected by centrifugation at 124000 *g* again. Fractions were collected, rapidly frozen in liquid N_2 and stored at -70ºC.

2.2. Purification of the ryanodine receptor complex

HSR vesicles containing ryanodine receptor complex were resuspended in 9 ml medium to a final protein concentration of ≈3 mg/ml. In order to solubilize the RyRs 1% CHAPS (v/v) was present in the same solution. After incubation (2 h, 4˚C) insoluble proteins were removed by centrifugation at 59000 g, and subsequently the resulting supernatant (3-3 ml) was layered on the top of 10-28 % sucrose gradient (1 „hot" + 2 „cold"). The extent of ryanodine receptor solubilization and the subsequent migration distance of the sulobilized receptor in the sucrose gradient was monitored by labeling one part (3 ml) of the solubilized sample with [^{3}H]ryanodine. Unlabeled and [^{3}H]ryanodine-labeled solubilized SR membranes were centrifuged through identical sucrose gradient for 16 h at 90000 g in a swing out (SW-27) Beckman rotor. Fractions of the unlabeled gradient corresponding, by sucrose density, to the peak of the [^{3}H]ryanodine-labeled receptors were collected. Aliquots of the fractions of the solubilized receptor were collected from the sucrose density gradient and were visualized by SDS/PAGE using Laemmeli type 10% linear gel.

2.3. Planar lipid bilayer measurements

Solubilized ryanodine receptor molecules were incorporated into planar lipid bilayer and measurements were carried out under voltage clamp conditions. The bilayer was formed across a 250 µm aperture from a mixture of L-α-phosphatidylethanolamine, L-α-phosphatidylserine and L-α-phosphatidylcholine in the ratio of 5:4:1 dissolved in n-decane (20 mg/ml). Reconstitution was initiated in symmetric solution in the presence of 50 µM ionized („free") calcium. During measurements holding potential was set between 40-80 mV. Small aliquots of the solubilized receptor were added to one side of the bilayer chamber defined as the cytoplasmic (*cis*) side, meanwhile the other chamber was regarded as luminal (*trans*) side. The correct orientation of the channel was checked by decreasing Ca^{2+} concentration (238 nM Ca^{2+}) at the *cis* side or by adding ryanodine to the same side.

Electrical signals were filtered at 1 kHz through an 8-pole low-pass Bessel filter and digitized at 3 kHz using Axopatch 200 and pCLAMP 6.03 (Axon Instruments, Union City, CA, USA). The free Ca^{2+} concentration was calculated using the computer program and affinity constants

published by Fabiato. Open probabilities were calculated using the common 50% criteria with a medial dead zone of 5%. Current amplitude distribution was analyzed using the Origin software (Microcal Software, Northampton, MA, USA). The LLSS ratio was calculated as the fraction of time that the channel spent in the LLSS compared to the total recording time. Records were analyzed using Axoscope 10 software.

2.4. $^{45}Ca^{2+}$ flux measurements

Using rapid filtration technique we can study the calcium content of SR lumen accordingly if the intraluminal calcium content is known we can determine the rate of calcium release. HSR vesicles were passively loaded for 1 h at room temperature in a medium containing $^{45}Ca^{2+}$. Vesicles were spread on filter papers type: DA, pore size: 0.65 µm. Calcium efflux was initiated for time duration of 20-300 ms by the stream of measuring buffer. The radioactivity remaining on the filter was determined by liquid scintillation method (Packard TRI-Carb 2200CA liquid scintillation spectrophotometer).

2.5. Ryanodine binding

Ryanodine binds to the opened state of calcium channel, so the rate of ryanodine-binding gives us information about activity of calcium channel. Ryanodine-binding assay was carried out using [^{3}H]ryanodine. Aliquots (50µl) containing 25 µg protein (rabbit HSR vesicle) were incubated at 37ºC for 2 hours. For measurements of gadolinium effect, Gd^{3+} was added 30 min before the addition of tritiated ryanodine. Nonspecific binding was determined in the presence of 50-100 µM ryanodine (unlabeled), which had been added to the incubation mixture prior to the ligand. The reaction was terminated by filtering the samples on BIO-DOT 96 well filter apparatus using Millipore 50 filter paper. This was followed by washing and drying. The radioactivities of filter papers were determined using liquid scintillation counter.

2.6. Measurements of SR Ca^{2+}-ATPase activity

Light sarcoplasmic reticulum vesicle fractions (LSR) isolated from rabbit skeletal muscle containing Ca^{2+}-ATPase in high density were used to determine the calcium pump activity. Hydrolytic activity was determined by coupled enzyme assay at 37 ˚C. The SR Ca^{2+}-ATPase produces ADP from ATP. ATP generates pyruvate from phoshoenol-pyruvate by pyruvate-kinase and at the final step lactate-dehydrogenase produces lactate from pyruvate by NADH.

Activity was determined in a medium containing 100 mM KCl, 0.5 mM $MgCl_2$, 20 mM Tris HCl (pH=7.5), 7.5 U/ml pyruvate-kinase, 18 U/ml lactate-dehydrogenase, 0.42 mM phoshoenol-pyruvate, 0.2 mM NADH-t and 2 μM A23187 (a Ca^{2+} ionophore). Total hydrolytic activity was measured as the decrease of optical density at the NADH absorbance wavelength (340 nm). Calcium pump activity calculated from the slope of decreasing absorbance (results were normalized to protein concentration). The specific activity of calcium pump was determined by applying 5 μM thapsigargin (specific inhibitor of SR Ca^{2+}-ATPase). In the medium the free calcium concentration was about 2 μM (At this calcium concentration the pump has the maximal activity).

3. Results

3.1. The effect of maurocalcine

3.1.1. The maurocalcine modifies the gating of the channel

The effect of the MCa on the channel function was tested using RyR1 incorporated into planar lipid bilayer. Applying 50 nM MCa on the cytoplasmic (*cis*) side of the channel induced long-lasting subconductance states (LLSS), which corresponded to about 60% of the full conductance. In case of the wild-type MCa, the subconductance state displayed an average LLSS with a time duration several thousand times longer than that of the mean open time (τ_o) of the unmodified channel. Between LLSS – in the presence of the peptide – the channel showed normal gating with increased activity. This manifested as an approximately 10-fold increase in the open probability during the inter-LLSS periods in the presence of 50 nM MCa *cis* ($P_{o,\,control}$ = 0.02 ±

0.007; $P_{o,\,MCa}$ = 0.21 ± 0.016). Interestingly, the MCa-modified channel retained its ryanodine sensitivity and in the presence of ryanodine (1 μM) and MCa (200 nM) we could simultaneously registrate the effect of ryanodine and MCa. This observation suggests that MCa and ryanodine bind to the different region of the channel.

3.1.2. Effect of mutation on gating

Synthetic MCa derivatives activate the P_o of the isolated RyR and induce subconductance states, in a similar fashion – but to different extents – as the wild-type MCa. Replacing ^{24}Arg with Ala ([Ala^{24}]MCa mutant) in MCa's sequence essentially abolished the MCa-induced subconductance states. Mutants [Ala^{20}]MCa, [Ala^{22}]MCa and [Ala^{23}]MCa exhibited consequently shorter and shorter transitions to the subconductance state, in accordance with their putative spatial distance of their mutation with respect to residue 24. In contrast, replacing amino acid residue in position 19 and 8 by Ala yielded almost the same LLSS as the wild-type MCa did, because Lys8 or Lys19 are located from basic cluster far away.

3.1.3. Polarity dependence of the effects of MCa

The parameters of the LLSS events induced by MCa and its mutants depended on the polarity, that is, on the direction of the current. When the charge carrier (K^+ or Ca^{2+}) moves from the *trans* to the *cis* side the current direction is identical to that when calcium is released from the SR ("physiological direction"). Using membrane potentials (negative holding potentials) to drive the ions in the physiological direction, the LLSS ratios for every mutant were found to be lower as compared to the opposite polarity ("reverse direction").

In the presence of toxins current records were also acquired at the same polarity, but different voltage steps. The effect of the peptides, in this respect, seemed to be dependent on the actual value of the holding potential in the voltage range of 20-120 mV because the raising of membrane potential increases the relative conductance of subconductance states ($LLSS_{rel.cond.}=I_{llss}/I_{max}$).

3.1.4. MCa and its mutants alter channel gating in different ways

The effects of the toxins on the LLSS ratio – the relative time the channel spends in LLSS – and on the length of these events were studied in the presence of 50 μM ionized calcium. In all studied concentrations (50 nM, 200 nM, and 500 nM) the most effective toxin was the wild type and [Ala^{24}]MCa mutant had no effect. As the site of the mutation was closer and closer to the critical residue 24, the LLSS ratio became smaller and smaller. The length of LLSS is the longest at wild type toxin and shortest at [Ala^{24}]MCa. Furthermore, the place of the mutation affected the average LLSS event length more substantially than that of the LLSS ratio, particularly at [Ala^{8}]MCa and [Ala^{19}]MCa at which the changes in LLSS-length are much more effective than LLSS-ratio. So the reason of the reduction of LLSS-ratio seems to be lowering of LLSS-length and not the frequency of the events. Note also that the LLSS ratio as well as the frequency of the events increased along with the peptide concentration. On the one hand the toxins increase the LLSS-ratio in concentration dependent manner, on the other hand the average length of the LLSS events seemed to be independent of the peptide concentration.

3.1.5. The effect of toxin is independent to calcium concentration

The effect of maurocalcine and its mutants were investigated not only at 50 μM but at 240 nM free calcium concentration. In the presence of 240 nM free calcium the MCa induced long lasting subconductance states. At low calcium concentration the wild type was the most effective peptide and the [Ala^{24}]MCa practically was ineffective. The wild type peptide induced the longest average LLSS events length. The polarity-dependence effect of toxins was also demonstrated at 240 nM calcium. This data suggests that calcium concentration has no effect on MCa-RyR interaction so the effects of toxins are independent from calcium concentration.

3.1.6. Maurocalcine and its mutants do not have affect on the activity of SR Ca^{2+}-pump

Coupled enzyme assay was used to determine the effect of maurocalcine and its mutants on SR calcium pump activity. Measurements were carried out three different concentrations (50 nM, 200 nM, 500 nM). The calculated activities in the presence of toxins were normalized to control (in the absence of toxin) activities. Data points suggested that wild type toxin (MCa) and its

mutants ([Ala^{8}]MCa, [Ala^{19}]MCa, [Ala^{20}]MCa, [Ala^{22}]MCa, [Ala^{23}]MCa, [Ala^{24}]MCa) do not have affect on the activity of SR Ca^{2+}-pump in the studied concentration range.

3.2. Effect of gadolinium on RyR1

3.2.1. Inhibition on cytoplasmic side

Gadolinium applied, in the micromolar concentration range on the *cis* side, decreased the probability of the channel opening (P_o). At 13.3 μM Gd^{3+} the RyR1 was in its almost completely closed state. The inhibition was reversible; after adding EGTA (equimolar with Gd^{3+}) the channel almost completely regained its original (control) activity. The open probability of RyR1 was calculated in the absence and presence of Gd^{3+}. The open probability of the channel as a function of the cis gadolinium concentration was fitted by the Hill equation resulting in a half-inhibitory concentration of 5.6 ± 0.3 μM for Gd^{3+} and a Hill coefficient of 4.7 ± 0.8.

3.2.2. Inhibition on luminal side

We have also tested the effect of Gd^{3+} on the luminal side of the calcium channel. To decrease the possibility of the applied Gd^{3+} acting on the cytoplasmic site of RyR1 after passing through its conducting pore, the EGTA-Ca buffer was applied on the *cis* side. Gd^{3+} inhibited the channel activity from the *trans* side in a reversible and concentration dependent manner. By addition of equimolar EGTA on the *trans* side of the channel resulted in the recovery of the RyR1 from the inhibition in a few seconds, which supports the idea that the Gd^{3+} binds to binding sites located on the *trans* side and does not pass through conducting pore of RyR1. The average of normalized P_o were plotted against the *trans* gadolinium concentration and the Hill equation resulting in a half-inhibitory concentration of 5.4 ± 0.2 μM for Gd^{3+} and a Hill coefficient of 4.3 ± 0.6.

Open time histograms were constructed using the same represantive current records from which the open probabilities were calculated. Histograms could be fitted in a satisfactory way by a single exponential. Gadolinium decreased the open time constant (τ_o) of the reconstituted ryanodine receptor in a concentration dependent manner acting on both side.

The voltage dependence of the inhibitory effect of the gadolinium was also investigated in four experiments. Gadolinium (2 μM) was applied at the *trans* side of the channel and current records were acquired at different voltage steps. Open probabilities were calculated and normalized in the absence of the gadolinium. The ratios of the normalized P_o value to the average P_o value during the individual experiments were plotted against the transmembrane potential differences. Data produced by linear regression support that there is no voltage-dependence of the inhibition. This finding proves that gadolinium exerts its effect from the luminal side of the channel and does not penetrate to the *cis* side.

3.2.3. The effect of accessory proteins

Using single channel technique we could study the activity of purified calcium channel. We wanted to elucidate the roles of accessory proteins located in the junctional membrane to see whether these proteins/peptides modify the RyR- Gd^{3+} interaction. So we investigated the effect of gadolinium on heavy SR vesicles as well.

The effect of gadolinium on the ADP-caffeine induced calcium release from passively loaded HSR vesicles was measured by the rapid filtration technique. The radioactivity was plotted against the contact time in the presence or absence of Gd^{3+}. Data points could be fitted in a satisfactory way by a single exponential. In the presence of gadolinium (20 μM) the time constant of release increased as a result of the inhibitory effect of Gd^{3+}. The total amount of the released calcium did not change.

The effect of gadolinium on [^{3}H]ryanodine binding to the HSR was also investigated. Gadolinium (5μM) increased the dissociation constant of ryanodine-binding while the value of B_{max} did not change significantly ($p<0.05$). Gadolinium dependence of ryanodine-binding in the presence of 18 nM [^{3}H]ryanodine was determined at various gadolinium concentrations. Data points were fitted by Hill equation with kinetic parameters of K_d=14.7±0.7 nM and n_{Hill}=3.16±0.42 for Gd^{3+}-RyR interaction.

4. Discussion

4.1. Maurocalcine modifies the activity of the channel

Earlier studies revealed that maurocalcine induces Ca^{2+}-release from SR vesicles and generates subconductance states on purified RyR1 incorporated into planar lipid bilayer. In single channel measurements applying 50 nM MCa on the cytoplasmic (*cis*) side of the channel induced long-lasting subconductance states (LLSS), which corresponded to about 60% of the full conductance. Between LLSS – in the presence of the maurocalcine – the channel showed normal gating with increased open probability. In the presence of wild type toxin the duration of LLSS is about 10-20 s contradiction to usual 300-600 μs means open time of the channel. The rise of activity of channel and the length of LLSS resulting high LLSS-ratio can induce significant Ca^{2+}-release from SR vesicles as revealed by earlier studies. Another group of our department studied the effect of maurocalcine on elementary Ca^{2+} release events (ECRE) in rat and frog skeletal muscle. After the addition of toxin the amplitude of ECRE decreased without any change in spatial half-width of events. The reduction of amplitude of ECRE parallel to the appearance of long lasting subconductance states originating from lipid bilayer experiments. In the presence of maurocalcine the channel preserves ryanodine sensitivity as it was observed in single channel measurements. In the presence ryanodine and MCa simultaneously (1 μM ryanodine + 50 nM MCa) both of them induce subconductance states. The channel oscillated between two subconductance states induced by MCa and ryanodine. The apparently free gating of the channel between these two states ensures the state into which MCa transforms the channel is independent of that induced by ryanodine.

4.2. Effect of mutations

It was revealed that basic amino acids of $IpTx_a$ and the structurally similar domain A of DHPR play role in RyR-toxin interaction. The substitution of only one amino acid in the peptides mentioned above resulted the loss of their activity. Another group of our department showed that mutations altered the properties of spontaneous Ca^{2+} release events. The [Ala^{8}]MCa and [Ala^{19}]MCa same as wild type toxin induced long openings events which are not similar to those

under control conditions. In contrast in the presence of [Ala^{22}]MCa the duration of elementary events was shorter and the [Ala^{24}]MCa had no effect on the occurrence of ECRE. In single channel experiments the effect of [Ala^{8}]MCa and [Ala^{19}]MCa was similar to wild type toxin, but the efficiency of [Ala^{20}]MCa, [Ala^{22}]MCa, [Ala^{23}]MCa to evoke subconductance states of RyR1 decreased in this order whilst the [Ala^{24}]MCa was practically ineffective. The data arising from the measurement of elementary Ca^{2+} release events and single channel measurements are in good agreement. Our and collaborators data suggest that ^{24}Arg is the critical amino acid in the effect of MCa. Mutants exhibited consequently shorter and shorter transitions to the subconductance state, paralleling the putative three dimensional distance of their mutation with respect to residue 24. The basic amino acids (19 **KKC**K**RR** 24) located on the surface of MCa are responsible for efficacy similar to $IpTx_a$ and domain A.

4.3. Effect of MCa is polarity-dependent

Earlier studies showed that $IpTx_a$ – whose sequence is partially identical to MCa – exerts a voltage- and polarity-dependent effect on RyR1. Our data suggest that MCa and its mutants induce LLSS events on the RyR1 which effect is membrane potential and current direction dependent. When the charge carrier (K^+ or Ca^{2+}) moves from the *trans* to the *cis* side the current direction is identical to that when calcium is released from the SR ("physiological direction"). Using negative holding potentials to drive the ions (K^+ or Ca^{2+}) in the physiological direction, the LLSS ratios for each mutant were found to be lower. In contrast using positive holding potential ("reverse direction") the LLSS ratios for each toxin were higher. In the presence of toxins current records were acquired at different voltage steps. After addition of toxin the raising of membrane potential increases the relative conductance of subconductance states ($LLSS_{rel.cond.}=I_{llss}/I_{max}$). These data suggest that the effect of MCa and its mutants depends on the actual value of the holding potential in the voltage range of 20-120 mV.

4.4. MCa and its mutant have no any effect on SR Ca pump

The toxins (MCa, [Ala^{8}]MCa, [Ala^{19}]MCa, [Ala^{20}]MCa, [Ala^{22}]MCa, [Ala^{23}]MCa, [Ala^{24}]MCa) did not modify the hydrolytic activity of SR Ca^{2+}-ATPase in studied concentration

range (50 nM, 200 nM, 500 nM). Maurocalcine has strongly basic characteristic (on physiological pH +7). Gly and Lys amino acid residues play role in appearance of basic cluster. Data suggests that SR calcium pump has no any sequence which is able to bind to the maurocalcine. We suggest that SR calcium pump does not play role in the effect of maurocalcine that is in the altering of calcium homeostasis of cells.

4.5. Gadolinium inhibits the activity of RyR1

RyR1 were reconstituted into an artificial lipid bilayer and the effect of gadolinium was investigated from *cis* and *trans* side in order to elucidate from which side gadolinium acts. In single channel experiments gadolinium inhibited the channel concentration dependent manner on the both side. The half-effective concentrations were about 5μM and the Hill-coefficient were about 4 for both sides which supports that there are four binding site with similar affinity of gadolinium on the cytoplasmic and also on the luminal side of the ryanodine receptor. Although in single channel experiments the luminal Ca^{2+} concentration has an effect on the activity of the RyR1, no luminal cation binding site has been described so far.

No voltage–dependence of the inhibitory effect of the gadolinium was observed in the single channel experiments. When gadolinium was applied on *trans* side, the channel was inhibited in spite of addition 100 μM EGTA on *cis* side. The affinity of the Gd^{3+} compared to the EGTA is a million-fold larger than that of the Ca^{2+}. The fast recovery (≈10 s) of the RyR1 from inhibition after adding EGTA to the *trans* side also make it probable that Gd^{3+} ions were removed from the luminal binding sites of RyR1. Experiments were carried out to investigate if there is any competition between the Gd^{3+} and Ca^{2+} in binding to high and low affinity calcium-binding regulatory sites of the RyR1. In the single channel experiments, in the presence of 4.5 μM Gd^{3+} *cis*, elevating free Ca^{2+} concentration *cis* up to 1 mM did not result in any significant change in the P_o of the channel. Consequtely applied 50 μM EGTA caused slight recovery of RyR1, because this concentration of EGTA decreased the Gd^{3+}-concentration to nominally zero whereas the calcium concentration decreased only by some tens of μM. If competition had occurred between Gd^{3+} and Ca^{2+} addition 1 mM calcium should have increased the activity of the channel to the control value. After applying calcium the activity of the gadolinium inhibited

calcium channel did not change significantly so this observations means that *cis* Gd^{3+} does not inhibit the channel by binding to the high affinity calcium binding site.

The effect of gadolinium on RyR1 calcium release was measured by rapid filtration and ryanodine binding technique. In the presence of 20 μM Gd^{3+} the rate of release decreased. This concentration of Gd^{3+} increased the time constant of the calcium release without affecting the total amount of calcium released. The increasing of the time constant of the calcium release suggests that Gd^{3+} inhibits the activity of SR calcium release channel.

Gadolinium (5 μM) increased the dissociation constant of ryanodine binding while the value of B_{max} did not change significantly. The gadolinium dependence of ryanodine binding of calcium channel suggests that gadolinium inhibits the activity of RyR1 concentration dependent manner. Our data suggest that RyR1 is able to bind Gd^{3+} on both side (*cis* and *trans*). The Gd^{3+} inhibition on RyR1 was reversible and concentration dependent on both sides with similar n_{Hill} and EC_{50} values. The *cis* binding sites seem not to be identical with known *cis* calcium-binding regulatory sites.

5. Abstract

Maurocalcine (MCa), a 33 amino acid peptide toxin obtained from scorpion venom, has been shown to interact with the isolated skeletal-type ryanodine receptor (RyR1) and to strongly modify its calcium channel gating. In this study, we explored the effects of MCa and its mutants (Lys8Ala, Lys19Ala, Lys20Ala, Lys22Ala, Lys23Ala and Lys24Ala) on single channel currents through RyR1 in artificial lipid bilayer. The effect of gadolinium ions on purified calcium channel (RyR1) and heavy SR vesicles (HSR) were studied using single channel experiments, $^{45}Ca^{2+}$ flux measurements and [^{3}H] ryanodine binding assay. Individual LLSSs last even for several seconds and the average length of these events and the frequency of their occurrence are altered in different mutations, according the spatial distance of the mutated amino acid from the critical residue, residue 24. If the mutated residue is more and more far away (distant) from this critical residue, the length and the frequency of these LLSSs lessens resulting in lower LLSS ratio too. The effect is strongly dependent on the direction of the channel current (the polarity). If the direction of the cation current is the same as in case of calcium release, the toxin is about 8-10 fold less effective compared to the opposite current direction. The toxin and its mutants induce similar effect at 50 μM and at 240 nM free calcium concentration. Here we show, that effect of the toxin is governed by the large single charged surface formed by the residues ^{20}Lys, ^{22}Lys, ^{23}Arg, ^{24}Arg, ^{8}Lys as shown by the effect of the 19 mutant, which exhibits almost identical effect with the wild type.

In single channel experiments the gadolinium inhibited the activity of calcium channel concentration dependent manner on both (cis and trans) side. It inhibited the calcium release from SR vesicles and [^{3}H] ryanodine binding. The inhibition of channel did not have any voltage-dependence. Our results suggest that there are sites on cis and trans side of the RyR1 that are able to bind Gd^{3+} causing inhibition of channel with similar parameters (Hill coefficient, IC_{50}).

PUBLISHED DATA RELATED TO THE RESULTS OF THE PRESENT THESIS:

B. Lukács, M. Sztretye, J. Almássy, S. Sárközi, B. Dienes, K. Mabrouk, C. Simut, L. Szabó, P. Szentesi, M. De Waard, M. Ronjat, I. Jóna and L. Csernoch (2008): Charged surface area of maurocalcine determines its interaction with the skeletal ryanodine receptor. *Biophysical Journal* accepted for publication. **IF: 4.757**

S. Sárközi, Cs. Szegedi, **B. Lukács**, M. Ronjat and I. Jóna (2005) Effect of gadolínium on the ryanodine receptor/sarcoplasmic reticulum calcium release channel on skeletal muscle. *FEBS Journal* 272. 464-471. **IF: 3.260* (3.033)**

OTHER PUBLISHED DATA:

S. Sárközi, J. Almássy, **B. Lukács**, N. Dobrosi, G. Nagy and I. Jóna (2007) Effect of natural phenol derivatives on skeletal type sarcoplasmic reticulum Ca2+-ATPase and ryanodine receptor. Journal of Muscle Research and Cell Motility 28(2-3). 167-174. **IF: 0.944**

ABSTRACTS:

B. Lukács, G. Nagy, N. Dobrosi, I. Gherasim, I. Jóna, (2005): Effects of natural derivatives of phenol on calcium transport of sarcoplasmic reticulum. *Journal of Muscle Research and Cell Motility* 26: P61

B. Lukács, J. Almássy, E. Esteve, Cs. Szegedi, (2003): Effect of maurocalcine on calcium release channel-ryanodine receptor. *Journal of Muscle Research and Cell Motility* 24: P357

I. Jóna, J. Almássy, M. Ronjat, **B. Lukács**: Effect of Maurocalcine on RyR1 and RyR2 is Substantially Different. *Biophysical Journal 2007 January, 87a*

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LECTURES AND POSTERS:

Balázs Lukács: Effect of thymol and carvacrol on the sarcoplasmic reticulum ion transport. TDKCongress, Debrecen, 2001 (lecture)

Balázs Lukács, Csaba Szegedi, Sándor Sárközi, István Jóna: Effect of natural derivatives of phenol on ionic transport of sarcoplasmic reticulum. Membrane Transport Congress, Sümeg 2001 (poster)

Csaba Szegedi, **Balázs Lukács**, István Jóna: Effect of thymol and carvacrol on the sarcoplasmic reticulum ion transport. Channelopathies 2001 Conference, Sheffield, 2001 (poster)

Balázs Lukács: Effect of thymol on the sarcoplasmic reticulum calcium transport. TDKCongress, Debrecen, 2002 (lecture)

István Jóna, János Almássy, **Lukács Balázs**, Szegedi Csaba, Sárközi Sándor: Regulation of skeletal type calcium release channel. 68th Annual Meeting of the Hungarian Physiological Society, Debrecen, 2004 (lecture)

Csaba Szegedi, János Almássy, **Balázs Lukács**, István Jóna: Effect of maurocalcine on skeletal type calcium release channel (RyR1). 68th Annual Meeting of the Hungarian Physiological Society, Debrecen, 2004 (lecture)

Balázs Lukács: Effect of a scorpion toxin (maurocalcine) on RyR1. Ph.D. Congress, Debrecen, 2005. (lecture)

Balázs Lukács, János Almássy, Sándor Sárközi: Effect of a scorpion toxin (maurocalcine) on the calcium transport of skeletal muscle. 69th Annual Meeting of the Hungarian Physiological Society, Budapest, 2005 (lecture)

János Almássy, **Balázs Lukács**, Sándor Sárközi: Effect of calcium chelator (TPEN) on the activity of ryanodine. 69th Annual Meeting of the Hungarian Physiological Society, Budapest, 2005 (lecture)

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Balázs Lukács, János Almássy, István Jóna: Effect of scorpion toxin (maurocalcine) and domain A on the ryanodine receptor. 71st Annual Meeting of the Hungarian Physiological Society, Pécs, 2007 (poster)