DOCTORAL (Ph.D.) THESIS

Ryanodine receptor related changes of skeletal muscle function in chronic heart failure

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1. INTRODUCTION

The history of muscle research goes back to the experiment of Galvani in the 18th century, when on a stormy evening he connected a long metallic wire to a spinal nerve exposed in a frog preparation, and put outside pointing toward the sky. According to his expectation the preparation contracted strongly in conjunction with lightning. Since then the subject became a separate field of medical science which involves the issue of excitation-contraction coupling (ECC).

ECC comprises the train of steps leading to muscle contraction via depolarization of the surface membrane of the muscle fiber and transient elevation of intracellular Ca^{2+} level. The process goes on with the cooperation of two Ca^{2+}-channels: the L-type Ca^{2+}-channel also called dihydropyridine receptors located in the transversal tubules (TT) of the sarcolemma, and the ryanodine receptor-Ca^{2+} release channels (RyR) of the sarcoplasmic reticulum (SR). ECC happens in a different way in skeletal and cardiac muscle. The common step of ECC in both striated muscle types is the Ca^{2+} release through the activation of RyRs. In skeletal muscle DHPR does not open during action potential due to the relatively long activation time, so does not functions as a channel, but it forms a direct allosteric connection with opposed RyRs in the SR. DHPR shows conformational change during depolarization and mediates the signal of the sarcolemma onto RyRs to open them. Ca^{2+} released from SR through directly coupled RyRs activates the neighboring DHPR-uncoupled RyRs. The latter process is referred as Ca^{2+}-induced Ca^{2+} release (CICR). In cardiac muscle direct mechanical coupling between DHPR and RyR does not exist but DHPR opens during the long action potential of cardiac myocyte, entering Ca^{2+} from extracellular space into the cleft between transversal tubule and SR. Then Ca^{2+} causes CICR.

Ca^{2+}-release is confined to terminal cisternae and remains local due to the tight spatial relation of TT and SR membranes which enables isolated function. This functional unit of Ca^{2+}-release in a single terminal cisterna is termed as spark.

Accordingly the global Ca^{2+} transient of muscle fiber is the sum of sparks sincronized by action potential in time.

The result of the process detailed above is the elevation of $[Ca^{2+}]$ and consequent activation of the contractilic apparatus.

1.1. The ryanodine receptor

Ryanodine is a plant alkaloid that binds SR Ca^{2+} release channel with high affinity. In single channel experiments on RyRs reconstituted into artificial lipid bilayers ryanodine induces sustained partially open- so called subconductive states of the channel. The phenomena is so characteristic that it makes ryanodine to be the most useful and reliable tool in RyR identification. The functional release channel is a homotetramer. Each monomer comprises ~5400 amino acid residues with a molecular mass of 565 kDa. In mammals there are three isoforms of RyR (RyR1, RyR2, RyR3) with tissue specific distribution: RyR1 is predominantly expressed in skeletal muscle (skeletal type RyR), while RyR2 is in heart muscle and almost all over in the brain (cardiac type RyR). RyR3 was first described in endothelial cells and in the brain, but it is universally expressed.

According to the results of Williams' group RyR is a high conductance poorly selective cation channel with ohmic behaviour, which results in a voltage independent, linear current-voltage relationship. Despite functioning as the Ca^{2+} channel of the SR, it is permeable to multiple physiologically relevant cations. For instance it has surprisingly high conductance for the monovalent cations of group 1a, while it has lower for divalent cations (maximal conductance for K^+ is ~900 pS for Ca^{2+} is 135 pS). It does not make distinction among monovalent cations, shows significant preference to divalent cations but shares almost the same selectivity among them. Relatively low selectivity does not have significant relevance in *vivo*, because concentration gradient through SR membrane is not established for monovalent cations and membrane potential is not (or only transiently) generated during Ca^{2+} release due to high K^+ and Cl^- channel activity of the SR. This is why mainly Ca^{2+} flows through RyRs during Ca^{2+} release.

1.1.1. The regulation of ryanodine receptor

RyR is controlled by the membrane potential of the sarcolemma (DHPR-RyR1 direct coupling) and soluble factors of the cytoplasm and SR lumen (Ca^{2+}, Mg^{2+}, and ATP)

Action of Ca^{2+} on RyR function is biphasic: in nanomolar and millimolar range the open probability (P_o) of RyR is close to 0, and at ~100 µM [Ca^{2+}] P_o is maximal. Hill-fit of the ascending and descending limbs of the resulting bell shaped curve gives Hill coefficients of unity for both, which indicates the involvement of two different Ca^{2+}. One of them is a high-affinity (Kd≈10 µM), activatory, while the other is a low-affinity (Kd≈300 µM) inhibitory binding site. The Ca^{2+}-sensitivity of RyR is adjusted jointly by ATP and by Mg^{2+}.

ATP is considered as the most efficient endogen agonist of the RyR (Kd=350 µM, N_{Hill}=2) which significantly increases channel P_o even in „Ca^{2+}-free" solutions. The importance of ATP in ECC is that direct activation of RyR by DHPR alone is not enough to evoke Ca^{2+} release, but soluble agonists such as ATP are required as well. At intracellular ATP and Ca^{2+}-concentrations (8 mM and 100 nM respectively) the P_o of RyR is maximal, which rises the question that what keeps RyR channels closed in the resting muscle.

The answer is Mg^{2+} and the DHPR itself.

DHPR consists of five subunits (α_1, α_2, β, γ, δ). One of the loops connecting the II. and III. transmembrane domain of the α_1 subunit have a key role in direct coupling. A peptide (peptide A) sharing identical sequence with the loop between the 667-696 amino acid residues activates both isoforms of RyR in low concentrations. The criteria of the action of peptide A on RyR is a segment containing basic amino acid residues. Some toxins' sequence mimics the primary structure of peptide A which makes these toxins valuable research tool in ECC research. In a scorpion toxin, maurocalcine (MCa) homologue sequences together with other basic amino acid residues construct a large positively charged area on the 3D surface.

According to the previous studies of our group MCa increases the P_o of RyR and induces long lasting subconductive states (LLSS) of the channel corresponding to the ~40% of maximal conductance of the channel. Polarity dependence of the effect of MCa indicates electrostatic interaction in the pore. Changing certain charged amino acids to uncharged alanin in MCa sequence reduces efficency of MCa. The central role of arginin in the position 24 is indicated by a substituent lacking abitity to bind RyR.

Although RyR is controlled by the DHPR, fine tuning is done by other associated proteins. The next section is about them.

1.1.1.1. The FK-506-binding protein (FKBP)

FKBP binding of RyR is isoform specific: RyR1 is modulated by FKBP12 while RyR2 binds FKBP12.6. The stochiometry is 1 per RyR subunit. Macrolide administration (FK506, rapamicin) causes dissociation of FKBP from RyR which is associated with uncoordinated RyR gating caracterised by subconductive states of channel current. In addition RyR shows increased P_o due to increased sensitivity to Ca^{2+} activation. Accordingly FKBP stabilises closed channel state. Moreover contribution to coupled gating of nearby RyR channel complexes was suggested. It enables simultanious opening-closing of the channels. So, dissociation of FKBP leads to independent, impaired coupled gating of RyR channels. In certain heart diseases FKBP dissociates, consequently RyR gating becomes stochastic, which results in arythmogen leaky cahnnels. ECC gain and the amplitude of Ca^{2+}-transient reduces that can cause heart failure.

1.1.1.2. Phosphorilation

During the activation of simpathetic nervous system (during exercise or fight or flight reaction) catecholamines result in cAMP-dependent protein kinase (PKA) activation, which leads to activation of proteins involved in the regulation of Ca^{2+} release and uptake in the heart. This process raises Ca^{2+} transient's amplitude and contractility.

In chronic heart failure (CHF) (insufficient function of the heart) simpathetic activity is elevated and the level of circulating catecholamines is sustained, which helps to maintain the mean arterial pressure, but has harmful long-term effects such as ventricular hypertrophy/dilation. Recent reports suggest that modified Ca^{2+} handling, impaired ECC, Ca^{2+}-release and reuptake are responsible for the functional changes of muscle in HF which are the consequence of impaired phosphorilation of RyR. HF is caracterised by low SERCA activity, and reduced Ca^{2+}-transient amplitude.

There are data suggesting that phosphorylation state of RyR2 in CHF exceeds physiological levels (hyperphosphorylated RyR) which causes FKBP dissociation from RyR. FKBP-depleted RyR opening is desincronised, RyRs are uncoupled the coupling gain is low, and because of altered Ca^{2+}-sensitivity RyR leaks in diastole. Leaky channels are the source of arythmias and SR Ca^{2+} depletion. These results together can explain the symptoms listed above.

Exercise intolerance is one of the major hallmarks of CHF. Numerous studies have explained different aspects of skeletal muscle dysfunction in clinical and experimental heart failure. The low cardiac output and reduced muscle blood flow for a given work rate, a decreased skeletal muscle mass, and disorders at the cellular and subcellular level have been identified as contributing causes to altered muscle aerobic metabolism during exercise. However, insufficient cardiac output and/or muscle blood flow are unlikely to cause the abnormal skeletal muscle metabolism that occurs during exercise in CHF. Reduced twitch tension, suppressed sarcoplasmic reticulum (SR) function including anomalous calcium release from the SR, altered calcium reuptake, and SR calcium load, is associated with decreased muscle contractility in HF. Recently homologous processes were suggested in skeletal muscle and heart in HF.

A potential therapeutic agent K-201 has been suggested due to its antiarrhythmogenic action. K201 evokes its cardioprotective effect through stabilizing RyR gating with increasing the affinity of FKBP to RyR. K201 also potently reduce skeletal muscle fatigue and weakness *in vivo*.

The aim of the present study beside the identification of putative functional Ca^{2+}-release channel (RyR1) changes contributing to the symptomes was to elucidate the effect of a new drug K201 on channel gating. K201 has been suggested as a potential therapeutic agent in HF due to its antiarhythmogenic action and ability to avoid muscle weakness in HF model. For these purpose single RyR1 channels from rats with HF were reconstituted into planar lipid bilayer and the gating behaviour was studied under voltage-clamp conditions.

Recently our group's attention was attracted by peptide toxins particularly MCa which contains a motif similar to the DHPR loop involved in direct coupling. Due to the homology MCa is a useful molecular tool in ECC research.

In single channel experiments we previously described the detailed action of MCa on skeletal type RyR, identified the determinant amino acids and estimated the significance of them in the electrostatic binding to RyR1.

We continued our experiments on cardiac type RyR since the question if MCa has any isoform specific action come up. The potential differences were expected to give some new ideas about skeletal muscle ECC.

2. MATERIALS AND METHODS

2.1. Animal model

Male Wistar rats were subjected to coronary artery ligation to produce PMI, and sham-operated animals served as controls. After left-median thoracotomy, the left anterior coronary artery was occluded at the most proximal position. PMI rats were used 24 weeks after surgery and were arbitrarily selected according to the visible level of remodeling estimated by the size (at least 50%) of the fibrotic area on the left ventricle.

2.2. Heavy SR vesicle preparation

Heavy SR (HSR) vesicles -containing sealed vesicles formed from membrane fragments of the terminal cisternae of the SR - were isolated from rat skeletal muscle. After a homogenization crude microsomes were collected by centrifugation at 40,000×g. The pellet was resuspended and loaded onto a 20–45% linear sucrose gradient. HSR and LSR vesicles were extracted from the continuous sucrose gradient and collected by centrifugation. Vesicles were immediately used for RyR purification or aliquoted and rapidly frozen in LN2 and stored at -70°C for further use.

2.3. RyR reconstitution and single-channel recording

Measurements of channel activity were carried out using purified RyR incorporated into planar lipid bilayers. RyR was purified from rat SR vesicles. The bilayers were formed using phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine in a ratio of 5:4:1 dissolved in decane up to the final lipid concentration of 20 mg/ml. Bilayers were formed across a 250 μm diameter aperture of a Delrin cap using a symmetrical buffer solution (250 mM KCl, 100 μM K2H2EGTA, 150 μM $CaCl_2$, 20 mM PIPES, pH 7.2). A small aliquot of purified RyR was added into the chamber which was defined as the *cis* (cytoplasmic) side, whereas the other chamber was labeled as the *trans* (luminal) side and was kept on ground potential. To ensure the orientation of the incorporated RyR, we tested the effect of Ca^{2+} on both sides. After successful incorporation of the RyR channel, free calcium concentration in the cis chamber was adjusted to 238 nM by the addition of EGTA. Electrical signals were filtered at 1 kHz through an eight-pole lowpass Bessel filter and digitized at 3 kHz using Axopatch 200 and pCLAMP 6.03 (Axon Instruments, Union City, CA, USA). Open probabilities were calculated using the common 50% criteria with a medial dead zone of ±2.5%. Current amplitude distribution-from the all-point histograms-was analyzed using Origin (Microcal Software, Northampton, MA, USA). Partial open probabilities of the S1 (PS1) and of the S2 substate (PS2) were calculated using the amplitudes of the all point histograms, such as the ratio of the amplitude

of S1 to the sum of the amplitudes of the four possible states (open + S1 + S2 + closed, called as "total"). The expression "physiological" current direction is used when the cations (Ca^{2+} or K^+) move as the calcium ions move during calcium release (from the SR), while "reverse" direction denotes when cations move in the opposite direction.

2.4. Calcium flux of HSR vesicles

HSR vesicles were actively loaded with calcium, and the calcium efflux was determined by measuring the extravesicular calcium concentration using a spectrofluorometer modified for absorption measurements by monitoring the transmittance at 710 and 790 nm and calculating the corrected absorbance change (A710−A790). Vesicles were actively loaded with calcium in the cuvette by the addition of 1 mM ATP followed by the addition of the appropriate amount of $CaCl_2$. The K-201 was added to see whether there is an increase of the calcium leak.

3. RESULTS

3.1 Results on postmiocardial infarcted rat RyR1

3.1.1. Conductance of the rat SR calcium-release channel

Channels were incorporated at 50 μM free Ca^{2+} *cis* and *trans*, to get open probabilities high enough to detect the incorporation immediately. The charge carrier was 250 mM K^+, and the channel current was recorded as described in "Materials and methods," using positive and negative holding potentials. In some experiments, stepwise changes in holding potential were applied to construct the I–V characteristics of the channel. The average conductance of the control channels was 535±32 pS. However, in PMI rats, at negative potentials, the average conductance of RyR was not significantly different from the control (575± 31 pS, $p>0.2$), while reaching a significant increase (785±28 pS, $p<0.001$) at positive potentials. It should be stressed that only a group of channels isolated from PMI

animals displayed this increased conductance at positive potentials. To present this dual behavior frequency, histograms were constructed including all channels, and the conductance for each condition was also determined by fitting a single (control) or a double Gaussian peak (PMI) to the distributions. At positive holding potentials, the estimated conductance -represented by the center of the distribution- was 520.3±2.1 pS for the control while 519.5±3.2 and 793.1±5.7 pS for the PMI channels. At negative holding potentials, the Gaussian fits gave for channels conductance 529.2±3.8 pS under control conditions and 573±4.3 pS for the PMI rats.

3.1.2. Regulation of rat RyR activity by cis Ca^{2+}

Ca^{2+} concentration (in the pCa=3-8 range) was adjusted on the *cis* side of the bilayer by the addition of the required amounts of EGTA or $CaCl_2$ stocks. The open probability was calculated from single-channel records at different Ca^{2+} concentrations after reaching equilibrium. The solubilized and incorporated RyR was most active in the 50-100 μM cis Ca^{2+} range in both animal groups. Average mean open time at 50 μM Ca^{2+} cis was 0.436±0.057 (control) and 0.473±0.041 ms (PMI), while at 472 nM Ca2+ cis, it was 0.417±0.054 (control) and 0.431±0.041 ms (PMI). These differences are not significant ($p>0.8$). Raising the cis calcium concentration from 50 to 500 μM Ca^{2+} cis, the mean open time significantly ($p<0.02$) decreased to 0.247±0.032 (control) and to 0.221±0.027 ms (PMI). Decreasing the calcium concentration from 50 μM Ca^{2+} cis into the submicromolar range drastically reduced Po , mainly because of the lesser number of open events and not to the decrease in the mean open time. Similarly, if Ca^{2+} cis was increased above 100 μM, the open probability decreased. This was, on the other hand, not only the result of the decreased number of open events but also of the decrease in the mean open time. Fitting the open probabilities using the Hill equation revealed Hill coefficients close to unity for the ascending (0.84±0.07 in sham-operated and 0.92±0.12 in PMI rats) and for the descending (1.19±0.1 in control and 1.03±0.27 in PMI rats) limbs in agreement with the presence of one activatory and one

inhibitory calcium binding site. There was only a slight difference between the two animal groups in the half activatory concentrations (12.3±1.3 and 7.1±1.3 μM, in sham-operated and PMI rats, respectively). However, the half inactivatory concentration was moved to considerably higher values in PMI rats (274.8±23.4 vs 675.8±15.9 μM, in control and PMI rats, respectively) showing that the calcium sensitivity of inactivation is particularly reduced in PMI rats.

3.2. Effect of K201 on channel gating

The effect of K201 on channel gating was tested using solubilized RyR1 from control rats incorporated into planar lipid bilayer. At 10 μM, the drug drastically changed the channel gating. Under normal conditions, RyR1 channel exhibits two states (open and closed); after the addition of 10 μM K201 to the bath, the channel changes its gating mode, showing two subconductance states denoted as S1 and S2 in addition to the open and closed states. In this concentration range, this change is accompanied by a decreased probability of both the open and the closed states. Increasing the drug concentration, the probability of the S1 and the S2 states increased at the expense of both the open and the closed states up to 50 μM K201 concentration. At this concentration, compared to the control case, the relative reduction of the open state was much more pronounced than that of the closed state. With any further increase in K201 concentration, the probability of the S1 and the S2 states decreased again, and also, the open state became less and less favorable for the RyR1, so the overall open probability of the channel substantially decreased. Apart from the higher starting P_o at the given free [Ca^{2+}[, these experiments revealed no difference between control and PMI channels, showing very similar subconductance states. Plotting the open probability of the channel using the common 50%±2.5% dead zone as a function of K201 concentration exhibits a decrease of the open state probability. This inhibition is characterized by a half inhibitory constant of IC50=25.1±2.7 μM and nHill=1.74± 0.28 Hill coefficient for the control and IC50=22.0±1.0 μM and nHill=1.98± 0.17 Hill coefficient for the PMI channels. These data reveal that both the control and the

PMI RyR1 have two binding sites for K201, and the half inhibitory concentration values are almost identical. The decrease of the probability of the full open event is due to the induction of the S1 and S2 subconductance states at the expense of the open events up to about 50 μM K201 concentration. Analyzing the concentration dependence of the probability of the S1 and of the S2 substates using control and PMI channels show a biphasic character. The probability of the S1 subconductance state increases with increasing K201 concentration up to 50 μM K201-reaching about 0.259±0.053 in the control channel and 0.196±0.041 in the case of the PMI channel. Further increases in drug concentration resulted in a decrease of the S1 partial probability down to 0.02±0.006 at 300 μM K201 for control and also for PMI channels. Statistical analysis shows that the corresponding partial probabilities do not differ for S1 and S2 when comparing PMI to control rats. The overall concentration dependence of the probabilities of S1 and S2 substates shows similar characteristics. The two substates are generated at the expense of the open state. Calcium release experiments were carried out to verify the single channel data measuring the initial rate of release from actively loaded SR vesicles. Calcium release was evoked by the addition of 10 μM K201. This K201 concentration was chosen because it introduces the (S1 and S2) substates while decreasing only slightly the common (50%±2.5% criteria) open probability. These initial release data reckon that the S1 and S2 subconductance states seen on the bilayer records are indeed calcium-conducting conformations, i.e., the calcium release channel is able to transport calcium ions in these substates.

3.3. The Effect of maurocalcine on RyR2

I next studied the effect of MCa on the gating properties of RyR2. Purified RyR2 was incorporated into an artificial lipid bilayer and MCa was added to the *cis* chamber which corresponds to the cytoplasmic face of the RyR2 channel. We previously showed that MCa affects the RyR1 gating behaviour by inducing: (i) an increase of the opening probability (P_o), and (ii) the appearance of characteristic LLSS (long-lasting subconductance states). These LLSS correspond to the opening

RyR1 at an intermediate conductance state for extremely long periods of time, up to a few seconds Current recordings were performed by imposing two ionic current directions, corresponding to (i) the physiological direction, from the RyR2 luminal side (*trans* chamber) to the cytoplasmic side, or (ii) the reverse direction, from the RyR2 cytoplasmic side to the luminal side. Physiological current direction corresponds to ionic flux from the luminal face of the RyR to its cytoplasmic face, whereas reverse current direction corresponds to an ionic flux from the cytoplasm face of the RyR to its luminal side. In the reversed direction of the ionic current, addition of MCa increased the P_o and induced the appearance of LLSS events on RyR2. Average single LLSS duration was 192.7±14.7 ms and frequency was 136,3±53,2/minute for RyR2. These results presented above show major differences in the effect of MCa on RyR2 compared with its effect on RyR1, which I discuss later.

4. DISCUSSION

4.1. Functional alterations of RyR1 in HF

Our group investigated, the steps in EC coupling of the skeletal muscle of control and PMI rats. Intramembrane charge movement and the depolarization- activated Ca^{2+} transients and spontanious elementary Ca^{2+} release events (ECRE, sparks and embers) were studied as well. There were no differences in the voltage dependence of the intramembrane charge movement between the sham-operated and PMI rats suggesting that the function and voltage dependence of the skeletal muscle-type DHPR are not altered after myocardial infarction. However, the global Ca^{2+} transients evoked by membrane depolarization displayed a characteristic reduction in the average amplitude and a lengthening of the rising phase, apparent as an increase in the TTP values, in PMI rats. There are at least three reasons that could underline this alteration:

(1) the coupling between the DHPR and the RyR is modified,

(2) the function, conductivity, and/or Ca^{2+} sensitivity of the RyR is altered, or

(3) the calcium content of the SR is reduced under the diseased condition.

To answer these questions, the elementary Ca^{2+}-release events and the function of the RyR were studied using confocal microscopy and lipid bilayer technique.

Both types of ECRE, sparks (short release with high amplitude) and embers (longer response with low amplitude), were detected after myocardial infarction; however, the relative proportion of the latter was considerably increased. In addition, the amplitude of the events was reduced in PMI rats, and moreover, the Ca^{2+} fluxes during the events were also depressed. Consistent with the increased time to peak of the global calcium transients in PMI rats, the rise time of the calcium sparks was also significantly lengthened in these animals. These observations are consistent with a reduced Ca^{2+} content of the SR or with alterations in RyR function. These results are consistent with the observations of Ward et al. who showed alterations in the spatiotemporal properties of spontaneous Ca^{2+} sparks in muscle from PMI rats. Populations of sparks from EDL fibers were significantly lower in amplitude and had slower temporal kinetics in CHF muscle, without evidence of alteration in SR Ca^{2+} load. Ward et al. also demonstrated in single channel experiments that RyRs are more active and show subconductive states indicating FKBP12 dissociation which was confirmed with western blott analysis. RyR1 in PMI muscle was PKA-hyperphosphorylated and depleted of FKBP12. They suggested that the altered global and local Ca^{2+} signaling seen in the PMI model results from a PKA-dependent mechanism inducing a disruption of the FKBP12 interaction with the RyR1 and RyR1-DHPR.

Our data on the single channel characteristics showed that the voltage dependence of the RyRs is altered in PMI rats in positive potentials. However, there is no difference in the voltage dependence between the two groups in the physiological (more negative) voltages. In addition, we did not observe any clear subconductance states in RyR gating questioning any significant depletion of FKBP12 of RyR1. One could, however, speculate that while the depletion of FKBP12 seen by Ward et al. was not enough to disrupt intersubunit interactions, it was sufficient to alter the coupling between neighboring channel complexes. This would explain the reduced number of channels seen in the elementary events of calcium release.

Another difference in the biophysical properties of the RyR is the Ca^{2+} dependence of the channel activation and inactivation. The Ca^{2+} dependence of the activation of RyRs isolated from PMI rats is slightly shifted to the left; the Ca^{2+} dependence of the channel inactivation is shifted to higher cytoplasmic Ca^{2+} concentrations meaning that the Ca^{2+} sensitivity of the channel is reduced in PMI rats. These data suggest that the function, conductivity, and Ca^{2+} sensitivity of the RyR are altered in PMI rats. Our data indicate that the single channel conductance of the control rats is around 520 pS in agreement with previous reports from our as well as from other laboratories. On the other hand, higher conductance-around 800 pS-has also been reported in a partially similar ionic environment. One explanation might be that in those measurements, HSR vesicles were fused with the bilayer with luminal [Ca^{2+}] of 53 mM, while we use CHAPS-solubilized RyR1 with luminal [Ca^{2+}] of 50 μM. In addition, the different redox state of the preparations may also contribute to the observed differences. Altered open probability and gating behavior of RyR1 of PMI rats were reported earlier and attributed to the different levels in their phosphorylation or that of FKBP without significant changes in the calcium current through RyR1 driven by the calcium gradient. In addition, PKA phosphorylation was reported to markedly increase the P_o and significantly elevate the specific conductance of RyR2 with an increase of about 39% in the single channel conductance. However, no rectifying effect of PKA phosphorylation was observed. Our data shows an increase of about 46% in the single channel conductance in a rectifying fashion; that is, increased conductivity is present only at positive voltages, when cations move from the cytoplasmic toward the luminal side of the channel. Taken together, our data are not inconsistent with the following framework of events. Because RyR conductance at negative voltages was found to be unaltered and SR calcium load was previously reported unchanged, the reduced amplitude and SM of calcium sparks together with an increased proportion of embers clearly indicates that in CHF, the number of calcium-release channels that open during a spontaneous calcium-release event is considerably reduced. Although the increased rise time of the calcium sparks may

intrinsically originate from their smaller amplitude, the altered Ca^{2+} concentration dependence of RyR inactivation observed in these experiments could also contribute. All in all, the reduction in functioning calciumrelease channels, their altered kinetics, and modified recruitment readily explain the suppression and the prolonged TTP of the global calcium transient in this animal model. These specific alterations in EC coupling could thus play a significant role in the skeletal muscle-specific force decrements and reduced exercise tolerance seen in humans and in experimental models of myocardial overload. Moreover, because cellular Ca^{2+} homeostasis has been shown to play a crucial role in the determination of muscle fiber type, these alterations in RyR1-dependent Ca^{2+} release may play a determinant role in the remodeling of the skeletal muscle that occurs secondary to chronic myocardial overload and CHF. These alterations in the Ca^{2+} handling of the skeletal muscle may answer the reduced contractility of skeletal muscle in patients with CHF, because our results are consistent with the idea that either the averaged number of RyR channels that open during a calcium-release event or the driving force for Ca^{2+} to leave the SR is (or both are) reduced in CHF.

4.2. Effect of K201 on RyR1

The derivative K201 demonstrates cardioprotective and antiarrhythmic effects. These have, at least partially, been attributed to the suppression of SR calcium efflux associated with RyR stabilization through FKBP. Although the interaction of K201 and the cardiac isoform of RyR has been described, its effect on RyR1 and the consequent alterations in skeletal muscle function remain elusive. Here, we demonstrate that K201 induces characteristic subconductance states in RyR1 and alters the elementary events of SR calcium release by reducing the frequency of sparks and increasing the occurrence of embers. These changes result in a decreased open probability of RyR with increasing K201 concentration, with an apparent K_d around 25 μM, if the open probability is defined in the usual way. Interestingly, RyR1 prepared from PMI rats behaves indistinguishably from control channels with respect to its K201 sensitivity.

4.2.1 Subconductance states of RyR1 in the presence of K201

To understand whether the effects of K201 on RyR1 could also be interpreted as decreasing the activity of leaky channels, similar to that in cardiac failure, one must consider the properties of the subconductance states induced by the drug. These states-with an approximate conductance of 13% and 24% of the fully open state-are clearly different from those induced either by ryanodine or the removal of FKBP12. Not only are the conductances of these states different from those mentioned above, but their appearance is voltage dependent, namely, they are not seen at negative voltages. Although this voltage dependence might seem to be a peculiarity, its importance is pivotal to interpret the results for in vivo conditions. If the membrane potential of the SR is negative enough, the subconductance states are not present, and the drug acts clearly as a stabilizer. If these states, on the other hand, are generated under in vivo conditions, the drug induces an extra, albeit small leak from the SR. It is not without precedent that the action of a substance depends on the direction of the membrane potential, i.e., on the direction of the movement of the charge carrier. These characteristics-a special kind of rectification-is essentially identical if the current is elicited by a voltage difference or by a concentration gradient. Although transient changes in membrane potential might be associated with the opening of RyR1 during the course of EC-coupling at the terminal cisternea of the SR and to a much lesser extent at the longitudinal part of the SR during the reuptake of the calcium, it is widely accepted that the SR has no permanent membrane potential. Unfortunately, the exact membrane voltage where the subconductance states appear cannot be determined using the bilayer technique due to the noise on the records in the −25 and +25 mV voltage range, rendering the physiological interpretation difficult. However, two independent observations seem to favor the presence of these substates at or around 0 mV SR membrane potential. First, calcium flux data demonstrated an increased calcium efflux from SR vesicles upon the addition of K201. Second, embers, the elementary calcium release events associated with the opening of a single RyR1,

showed resolvable levels within a single event. Although neither observation is conclusive, taken together, they indicate that the subconductance states are indeed present on calcium release channels in their native environment.

4.3. Effect of MCa on RyR2

In the present study, we evaluated the putative activation of RyR2 by MCa. We demonstrate that MCa is able to interact directly with purified RyR2. This interaction involves at least two domains homologous with those of RyR1 identified previously as MCa-binding sites. We show that MCa fails to promote Ca^{2+} release from cardiac HSR. Our results revealed major differences in the effect of MCa on RyR2 compared with its effect on RyR1: (i) in the physiological direction of the ionic current, neither LLSS events nor significant change of P_o were observed after addition of MCa on RyR2, and (ii) in the reversed direction of the ionic current, addition of MCa induced the appearance of LLSS events on RyR2 different from those observed on RyR1. Indeed, a statistical comparison of RyR1 and RyR2 LLSS induced by MCa in the reverse direction shows a much shorter duration of RyR2 single LLSS (192.7±14.7 ms for RyR2 compared with 12037±875 ms for RyR1). In the reverse direction current condition, no significant change of P_o value (measured during inter-LLSS periods) was observed in the presence of MCa.

In previous work, Ronjat et al characterized MCa as one of the most powerful effectors of RyR1 and identified two discrete domains (F3 and F7) of RyR1 responsible for the binding of MCa. In the present study, they show that despite the fact that MCa interacts with RyR2 on the domains homologous with F3 and F7, MCa is unable to induce the characteristic functional modifications that it promotes on RyR1. These results suggest that, whereas F3 and F7 domains are directly involved in the control of the RyR1 gating process, they exert a completely different control on RyR2 gating behaviour. Therefore MCa presents specificity for the skeletal compared with the cardiac RyR isoform in terms of functional effect, although it does not show specificity in terms of interaction. To our knowledge

MCa is the first molecule shown to interact with the homologous sequences of RyR1 and RyR2, and yet presents a completely different effect on each isoform. A significant effect of MCa on RyR2 conductance was observed when the imposed current was in the opposite direction compared with the physiological situation during Ca^{2+} release (positive potential). The subconductance events induced by MCa in these conditions were however much shorter than those observed on RyR1 at both positive and negative potentials. In its primary amino acid sequence, MCa presents a cluster of basic residues. Structural data show that these residues form a positively charged surface. Consequently, at positive potential, the electric field could provoke an accumulation of MCa at the vicinity of the pore region of RyR2 responsible for the conductance reducer effect observed.

The effects of MCa on RyR1 indicate that MCa strongly activates RyR1 channel opening. This effect could result from the direct implication of the MCa-binding sites (i.e. the F3 and F7 domains) in the pore-forming region of RyR1 or from the destabilization, following MCa binding, of an intramolecular 'brake' that would, through a distance effect, allow RyR1 opening. In the first hypothesis, the absence of an MCa effect on RyR2 would suggest a completely different three-dimensional topology of RyR2 channel moiety, excluding the homologous domains of F3 and F7 from the pore region. In the second hypothesis, the lack of MCa effect on RyR2 would highlight the presence on RyR2 of a different intra-molecular brake insensitive to MCa binding. Previous studies using chimaeric RyRs have shown that the functional importance of a specific domain of RyR1 differs when this domain is expressed in a RyR3 or RyR2 background, revealing that several domains of RyRs are involved in the channel-gating process. All together these results seem to favour the second hypothesis, the difference in MCa effect on RyR1 and RyR2 being due to the lack of signal transduction from the MCa-binding domains to the pore domain in RyR2.

Interestingly, it was recently demonstrated that MCa shares common binding sites on RyR1 with a domain of the cytoplasmic II-III loop of the DHPR α_1 subunit. Based on the effect of MCa on Ca^{2+} sparks in skeletal muscle cells and on the

closure kinetics of RyR1 following the repolarization of the plasma membrane, we proposed that this domain of the DHPR could be a regulator of the RyR1 internal brake. Therefore the absence of an effect of MCa on RyR2 could reflect a physiological difference in the role of the corresponding domain of the cardiac DHPR α_1 subunit in the control of RyR2 gating behaviour.

5. SUMMARY

In heart failure (HF) exercise intolerance characterized by skeletal muscle weakness and fatigue develops that could not be explained by the reduced muscle perfusion but rather by reduced Ca^{2+} content of the sarcoplasmic reticulum (SR) and the consequent reduction of calcium transients' amplitude, both indicating impaired calcium transport mechanisms. Our aim beside the identification of putative functional Ca^{2+}-release channel (RyR1) changes contributing to the symptoms was to elucidate the effect of a new drug K201 on channel gating. K201 has been suggested as a potential therapeutic agent in HF due to its antiarrhythmogenic action and ability to avoid muscle weakness in HF model animals. For these purpose single RyR1 channels from rats with HF were reconstituted into planar lipid bilayer and the gating behavior was studied under voltage-clamp conditions.

Significant portion of RyRs showed ~50% higher conductance compared to RyRs from control rats and the voltage dependence of the channel conductance was, showing still ohmic but rectifying, polarity dependent conductance. Altered Ca^{2+}-dependency of channel activity was also observed on RyRs from HF afflicted rats, such as reduced sensitivity to calcium dependent inactivation, which can lead to SR depletion.

K201 induced two subconductance states corresponding to approximately 24% (S1) and 13% (S2) of the maximum conductance. Dependence of event frequency and of time spent in S1 and S2 on the drug concentration was biphasic both in control and in PMI rats, with a maximum at 50 μM. At this concentration, the channel spends 26±4% and 24±4%, respectively, of the total time in these subconductive states at positive potentials, while no subconductances are observed at negative potentials. Taken together K201 action on RyR1 can be interpreted as a definite inhibition.

I also investigated the effect of a 33 amino acid toxin maurocalcine (MCa) on the gating properties of RyR from canine heart. MCa is a suitable research tool of electromechanical coupling because it mimics a DHPR segment responsible for allosteric coupling between DHPR and RyR in skeletal muscle. Our aim was to describe the potential differences of our results obtained on skeletal (RyR1) and cardiac (RyR2) type RyR in the presence of MCa. MCa induced long lived subconductive states (LLSS) of RyR2 channel, just like it did in the case of RyR1 with a slight difference: the duration of LLSSs are shorter and the frequency of LLSSs are higher compared to RyR1, indicating weaker electrostatic forces. These results highlight a different role of the MCa-binding domains in the gating process of RyR1 and RyR2.

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Data labeled with * are valid for 2007 because impact factors for 2008 are not available at present.