Thesis for the degree of Doctor in Phylosophy (Ph.D)

Modulation and alteration of the elementary calcium release events under normal and pathological conditions

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Introduction

Skeletal muscle structure and its physiological relevance

The skeletal muscle is responsible for locomotion - the physical movement of our body and is specialized for rapid force production. The cellular units of skeletal muscle are the myofibers, which are coordinated directly or indirectly by the nervous system and are capable of highly complex actions (e.g. contraction). In a muscle fiber the myofilaments are organized into myofibrils which are surrounded by the sarcoplasmic reticulum (SR). The transverse or T-tubules are plasma membrane invaginations and along with the terminal cisternae form the triads, which regulate the Ca^{2+}-release and transport. The terminal cisternae are the main site of Ca^{2+} storage in the muscle fiber and thus the triad is the structural core of the excitation contraction coupling (ECC).

Excitation-contraction coupling

The stimulation of the muscle fiber by the motor neuron leads to muscle contraction. The main steps linking both processes are well understood and are collectively referred to as excitation-contraction coupling. Briefly, changes in membrane permeability at the neuromuscular junction result in action potential propagation across the muscle fiber membrane system. The membrane depolarization triggers calcium release from intracellular stores causing a fast increase in the cytosolic free Ca^{2+} concentration. Subsequently, the released calcium binds to troponin initiating the muscle contraction. The calcium is eventually transported back to the intracellular stores promoting the muscle relaxation

The depolararization of the plasma membrane and the T-tubules is sensed by the dihydropyridine receptor (DHPR), an L-type voltage-gated Ca^{2+} channel present at those membranes. The DHPR charge movement promotes the activation of Ca^{2+} release from the SR, likely through a mechanical coupling with the ryanodine receptor calcium release channel (RyR) present at the

membrane of the SR terminal cisternae. The activation of RyR results in the increase of the cytoplasmic free [Ca^{2+}] which can activate the neighboring DHPR-uncoupled RyRs. The latter process is referred as **C**alcium **I**nduced **C**alcium **R**elease (CICR). In mammalian skeletal muscle there are three isoforms of RyR (RyR1, RyR2 and RyR3) with tissue specific distribution: RyR1 is predominantly expressed in skeletal muscle, RyR2 in heart muscle and almost all over in the brain, while RyR3 is universally expressed.

The elementary calcium release events

Ca^{2+}-release is confined to terminal cisternae and remains local due to the tight spatial relation of T-tubules and SR membranes which enables isolated function. The openings of one or some RyRs, that underlie the global calcium release and transient calcium concentration ($[Ca^{2+}]_i$) increase, are termed **E**lementary **C**calcium **R**release **E**events (ECRE). They can be subdivided into *spark*s and *ember*s. The *spark* is characterized by the relatively large amplitude, and short duration, while the *ember* is an event with long duration and small amplitude. In embryonal skeletal muscle the production of *spark*s require the presence of both RyR1 and RyR3. The adult muscle (which loses RyR3 during development) can not produce *spark*s under intact conditions.

Characteristic parameters

Today it is well known that the *spark* reflects the coupled gating of a RyR cluster, and as a consequence it carries essential information on the SR calcium release mechanism. The characteristic parameters of the *spark*s are the amplitude (A) expressed as $\Delta F/F_0$, the spatial half-width measured at the time of the peak (**F**ull **W**idth at **H**alf **M**aximal amplitude, FWHM), the **F**ull **T**ime at **H**alf **M**aximum (FTHM), the duration and rise time. In simple cases, when there are no channel reopenings, the rise time is a good measure of the SR release duration, while the signal mass index (SM(t)) is a measure of the released Ca^{2+}.

Chronic hear failure and skeletal muscle weakness

The **c**hronic **h**eart **f**ailure (CHF) is characterized by an energetic failure of both cardiac and skeletal muscles. In CHF, skeletal muscles develop a weakness that is not associated to an impaired circulatory function but rather alterations in the skeletal muscle fibers themselves. Recent reports suggest that modified Ca^{2+} handling, impaired ECC, Ca^{2+}-release and reuptake are responsible for the functional changes of muscle in heart failure which are the consequence of impaired RyR phosphorilation. Exercise intolerance is one of the major hallmarks of CHF. Some authors speculate that CHF causes specific decrease in skeletal muscle oxidative capacity, possibly because of impaired mitochondria function that's causing diminished exercise capacity. Also, there are suggested functional alterations, like reduced twitch tension and supressed SR function including anomalous calcium release form the SR, altered calcium reuptake and SR calcium load.

TPEN

N,N,N',N'- tetrakis (2-pyridylmethyl) etylenediamine (TPEN) was first mentioned in the chemical literature by Anderegg és Wenk (1967) but its properties as a membrane-permeable heavy-metal ion chelator were first used in experiments in biological systems by Arslan and colleagues (1985).

TPEN has very high affinities for heavy metals like Mn^{2+}, Fe^{2+}, Zn^{2+} but low affinities for Mg^{2+} and Ca^{2+} (K_ds are $10^{-10.3,}$ $10^{-14.6}$, $10^{-15.6}$, $10^{-1.7}$ and $10^{-4.4}$, Arslan et al., 1985). The facts that free TPEN is uncharged and membrane-permeable makes this compound particularly suitable for rapidly and reversibly chelating intracellular heavy metals and luminal calcium, where the free Ca^{2+} concentration is comparable with the K_d of TPEN, without influencing the cytosolic $[Ca^{2+}]$ that falls in the nanomolar range.

Even though several aspects of the effect of TPEN on cells have already

been examined, often resulting in conflicting conclusions, the interaction of one of the key Ca^{2+}-handling proteins, the RyR and TPEN has still been obscure.

Aims of the experiments

The work presented here deals with the study of certain steps of the excitation-contraction coupling mechanism under various intraluminal Ca^{2+}-concentrations. We were interested in the comparative study of the elementary calcium release events kinetics under normal and pathological conditions.

The problem can be approached from two directions: by increasing the SR Ca^{2+}-content or by decreasing it. In the present work we investigated the latter case, for which we used a membrane permeable, low affinity heavy metal chelator, namely N,N,N,N –tetrakis-(2-pyridylmethyl)-ethilendiamine (TPEN). This compound can rapidly buffer the intraluminal $[Ca^{2+}]$ and can be removed with a simple wash out.

Buffering the SR calcium will lead to the decrease of the amount released, process that can be well followed on the level of ECRE. We wanted to collect evidence that the alteration of the intraluminal $[Ca^{2+}]$ will affect the number of simultaneously opening Ca^{2+}-channels.

The work was performed on two different systems:

1. a myoblast cell line (C2C12 cells) was used to record whole cell calcium transients in the presence of different TPEN concentrations.

2. enzymatically (rat) or mechanically (frog) isolated, saponin treated single fibers were used to record elementary calcium release events.

In chronic heart failure along with the major hallmark of muscle weakness and fatigue, several studies have reported alterations in the ECC process and calcium handling mechanism. In the second part of my thesis, I performed a comparative study of the ECRE properties recorded on fibers obtained from healthy (smam operated) and a postmyocardial infarcted rat animal model (PMI).

Materials and methods

1. Cell and cell culture

1.1. Culturing the C2C12 mouse skeletal muscle cell line: Cells were cultured on **D**ulbecco **M**odified **E**agles's **M**edium (DMEM) containing 15% FCS, antibiotics and antimycotics. The cells were incubated at 37°C, 5% CO_2. Differentiation was induced by DMEM containing 5% FCS and 5% HS.

1.2. Preparation of single muscle fibers: Single fibers were either isolated enzymatically (collagenase, type I, Sigma, 1-$1^{1/2}$ hour at 37ºC) from the *m. digitorum communis* of the rat or dissected manually from the *m. semitendinosus* of the frog (*Rana esculenta*). Isolated skeletal muscle fibers were mounted into a chamber with glass bottom and treated with a modified relaxing solution containing 0.002% (for rats) or 0.004% (for frogs) saponin, respectively, for 2-3 minutes. Permeabilization of the surface membrane was monitored by the addition of 50 μM fluo-3 into the solution while imaging the fiber. This solution was then exchanged to an internal solution (K-glutamate or K_2SO_4-based solution supplemented with 0.1μM fluo-3) in which the experiments were carried out.

1.3. The postmyocardial infarcted animal model: Male *Wistar* rats were subjected to coronary artery ligation to produce PMI, and sham operated animals served as controls. The rats (180 g-220 g) were anesthetized (pentobarbital, 60 mg/kg) and ventilated. After left-median thoracotomy, the left anterior coronary was occluded at the most proximal position. Sham operated animals were subjected to the same surgical operation without any ligation of the coronary artery. PMI rats were used ~24 weeks after surgery and were arbitrary selected according to the visible level of remodelling estimated by the size (at least 50%) of the fibrotic area of the left ventricle.

2. Following the changes in $[Ca^{2+}]_i$

2.1. Single cell fluorescent calcium measurements: Fura-2 loaded into cells was alternately excited by 340- and 380 nm light by the DeltaScan™ apparatus of **P**hoton **T**echnology **I**nternational (PTI). Emitted light was detected at 510 nm by a photomultiplier. The ratio of the fluorescence measured after excitation at the two wavelengths ($R=F_{340}/F_{380}$) provided the intracellular calcium concentration. The parameters of the transient changes in the $[Ca^{2+}]_i$ (amplitude, latency, maximal rate of rise, time constant of decay, time to peak, full width at half maximum) were determined by a computer program written in our laboratory for this purpose (PTIana).

2.2. Calculation of the calcium flux (FL): Calcium flux was defined as the flux of calcium ions into the myoplasm from the extracellular space and the intracellular stores. FL was determined according to the formula $FL=d(Ca_{tot}+Ca_{transzp})/dt$, where Ca_{tot} denotes all the calcium in the myoplasm, $Ca_{transzp}$ stands for the amount of calcium trasnported by the calcium removal mechanisms (i.e. the pumps), which is proportionate to the relative saturation of the pumps ([Ca-pump]/[pump]), where the proportionality coefficient is the maximal rate of removal (PV_{max}). PV_{max} was determined separately for each cell examined, using an exponential fit on the descending segment of a KCl-evoked calcium transient, at least three seconds after the peak.

2.3. Confocal miscroscopy: Spontaneous calcium release events were recorded on the fluo-3 loaded cells with a laser scanning miscroscope (LSM 510 META, Zeiss, Oberkochen,Germany). Line-scan images (x,t) were taken with the following scanning parameters: 0.15 ms/line, 512 pixel/line (0.142 µm pixel) using a 63x water immersion objective (N.A=1.2) parallel to the fiber axis. Fluo-3 was excited with an argon ion laser (at 488 nm, 5% laser intensity), the emitted light was collected through a band-path filter and digitized at 12 bit.

The software: The analysis of the data was performed using an automatic computer detection method, developed in our department. The program

identified elementary events as regions with fluorescence above a relative threshold, calculated from the noise in the images, and having amplitudes greater than 0.2 $\Delta F/F_0$ (*spark*s) and 0.05 $\Delta F/F_0$ (*ember*s).

3. The lipid bilayer method

3.1. SR vesicle preparatiom: **H**eavy **S**arcoplasmic **R**eticulum (HSR) vesicles were isolated from *m. 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 40000 g, 30 min. After removing the actomyosin contamination, by dissolving it in 600 mM KCl, a microsome fraction was collected at 109000 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 h. HSR and LSR vesicles were resuspended and collected by centrifugation at 124000 g again. Fractions were collected, rapidly frozen in liquid nitrogen and stored at -70°C until further use.

3.2. Purification of the RyR complex: HSR vesicles containing ryanodine receptor complex were resuspended in 9 ml medium to a final protein concentration of ~ 3mg/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 subsequrntly the resulting supernatant (3-3 ml) was layered on the top of 10-28 % sucrose gradient. The extent of ryanodine receptor solubilization and the subsequent migration distance of the solubilized receptor in the sucrose gradient was monitored by labeling one part (3 ml) of the solubilized sample with [H^3] ryanodine. Unlabeled and 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 [H^3] 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.

3.3. Measurements of SR Ca^{2+}-ATPase activity: **L**ight **S**arcoplasmic **R**eticulum (LSR) vesicle fractions 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 phosphoenol-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 Mg Cl_2, 20 mM Tris HCl (pH=7,5), 7,5 U/ml pyruvate-kinase, 18 U/ml lactate-dehydrogenase, 0,42 mM phosphoenol-pyruvate, 0,2 mM NADH and 2 μM A23187 (Ca^{2+} ionofore). Total hydrolytic activity was measured as the decrease of optical density at the NADH absorbance wavelength (340 nm). Calcium pump activity was calculated from the slope of decreasing absorbance. The specific activity of calcium pump was determined by applying 5 μM thapsigargin. In the medium the free calcium concentration was about 2 μM.

4. Statistical analysis

To compare groups of experimental data Student's t-test was used, significant difference was declared at $p<0,05$.

Results

1. Effects of TPEN on C2C12 mouse myoblasts

1.1. The effectiveness of various TPEN concentrations: First, the possible differences in the mode of action of various concentrations of TPEN were tested on cultured C2C12 mouse myotubes. The response to a 100 mM KCl-evoked depolarization was used to test for cell viability.

On 18 % (28 cells) of the cells tested, the application of 5 μM TPEN caused a spontaneous Ca^{2+}-transient while 20 μM TPEN had similar effect on 50% (14 cells) of the cells. 50 μM TPEN caused a transient increase in $[Ca^{2+}]_i$ (a Ca^{2+} transient) in 9 out of 14 cells tested (64 %), and in 15 applications out of the 26 trials (58 %). In contrast to the capability of cells to respond to 50 μM TPEN, the application of 500 μM of the drug never induced any detectable Ca^{2+} transient in any of the cells tested (21 applications in 13 cells).

These results indicate that 50, but not 500 μM TPEN can activate the RyR protein complex in C2C12 myotubes. However, the lack of the ability of 500 μM TPEN to induce any detectable Ca^{2+} release does not mean that it failed to affect Ca^{2+} release altogether.

1.2. Effects of TPEN on the Ca^{2+}- removal from the myoplasm: The ability of the SR Ca^{2+} pump to decrease $[Ca^{2+}]_i$ was assessed by determining the time constants of the declining phases of transients and the underlying Ca^{2+} transport capacity of the pump.

Two parameters were changed in the presence of 500 but not by 50 μM TPEN. Namely, the amplitude of the transient decreased and the return of $[Ca^{2+}]_i$ to the resting level was slowed. To quantify the latter action, single exponential functions were fitted to the declining phase of the KCl-induced Ca^{2+} transients and the time constants (τ) were determined. The time constant of decay in the absence of TPEN (3.38±0.28 s, n=17) was not altered by 50 but was significantly increased by 500 μM TPEN.

Following a depolarization, the calculated maximal rate of Ca^{2+} extrusion from the cytoplasm by the pump was found to be 444±83 μMs^{-1} (n=10) when TPEN was not present. Normalizing the data obtained after the addition of the buffer to those of the control transient of the same cell we found that 50 μM TPEN slightly, although not significantly increased the relative PV_{max} (to 113.5±6.9 %, n=7). In contrast, 500 μM of the buffer caused a significant inhibition of the pump, with the relative PV_{max} dropping to 72.8±8.6 % of the control (n=15).

1.3. The effects of caffeine and KCl-depolarization in the presence of TPEN: Higher concentrations of TPEN seemed to suppress not only the removal of Ca^{2+} from the myoplasm but also the peak of the depolarization-induced Ca^{2+} transient and the underlying Ca^{2+} flux. The notion that 500 but not 50 μM TPEN putatively inhibits the Ca^{2+} release from the SR was further investigated by examining agonist- and KCl-evoked Ca^{2+} transients. We found that repetitive applications of 15 mM caffeine or 100 mM KCl in the extracellular solution of C2C12 myotubes resulted in non-desensitizing Ca^{2+} transients.

The presence of 50 μM TPEN was found to leave the relative amplitudes of both the KCl- and caffeine-evoked Ca^{2+} transients practically untouched, whereas 500 μM decreased them to 28.3±5.6% of control in the case of caffeine and to 10.3±3.2% of control in the case of KCl. This effect of TPEN was clearly not due to the presence of the solvent (0.5% DMSO – the highest concentration used in this study), as the relative amplitude of the KCl-evoked Ca^{2+} transients (1.21±0.05 and 1.13±0.1 in control and in the presence of DMSO; not significantly different from each other) were unaffected.

In sumarry, 50 μM TPEN hardly affected the activation of the RyR protein complex by its agonist caffeine or by depolarization. On the contrary, 500 μM of the drug dramatically decreased the amount of Ca^{2+} released from the internal stores.

1.4. Effects of TPEN on the amplitude of KCl-depolarization induced Ca^{2+}-transients in the presence of physiological and low extracellular $[Ca^{2+}]$:

Another series of experiments demonstrated that decreasing the Ca^{2+} content of the extracellular solution by more than half without changing the concentration of TPEN left the effect of the drug unaffected. 250 μM TPEN inhibited the repeated KCl-depolarizations from releasing as much Ca^{2+} as the control first stimuli, causing drops in relative amplitudes to 0.75±0.06 ($[Ca^{2+}]_e$=1.8 mM) and 0.86±0.05 ($[Ca^{2+}]_e$=0.8 mM) on average. These values were not statistically different from each other. On the other hand, statistically significant difference was found between either of the above-mentioned sets of data and the data obtained in 500 μM TPEN at $[Ca^{2+}]_e$=1.8 mM (0.44±0.05), even though the free TPEN concentration in the 250 μM solution at $[Ca^{2+}]_e$=0.8 mM is equal to the free TPEN concentration in the 500 μM at $[Ca^{2+}]_e$=1.8 mM, both calculating to be around 40 μM.

Thus these experiments suggest that it is the total rather than the free TPEN concentration that determines the effect as far as the modification of depolarization-evoked Ca^{2+} transients is concerned.

2. Effects of TPEN on enzymatically isolated rat muscle fibers

Since on intact skeletal muscle fibers the ECRE are scarce, we used saponin treated (0.002%) cells and sulfate based solutions (which is known to enhance the SR Ca^{2+}-release, without altering the ECRE parameters overall). As expected, both calcium *spark*s and *ember*s were readily observed under control conditions. Surprisingly, the addition of TPEN resulted in suppression rather than augmentation of spontaneous activity. A total of 574, 159 and 197 *spark*s and 154, 6 and 0 *ember*s were detected and analyzed under control conditions, and in the presence of 50 and 500 μM TPEN, respectively.

In control, *spark*s occured with an appreciable frequency of 0.032±0.005s^{-1}sarcomere^{-1} and displayed all the characteristics parameters

described earlier in the literature. Although the frequency did not change significantly in the presence of 50 μM TPEN ($0.031\pm0.014 s^{-1}$sarcomere^{-1}, $p>0.9$), increasing the concentration of the buffer to 500 μM significantly reduced the frequency ($0.021\pm0.026 s^{-1}$sarcomere^{-1}, $p<0.05$). Together with the decrease in the number of events their morphology was also altered by TPEN with significant suppression of event amplitude, duration and FWHM for the latter concentration.

3. Effects of TPEN on mechanically isolated frog muscle fibers

3.1. Effects of TPEN in low $[Mg^{2+}]_i$: A total of 4365, 9885 and 286 *spark*s were detected and analyzed under control conditions and in the presence of 50 and 200 μM TPEN, respectively. Low concentrations of the buffer (50 μM) clearly enhanced, while higher concentrations (200 μM) inhibited the spontaneous activity of the fibers as indicated by changes in most of the parameters of Ca^{2+} *spark*s from which the most striking was the change in event frequency. While under control conditions the frequency was 0.23 ± 0.05 s^{-1}sarcomere^{-1}, in the presence of 50 μM TPEN it increased to 143% of control. 200 μM of the drug, on the other hand, decreased it to 39 % of control. Not only did the application of 50 μM TPEN modify the frequency, it also clearly altered the morphology of Ca^{2+} *spark*s, namely, the presence of the drug gave rise to propagating Ca^{2+} *spark*s. The velocity of propagation of Ca^{2+} release could be calculated from the cotangent of the angle of the *spark*'s wave front. Its average was found to be 273 ± 7 μm/s (n=62), in good agreement with previous results.

The characteristic parameters of Ca^{2+} *spark*s were also altered by the buffer. Control values for the duration (41.3 ± 0.2 ms), FWHM (2.12 ± 0.01 μm) and amplitude (0.92 ± 0.005) were modified to 102.3%, 113.6% and 84.7% of the control by 50, and to 73.4%, 82% and 70.6% of the control by 200 μM TPEN, respectively. The increase in FWHM in 50 μM TPEN presumably reflects an

increased number of neighboring release channels that were opened by the released Ca^{2+}, that is, a more effective CICR during the event.

In addition, propagating events were never observed in the presence of 200 μM TPEN. These clearly indicate that the effects of the higher concentration cannot simply be attributed to an increase in the buffering capacity, rather to a qualitatively different effect at 200 μM of TPEN.

3.2. Effects of TPEN in elevated $[Mg^{2+}]_i$: To further the concept that the presence of CICR is important in the effect 50 μM TPEN, measurements were carried out after slightly elevating $[Mg^{2+}]_i$ (to 5.5 mM) in the internal solution to obtain a condition which is known to suppress CICR. 50 μM TPEN had essentially the opposite effects to those measured at lower $[Mg^{2+}]_i$. The buffer suppressed rather than increased both the frequency and the FWHM of Ca^{2+} *spark*s. In addition, a slight increase in event amplitude was also observed.

4. Effects of TPEN on isolated RyRs

Our data suggested that TPEN might directly influence the activity of the calcium release channel itself. To test this hypothesis isolated Ca^{2+} release channels (RyR1 tetramers) were incorporated into planar lipid bilayers and the properties of channel gating were determined.

At 470 nM (ionized) Ca^{2+} concentration the channels displayed low open probability (P_o=0.0076 ± 0.0018). Following the addition of 50, 100, and 200 μM TPEN the open probability increased, demonstrating a direct activation of the Ca^{2+} release channel by the drug at negative membrane potentials.

At positive potentials, +85 mV, the effect of TPEN was quite opposite, since the addition of 50 μM of the drug resulted in a substantial decrease in open probability from $P_{o,contr}$ = 0.208 ± 0.024 to $P_{o,TPEN}$ = 0.0014 ±0.002 while at –85 mV the activation was still present.

5. Effects of TPEN on the SR Ca^{2+}-pump

Our data on C2C12 cells were suggesting that TPEN might interfere with the reuptake of calcium into the SR since it slowed the decay of the calcium transients following a KCl-induced depolarization on C2C12 cells. To confirm that this was indeed due to a direct interaction of TPEN with the calcium pump, the activity of the SERCA pump was examined in isolated preparations.

To estimate the magnitude of the effect, the hydrolytic activity of HSR vesicles was determined at different TPEN concentrations. In 5 independent experiments we found that TPEN inhibits the ATPase activity of the calcium pump.

6. Altered elementary calcium release events on PMI rat fibers

Under control conditions (sham operated rats), ECRE occurred with an appreciable frequency of 0.038 ± 0.001 s^{-1} $sarcomere^{-1}$ (733 images from 10 fibres) and displayed all the morphological characteristics described earlier. In PMI rats the frequency increased slightly but not significantly to 0.041 ± 0.001 s^{-1} $sarcomere^{-1}$ (522 images from 8 fibres, $p > 0.3$). On the other hand, the ratio of *spark*s to *ember*s was altered. While in control animals 73.8% of the events were *spark*s, this was decreased to 55.1% in PMI rats.

A total of 1284 *vs* 937 *spark*s from 10 *vs* 8 fibres (6 *vs* 4 animals) were analyzed under control conditions and in PMI rats, respectively. In PMI rats the average amplitude of *spark*s decreased by 40% leaving the FWHM essentially constant. The decrease in *spark* amplitude was attributable to a decrease in the number of large *spark*s. Together with the decrease in amplitude the rise time of the *spark*s increased significantly. The amplitude of *ember*s was also decreased in PMI rats, by 20%, and there was a small increase of FWHM comparing the values obtained from sham-operated rats.

To estimate the calcium flux during the events, the rate of production of SM(t) was determined using the equation:

$SM(t)=1{,}206\cdot A(t)\cdot FWHM(t)^3$

Signal mass production was estimated as the slope of the straight line fitted to the rising phase of SM(t). Selecting the 10 largest *spark*s, assumed to represent events closest to the confocal plane, the average rate of signal mass production calculated from control *spark*s was significantly greater than that of PMI rats (2.59 ± 0.24 and 1.42 ± 0.25 $\mu m^3\ ms^{-1}$, respectively; $p<0.01$).

Discussion

1. Effects of TPEN on the Ca^{2+}-homeostasis of C2C12 cells

Single cell $[Ca^{2+}]_i$ measurements indicate that low concentrations of the drug (20 μM, 50 μM) evoke a transient release of Ca^{2+} into the myoplasm in the majority of cells and at most attempts. On the other hand, high concentrations (500 μM) of TPEN not only fail to evoke Ca^{2+} transients, but they seem to hinder the depolarization- as well as the caffeine-induced release of Ca^{2+} from the SR. Moreover, the removal of Ca^{2+} from the myoplasm following a transient increase is also inhibited by this concentration of the buffer.

To understand the mode of action of TPEN on the RyR protein complex, the first question is whether the free or the Ca^{2+}-bound form of the buffer exerts the effects. Our experiments carried out after decreasing the extracellular $[Ca^{2+}]$ proved that it is the amount of the total rather than the free or Ca^{2+}-bound TPEN concentration that determines the effect of the drug on Ca^{2+} release. On the other hand, we do not know exactly how increasing the total TPEN concentration switches to inhibition following its enhancing effect at low concentrations.

Although an appealing possibility, we at this point cannot conclude that while low concentrations activate, high concentrations of the buffer directly inhibit the RyR protein complex. Our results are not sufficient to exclude the possibility that high concentration of TPEN would accumulate in the SR as membrane impermeable Ca^{2+}-TPEN and produces all the experimental results we observed by simply decreasing the amount of releasable intraluminal Ca^{2+}. The thus lowered intra SR $[Ca^{2+}]$, together with the simultaneous blockage of the Ca^{2+} pump, could explain our observations. It is certain, however, that either the intra SR buffering of Ca^{2+} or the direct inhibition of the RyR protein complex is present (or both are) besides the inhibition of the pump, since the sole inhibition of the SERCA pump should result in an increase in $[Ca^{2+}]_i$ due to

leakage from the SR, similarly to what is seen upon the administration of other blockers of the SR Ca^{2+} pump, like thapsigargin.

Despite the uncertainties as to the mode of action of the drug, our experiments clearly prove that it is the total rather than the free TPEN concentration that determines its capacity to alter Ca^{2+} transients. The range of putative effects as well as that of the effective concentrations of TPEN is wide, and their understanding seems essential in elucidating certain aspects of cellular Ca^{2+} homeostasis.

2. Effects of TPEN on permeabilized fibers

Our experiments on skeletal muscle fibers indicated that 50 μM TPEN is able to promote spontaneous Ca^{2+} release events. The buffer not only increased the frequency of Ca^{2+} *spark*s but it also induced propagating events on frog skeletal muscle fibers. Importantly, these effects were neither seen on mammalian skeletal muscle nor did they appear on frog fibers when $[Mg^{2+}]_i$ was elevated to 5.5 mM. These observations clearly indicate that TPEN promoted CICR, that is, the further activation of RyR by calcium. In line with this observation the buffer at 50 μM had a more pronounced effect on the isolated channel incorporated into planar lipid bilayers when the *cis* $[Ca^{2+}]$ was 50 μM as compared to that when it was 470 nM.

Further increasing the concentration of TPEN had, in contrast, an inhibitory effect on Ca^{2+} release events even on frog skeletal muscle at the lower $[Mg^{2+}]_i$ tested. This manifested in the decrease in event frequency as well as in the suppression of the amplitude and FWHM of Ca^{2+} *spark*s. At least three independent effects could be responsible for this observation. If increased TPEN concentration would further stimulate the opening of RyR it could result in the depletion of calcium in the SR. TPEN could inhibit the reuptake of calcium into the SR again resulting in the depletion of the internal stores. Finally, the buffer

could have an inhibitory effect on RyR at higher concentrations. Measurements conducted on isolated systems gave indication for all above possibilities.

3. Effects of TPEN on isolated RyRs

Examination of isolated RyR in lipid bilayers revealed that at negative membrane potentials increasing the concentration of TPEN on the *cis* side results in greater open probability of the RyR when *cis* [Ca^{2+}] was low.

Our data clearly establish that TPEN inhibits the hydrolytic activity of the SR Ca^{2+} pump. This inhibition, however, occurs at relatively high, EC_{50} is greater than 600 μM, concentrations of the buffer.

Taken together, the experiments on Ca^{2+} release events and on lipid bilayers suggest a possible mode of action for the buffer. Assuming that TPEN sensitizes RyR towards both the activating and inactivating Ca^{2+}, increasing TPEN concentrations would generate an increasing leftward shift of the bell-shaped Ca^{2+}-activation and -inhibition curve described in numerous reports (e.g. Sarkozi et al, 2000). At low [Ca^{2+}], one, therefore, obtains an increasing activation with increasing buffer concentration describing only the rising phase of the bell-shaped curve. At higher [Ca^{2+}] the declining phase of the Ca^{2+}-activation and -inhibition curve of the RyR is also reached, that is, an inhibition at higher TPEN concentrations is observed.

4. Elementary calcium release events on PMI rats

Both types of the events, *spark*s and *ember*s, were detected following myocardial infarction, however, the relative proportion of the latters was considerably increased.

Our data about the elementary Ca^{2+} release shows a complex modification of the Ca^{2+} handling in PMI rats. One of the major things the probability of the *spark*s and *ember*s of the total elementary events is extremely different in the two animal groups. In the sham-operated rats only 26% of the events were long

events with reduced amplitude (*ember*), but this parameter increased to 45% in PMI rats. This data suggest minimum two statements: 1) the SR Ca^{2+} content reduced in PMI rats and 2) the intra- and intermolecular connections in the RyR are modified after myocardial infarction. The changed signal mass and amplitudes of events suggest these statements also.

It is tempting to speculate that the specific alterations in EC coupling seen here could 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 fibre type, these alterations in RyR1-dependent Ca^{2+} release may play a determinant role in the remodeling of 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.

Summary

The aim of our experiments was the investigation of the calcium homeostasis of cultured skeletal muscle myotubes and adult mammalian and amphybian skeletal muscle fibers in the presence of a low affinity heavy metal chelator – TPEN. We were also interested in studying the properties of elementary calcium release events in a chronic heart failure rat animal model.

On C2C12 mouse myotubes in culture, we have shown that low concentrations (20, 50 μM) of TPEN increased the responsivness and the amplitude of Ca^{2+}-transients. In contrast, when applying the drug in higher concentrations (500 μM), it seemed to suppress not only the removal of Ca^{2+} from the myoplasm but also the peak of the depolarization- and agonist-induced Ca^{2+} transients and the underlying Ca^{2+}-flux.

Further experiments carried out on mammalian and amphybian single skeletal muscle fibers were meant to investigate the properties of the building blocks of the global Ca^{2+}-release, namely the elementary calcium release events (ECRE). On amphybian skeletal muscle fibers we have shown that in the presence of lowered $[Mg^{2+}]_i$, low concentraction of TPEN (50 μM) altered the characteristic parameters of ECRE, increasing the frequency and the FWHM, decreasing the amplitude, but most importantly, evoking travelling *spark*s. These travelling *spark*s (also called *macrospark*s) were never observed in case of mammals or when using slightly increased $[Mg^{2+}]_i$.

Several studies have reported that under pathological conditions, like chronic heart failure, characterized with altered Ca^{2+}-homeostasis properties, the ECREs are showing modified freqvencies. In our hands, confocal microscopy experiments carried out on single skeletal muscle fibers obtained from a CHF animal model, confirmed these observations. The *spark-ember* ratio changed dramatically compared to the control group, along with the number of

simultaneously opening channels, which were significanly decreased in the PMI rats.

Taken all this together, we think that our data can contribute to the better understanding of the putative effects of TPEN on the calcium release mechanism, and with this to elucidate certain aspects of Ca^{2+}-homeostasis in skeletal muscle cells under normal and pathological conditions.

Publications

Note: The author used her maiden name (Fehér) before March 2007.

Publications, lectures and posters used in these theses.

PUBLICATIONS:

Sztretye M., Almássy J., Deli T., Szentesi P., Jung C., Dienes B., Simut C.A., Niggli E., Jóna I., Csernoch L. (2009): Altered sarcoplasmic reticulum calcium transport in the presence of the heavy metal chelator TPEN. *Cell Calcium,* doi:10.1016/j.ceca.2009.10.002 [IF:4,481]

Sztretye M., Deli T., Szentesi P., Szigeti Gy.P., Csernoch L. (2008): Effect of TPEN on the calcium release of cultured C2C12 mouse myotubes. *J. Muscle Res. Cell Motil.* 28(7-8):421-8 [IF:1,731]

Szigeti Gy. P., Almássy J., **Sztretye M.**, Dienes B., Szabó L., Szentesi P., Sárközi S., Jóna I., Vassort G., Csernoch L. (2007): Alterations in the calcium homeostasis of skeletal muscle from postmyocardial infarcted rats. *Pflügers Arch.* 455(3):541-53. [IF: 3,842]

LECTURES

Sztretye M., Szentesi P., Csernoch L. (2008): Effect of the low affinity Ca^{2+} chelator TPEN on Ca^{2+} release from the sarcoplasmic reticulum in skeletal muscle. *Journal Club*, Rush University Medical Center, Department of Molecular Biophysics & Physiology, Section of Cellular Signalling, Chicago, USA.

Fehér M., Szentesi P., Csernoch L. (2007): A vázizom kalciumhomeostasisának vizsgálata miokardális infarktuson átesett patkányokon. University of Debrecen, Medical School and Health Science Center, Ph.D. conference, Debrecen.

Fehér M., Szentesi P., Csernoch L. (2006): Tovaterjedő elemi kalciumfelszabadulási események egy alacsony affinitású kalciumpuffer (*TPEN*) jelenlétében harántcsíkolt izmokon. University of Debrecen, Medical School and Health Science Center, Ph.D. conference, Debrecen.

Fehér M., Csernoch L. (2006): Effects of TPEN on Excitation-Contraction Coupling in mammalian and amphibian skeletal muscle fibers. *IHP Training Unit on Animal Welfare and Animal Models*, University of Siena, Italy.

POSTERS:

Szigeti Gy.P., **Fehér M.**, Szentesi P., Dienes B., Szabó L., Csernoch L. (2006): Changes in the calcium homeostasis of skeletal muscle from postmyocardial infarcted rats. *New frontiers in basic cardiovascular research conference*, Debrecen.

Fehér M., Dienes B., Szabó L., Szentesi P., Csernoch L. (2006): The study of skeletal muscle fibers calcium homeostasis in the presence of a low affinity calcium chelator. *Magyar Élettani Társaság LXX Vándorgyűlése (LXXth Meeting of the Hungarian Physiological Society)*, Szeged.

Szentesi P., **Fehér M.**, Almássy J., Dienes B., Jóna I., Csernoch L. (2006): Altered sarcoplasmic reticulum calcium transport in the presence of the heavy metal chelator TPEN. *Gordon Conference*, Colby-Sawyer College New London, USA.

Publications, lectures and posters NOT used in these theses.

PUBLICATIONS:

Sztretye M., Manno C., Ríos E. (2009): Buffering by BAPTA promotes intracellular calcium release operated by membrane depolarization in mouse muscle. *J. Physiol* (submitted). [IF: 4,540]

Bannwarth M., Corrêa Jr I. R., **Sztretye M.**, Pouvreau S., Fellay C., Aebischer A., Royer L., Ríos E., Johnsson K. (2009): Indo-1 derivatives for local calcium sensing. *ACS Chemical Biology*. 4(3):173-190. [IF: 4,741]

Fodor J., Gönczi M., **Sztretye M.**, Dienes B., Oláh T., Szabó L., Csoma E., Szentesi P., Szigeti Gy.P., Marty I., Csernoch L. (2008): Altered expression of triadin 95 causes parallel changes in localized Ca^{2+} release events and global Ca^{2+} signals in skeletal muscle cells in culture. *J. Physiol.* 586(Pt 23):5803-18. [IF: 4,580]

Lukács B., **Sztretye M.**, Almássy J., Sárközi S., Dienes B., Mabrouk K., Simut C.A., Szabó L., Szentesi P, De Waard M., Ronjat M., Jóna I., Csernoch L.

(2008): Charged surface area of maurocalcine determines its interaction with the skeletal ryanodine receptor. *Biophys. J.* 95(7):3497-509. [IF: 4,627]

Almássy J., **Sztretye M.**, Lukács B., Dienes B., Szabó L., Szentesi P., Vassort G., Csernoch L., Jóna I. (2008): Effects of K-201 on the calcium pump and calcium release channel of rat skeletal muscle. *Pflügers Arch.* 457(1):171-83. [IF: 3,842]

Deli T., Varga N., Ádám A., Kenessey A., Rásó E., Puskás L. G., Tóvári J., Fodor J., **Fehér M.**, Szigeti Gy.P., Csernoch L., Tímár J. (2007): Functional genomics of calcium channels in human melanoma cells. *Int. J. Cancer.* 121(1):55-65. [IF: 4,555]

LECTURE:

Fodor J., **Sztretye M.**, Dienes B., Gönczi M., Szentesi P., Csernoch L. (2007): Suppressed Trisk 95 expression modifies E-C coupling in rat primary myotubes. *Magyar Élettani Társaság LXXI Vándorgyűlése (LXXIst Meeting of the Hungarian Physiological Society)*, Pécs.

POSTERS:

Sztretye M., Yi J., Royer L., Zhou J., Knollmann B., Allen P.D., Protasi F., Ríos E. (2010): D4cpv-Casq1. A novel approach to targeting biosensors yields detailed dynamic imaging of calcium concentration inside the sarcoplasmic reticulum of living cells. *Biophysical Society 54rd Annual Meeting*, San Francisco, California, USA.

Sztretye M., Royer L., Manno C, Zhou J., Knollmann B., Allen P.D, Protasi F., Ríos E. (2010): Ca depletion and ablation of calsequestrin similarly increase the evacuability of the Ca store of skeletal muscle. *Biophysical Society 54rd Annual Meeting*, San Francisco, California, USA.

Manno C., **Sztretye M.**, Ríos E. (2010): Effects of high [BAPTA] inside mouse muscle fibers reveal a role of calcium in the termination of voltage-operated calcium release from the SR. *Biophysical Society 54rd Annual Meeting*, San Francisco, California, USA.

Sztretye M., Yi J., Royer L., Pouvreau S., Zhou J., Ríos E. (2009): Targeting biosensors for the monitoring of calcium inside the sarcoplasmic reticulum of adult murine skeletal muscle. *Gordon Conference*, Waterville Valley, New Hampshire, USA.

Sztretye M., Pouvreau S., Bannwarth M., Corrêa Jr I. R., Fellay C., Aebischer A., Royer L., Johnsson K., Ríos E. (2009): Indo-1 hybrid biosensors for calcium monitoring in cellular organelles. *Biophysical Society 53rd Annual Meeting*, Boston, Massachusetts, USA.

Szentesi P., **Sztretye M.**, Dienes B., Németh R., Jenes Á., Vincze J., Kertai P., Csernoch L. (2008): Effects of KOENZIM Q10 in fluvastatin therapy induced modified calcium homeostasis in rat skeletal muscle fibers. *Magyar Élettani Társaság LXXII Vándorgyűlése (LXXIInd Meeting of the hungarian Physiological Society)*, Debrecen.

Gönczi M., Fodor J., **Sztretye M.**, Dienes B., Szabó L., Szentesi P., Csernoch L. (2007): The role of triadin 95 on calcium homeostasis of skeletal muscle cells. *Joint meeting of The Slovak Physiological Society, The Physiological Society and The Federation of European Physiological Societies*, Bratislava, Slovakia. (Abstract: *Acta Physiologica* 2007. Vol.191, Supplement 658).

Dienes B., Fodor J., Bihari J., Oláh T., **Sztretye M.**, Gönczi M., Szentesi P., Csernoch L. (2007): Trisk 95 overexpression in rat primary myotubes suppresses E-C coupling mechanism. *Magyar Élettani Társaság LXXI Vándorgyűlése (LXXIst Meeting of the Hungarian Physiological Society),* Pécs.

Csernoch L., Fodor J., **Fehér M.**, Dienes M., Deli T., Szentesi P., Szabó L., Marty I. (2007): Suppressed SR calcium release and modified elementary calcium release events in Triadin-overexpressing cultured myotubes, *Biophysical Society 51st Annual Meeting*, Baltimore, Maryland, USA (Abstract: *Biophys. J.,* 92 Suppl., 79a).

Szentesi P., Almássy J., **Fehér M.**, Dienes B., Simuţ C.A., Jóna I., Ronjat M., Csernoch L. (2006): Mutations in the scorpion toxin maurocalcine alter its ability to modify the calcium release events in frog skeletal muscle, *XXXVth European Muscle Conference*, Heidelberg, Germany (Abstract: *J. Muscle Res. Cell Motil.* 27:516-517).

Szentesi P., Fodor J., **Fehér M.**, Dienes B., Szabó L., Csernoch L. (2006): Triadin modifies EC-coupling in C2C12 myotubes. *XXXVth European Muscle Conference*, Heidelberg, Germany.

Csernoch L., Lukács B., **Fehér M.**, Dienes B., Szentesi P., Jóna I. (2006): Mutations in the scorpion toxin maurocalcine alter its ability to modify the calcium release events in frog skeletal muscle. *Gordon Conference*, Colby-Sawyer College New London, USA.

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