

Short Thesis for the Degree of Doctor of Philosophy (PhD)

**Alteration of the Ca^{2+} -homeostasis in skeletal muscle in
case of changes in the expression of regulatory proteins**

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**UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR MEDICINE**

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the expression of regulatory proteins**

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Introduction

The skeletal muscle

In vertebrates the skeletal muscle system has a prominent role in performing different movements. During differentiation the single muscle cells (myoblasts) fuse into multinucleated myotubes, and then form elongated, cylindrical muscle fibers. Contraction of the fibers is evoked by the process termed excitation-contraction coupling (ECC).

In skeletal muscle ECC begins when the depolarization of the transverse (T-)tubular membrane initiates the release of calcium from its intracellular store, the sarcoplasmic reticulum (SR) via specialized Ca^{2+} release channels known as ryanodine receptors (RyR). RyR activation is achieved by the depolarization-evoked conformational change of the dihydropyridine receptors (DHPR) localized in the T-tubular membrane. Calcium ions then bind to the regulatory sites on troponin-C to initiate a conformational change which in turn enables the binding of myosin to actin and, ultimately, results in muscle contraction.

The myostatin

Myostatin, also known as growth/differentiation factor- 8 (GDF-8) is a member of tumour growth factor β (TGF- β) family. This protein is a homodimer with a molecular weight of 25 kDa and a disulfide bond between the monomers at the C-terminal regions. Myostatin circulates in the blood in a latent form with an additional noncovalently bound propeptide at the N-terminus. Proteolytic cleavage of the propeptide by the bone morphogenetic protein (BMP)-1/tolloid family of metalloproteinases is necessary for activation of protein function. The role of myostatin in skeletal muscle was discovered using the method of gene disruption in mice. Mstn null animals showed significant increase in muscle mass (up to two-fold) and decrease of fat tissue compared to the wild type. Similar effects were observed in the presence of natural mutations of Mstn in cattle, sheep, dogs and humans and upon the inhibition of the protein function in adult mice. At the same time, overexpression of Mstn led to the reduction of muscle mass suggesting myostatin to be a negative regulator of skeletal muscle growth. During embryogenesis, myostatin is exclusively expressed in skeletal muscle to control the differentiation and proliferation of the myoblast, but in adulthood, it is not only restricted to skeletal muscle but also detected in other tissues (e.g. heart, adipose tissue, mammary gland).

Myostatin is an extracellular cytokine, and as many other members of the TGF- β family, it mediates the signal through activin receptors. Active myostatin mostly binds to the ActRIIB and engages the signalling cascade leading to the inhibition of myoblast differentiation and

proliferation. ActRIIB can mediate other signalling pathways with diverse affinity for ligands—high for activin A and GDF11, low for BMP-2 and BMP-7. These ligands are responsible for several cell responses by activating different members of the Smad family of transcription factors. Activin receptors are transmembrane threonine/serine kinases divided in two types. Type I receptor (ALK 4 and ALK 5 for myostatin) has the unique GS domain located closely to the intracellular space and adjacent to the kinase domain which is absent in the second type (ActRII). Binding of myostatin with ActRIIB causes its assembly with type I receptor and phosphorylation of its GS domain. Therefore, the signal of myostatin is mediated through activated complex of two receptors. Myostatin signalling through ActRIIB is crucial for the regulation of muscle growth. The natural defect in ActRIIB sensitivity in humans leads to a significant increase in muscle mass. The same effect was observed in the experiments that used blockade of the murine receptor. In the characterization of the ActRIIB role in myostatin signalling, the soluble form of this receptor (sActRIIB) is used. sActRIIB is a fusion protein of the receptor extracellular domain with immunoglobulin Fc. It acts as a decoy receptor for myostatin. Healthy mice treated with sActRIIB showed a 60% increase in muscle mass, just 2 weeks after treatment initiation.

Muscle fibres during development are formed by the fusion of myoblasts. These cells express the transcription factor termed myoblast determination protein (MyoD) which together with other myogenic regulatory factors is involved in the determination and differentiation of skeletal muscle. Myostatin - originally named growth and differentiation factor-8 (GDF-8) -, a member of the transforming growth factor β (TGF- β) superfamily has emerged as a potent negative regulator of skeletal muscle growth. It plays a key role in skeletal muscle homeostasis and has been well described since its discovery. During embryogenesis, myostatin is exclusively expressed in skeletal muscle to control the differentiation and proliferation of the myoblasts. It mediates the cell signalling cascade through activin receptors in the muscle, which interfere with MyoD activity and expression. Recently, myostatin has also been shown to inhibit Akt, a kinase that is sufficient to cause muscle hypertrophy, in part through the activation of protein synthesis.

Myostatin was first identified when null-mutant knock-out mice exhibited a huge increase in skeletal muscle mass due to hypertrophy and hyperplasia. Mutations causing hyper muscularity in the double-muscled Belgian Blue and Piedmontese cattle breeds were also found in the myostatin gene and the presence of modifier genes was also indicated in these spontaneous mutants. In a similar finding, whippet dogs with excessive muscle growth were found to have

a heterozygous naturally occurring mutation. Hypermuscular mice with naturally occurring mutations in the myostatin gene, including the so called ‘Compact’ (*Cmpt*) mouse strain, were also found by several groups.

Sequencing of the myostatin gene of the *Cmpt* mouse revealed a 12-bp deletion – thus the name MstnCmpt-dl1Abc – in the propeptide region of the myostatin precursor. This deletion together with the Compact trait can be considered as the mutation responsible for the hypermuscular phenotype. In homozygous *Cmpt/Cmpt* mutants, the activity of mature myostatin is present, enabling genes modifying the expression of myostatin or modulating downstream signalling to have a significant influence on the hypermuscular phenotype.

Amthor et al. (2007) showed, that despite a larger muscle mass relative to age-matched wild types, there was no increase in maximum tetanic force generation, rather, when expressed as a function of muscle size (specific force) EDL muscles of myostatin knock-out (Mstn^{-/-}) and *Cmpt* mice were weaker than wild-type muscles. Muscles from Mstn^{-/-} animals had a marked increase in the number of type IIb fibres as compared to wild-type controls. They suggested that in case of myostatin deficiency decreased force production is accompanied by loss of oxidative characteristics of skeletal muscle.

It is, therefore, reasonable to suggest that modification in one or more of the events leading to shortening (i.e. in ECC) could be the underlying reason for the above described alterations in muscle performance of *Cmpt* mice. Especially interesting in this respect is the fact that RyR-s are highly sensitive to changes in the redox state of the myoplasm. Nevertheless, the steps in ECC have not yet been investigated in these animals.

The connection between MTM1 and PtdInsPs

The function of phosphoinositides had for a long time remained limited to the possible role of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) as a substrate of phospholipase C to produce inositol-trisphosphate (Ins(1,4,5)P₃) and diacylglycerol. The last decade has seen a remarkable expansion of the concept so as to now include the role of PtdInsPs phosphorylated in the 3-OH position, which control a wide array of cellular functions including membrane trafficking, cytoskeleton remodeling, protein docking, and membrane transport, and numerous studies have also. This basic functional scheme is either known or presumed to be controlled, regulated or modulated by a wide array of accessory proteins and messenger molecules. The possible role of PtdInsPs in the regulation of Ca²⁺ homeostasis and excitation–contraction (E–C) coupling in muscle remained elusive until recent years. Indeed, if one excludes the

controversies related to the possible role of the Ins(1,4,5)P₃ pathway, only very few studies in the 1990s suggested that PtdIns(4,5)P₂ could affect the SR Ca²⁺ release process. However, recent data from transgenic models of PtdInsP-phosphatase deficiency, specifically MTM1 (myotubularin) and parent proteins ("myotubularin and myopathy related" phosphatases [MTMRs]) provided completely unanticipated insights into the field: in humans, more than 200 mutations of the gene encoding MTM1 are associated with X-linked myotubular myopathy, characterized by very severe muscle weakness and consequent fatal outcome, often within weeks following birth. The link between MTM1 deficiency and muscle weakness remained obscure until muscle fibres from MTM1-deficient mice were shown to suffer from a critically defective E-C coupling process. In mouse, MTM1-deficient muscle fibres yield defects in the internal membrane system structure and organization, reduced levels of DHPR and RyR1 and a strong decrease in the amplitude of voltage-activated cytosolic Ca²⁺ signals. This functional alteration is certainly the direct cause of muscle weakness in the affected patients. Results by Al-Qusairi et al. also demonstrated that SR Ca²⁺ content and cytosolic Ca²⁺ removal capabilities are unaltered in MTM1-deficient muscle fibres. At the functional level, the disease status thus appears to specifically affect the SR Ca²⁺ release process.

Aims

The effect of Mstn on the EC-coupling

To understand the apparent discrepancy between increased muscle mass and reduced specific force reported in the literature for *Cmpt* mice we hypothesised that ECC is also modified in these animals. To this end, we give an in depth investigation of muscle function by comparing the physical endurance of control and *Cmpt* mutant mice as well as changes of ECC. For the latter, intracellular calcium concentration measurements were conducted using electrical stimulation or prolonged depolarization of enzymatically isolated fibres loaded with fluorescent calcium indicators while the contractile activation was studied on chemically skinned fibre segments. We demonstrate that reduced SR Ca²⁺ release underlies the suppression of specific force. Excitation-contraction coupling (ECC) in skeletal muscle

The effect of MTM1 and the PtdInsPs on the EC-coupling

E-C coupling defect results from altered PtdInsPs metabolism due to the loss of MTM1 PtdInsP-phosphatase activity. It is supported by results from Shen et al. showing that loss of another PtdInsP-phosphatase (MTMR14) is responsible for alterations of intracellular Ca²⁺

homeostasis in muscle, attributable to defective regulation of RyR1 channel activity by PtdInsPs. Recent data also indicate that muscle weakness associated with aging may be due to altered Ca^{2+} homeostasis because of a reduced level of MTMR14. This trend of data thus tends to suggest a role for PtdInsPs in the regulation of skeletal muscle Ca^{2+} homeostasis and E–C coupling under normal conditions and a role for alteration of this regulation in certain muscle diseases. We looked here for a direct evidence that PtdInsPs are physiologically involved in the regulation of Ca^{2+} and E–C coupling in normal muscle. We demonstrate that MTM1 main PtdInsP substrates inhibit the function of E–C coupling while maneuvers intended to reduce the level of these 3-OH phosphorylated PtdIns have no effect.

Materials and Methods

Animal care

Animal experiments conformed to the guidelines of the European Community (86/609/EEC). The experimental protocol was approved by the institutional Animal Care Committee of University of Debrecen (22/2011/DE MAB). The mice were housed in plastic cages with mesh covers, and fed with pelleted mouse chow and water *ad libitum*. Room illumination was an automated cycle of 12 h light and 12 h dark, and room temperature was maintained within the range of 22–25°C. When necessary mice were sacrificed by cervical dislocation.

Mating and genotyping *Cmpt* mice

The *Cmpt* mice are originated from Dr. Müller's lab. To monitor the *Cmpt* mutation, heterozygous mice were obtained by mating wild-type C57BL/6 and *Cmpt* mice. Tail-tip biopsy was performed for genotyping.

In vivo experiments

Voluntary activity wheel measurement

12–16 week old *Cmpt* ($n=8$) and C57BL/6 ($n=8$) mice were singly housed in a cage with a mouse running wheel (Campden Instruments Ltd., Loughborough, UK). Wheels were interfaced to a computer and revolutions were recorded in 20 minutes intervals, continuously for 14 days. The average and the maximal speed, the distance and the duration of running was calculated for individual mice and then averaged by strains.

Forepaw grip test

12–16 week old *Cmpt* ($n=5$) and C57BL/6 ($n=11$) mice were held perpendicular to the bar connected to a capacitive force transducer, so that they did not reach at an angle during the trials. The hind limbs were not allowed to touch any surfaces. When the unrestrained forepaw

was brought into contact with the bar of the grip test meter, the animals reliably grasped the bar, and the animal was then gently pulled away from the device. The grip test meter then measured the maximal force before the animal released the bar. We allowed the mouse to grip the bar fully. Force responses were digitized at 2 kHz and stored via an online connected computer.

***In vitro* experiments**

Isolation of single skeletal muscle fibres

Experiments were carried out on skeletal muscle fibres from the *flexor digitorum brevis* (FDB), *extensor digitorum longus* (EDL) and soleus (SOL) muscles of the mice. Single muscle fibres were enzymatically dissociated in calcium free modified Tyrode's solution (in mM, 137 NaCl, 5.4 KCl, 0.5 MgCl₂, 11.8 Hepes, pH 7.4) containing 0.2% Type I collagenase (Sigma, St. Louis, USA) at 37°C for 50-55 minutes.

To release single fibres muscles were triturated gently in modified Tyrode's solution supplemented with 1.8 mM CaCl₂. The fibres were then mounted on laminin-coated cover slip floors of culture dishes and kept at 4°C until use.

Resting membrane potential measurement

FDB muscle fibres were superfused with oxygenized Tyrode's solution (in mM, 137 NaCl, 5.4 KCl, 0.5 MgCl₂, 1.8 CaCl₂, 11.8 Hepes-NaOH, 1 g/L glucose, pH 7.4) at room temperature (22°C). Only the cells with intact surface membrane and clear cross striations were used. Membrane potentials were recorded using 3M KCl filled sharp glass microelectrodes having tip resistances between 30 and 40 MΩ. The electrodes were connected to the input of a Multiclamp-700A amplifier (Axon Instruments, Foster City, CA, USA) under current clamp conditions. Membrane potentials were digitized at 100 kHz using Digidata 1322 A/D card (Axon Instruments) under software control (pClamp 9.2, Axon Instruments, Foster City, CA, USA) and stored for later analysis.

Ca²⁺ sensitivity of the contractile proteins

Ca²⁺-dependent active isometric force and its Ca²⁺-sensitivity, and Ca²⁺-independent passive force of permeabilized EDL and soleus muscle fibres were determined by a mechanical measuring system at 15°C as described earlier (Papp *et al.*, 2002; for additional details see Supplementary material). Briefly, Ca²⁺-contractures were evoked by transferring the fibres from Ca²⁺-free relaxing solution (in mM, 37.34 KCl, 10 BES, 6.24 MgCl₂, 7 EGTA, 6.99 Na₂ATP, and 15 sodium creatinin-phosphate, pH 7.2) to activating solutions (relaxing solution supplemented with Ca) of gradually increasing [Ca²⁺]. During single Ca²⁺-contractures, when

the force reached the maximal value, a quick release-re-stretch manoeuvre was performed in the activating solution. Isometric force values were normalized for the maximal Ca^{2+} -activated active force, and Ca^{2+} -force relations were plotted to determine the Ca^{2+} -sensitivity of isometric force production.

Whole-cell intracellular Ca^{2+} concentration measurement

Changes in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) were monitored using Fura-2 as described previously. Briefly, isolated FDB fibres were mounted on laminin-coated cover slips and loaded with 5 μM Fura-2 AM for 60 min.

Detection of the changes in $[\text{Ca}^{2+}]_i$ using confocal microscopy

Individual action potentials were evoked by applying supra-threshold 2 ms long square pulses (S88 Stimulator, Grass Technologies, Warwick, RI, USA) through a pair of platinum electrodes placed closed to the fibre. Tetanic depolarisations were initiated using trains of supra-threshold 2 ms long square pulses with a frequency of 100 Hz within the train. Each train lasted for 500 ms. Depolarisation-evoked calcium transients were measured at 22°C using a confocal laser scanning microscope system (Zeiss 5 Live, Oberkochen, Germany) after loading the fibres with 20 μM Rhod-2 AM for 15 minutes at room temperature. Line-scan images (512 pixels/line) were used to monitor the fluorescence intensity changes at 1 ms/line and using a 40x water immersion objective. Rhod-2 was excited with a HeNe ion laser at 543 nm, emission was detected with a 550 nm long pass filter. To obtain the time-course of Rhod-2 fluorescence change (F_{rhod}), corresponding data points (usually 10-15) in the line-scan images were averaged in the spatial domain. Resting fluorescence was determined as the average fluorescence before the depolarization.

Detection of calcium release events

After enzymatical dissociation, FDB fibres were permeabilized using 0.01% saponin for a few seconds. Spontaneous calcium release events were visualised by 50 μM Fluo-3 using a confocal laser scanning microscope (Zeiss 5 META, Oberkochen, Germany) at 22 °C. Images containing spontaneous calcium release events were analysed using an automatic event detection program, which calculated the amplitude ($\Delta F/F_0$), full width at half maximum (FWHM), rise time and duration of the identified events.

Calculation of calcium release from the SR

The Ca^{2+} release flux (R_{rel}) was defined as the sum of Ca^{2+} fluxes entering the myoplasmic space and that transported back into the calcium storage organelle.

***In vivo* transfection**

An expression plasmid encoding wild-type mouse MTM1 fused N-terminally to mCherry was constructed using the pmCherry-C1 vector (Clontech, Mountain View, CA, USA). Swiss OF1 male mice that were 1–2 months old were used. Transfection was performed in the FDB and interosseus muscles of the animals. Details of the procedure used for in vivo electroporation are given in Electronic supplementary material. Experimental observations and measurements were carried out 7–10 days later.

Electrophysiology

An RK-400 patch-clamp amplifier (Bio-Logic, Claix, France) was used in whole-cell voltage-clamp configuration. Fibres were bathed in the TEA-containing extracellular solution. Command voltage pulse generation was achieved with an analog–digital converter (Digidata 1440A; Axon Instruments, Foster City, CA, USA) controlled by pClamp 9 software (Axon Instruments). Voltage-clamp was performed with a microelectrode filled with the intracellular-like solution. The tip of the microelectrode was inserted through the silicone, within the insulated part of the fibre. Analog compensation was systematically used to decrease the effective series resistance. Membrane depolarizing steps were applied from a holding command potential of -80 mV, unless otherwise specified.

Statistical analysis

Pooled data were expressed as mean \pm standard error of the mean (SEM). The differences between control and *Cmpt* animals were assessed using one way analysis of variance (ANOVA) and all pair-wise multiple comparison procedures (Student-Newman-Keuls Method). *F*-test was used to test the significance and $p < 0.05$ was considered as statistically significant difference.

Results

The effects of myostatin

In vivo experiments

Cmpt mice displayed great hypermuscularity and their weight was more than two times greater than that of control animals. To check the *in vivo* muscle performance of the adult animals, 12–16-week-old mice were used from both groups in grip tests. As expected, based on the increased muscle mass, *Cmpt* mice performed significantly better than the wild type animals in these tests. That is, the maximal force that they could produce was significantly greater than that of control animals. However, after normalization to body weight – to compensate for the increased muscle mass – wild type mice displayed significantly greater relative force as compared to the *Cmpt* animals.

Experiments with the voluntary wheel gave similar results. Control mice performed significantly better given both the average and maximal speed and the total distance covered. In addition, they spent more time in the wheel than *Cmpt* mice did. Overall, these experiments clearly indicated, that although *Cmpt* mice were bigger and stronger, their strength was less than what one would expect from the increase in muscle mass. To clarify the underlying mechanisms the steps in ECC were investigated in detail.

In vitro experiments

Resting membrane potential

To exclude the possibility that the electrical properties of the cell membrane were substantially altered, the resting membrane potential of FDB muscle fibres from 4 control and 3 *Cmpt* animal was determined. Although, its average value proved to be slightly depolarized in *Cmpt* animals (-77.8 ± 0.2 mV, n=18) as compared to control mice (-79.2 ± 0.3 mV, n=13), we do not consider this difference physiologically relevant.

Calcium sensitivity of the contractile system

As the next step, the possible alterations in the contractile machinery were studied in both *Cmpt* and control mice. Force development during isometric contractions was measured in 31 fibre segments (segment length 80.7 ± 5.1 μm) dissected from soleus and EDL fibres of 4 wild type and 4 *Cmpt* mice. There was no significant difference in these values between wild type and *Cmpt* animals.

Changes in intracellular Ca^{2+} concentration in *Cmpt* mice

Since the calcium sensitivity of the contractile proteins were similar in both groups of animals, the possible effects of the *Cmpt* mutation on the calcium homeostasis was examined in isolated, single FDB skeletal muscle fibres of both types of mice. 12-16-week-old wild type and *Cmpt* mice were used for functional experiments. Figure 3A presents calcium transients measured on these fibres evoked by depolarization using 120 mM KCl in the presence of normal (1.8 mM) extracellular calcium concentration. The *Cmpt* mutation had little, if any, effects on the resting $[\text{Ca}^{2+}]_i$ suggesting that the calcium leak from and the extrusion into the extracellular environment was unaffected under these conditions. On the other hand, depolarization-evoked calcium transients were almost 50% higher in control than in *Cmpt* fibres.

Action potential-evoked calcium transients in FDB fibres

Field stimulation-induced global calcium transients were also measured on FDB fibres. These calcium transients were visualized using laser scanning confocal microscopy in the line-scan mode. Note that the spatial profile of the transients was homogenous in both strains indicating a normal propagation of the depolarisation in the T-tubular system.

To analyse the calcium transients, the line-scans were normalized and 10-15 points of the images in the space domain were averaged. The analysis of these transients revealed that the average amplitude of the calcium transients in *Cmpt* fibres were almost identical during the single stimulation ($2.37 \pm 0.12 \text{ F/F}_0$, $n=25$ in control; $2.50 \pm 0.15 \text{ F/F}_0$, $n=26$ in *Cmpt*, $p>0.4$) but significantly reduced during the train stimulation as compared to controls.

During the measurements either 6 or 3 calcium transients were recorded in case of single or train stimulations, respectively. There was no significant decrease in the amplitude of the consecutive transients neither in the single nor in the train depolarisations in either animal groups.

To further analyse the calcium release from the SR, first the normalized fluorescence values of the train depolarisations were transformed to calcium concentration, and then the total amount of calcium released ($[\text{Ca}]_T$) and the calcium release flux (R_{rel}) through the RyRs was calculated. The change in intracellular calcium concentration as well as the total calcium released into the myoplasm, and the corresponding calcium release flux was reduced in *Cmpt* mice. R_{rel} reached its peak 9 ms after the onset of the depolarization and was found to be significantly smaller in the *Cmpt* strain.

Elementary calcium release events in *Cmpt* fibres

To understand the underlying reasons for the suppressed SR calcium release, localized calcium release events were measured in control and in *Cmpt* fibres using high speed laser scanning confocal microscopy. The fibres displayed calcium sparks (calcium release events with large amplitude and short duration) and embers (events with small amplitude and long duration). It should be noted, that the number of calcium sparks was far greater than that of embers. Although fibres from *Cmpt* mice generated calcium sparks and embers as control cells did, the characteristic parameters of these events were altered. Plotting the amplitude histograms of sparks revealed that the *Cmpt* mutation was associated with an increase in the relative frequency of calcium sparks with smaller amplitudes. Pooled data show that on average the amplitude of the calcium sparks was significantly higher in control fibres than in *Cmpt* cells (0.165 ± 0.001 vs. 0.154 ± 0.002 ; $p<0.001$, respectively). Similarly, we observed a relatively higher amount of faster evolving sparks in *Cmpt* than in control mice, the average rise time showed a significant (30%, $p<0.01$) reduction (from 34.7 ± 0.9 to 24.3 ± 0.8 ms in control as compared to *Cmpt* mice, respectively). Although the overall shape of FWHM histograms remained unaffected in *Cmpt* animals the distribution was slightly shifted to the left. That is, the *Cmpt* mutation decreased the average FWHM significantly ($p<0.01$) from the control 1.92 ± 0.01 to 1.87 ± 0.01 μm . The distribution of event duration was also altered by the mutation showing a relatively high frequency of shorter events in *Cmpt* mice. In addition, and in accordance with the above, the frequency of the spontaneous calcium release events was also significantly ($p<0.001$) reduced in fibres from *Cmpt* mice (6.2 ± 0.5 Hz) as compared to the control group (33.0 ± 2.7 Hz).

PtdIns(3,5)P₂ and PtdIns(3)P depress voltage-activated Ca²⁺ release

Since MTM1 deficiency produces a severe impairment of E–C coupling, we were specifically interested in the effect of substrates and products of its phosphatase activity. Soluble forms of PtdIns(3,5)P₂, PtdIns(3)P, PtdIns(5)P or PtdIns were thus micro-injected in separate batches of single isolated muscle fibres and intracellular Ca²⁺ was measured under voltage-clamp conditions. PtdInsPs injection was performed so as to reach a final concentration expected to be within the 100 μM range, matching the maximum levels of PtdInsPs shown to affect [³H] ryanodine binding to SR as well as single channel activity of RyR1 in bilayer. Mean resting [Ca²⁺] did not differ between control fibres (0.16 ± 0.02 μM , $n=16$) and any of the PtdInsPs-injected fibre group, but was significantly elevated in fibres injected with either PtdIns(3,5)P₂ (0.21 ± 0.02 μM , $n=8$) or PtdIns(3)P (0.20 ± 0.02 μM , $n=8$) as compared to fibres injected with PtdIns(5)P (0.14 ± 0.01 μM , $n=7$) or PtdIns (0.12 ± 0.02 μM , $n=6$). In order to evaluate the effect of the different PtdInsPs on the properties of Ca²⁺ transients and Ca²⁺ release, fibres were

stimulated by a voltage-clamp protocol consisting in successive 20-ms-long depolarizing pulses of increasing amplitude from -80 mV. This protocol was repeated five times in each fibre and the traces were averaged. While testing the effect of PtdInsPs on Ca^{2+} transients, we questioned the possibility that voltage-dependent inactivation of Ca^{2+} release would be affected. To test for this, we measured indo-1 transients in response to a pair of 20-ms long pulses to -20 and 0 mV from increasing levels of holding potential. The two pulses were used in order to ensure that heavy saturation of indo-1 for the largest pulse would not hinder detection of a decrease in the Ca^{2+} transient amplitude when changing the holding potential towards more depolarized levels. The pulse protocol and the corresponding mean ($\pm \text{SEM}$) indo-1 saturation traces obtained in control fibres are shown in In each fibre, the peak Ca^{2+} transient amplitude calculated from the indo-1 signal was normalized to the initial value measured from the holding potential of -80 mV. We found no indication that any of the four tested PtdInsPs affected the inactivation process, where the mean normalized peak amplitude of the Ca^{2+} transient versus the holding potential in the different groups of fibres are shown. Fitting a Boltzmann function to the data in each fibre gave mean values for half-inactivation voltage and steepness factor that did not differ between control fibres and any of the four groups of PtdInsPs-injected fibres.

PtdIns(3,5)P₂, PtdIns(3)P and PtdIns(5)P depress the frequency of ECREs in permeabilized muscle fibres

Measurement of spontaneous ECREs in permeabilized muscle fibres provides a way to study the functional properties of the ryanodine receptor in its native environment, freed from the DHPR control. We used this approach to test whether PtdInsPs would directly affect RyR-mediated Ca^{2+} release activity and get insights into the underlying mechanism. Spontaneous ECREs were measured using fast confocal (x,y) imaging, 15 min following equilibration of fibres with a given PtdInsP and properties of the events were

compared to those of fibres from the same muscles equilibrated for 15 min with the standard experimental solution. Each frame represents the cumulated ECRE activity recorded during a series of 200 consecutive images, covering a period of approximately 13 s. There was a substantial reduction of the number of detected ECREs in the fibres equilibrated with PtdIns(3,5)P₂, PtdIns(3)P and PtdIns(5)P, the effect being most important with PtdIns(3,5)P₂. ECRE frequency was strongly depressed in the presence of each of the three tested phosphorylated forms of PtdInsP with the drop amounting to 98% in the presence of PtdIns(3,5)P₂. The mean ECRE amplitude was slightly but significantly increased in the presence of PtdIns(5)P whereas the FWHM along both the x and y directions was depressed by

~10% in the presence of PtdIns(3,5)P₂. Altogether, the tested PtdInsPs appeared to modestly, if any, affect the individual ECRE amplitude and spatial properties while severely impairing the occurrence of the events. From that series of measurements we could also check that the observed decrease in ECRE frequency in the presence of PtdIns(3,5)P₂, PtdIns(3)P and PtdIns(5)P was present right when we started taking measurements: for instance, during the first series of 200 images, the average ECRE frequency was depressed by 92% in fibres equilibrated with PtdIns(3,5)P₂ as compared to the frequency in their control counterparts.

Overexpression of MTM1 does not affect voltage-activated Ca²⁺ release

PtdIns(3,5)P₂ and PtdIns(3)P are the main substrates of the PtdInsP phosphatase MTM1. An alternative possibility for reducing the level of these two PtdInsPs was thus to overexpress MTM1; this was also of particularly strong interest since loss of activity of this phosphatase is responsible for severe pathological E–C coupling defects. The mCherry-MTM1 was distributed throughout the entire volume of the fibres and, interestingly, that the fluorescence yielded a clear transversally striated component forming double rows spaced by ~2 μm, reminiscent of the triadic region. This was confirmed by co-localization of the mCherry fluorescence with that of t-tubule stained with di-8 anepps. Although there was a tendency for a slight reduction in peak Ca²⁺ release in the fibres overexpressingMTM1 as compared to control fibres, the difference was not statistically significant. Fitting a Boltzmann function to the data in each fibre gave mean values for maximum peak Ca²⁺ release, voltage of half-activation and steepness factor of 32.5±3 μM ms⁻¹, -7.2±1 mV, 6.8±0.2 mV and 28.2 ±3 μM ms⁻¹, -5.6±1 mV, 6.5±0.4 mV in control and MTM1- overexpressing fibres, respectively.

Discussion

The effect of myostatin deficiency to the EC-coupling

Myostatin knock-out as well as *Cmpt* mice - despite their increased muscle weight - were found to have reduced specific force as compared to control animals. Albeit the fact that changes in fibre composition and consequently altered metabolic status of the muscles from these mice have been reported a clear explanation for the altered contractile function is not yet available. Here we, on the one hand, complement the observations previously made *in vivo*, and, on the other hand, provide evidence that alterations in calcium signalling but not in the activation of the contractile machinery underlie the reduced force production. The observation that the normalized force – force normalized to body weight – was found to be reduced in the grip-strength tests in *Cmpt* mice is consistent with the idea that the increased muscle mass was not accompanied by a parallel and proportional increase in overall force. Although it is likely that alterations in cardio-vascular and/or respiratory functions also contributed to the reduced physical performance in the activity wheel for these animals, based on previous reports as well as on our own findings mentioned above, changes, in the events that couple electrical excitation to contraction, are also part of the pathology.

We were unable to demonstrate any modification in the pCa dependence of force production as studied in chemically skinned fibre segments. Neither fast nor slow muscles displayed any change in their pCa-tension curves. In addition, the maximal attainable tension was also similar in control and *Cmpt* animals. On the other hand, action potential-evoked calcium transients were significantly reduced in mutant as compared to the control animals. This reduction in the increase in $[Ca^{2+}]_i$ during a train of action potentials which is the physiological stimulus during *in vivo* contractions – taken together with the unaltered pCa-tension relationship – readily explains the reduced overall tension. It should be noted, however, that the reduction in $[Ca^{2+}]_i$ and the consequently anticipated reduced force, based on the measured pCa-tension relationship, was proportionally less than the increase in muscle diameter (2.35 ± 0.19 vs. 6.93 ± 0.32 mm 2 in the control (n=13) and *Cmpt* (n=19) strains, respectively, for EDL, $p < 0.001$) not accounting fully for the observed greater strength in grip tests. SR calcium release flux was determined from the global calcium transients and a 33% reduction was observed in both the peak and the maintained steady flux. Since the peak-to-steady ratio was unaltered in *Cmpt* mice, that is, the waveform of the SR calcium release flux was essentially the same in the mutant and the control animals, the kinetics of activation and inactivation of the calcium release channels

was likely not affected. This was further strengthened by the findings that the time-to-peak of SR calcium release flux as well as the rate constant of inactivation, calculated by fitting an exponential function to the declining phase, were essentially unchanged. It should be noted at this point that a slight (but statistically not significant) increase in the time constant was indeed observed which is in line with previous reports that a reduced SR calcium release would initiate a slower inactivation. Furthermore, in accordance with the above, a clear reduction in the amount of calcium released from the SR during a train of action potentials was also detected. These observations leave three possibilities, or their combination, to explain the reported findings. First, there is a reduction in SR calcium content, second, there is a reduction in RyR permeability/conductance, and third, the number of activated RyR-s is reduced. It should be stressed, however, that the magnitude of these modifications should not be too extensive otherwise changes in the kinetics of inactivation would accompany the reduced SR calcium release. Indeed, the reduction in SR calcium release flux observed here was within these limits and was comparable to earlier studies where the effect of SR depletion on the SR calcium release waveform was investigated. Shifts in the redox state of the myoplasm from the control, to more reducing would result in a reduced activation, while shifts to more oxidative state would result in an increased resting SR leak and a consequent reduction in SR calcium content. To determine whether the RyR channels *per se* were affected in the mutant strain direct measurements of the channel activity would be needed. At the moment there is no data available in the literature on RyR-s isolated from *Cmpt* or *Mstn^{-/-}* mice. Here we attempted to get insight into channel function using Saponin-skinned fibres and determining the characteristics of calcium release events. Although these events should be regarded with caution since they do not appear with appreciable frequency in non-skinned fibres, their study is the only possibility to tackle channel behaviour in the native environment. Due to the above mentioned clear limitations, only the simplest properties were investigated in detail in this study. It is clear from the results that calcium release events occur with a lower frequency in *Cmpt* animals than they do in control mice. In addition, these events have a smaller amplitude and FWHM than their control counterparts. These observations are consistent with the idea that channels open less readily and release less calcium once they are opened in the mutant as compared to control mice. In particular, the reduced number of events and the amount of calcium released per event would readily explain why global calcium transients were smaller in the mutant animals. These findings would then suggest that the alterations that explain the *Cmpt* phenotype – in respect to the reduced force – reside, at least in part, in the calcium release process.

Considering the above, the most likely explanation for the reduced calcium release is a reduction in the SR content. This would then influence the release process through at least two routes. The activation of RyR depends on the calcium concentration inside the SR, namely, lower intraluminar calcium concentration results in a lower activation level of the channels. In addition, due to the lower SR calcium the current through the channels is also reduced. These effects combined would then explain the smaller frequency as well as the smaller amplitude of the calcium release events and, finally, the reduced calcium transients. Although, at the moment, we have no clear explanation how the mutation leads to the reduction in SR calcium content, all data are consistent with the idea that the reduced specific force of these mice is the consequence of this alteration.

The effect of changes in MTM1 expression on the EC-coupling

We show that intracellular application of either PtdIns(3,5)P₂ or PtdIns(3)P depresses global voltage-activated Ca²⁺ release in intact muscle fibres. Conversely, we also show that two experimental strategies intended to reduce the level of these two 3-OH phosphorylated PtdIns have no effect on Ca²⁺ release, suggesting that they are maintained at an ineffective low-level in regard to this effect, in normal conditions. Evidence for a role of PtdInsPs in the regulation of muscle Ca²⁺ homeostasis and/or E-C coupling is sparse: mainly, measurements of single RyR channel activity, Ca²⁺ release from SR vesicles and tension from skinned fibres suggested that PtdIns(4,5)P₂ has the potency to activate RyR1 but no clear physiological correlate was ever provided. Our interest here was more specifically focused on the PtdInsPs species that are substrates or hydrolyzed products of MTM1. In relation to these, a set of relevant data was collected by Shen et al. indicating that: (a) PtdIns(3,5)P₂ in cultured myotubes resides in the SR; (b) defects in myotubes Ca²⁺ signalling occur upon perfusion with PtdIns(3,5)P₂, PtdIns(3,4)P₂ or PtdIns(3)P; (c) all PtdInsPs bind to RyR1 with PtdIns(5)P yielding the highest binding activity; (d) PtdIns(3,5)P₂, in the 10–100 μM range, directly increases the activity of RyR1 as inferred for instance from the increased number of single RyR1 channel open events in reconstituted lipid bilayers. Although of strong relevance to the present issue, these results provided little insight into whether or not — and if so, how — PtdInsPs actually affect RyR1 function and E-C coupling in the native functional environment of an adult muscle fibre. We also examined the effect of PtdInsPs on Ca²⁺ release detected under the form of spontaneous elementary events. Normally, ECEREs in mammalian muscle do not occur at a frequency that allows appropriate analysis, under physiological conditions. On the other hand, conditions that substantially increase their frequency, such as discussed here after saponin permeabilization,

allow a connection between data measured in vitro (whether from vesicles or from artificial bilayers) and global calcium transients measured in intact muscle fibres. In other words, analysis of ECREs offers a way to address discrepancies of RyR1 function between a full in vitro situation and—as full as possible—physiological situation. Also considering that the physiological ECREs that build up the global Ca^{2+} transients in mammalian muscle fibres still remain poorly identified, this approach represents the best one can use to address elementary properties of RyR1-mediated Ca^{2+} release in situ. In qualitative agreement with the reduction of peak voltage-activated Ca^{2+} release by PtdIns(3,5)P₂ and PtdIns(3)P that we observed in intact fibres, we also found that the frequency of spontaneous Ca^{2+} release events was reduced by these two PtdInsPs in permeabilized fibres, although in contrast with data from intact fibres PtdIns(5)P also was effective under these conditions. According to the ECRE parameters, PtdInsPs effect on RyR1-mediated Ca^{2+} release is likely to result from a reduced occurrence of RyR1 channel opening than from an alteration of the channel conductance or of the number of coherently operating channels involved in a Ca^{2+} release event.

Summary

Myostatin, a member of the transforming growth factor β family was shown to be a potent negative regulator of skeletal muscle growth, as myostatin deficient mice have a great increase in muscle mass. Yet, the physical performance of these animals is not improved but suppressed. As an explanation, alterations in the steps in excitation-contraction coupling were hypothesized and tested for on mice with the 12-bp deletion in the propeptide region of the myostatin precursor ($Mstn^{Cmpt-dl1Abc}$ or $Cmpt$). In voluntary wheel running control C57/BL6 mice performed better than the mutant animals in both maximal speed and total distance covered. Despite the previously described lower specific force of $Cmpt$ animals, the pCa-force relationship, determined on chemically permeabilized fibre segments did not show any significant difference between the two mouse strains. While resting intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) measured on single intact *flexor digitorum brevis* (FDB) muscle fibres using Fura-2 AM was similar to control (72.0 ± 1.7 vs. 78.1 ± 2.9 nM, $n=38$ and 45), the amplitude of KCl-evoked calcium transients was smaller (360 ± 49 vs. 222 ± 45 nM, $n=22$) in the mutant strain. Similar results were obtained using tetanic stimulation and Rhod-2 AM which gave calcium transients that were smaller (2.42 ± 0.11 vs. $2.06 \pm 0.10 \Delta F/F_0$, $n=14$ and 13, respectively) on $Cmpt$ mice. SR calcium release flux, calculated from these transients showed a reduced peak (23.7 ± 3.0 vs. $15.8 \pm 2.1 \text{ mMs}^{-1}$) and steady level (5.7 ± 0.7 vs. $3.7 \pm 0.5 \text{ mMs}^{-1}$) with no change in the peak-to-steady ratio. The amplitude and spatial spread of calcium release events detected on permeabilized FDB fibres were also significantly smaller in mutant mice.

These results suggest that reduced SR calcium release underlies the reduced muscle force in $Cmpt$ animals.

Skeletal muscle excitation–contraction (E–C) coupling is altered in several models of phosphatidylinositol phosphate (PtdInsP) phosphatase deficiency and ryanodine receptor activity measured *in vitro* was reported to be affected by certain PtdInsPs, thus prompting investigation of the physiological role of PtdInsPs in E–C coupling. We measured intracellular Ca^{2+} transients in voltage-clamped mouse muscle fibres microinjected with a solution containing a PtdInsP substrate (PtdIns(3,5)P₂ or PtdIns(3)P) or product (PtdIns(5)P or PtdIns) of the myotubularin phosphatase MTM1. No significant change was observed in the presence of either PtdIns(5)P or PtdIns but peak SR Ca^{2+} release was depressed by ~30% and 50% in fibres injected with PtdIns(3,5)P₂ and PtdIns(3)P, respectively, with no concurrent alteration in the membrane current signals associated with the DHPR function as well as in the voltage dependence of Ca^{2+} release inactivation. In permeabilized muscle fibres, the frequency of

spontaneous Ca^{2+} release events was depressed in the presence of the three tested phosphorylated forms of PtdInsP with PtdIns(3,5)P₂ being the most effective, leading to an almost complete disappearance of Ca^{2+} release events. Results support the possibility that pathological accumulation of MTM1 substrates may acutely depress ryanodine receptor-mediated Ca^{2+} release. Overexpression of a mCherry-tagged form of MTM1 in muscle fibres revealed a striated pattern consistent with the triadic area. Ca^{2+} release remained although unaffected by MTM1 overexpression.

Publications



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Candidate: Dóra Bodnár
Neptun ID: QOKFTK
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List of publications related to the dissertation

1. Bodnár, D., Geyer, N., Ruzsnávszky, O., Oláh, T., Hegyi, B., Sztretye, M., Fodor, J., Dienes, B., Balogh, Á., Papp, Z., Szabó, L., Müller, G., Csernoch, L., Szentesi, P.: Hypermuscular mice with mutation in the myostatin gene display altered calcium signaling.
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Pflügers Arch. 466 (5), 973-985, 2013.
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