Short Thesis for the Degree of Doctor of Philosophy (Ph.D.)

Alteration of the Ca^{2+}-homeostasis in skeletal muscle in case of overexpression of regulatory and channel proteins

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The Examination takes place at the Department of Physiology, Medical and Health Science Center, University of Debrecen
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Introduction

Physiology of 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). Depolarization of the plasma membrane initiates a conformational change in the voltage sensors, the dihydropyridine receptors (DHPRs), of the surface membrane which in turn activate the ryanodine receptors (RyRs). Through these channels Ca^{2+} is released from the sarcoplasmic reticulum (SR), the intracellular Ca^{2+} store. Increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) activates the contractilic apparatus of the fibers and evokes contraction.

The inositol 1,4,5-trisphosphate (IP_3) pathway

In the early stage of muscle development – in myoblasts – the complex structures required for ECC are not yet functional. Nevertheless changes in $[Ca^{2+}]_i$ control a wide variety of other functions, including division, secretion, motility, differentiation and apoptosis. So in myoblasts – similarly to non-excitable cells – the other main Ca^{2+} release channels, the inositol 1,4,5-trisphosphate receptors (IP_3Rs) have a major role compared to RyRs. In response to different external stimuli (as the activation of bradykinin or vasopressin receptors), phospholipase C (PLC) β hydrolyses phosphatidylinositol 4,5 bisphosphate (PIP_2) in the plasma membrane to produce IP_3 and diacylglycerol (DAG). While DAG activates protein kinase C (PKC) isoforms, IP_3 binds to the Ca^{2+} release channel of the ER.

IP_3Rs are tetrameric molecules composed of large subunits, with a C-terminal end anchored in the membrane of the ER/SR, and a cytoplasmic N-terminal end. At least three isoforms are known with different tissue distribution

and regulation. Although its structure is highly similar to that of the RyR, only a few molecules are known to contribute to its regulation or to directly connect to the channel.

The 32 kDa skeletal muscle triadin (Trisk 32)

The triadin proteins were described in skeletal and heart muscle. In both tissues various isoforms are known, all of them are splice variants of the same gene. They localize in the SR, and have a short cytoplasmic N-terminal segment, a transmembrane α-helix, and a unique luminal C-terminal segment with different lengths. The larger – 95 and 51 kDa – skeletal isoforms appear in the later stages of muscle development and regulate RyRs. But the smallest – 32 kDa – triadin isoform (Trisk 32) appears in the myoblast stage and it does not localize in the triad, rather in the longitudinal tubule of the SR. It has been shown to connect to IP_3R, so its role in the regulation of the receptor was proposed, but until now this has not been proved with functional measurements. In the first part of my thesis the role of Trisk 32 in the regulation of IP_3R was examined.

Store-operated Ca^{2+}-entry (SOCE)

Activation of both IP_3R and RyR decreases the Ca^{2+} content of the SR. One part of the released Ca^{2+} is taken back to the SR by the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA), but the other part is removed from the cell. This would lead to the complete depletion of the SR if there was not a mechanism which enables the entry of extracellular Ca^{2+} for replenishing the stores. This mechanism is called store-operated Ca^{2+}-entry (SOCE). The role of SOCE is not only to refill the Ca^{2+} stores, but it contributes to Ca^{2+} signaling pathways and to the maintenance of the amplitude of the Ca^{2+} oscillations. SOCE has a significant role in skeletal muscle too, its decrease can cause myopathies.

At first the canonical transient receptor potential channels (TRPC) were proposed to be the pore-forming subunits of the SOC channels, but the evidence were controversial. The mechanism of SOCE is still not clear in all details, but the identification of the Ca^{2+} sensor of the SR, the stromal interaction molecule 1 (STIM1), and one of the most important store-operated Ca^{2+} channels, Orai1 helped a lot to clarify the issue. As a consequence of store depletion STIM1 molecules aggregate into puncta in the SR near the plasma membrane, open Orai1 channels, and enable Ca^{2+} to enter the cell. However in the latest articles it was described that STIM1 can also open TRPC1 channels, which can thus work as a store-operated path.

Transient receptor potential canonical 1 channel (TRPC1)

TRPC1 is a non-selective, Ca^{2+}-permeable cation channel which forms functional heterotetramers with other TRPC channel subunits. Its role in SOCE mechanism is not completely clear, different authors published different results concerning TRPC1 is a part of the STIM1-Orai1 system or independent of it. Some workgroups even question whether it is store-operated. In the second half of my thesis I investigated the role of TRPC1 in SOCE and tried to clear some of these contradictions.

Aims

Examination of the role of Trisk 32

From the four isoforms of skeletal muscle triadin, we have the least information about the physiological role of Trisk 32. Based on its localization in the longitudinal SR, and its co-localization with IP_3R it was proposed to have a role in the signaling pathway via IP_3R.

- Our first aim was to prepare an L6 myoblast cell line which stably overexpresses Trisk 32 and is suitable for examining the functions of the protein.

- We wanted to examine the expression of the overexpressed and the endogenous Trisk 32 in L6.G8 myoblasts, and their co-localization with IP_3R to strengthen the data obtained from adult fibers and transient transfected cultures in this cell line.
- We wanted to examine the effects of Trisk 32 overexpression on the activity of IP_3R with fluorescent $[Ca^{2+}]_i$ measurements and confocal microscopy. To this end we applied various agonists to activate the IP_3 pathway via different receptors, and IP_3R directly.

From our results we hoped that we can confirm the proposed role of Trisk 32 in the regulation of IP_3R.

Examination of the role of TRPC1

The role of TRPC1 in SOCE mechanism – in spite of the numerous articles in this topic – is still controversial. The relation of the protein to the other two known members of SOCE, STIM1 and Orai1 proteins, is still not clear. In our research we wanted to clarify the role of TRPC1 in differentiating myotubes.

- First we wanted to generate a TRPC1 overexpressing C2C12 cell line with which we could establish the function of TRPC1 as compared to control cells.
- Our most important question was to describe the role of TRPC1 in SOCE mechanism, so we wanted to perform functional $[Ca^{2+}]_i$ measurements to confirm that TRPC1 behaves as a store-operated channel.
- We wanted to examine the effect of TRPC1 overexpression on the expression of other proteins involved in Ca^{2+} homeostasis – especially STIM1 and Orai1 –, to investigate the connection between them.
- TRPC1 has a role in skeletal muscle differentiation. We wanted to examine whether it changes in our cultures.

Our results should contribute to explain the role of TRPC1 in SOCE mechanism and skeletal muscle differentiation, and its connection with the STIM1-Orai1 system.

Materials and methods

L6.G8 and C2C12 cell cultures

L6.G8 (further L6) and C2C12 myoblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were incubated at 37 ºC in a humidified incubator with 5% CO_2. Differentiation of C2C12 cells into myotubes was induced at 90% confluency by modifying the culture medium.

Liposome-mediated stable transfection

Transfection of the L6 cells was carried out with a pcDNA3.1 eukaryote expression vector cloned with Trisk 32 cDNA, while that of the C2C12 cells was carried out with a pcDNA3.1 eukaryote expression vector cloned with TRPC1 cDNA. Transfection was performed in Opti-MEM medium using Lipofectamine 2000 reagent. Cells were allowed to express the introduced genes for 48 h in growth medium then were selected in DMEM containing 1 mg/ml geneticin. After 14-15 days, single colonies were isolated. To obtain controls for the transfection, cells were mock-transfected by the empty pcDNA 3.1 vector. The efficiency of overexpression of the proteins in different clones was monitored by RT-PCR, immunostaining and Western-blot. Functional experiments were carried out on control and transfected L6 myoblasts and 5- to 6-day-old differentiated C2C12 myotubes.

RT-PCR

For RT-PCR analysis total RNA was isolated from the cultures with Qiagen RNeasy® Mini Kit and Trizol reagent. Reverse transcription was

performed by Omniscript reverse transcription kit and oligo(dT) primers. Amplifications of specific cDNA sequences were performed with specific primers that were designed based on nucleotide sequences published on the internet. PCR samples were examined on 2% agarose gel, stained with EZ-Vision Three reagent, or ethidium-bromide.

Immunostaining

This immunostaining protocol was used for both L6 and C2C12 cells to stain Trisk 32, IP_3R, B2 bradykinin receptor, V1a arginin-vasopressin (AVP) receptor, TRPC1, STIM1, and Orai1 proteins. Cell cultures were fixed with ice cold 4% paraformaldehyde or -20 ºC 100% methanol. Cells were permeabilized with 0.1% Triton X-100, and blocked with 1% bovine serum albumin (BSA). The cells were then incubated overnight at 4 ºC with the primary antibodies. Then fluorescein or Cy3 labeled anti-rabbit secondary antibody was applied. DAPI was used to make the nuclei visible. Images were taken using confocal microscope.

Western-blot analysis, co-immunoprecipitation

For the detection of Trisk 32, TRPC1, STIM1, Orai1, IP_3R-I, II, III, calsequestrin (CSQ), RyR, SERCA, nuclear factor of activated T-cells (NFAT1), and actin, total cell lysates and nuclear fractions were prepared from the cultures. Samples were sonicated, then 1/5 volume of 5-fold concentrated electrophoresis sample buffer was added, and were boiled for 5 min at 80 °C. Samples were transferred electrophoretically to nitrocellulose membranes. After blocking with 5% non-fat dry milk in PBS, membranes were incubated with the primary antibodies overnight at 4 ºC. After washing three times for 10 min with PBST (PBS supplemented with 0.1% Tween 20), membranes were incubated with a peroxidase-conjugated secondary antibody. Signals were detected by enhanced chemiluminescence reaction.

To detect the association between Trisk 32 and the III. subtype of IP_3R (IP_3R-III), co-immunoprecipitation experiments were performed by our collaborating partner, Sarah Oddoux. Control and Trisk 32-overexpressing L6 cells were solubilized, and immunoprecipitation was performed with antibodies against rat Trisk 32, using protein A immobilized on sepharose 4B. The immunoprecipitated proteins were then analyzed by Western blotting.

Apoptosis, viability, and proliferation assays

MitoProbe $DilC_1(5)$ Assay Kit was used to detect apoptosis in C2C12 cultures. Fluorescence, which was proportional to the mitochondrial membrane potential of the cells, was measured at 630-nm excitation and 670-nm emission wavelengths using FLIPR.

Viability of L6 and C2C12 cells was detected by MTT assay. Cells were incubated with MTT reagent for 2 hours, and then the concentration of formazan crystals was determined colorimetrically at 570-nm wavelength.

The extent of the proliferation of L6 cells was measured by radioactive ^{3}H-thymidine incorporation. Cells were incubated with 1 µCi/mL ^{3}H-thymidine containing medium for 16 hours, then proteins were precipitated with trichloroacetic acid, and after drying, radioactivity of the samples was detected with a scintillation solution.

To compare the differentiation of control and TRPC1-overexpressing cells, myoblasts and myotubes were cultured under the same conditions in Petri-dishes. Pictures were taken on each day of culturing. To calculate the rate of proliferation the number of nuclei were determined and the value was divided by the number of nuclei counted on the previous day. To obtain the fusion index the number of nuclei in multinucleated myotubes were compared to the total number of nuclei after induction of differentiation.

NFAT-reporter system, X-Gal staining

Activity of NFAT in C2C12 cells was examined by transfection with HSP-NRE lacZ plasmid. The transfected cells expressed β-galactosidase from the plasmid in function of NFAT activity. The cells were fixed, then 1 mg/ml X-Gal in reaction buffer containing $K_4Fe(CN)_6$ and $K_3Fe(CN)_6$ was placed on them. In the dependence of β-galactosidase activity, the cells became blue. Quantitative analysis was performed with ImageJ.

Whole cell calcium measurements

Changes in intracellular [Ca^{2+}] of the cells were measured with Fura-2-AM fluorescent dye. The cells were loaded with the dye for 60 min in a 37 ºC incubator. Excitation wavelength was alternated between 340 and 380 nm by a Photon Technology International (PTI) Deltascan dual wavelength monochromator, while the emission was monitored at 510 nm using a photomultiplier. $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence intensities ($R = F_{340}/F_{380}$) using an in vivo calibration ($R_{min} = 0.2045$, $R_{max} = 8.315$, $K_d\ \beta = 1183$):

$$[Ca^{2+}]_i=K_D\cdot\beta\cdot(R-R_{min})/(R_{max}-R).$$

Cells were continuously washed with normal (NTY) or calcium-free Tyrode solution using a background perfusion system. Test solutions were directly applied onto the cells through a perfusion capillary tube.

Confocal microscopy

Calcium transients were also monitored with the Zeiss LSM 510 META confocal laser scanning microscope. L6 myoblasts were incubated with 10 μM Fluo-4-AM for 1 h at 37 ºC. Calcium imaging was performed in calcium-free Tyrode's solution. Line-scan images were used to monitor the fluorescence intensity. Fluo-4 was excited with an argon ion laser. Cells were continuously washed by Ca^{2+}-free Tyrode's solution using a background perfusion system.

0.1 μM AVP solution dissolved in Ca^{2+}-free Tyrode's solution was directly applied to the cells using a local perfusion system. All measurements were performed at room temperature. Images were analyzed by using an automatic event detection program, developed in our department. Fluorescence of the images (F) was normalized to baseline fluorescence (F_0) and amplitudes of the transients were determined as $\Delta F/F_0$.

Results

Examination of the function of Trisk 32

Trisk 32 protein synthesis was amplified using a pcDNA3.1 plasmid-vector system. In order to select the clone showing the most significant Trisk 32 expression from the plasmid, immunocytochemistry, RT-PCR and Western-blot experiments were performed and the clone showing the highest expression of the protein was used for further experiments. Empty pcDNA3.1 vector transfected cells were used as a control of the transfection. Transfection with the empty or Trisk 32 containing vector did not alter the viability but significantly decreased the proliferation of the myoblasts in the same extent.

Trisk 32 augments Ca^{2+} release via IP_3R

The expression level of IP_3R-I and III was similar in control and transfected cells, while IP_3R-II was undetectable with Western-blot. The major isoform was IP_3R-III. The direct interaction of Trisk 32 and IP_3R was confirmed with double fluorescent labeling and co-immunoprecipitation in the transfected cells.

To examine the functional effect of the overexpression, IP_3 pathway was activated by the stimulation of V1 vasopressin and B2 bradykinin receptors. The presence of these receptors in the cells was shown by immunocytochemistry. Measurements were performed with confocal microscopy in line-scan mode on control and Trisk 32 overexpressing cells. IP_3 pathway was activated in the absence of $[Ca^{2+}]_e$ by the local application of 0.1 μM AVP. The amplitude of the AVP-evoked Ca^{2+} transients was significantly higher ($p<0.01$) in Trisk 32 overexpressing cells (3.25±0.23, n=34, expressed as F/F_0) compared to control (1.45±0.17, n=21) and empty vector transfected (2.02±0.23, n=25) myoblasts, but the amplitudes of the transients did not differ significantly between the control and empty vector transfected cells. Xestospongin C, the antagonist of IP_3R, significantly reduced the amplitude of these transients, which suggests that

the Ca^{2+} was released through these receptors. Similar results were obtained with single cell $[Ca^{2+}]_i$ measurements with the application of 20 μM bradykinin both in the presence of 1.8 mM $[Ca^{2+}]_e$ (the amplitude of the transients was 76±12 nM, n=23 in control, and 426±84 nM, n=27 in Trisk 32 overexpressing cells, $p<0.01$; the maximal rate of rise of the transients was 3.3±1.1 nM/s in control, and 35.1±9.6 nM/s in Trisk 32 overexpressing cells, $p<0.01$), and in the absence of $[Ca^{2+}]_e$ (the amplitude was 97±29 nM, n=31 in control, and 217±41 nM, n=21 in Trisk 32 overexpressing cells, $p<0.02$). It should be noted that these differences were not due to an increase in the resting $[Ca^{2+}]_i$ since it was essentially identical in control and Trisk 32 overexpressing cells (73±2 nM, n=31 and 74±2 nM, n=21, respectively, $p>0.9$) in the absence of $[Ca^{2+}]_e$.

When IP_3R was directly activated by the local application of 50 μM thimerosal, the amplitude (84.2±5.4 in control, 82.7±7.4 in empty vector transfected, and 83.1±4.8 nM in Trisk 32 overexpressing cells) and the maximal rate of rise (0.26±0.04, 0.25±0.01, and 0.27±0.04 nM/s, respectively) of the Ca^{2+} transients were essentially identical ($p>0.8$) in the three cell types.

Overexpression of Trisk 32 does not affect SOCE

One of the possible explanations of the higher Ca^{2+} transients following the activation of the IP_3 pathway was an increase in SOCE. Internal calcium-stores were thus emptied by the application of 2 μM thapsigargin (TG) in the absence of $[Ca^{2+}]_e$ The changes in $[Ca^{2+}]_i$ evoked by re-establishing the normal 1.8 mM $[Ca^{2+}]_e$ were then measured in control and in Trisk 32 overexpressing L6 myoblasts. Neither the amplitude (105±18 nM, n=22 and 126±22 nM, n=21, respectively, $p>0.4$), nor the maximal rate of rise (0.8±0.2 nM/s and 1.4±0.4 nM/s, respectively, $p>0.2$) of SOCE differed significantly in the two cell types. The decreased STIM1 expression and the unchanged TRPC1 expression were shown with Western-blot in the transfected cells. To assess whether a change in ER Ca^{2+} content could underlie some of the observed alterations, the

parameters of the thapsigargin-evoked Ca^{2+} transients were compared for control and Trisk 32 overexpressing cells. Neither the integral (27.0±3.1 mM·s, n=7 and 28.7±4.5 mM·s, n=15, respectively, p>0.8) nor the amplitude (146±19 nM, and 199±25 nM, respectively, p>0.1) of these signals were altered significantly by the transfection.

Activity of RyR does not change in Trisk 32 overexpressing myoblasts

The Ca^{2+} released via IP_3R can evoke a Ca^{2+}-induced Ca^{2+}-release (CICR) via RyR. To examine whether the overexpression of Trisk 32 affects via RyRs, 30 mM caffeine was used to activate these receptors. Only two thirds of the cells responded to caffeine and the amplitude of the Ca^{2+} transients did not differ significantly (p>0.3) in control (61±7 nM, n=19) and transfected (53±6 nM, n=20) cells. Moreover the expression of RyR could not be shown in these cells with Western-blot. When the Trisk 32 overexpressing cells were stimulated with AVP or bradykinin in the presence of 10 µM ryanodine, the antagonist of RyR, the amplitude of the transients did not differ significantly compared to those without the drug. That suggests that the role of CICR is negligible in these cells.

Examination of the function of TRPC1

Protein expression of TRPC1 was amplified with a pcDNA3.1 plasmid vector system in C2C12 myotubes. The clone overexpressing the protein in the highest amount was selected with RT-PCR, immunocytochemistry and Western-blot after 2 weeks of selection with geneticin. Cells transfected with empty pcDNA3.1 vector were used as a control of the transfection. For the functional measurements control and TRPC1 overexpressing, 5 day old differentiated myotubes were used. Neither the viability of the cells, nor the extent of apoptosis in the cultures changed as a result of the TRPC1 overexpression, as it was shown by MTT assay and $DilC_1(5)$ Assay.

Overexpression of TRPC1 enhances SOCE

To show the effects of TRPC1 overexpression on SOCE, the intracellular Ca^{2+} stores of the cells were depleted with 4 μM thapsigargin in Ca^{2+}-free solution, then changes in $[Ca^{2+}]_i$ were measured when the normal 1.8 mM $[Ca^{2+}]_e$ was restored. As a result of TRPC1 overexpression, the amplitude (129±6 nM, n=14 in control, and 210±19 nM, n=24 in TRPC1 overexpressing cells) and the maximal rate of rise (3.7±0.6 nM/s, n=20 in control, and 6.1±0.7 nM/s, n=24 in TRPC1 overexpressing cells) of SOCE was significantly increased in transfected myotubes. In the clones expressing TRPC1 in a lesser extent, the magnitude of SOCE correlated with the expression level of TRPC1. In the empty-pcDNA3.1 vector transfected myotubes SOCE was similar to that of the control cells (3.0±0.3 nM/s, n=12 the maximal rate of rise, and 155±10 nM, n=13 the amplitude; $p>0.3$). Resting $[Ca^{2+}]_i$ was similar in control and TRPC1 overexpressing cells, moreover there were not a significant difference in the amplitude of the thapsigargin-evoked Ca^{2+} transients, which suggests that the Ca^{2+} content of the SR did not change as a result of TRPC1 overexpression. When control and transfected myotubes were treated with the TRPC inhibitor YM-58483 during the application of thapsigargin, the differences in the properties of SOCE not only disappeared, but the maximal rate of rise of SOCE in TRPC1 overexpressing myotubes (1.1±0.2 nM/s, n=16) was even smaller compared to that of the control cells (1.8±0.3 nM/s, n=19). In the presence of the drug, SOCE was slightly but not significantly smaller in TRPC1 overexpressing cells compared to control, which suggests the reduced activity of the STIM1-Orai1 system in these cells.

The expression of STIM1 and SERCA decreased in TRPC1 overexpressing cells

To prove the previous hypothesis, the expression of the key proteins of the Ca^{2+} homeostasis was examined with Western-blot. The expression of STIM1 and SERCA decreased significantly, but the expression of actin, Orai1, RyR and

calsequestrin did not change. However it was shown by immunocytochemistry that the decreased amount of STIM1 is also capable to form puncta so it can remain functional.

To examine the physiological effects of the decreased SERCA expression, myotubes were depolarized with 120 mM KCl. Neither the amplitude of the Ca^{2+} transients (235±30 nM, n=16 in control, and 230±23 nM, n=26 in transfected cells) nor the flux of the Ca^{2+} release from the SR (188±21 µM/s, and 193±14 µM/s, respectively) altered significantly (p>0.8) in TRPC1 overexpressing myotubes, which suggests that neither the Ca^{2+} release via RyRs, nor the activity of the voltage gated Ca^{2+} channels changed in these cells. On the other hand, following the transients $[Ca^{2+}]_i$ declined more slowly and returned to a significantly higher level in the transfected myotubes (the difference in the resting $[Ca^{2+}]_i$ before and after the transients was 3.5±0.6 nM in control and 11.0±1.2 nM in TRPC1 overexpressing myotubes) indicating a decreased Ca^{2+} uptake capability of the SERCA pumps. This was quantified by extracting PV_{max}, which was clearly (p<0.01) decreased in TRPC1 overexpressing myotubes (342±20 µM/s) as compared to control (465±39 µM/s) in line with the decreased expression of SERCA.

TRPC1 overexpression alters the differentiation of the myotubes

Morphological alterations of cells in TRPC1 overexpressing cultures were observed during the differentiation and were compared to control cells. Cultured under the same conditions, multinucleated myotubes showed a delayed appearance and thinner diameter in TRPC1 overexpressing cultures. To quantify these alterations, the rate of proliferation and the fusion index were analyzed on each day of culturing. As a possible explanation of these morphological changes the decreased nuclear activity of the transcription factor NFAT1 was observed in TRPC1 overexpressing cells by the application of Western-blot and HSP-NRE NFAT reporter plasmid transfection together with X-Gal staining (p<0.05;

57.5±1.4 AU, n=75 in control, and 46.1±2.6 AU, n=45 in TRPC1 overexpressing myotubes).

Discussion

In my thesis two proteins were examined whose physiological roles are not fully understood. Both of them were proposed to have a role in regulating the Ca^{2+} homeostasis of the cells; one of them could be important in the Ca^{2+} release from the SR, while the other in the Ca^{2+} entry following store depletion (SOCE). We have shown that the 32 kDa isoform of skeletal muscle triadin (Trisk 32) increases Ca^{2+} release via IP_3Rs, while TRPC1 behaves as a Ca^{2+} channel in SOCE mechanism.

Trisk 32 increases the activity of IP_3R

In rat skeletal muscle four isoforms of triadin proteins can be shown, all of them are splice variants of the same gene. We have little information about the physiological role of Trisk 32. It was proposed to have a role in the maintenance of the sarcomere structure and in regulating the Ca^{2+} release via IP_3R.

In our study L6.G8 rat skeletal muscle myoblasts were used which could not form myotubes, so the IP_3 pathway could be examined in them under conditions where any interference from RyR-mediated calcium release is negligible. Trisk 32 was overexpressed with stable transfection in the cells, and the overexpression was confirmed with RT-PCR, immunocytochemistry and Western-blot. The co-localization of IP_3R and Trisk 32 was shown, and their direct association was confirmed with co-immunoprecipitation.

The functional effect of Trisk 32 overexpression was examined with confocal microscopy and single-cell $[Ca^{2+}]_i$ measurement. IP_3-mediated Ca^{2+} release was activated by the local application of arginine-vasopressin and bradykinin. During the confocal measurements the amplitude of the Ca^{2+} transients evoked by the local application of 0,1 μM AVP was significantly higher in Trisk 32 overexpressing cells, compared to control and empty vector transfected myoblasts. The amplitude and the maximal rate of rise of the Ca^{2+}

transients evoked by 20 µM bradykinin was also significantly higher in Trisk 32 overexpressing cells, both in normal $[Ca^{2+}]_e$, and in Ca^{2+}-free environment. The observation that the stimulation of both the V1 vasopressin and the B2 bradykinin receptors evoked a similarly increased response in Trisk 32 overexpressing cells suggested that the examined protein did not affect directly on these two membrane receptors.

When the IP_3R was activated directly by thimerosal, the amplitude and the maximal rate of rise of the Ca^{2+} transients were similar in control and transfected cells. One possible explanation of this observation was that the conductance of IP_3R did not change as a result of Trisk 32 overexpression, while the other explanation was that since thimerosal acts only on IP_3R-I, Trisk 32 does not regulate IP_3R-I, only IP_3R-III.

When the Ca^{2+} content of the ER decreases, a Ca^{2+} entry process is activated from the extracellular milieu to fill the intracellular stores. This well regulated Ca^{2+} uptake mechanism is called store-operated Ca^{2+}-entry. As a result of Trisk 32 overexpression the amplitude and maximal rate of rise of SOCE did not change significantly in spite of the decrease in the expression of STIM1.

The Ca^{2+} released via IP_3R can open neighboring RyRs, causing further Ca^{2+} release (Ca^{2+}-induced Ca^{2+} release). The activity of RyR was examined with the agonist (30 mM caffeine) and antagonist (10 µM ryanodine) of the receptor, and there were no significant differences between the two cell types. This can indicate that the overexpression of Trisk 32 does not alter the activity of RyRs or CICR in these cells.

Thus Trisk 32 not only co-localizes with, but directly contributes to the regulation of Ca^{2+} release via IP_3R.

TRPC1 overexpression increases SOCE

In the second part of my thesis our experiments examining the function of TRPC1 were described. Here the effect of TRPC1 overexpression was examined

on SOCE mechanism, which depends on the co-operation of several proteins (STIM1, Orai1 and TRPC1). STIM1 is the Ca^{2+} sensor in the ER, Orai1 is the Ca^{2+} channel of the surface membrane, while the role of TRPC1 – as a store-operated channel or a part of it – and its relation with the previous proteins is still not clear.

In our experiments TRPC1 was overexpressed in C2C12 mouse skeletal muscle cells with liposome-mediated transfection. The overexpression was confirmed with RT-PCR at mRNA level, and with immunocytochemistry and Western-blot at protein level.

As a result of TRPC1 overexpression the amplitude and maximal rate of rise of SOCE significantly increased in TRPC1 overexpressing C2C12 myotubes. This seemed to correlate with the extent of the overexpression. When an antagonist of TRPC channels, YM-58483 was applied, the maximal rate of rise of SOCE decreased significantly, while its amplitude decreased slightly but not significantly compared to control, which means that the overexpressed TRPC1 can be blocked, and it also suggests the decreased activity of the STIM1-Orai1 system.

In these cells the decrease in the expression of STIM1 and SERCA was confirmed with Western-blot. The latter can explain the observation that after the Ca^{2+} transients evoked by 120 mM KCl, the uptake of Ca^{2+} was slower and higher resting $[Ca^{2+}]_i$ was observable. The downregulation of these proteins could be a compensatory response to TRPC1 overexpression. The reduced STIM1 expression – although it was still capable of forming puncta – could decrease the other pathway of SOCE via Orai1, while the decrease of SERCA could protect the cells from overfilling the stores.

As a result of TRPC1 overexpression morphological changes were observed, differentiation of myoblasts started later and the myotubes were thinner compared to control cultures. These changes could be caused by the decreased nuclear activity of the transcription factor responsible for initiating the

fusion of myotubes, NFAT1. This was confirmed by isolating nuclear fraction of the cultures and performing Western-blot experiments. The activation of NFAT is a [Ca^{2+}] dependent process, so the SOCE – altered by the overexpression of TRPC1 –, or the higher resting $[Ca^{2+}]_i$ observed after the KCl-evoked Ca^{2+} transients could cause the decreased ability of the cells to differentiate. On the other hand the viability and the extent of apoptosis of the transfected cultures did not change significantly compared to control.

Our results suggest that enhancing the expression level of TRPC1 increases SOCE and has a negative feedback effect on both the STIM1 – Orai1 system and SERCA activation, suggesting a cooperation between these proteins.

Summary

In this work two proteins were examined which could contribute to the regulation of the IP_3 receptor (IP_3R) and store-operated Ca^{2+}-entry (SOCE) in the early stages of skeletal muscle differentiation.

Until our results the physiological role of the 32 kDa skeletal muscle triadin isoform (Trisk 32) was unknown. When overexpressed in L6 myoblasts its co-localization and direct interaction with IP_3R could be observed, and the functional consequences of this interaction were shown. When the IP_3 pathway was activated via B2 bradykinin or V1 vasopressin receptors, the Ca^{2+} transients were significantly greater in the Trisk 32 overexpressing cells as compared to control. This enhancement of the transients was not caused by the increase of the activity of ryanodine receptors or SOCE, but it is more likely that Trisk 32 directly affects the opening of IP_3R.

SOCE mechanism depends on the co-operation of several proteins. It is widely accepted that the transient receptor potential canonical channel 1 (TRPC1) has a role in the process, but its role is controversial. TRPC1 was overexpressed in C2C12 myotubes and it was shown that in the transfected cells the magnitude of SOCE increases proportionally with the extent of the overexpression. The reduced expression of stromal interaction molecule 1 (STIM1) – the Ca^{2+} sensor of the SR – and sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) was observed in these cells which could be a compensatory response to avoid the overloading of the stores. Differentiation of the TRPC1 overexpressing cells was also altered, which could be caused by the decreased nuclear activity of the nuclear factor of activated T-cells 1 (NFAT1). Our results suggest that TRPC1 contributes to SOCE mechanism and has a negative feedback effect on the STIM1 – Orai1 system.

Publications

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Candidate: Tamás Oláh

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List of publications related to the dissertation

1. **Oláh, T.**, Fodor, J., Oddoux, S., Ruzsnavszky, O., Marty, I., Csernoch, L.: Trisk 32 regulates IP3 receptors in rat skeletal myoblasts.
   Pflugers Arch. Epub ahead of print (2011)
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   IF:3.354 (2010)

2. **Oláh, T.**, Fodor, J., Ruzsnavszky, O., Vincze, J., Berbey, C., Allard, B., Csernoch, L.: Overexpression of transient receptor potential canonical type 1 (TRPC1) alters both store operated calcium entry and depolarization-evoked calcium signals in C2C12 cells.
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List of other publications

3. Varga, Z., Juhász, T., Matta, C., Fodor, J., Katona, É., Bartók, Á., **Oláh, T.**, Sebe, A., Csernoch, L., Panyi, G., Zákány, R.: Switch of voltage-gated k channel expression in the plasma membrane of chondrogenic cells affects cytosolic ca-oscillations and cartilage formation.
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4. **Oláh, T.**, Fodor, J., Ruzsnavszky, O., Berbey, C., Allard, B., Csernoch, L.: The Alterations of Store-Operated Calcium Entry in TRPC1-Overexpressing C2C12 Myotubes.
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DOI: http://dx.doi.org/10.1113/jphysiol.2008.160457
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List of conference presentations connecting to the topic of the thesis:

Oláh T: Trisk 32 regulates IP_3 receptors in rat skeletal myoblasts. PhD Symposium of the Doctoral School of Molecular Medicine. Debrecen, 2011.

Fodor J, **Oláh T**, Ruzsnavszky O, Oddoux S, Szentesi P, Marty I, Csernoch L: Role of Trisk 32, the 32 kDa triadin isoform, in the calcium homeostasis of skeletal muscle. XXXIX European Muscle Conference (EMC). Padova, Olaszország, 2010.

Oláh T: The alterations of store-operated calcium entry in TRPC1-overexpressing C2C12 myotubes. PhD Symposium of the Doctoral School of Molecular Medicine. Debrecen, 2010.

Oláh T: A Trisk 32 fehérje szabályozó hatása az IP_3 receptoron keresztül történő Ca^{2+}-felszabadulásra patkány eredetű L6 myoblastokon. Molekuláris Orvostudomány Doktori Iskola szimpóziuma. Debrecen, 2009.

List of posters connecting to the topic of the thesis:

Oláh T, Fodor J, Ruzsnavszky O, Berbey C, Allard B, Csernoch L: The alterations of store-operated calcium entry in TRPC1-overexpressing C2C12 myotubes. Biophysical Society 54th Annual Meeting, San Francisco, USA, 2010.

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Oláh T, Fodor J, Ruzsnavszky O, Tóth A, Marty I, Csernoch L: A Trisk 32 fehérje szabályozó hatása az IP_3 receptorokon keresztül történő Ca^{2+}-felszabadulásra patkány eredetű L6 myoblastokban. 39. Membrán Transzport Konferencia. Sümeg, 2009.