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Examination of proteins involved in the regulation of calcium homeostasis in differentiating mesenchymal cells

PhD THESIS

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DEBRECEN
2009.

Introduction

The structure and physiological features of hyaline cartilage

During the initial development of the embryo, the skeleton mostly consists of cartilage. Hyaline cartilage forms the scaffold of the bones, which develops by endochondral bone formation, and the epiphyseal cartilage, which is responsible for the longitudinal growth of the bones. In adulthood it covers the surface of the articular bones as an articular cartilage, ensuring the unique resistance during the whole life. In addition, hyaline cartilage can be found as a permanent cartilage in certain organs as the larynx, trachea, bronchus, forming their scaffold. Cartilage is an avascular tissue, therefore chondrocytes are supplied with nutrients by diffusion from the perichondrium and surrounding tissues. As a consequence, cartilage can be characterized as a bradytrop tissue with slow rate of metabolism. On the basis of the morphological features cartilage can be divided into three major groups:

-Hyaline cartilage, characterized by homogenic, amorf matrix.

-Elastic cartilage, which matrix contains elastic fibers.

-Fibrous cartilage, consisting of compact collagen bundles.

Only the round shaped chondrocytes represent the similarity among different types, embedded within the special extracellular matrix (ECM). In our present study we examined the hyaline cartilage, since a considerable part of the society are concerned in the alterations of articular cartilage like arthrosis and rheumatoid arthritis.

Experimental models for studying cartilage differentiation (high density, HD cell cultures)

In chicken HD mesenchymal cell cultures, chondrogenic mesenchymal cells differentiate into chondroblasts and then chondrocytes during a 6-day-long culturing period. The majority of chondroblasts, characterised by the ability of

production of a cartilage specific ECM, appear from culturing day 3. Cells of HD cultures secrete ATP into the culture medium as an autocrine mediator to facilitate and promote their own differentiation.

Intracellular regulation of cartilage differentiation

One of the key regulators of the signalling processes in chondrogenic cells is Sox9. Since the expression of collagen type II and the core protein of aggrecan are controlled by this transcription factor, Sox9 is often referred to as the master gene of chondrogenesis. On the other hand, MAP kinases (Erk 1/2), regulated by protein kinase C, are also important factors, which control the cell functions of proliferation or differentiation, apoptosis or survival. The tightly regulated level of cytosolic Ca^{2+} is involved in a number of signalling processes in differentiating cells. Among non-excitable cells, the role of intracellular Ca^{2+} in the differentiation process of keratinocytes and osteoblasts has been established. According to our previous studies the extracellular space proved to be the source of elevated cytosolic Ca^{2+}-concentration observed on day 3 of differentiation, and in the present work we aimed to prove the possible role of ionotropic purinergic receptors responsible for the Ca^{2+}-influx into chondrogenic cells.

The structure and physiological features of skeletal muscle

Skeletal muscle is responsible for different movements. The basic unit of skeletal muscle is the muscle fiber. The most important function of the fibers is contraction, as a result of a motor command from the central nervous system. The myosin and actin myofilaments are bundled into myofibrils, which are surrounded by a well-developed intracellular membrane system. The transverse or T-tubules are invaginations of the plasma membrane and the functional connection between the T-tubule and terminal cisternae are referred to as triads. The Ca^{2+}, which is neccessary for muscle contraction, is released from the

terminal cisternae, as a result of the electromechanical coupling. This mechanism is basically activated by the action potential, which propagates along the surface membrane of the muscle fiber and spreads into the T-tubules. As a consequence of depolarization, the voltage sensor dihydropyridine receptors (DHPR) are activated resulting in Ca^{2+}-release through ryanodin receptors (RyR) from sarcoplasmic reticulum (SR). Although the patological symptoms of the skeletal muscle are less frequent, they require an extensive effort from medical practice. The acquired or inherited muscle diseases like myotonies, progressive muscle dystrophies, atrophies are generally fatal before reaching the elderly ages.

Experimental models for studying the Ca^{2+}-homeostasis of the skeletal muscle

Two different models are available to examine skeletal muscle development. Either primary cultures of skeletal muscle prepared from satellite cells of adult muscle or immortalized cell lines as C2C12 mouse or L6 rat myoblasts can be used. In the early stage of myogenesis the Ca^{2+}-homeostasis of myoblasts is comparable to that of smooth muscle cells. During differentiation into myotubes the structure of the SR becomes more developed, making the voltage-induced Ca^{2+}-release (VICR) essential in skeletal muscle function.

The regulation of Ca^{2+}-release and the intracellular Ca^{2+}-events of the skeletal muscle

Trisk 95 (triadin 95), an integral SR membrane protein, has previously been shown to interact with both the Ca^{2+}-release channel and calsequestrin (CSQ), the low affinity Ca^{2+}-binding protein of the SR lumen. They seem to form a tri- or tetramolecular complex which also includes junctin. This close association suggests that triadin could influence RyR function. Primary myotubes readily generate Ca^{2+}-transients evoked by action potentials. The

opening of a single or a group of calcium release channels results in localized, elementary Ca^{2+}-release event (ECRE) termed Ca^{2+} spark or ember.

Aims

Serious disorders and alterations, which are incurable or may only be slightly improved by medical science or physiotherapy treatment, can be mentioned as being associated with both cartilage and skeletal muscle. In my thesis I report on my examinations and results obtained from these mesenchymal tissues. The exact regulation of intracellular [Ca^{2+}] is indispensable to intrauterine and postnatal development and proper function of such tissues. We studied the Ca^{2+}-homeostasis and the possible role of various proteins involved in this complex process. In case of cartilage tissue we examined the putative role of purinergic receptors in the increase of intracellular [Ca^{2+}] during chondrogenic differentiation. On the other hand, we studied the effect of Trisk 95 protein on Ca^{2+}-release by modifying its expression level in skeletal muscle. Using confocal laser scanning microscopy we applied line-scan technique to detect Ca^{2+}-release events in control and triadin overexpressing cells, as well as in cells with reduced triadin expression. We aimed to determine how the altered triadin expression modifies the parameters (amplitude, frequency, full width at half maximum) of the Ca^{2+}-release events. We also applied whole cell intracellular [Ca^{2+}] measurements, and the comparative examination of Ca^{2+}-transients evoked by different agonists was achieved.

Materials and Methods

Cell cultures

High Density (HD) cell cultures

HD cell cultures were prepared from distal parts of the limb buds of 4-day-old Ross hybrid chicken embryos (Hamburger–Hamilton stages 22–24), which were removed and chondrifying micromass cultures of mesenchymal cells were established. 15 or 30 μL droplets of the suspension containing 1.5×10^7 cells/mL were inoculated on round coverglasses placed into plastic Petri dishes. Cells were allowed to attach to the surface for 2 hrs at 37 °C. Day of inoculation is considered as day zero. Colonies were grown in Ham's F12 medium supplemented with 10 % foetal calf serum, antibiotics and antimycotics, and were kept at 37 °C in an atmosphere of 95 % air and 5 % CO_2 and 80 % humidity. The medium was changed on every second day.

Skeletal muscle cultures and transfection

C2C12 skeletal muscle cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin and were incubated at 37 Cº in a humidified incubator with 5% CO_2/95% O_2. Full length coding sequence of Trisk 95 was ligated into the EcoR1 restriction site of pcDNA 3.1 expression vector. Transfection was performed in Opti-MEM reduced serum content medium using Lipofectamine 2000 reagent for 2.5 h at 37 Cº. Cells were allowed to express the introduced genes for 48 h in growth medium then were selected in DMEM containing 1000 μg/ml geneticin. After 14-18 days, single colonies were isolated. Differentiation of transfected cells was induced at 80% confluency by changing the culture medium for DMEM supplemented with 5% horse serum (HS). The efficiency of Trisk 95 overexpression was monitored by

immunostaining. Functional experiments were carried out on 5-7 days-old differentiated myotubes.

Primary cultures of skeletal muscle cells were obtained from 1 day-old mice or 5-10 days-old rats. Animals were sacrificed following approved protocols of the Animal Care Committee of the University of Debrecen. Skeletal muscle was collected from the hind limbs, put in Hank's solution and then cut into small pieces. The muscle tissue was dissociated at 37 Cº using 0.75 mg/ml collagenase type I, 1.5 mg/ml trypsin in a calcium/magnesium free phosphate buffer. After filtration and centrifugation the cells were seeded onto cover-slips in a proliferation medium (HAM F12 supplemented with 15% FBS and penicillin-streptomycin). On the third day of culturing the medium was exchanged to DMEM supplemented with 5% HS to allow differentiation of the myotubes. In this system adenoviral transfection was used to modify triadin expression. Two types of adenoviruses were used, a control virus (rAdV5-DsRed) with the cDNA of the red fluorescent protein and the test containing the full-length sequence of skeletal muscle Trisk 95 (rAdV5-Trisk 95-ires-DsRed). All the transgenes were controlled by the CMV promoter. After 48 h proliferation, cells were incubated with the viruses for 10 h in HAM F12 without serum (the cell nucleus to virus ratio was 1:40) then differentiation was induced by changing the medium. Confocal microscopy studies were performed after 55-60 hours of infection/differentiation.

The presence of Trisk 95 was determined by immunostaining at different stages of differentiation (2, 3, 4 days in differentiation medium). RNA interference technique was applied in order to reduce endogenous Trisk 95 expression. The chosen shRNA cassette sequence besides the sense (5'GGCAAAGATGTAAAGCCTAAA) and antisense (5'TTTAGGCTTTACATCTTTGCC) region contains a loop and termination sequence resulting in a hairpin siRNA. The shRNA transfection was carried out after 30-36 h of differentiation with the

application of Lipofectamine 2000 reagent in Opti-MEM transfection medium. Ca^{2+}-imaging studies were also performed using a confocal microscope.

Immunocytochemical staining of P2X receptors and Trisk 95 protein

Cultures were washed twice with PBS and fixed in 4 % paraformaldehyde for 15 min at 4 °C. After washing in PBS, cells were permeabilized with 0.1 % Triton X-100 in PBS for 30 min. Non-specific binding sites were blocked by 30 min preincubation in 1 % bovine serum albumin (BSA) in PBS, followed by incubation with the primary antibodies at 4 °C overnight. Subsequently, the cultures were washed three times with PBS for 10 min, and were incubated with a FITC-conjugated anti-rabbit IgG for 1 h. Cultures were mounted with Vectashield® mounting medium containing DAPI. Control experiments were carried out with primary antibodies incubated with their control peptides according to the instructions of the manufacturer.

Preparation of cell extracts

HD cultures were harvested on each day of culturing. Cell pellets were suspended in homogenization buffer and then were snap-frozen in liquid nitrogen, and were stored at –70 °C. Samples were sonicated for four times 30 sec by 50 cycles. For Western blot analyses, total cell lysates and plasma membrane fractions were used. For isolation of plasma membrane fraction of HDC, sonicated samples were centrifuged at 50,000 ×*g* for 90 min at 4 °C. Pellet was triturated continuously in 50 μL homogenization buffer supplemented with 1 % Triton X-100 at 4 °C. After 1 h of trituration samples were centrifuged again at 50,000 ×*g* for 55 min at 4 °C, and supernatant containing plasma membrane fraction was used for Western blot analyses.

Western-blot analysis

Total cell lysates and plasma membrane fractions were examined by Western blot. Samples for SDS-PAGE were prepared by the addition of 1/5 volume of 5-fold concentrated electrophoresis sample buffer About 50 μg of protein was separated by 7.5 % SDS-PAGE gel, then proteins were transferred electrophoretically to nitrocellulose membranes. After blocking in 5 % non-fat dry milk in PBS, membranes were incubated with primary antibodies raised against the carboxy termini of P2X-receptors (Alomone Labs, Jerusalem, Israel) and $P2Y_4$ receptor (Sigma, Budapest, Hungary), amino terminus of $P2Y_1$ receptor (Sigma, Budapest, Hungary) and 3rd intracellular loop of $P2Y_2$ receptor (Alomone Labs, Jerusalem, Israel) overnight at 4 °C. After washing three times for 10 min with PBST, membranes were incubated with a secondary antibody, anti-rabbit IgG. Signals were detected by enhanced chemiluminescence reaction.

RT-PCR analysis

For RT-PCR analysis total RNA was isolated from cells of HD culture of various ages using Quiagen RNeasy® Micro Kit. After reverse transcriptase reaction amplifications of specific cDNA sequences were performed with specific primers that were designed based on published chicken nucleotide sequences. PCR products were analysed using ethidium bromide-stained agarose gel.

Administration of extracellular ATP, ADP, UDP, UTP and suramin

ATP (100 μM), ADP, UDP, UTP (180 μM) and suramin (10 μM) were administered to cells of HDC on various days of culturing. The nucleotides and suramin were diluted in the culture medium. Effects on metachromatic cartilage matrix formation were examined by metachromatic staining with dimethylmethylene blue and toluidine blue.

Determination of extracellular ATP in the culture medium

Concentration of extracellular ATP secreted by cells of high density cell cultures was determined using ATP-Bioluminescent Assay Kit. Concentration of ATP secreted by cells of HDC into the culture medium was determined at approximately the same period of each culturing day. The medium was changed every day following measurements. 50 μL of the culture medium (pH adjusted to 7.8) was used to determine the amount of ATP in the culture medium in 2 parallel experiments. Background light emission was determined using blanks (both sterile water and Ham's F12 culture medium) and new standard curve was prepared each day prior to measurements (concentrations of ATP standard solutions were as follows: 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} M). Luminescence of samples was determined using a microwell plate reader.

Single cell [Ca^{2+}] measurements

Measurements were performed on chondrogenic and skeletal muscle cultures using the Ca^{2+}-dependent fluorescent dye Fura-2. Excitation wavelength was alternated between 340 and 380 nm by a dual wavelength monochromator (Deltascan, Photon Technology International), while the emission was monitored at 510 nm using a photomultiplier. The intracellular [Ca^{2+}] was calculated from the ratio of fluorescence intensities (R= F340/F380). Test solutions were directly applied to the cells through a perfusion capillary tube with an internal diameter of 250 μm at a 1.5 μL/s rate, using a local perfusion system. All measurements were performed at room temperature. Data were statistically analysed by Student's *t*-test.

Confocal-measurements

Localized Ca^{2+}-release events were monitored with the LSM 510 META confocal laser scanning microscope. C2C12 myotubes were loaded with Fluo-3, and myotubes from primary cultures were incubated with 10 μM Fluo 4-AM. Two dimensional (x-y) and line-scan images were used to monitor the fluorescence intensity. Line-scan images were recorded at 1.54 ms/line and 512 pixels/line using a 63x water immersion objective. Fluo-3 or 4 was excited with an argon ion laser. Images were analysed using an automatic event detection program, which calculated the amplitude (ΔF/F0), full width at half maximum (FWHM), rise time and duration of the identified events.

Results

Primary, HD cell cultures

Cells of HD cultures respond to extracellular ATP by elevating intracellular $[Ca^{2+}]$

ATP at a constant concentration of 180 μM was administered to the close proximity of cells of HD culture on various days of culturing. Administration of ATP could induce a transient increase in intracellular $[Ca^{2+}]$ in cells measured in Ca^{2+}-containing bathing solution. Note that both the amplitude of the average response (maximal increase in intracellular $[Ca^{2+}]$) and the time of exposure to ATP needed to induce the transient exhibited a differentiation-dependent pattern. Striking differences were also observed regarding the number of cells responding to ATP. While most of the cells (90 %) responded to ATP in 3-day-old cultures, essentially none did at day 1 of culturing. Administration of ATP on other days could also induce Ca^{2+}-transients, but the proportion of cells that responded was hardly comparable with that on day 3. To establish whether metabotropic or ionotropic purinergic receptors were responsible for these effects, ATP was administered to cells in a Tyrode's solution lacking Ca^{2+}. In the 30 cells examined, no response was detected on either days of culturing in

the absence of extracellular Ca^{2+}. This observation firmly supported our theory that influx of extracellular Ca^{2+} was needed to evoke the effect of extracellular ATP and the receptor of ATP was a member of the ionotropic purinergic receptor family (P2X).

To find candidates among P2X receptors, their non-specific antagonist suramin was tested on cells of HD culture. It is known that suramin inhibits all P2X receptors except $P2X_4$ and $P2X_6$. Cells treated with suramin (10 μM) showed no significant alteration in the intracellular [Ca^{2+}] following the administration of ATP. Repetitive administration of ATP could induce repetitive and transient elevations in intracellular [Ca^{2+}] in 3-day-old HD culture. These observations raised the possibility of the presence and function of $P2X_4$ and/or $P2X_6$ receptor subtypes.

Although the above results clearly suggested that P2X receptors play the decisive role, we also carried out experiments to obtain data on the function of metabotropic P2Y receptors. Prior to the administration of agonists of P2Y receptors, we intended to examine whether intracellular Ca^{2+} stores are present and contain releasable Ca^{2+} by the activation of IP_3 pathway. Since bradykinin receptors are known to activate this process and are described as being expressed by chondrocytes, therefore bradykinin was administered to 3-day-old cells at a concentration of 20 μM. A slight elevation (30 nM) was observed in 60% of cells proving the presence and active functioning of IP_3 signalling ADP, UDP and UTP non-specific agonists of P2Y receptors were administered to cells of HD culture at a concentration of 180 μM on day 3 of culturing. Slight elevation of cytosolic Ca^{2+} was detected only in 50% of cells measured during the administration of UTP. On the other hand, administration of ADP and UDP did not result in any significant Ca^{2+}-transients.

Chondrogenic mesenchymal cells express various P2X and P2Y receptor subtypes during differentiation

To identify the presence and expression pattern of various purinergic receptors during chondrogenic differentiation of chicken mesenchymal cells, RT-PCR reactions were performed. mRNA sequences of chicken P2X receptors, but not of $P2X_6$ (not yet published) as well as $P2Y_1$, $P2Y_3$, and $P2Y_5$ receptors were downloaded from GenBank and specific primer pairs for each mRNA sequence were designed for amplification. Amplimers of expected sizes were identified for all the available mRNAs, except for $P2X_2$, where only very weak signals were detected. $P2X_4$ and $P2X_5$ receptor subtypes showed the strongest expression levels. Both receptors were expressed markedly on day 1 then the signal became gradually weaker. mRNAs of $P2Y_1$, $P2Y_3$ and $P2Y_5$ showed constant expression levels throughout the culturing period.

Western blot analysis showed a different expression profile for the different P2X receptor subtypes. We could not detect the $P2X_2$ receptor subtype, and no signals were visible for $P2X_3$ and $P2X_6$, either in total lysates or in isolated plasma membrane fractions. Nevertheless, protein expression of $P2X_4$ receptor subtype proved to be the most interesting. While in total cell lysates it showed a rather variable profile, in isolated plasmamembrane-fractions it first appeared on day 3 with a strong band, and by days 4 and 6 its expression rapidly diminished. We also detected the expression of metabotropic purinergic receptors in HD culture. As $P2Y_3$ and $P2Y_5$ receptors are not regarded as functional members of this family of receptors, we investigated only $P2Y_1$, $P2Y_2$ and $P2Y_4$ protein in total cell lysates and plasma membrane fractions. On day 1, the protein of $P2Y_1$ was not expressed by cells of HD culture, then it was present at a constant level both in total cell lysates and in plasmamembrane-fractions until day 6, when it showed a small decline. The $P2Y_2$ receptor protein was found to be expressed in a peak like pattern in total lysates with strongest signals on days 2-4. However, we detected a constant level of expression in the plasma

membrane fraction with the exception of day 6, when the signal became weaker. We could only detect specific signals for $P2Y_4$ receptor in the plasma membrane fraction with a stronger band on day 2, but no immunopositivity was observed in total lysates.

Administration of extracellular ATP on day of differentiation increases matrix production

ATP was administered at various concentrations to cells of HD cultures on day 3 of culturing. At the concentration of 100 μM, extensive matrix production occurred by day 6 demonstrated by both DMMB and TB stainings. mRNA levels of collagen II and the core protein of aggrecan also reflected the slightly higher rate of matrix production under the effect of ATP. Although mRNA expression level of Sox9 did not change, protein expression of this transcription factor became higher as a result of the administration of ATP. Treatment of HD cultures with ATP on days 2 and 4 of culturing did not alter the cartilage matrix production. Administration of the non-specific P2X receptor antagonist suramin alone or combination with ATP did not cause any significant alteration in the amount of cartilage matrix produced by the end of the 6-day-old culturing period. Moreover, the ATP-stimulated Sox9 expression was not affected by suramin treatment, further supporting our theory that $P2X_4$ receptor could be involved in the transmission of the chondrogenesis promoting effect of extracellular ATP.

Administration of ADP, UDP and UTP to the culture medium has no effect on cartilage formation

We examined the administration of the nucleotides on cartilage matrix production of HD culture to elucidate a putative role of metabotropic purinergic receptors. The nucleotides applied at a concentration of 180 μM into the culturing medium on day 3 did not exert any effect on the amount of cartilage

matrix produced by the end of the 6-day-long culturing period as revealed by metachromatic staining.

Cells of HD cultures secrete ATP into the culture medium

The chondrogenic mesenchymal cells secrete ATP into the culture medium as an autocrine mediator to promote and facilitate their own differentiation. To investigate this, the culture medium was removed from the cells of HD cultures on each day of culturing and ATP assays were performed. We found that on each day of culturing a small amount of ATP was detectable in the culture medium in the range of 2–10 nM, which is comparable to data measured in culture medium of other non-excitable cells.

Skeletal muscle cultures

Ca^{2+}-transients in triadin overexpressing C2C12 cells

The effect of trisk 95 overexpression was examined in a mouse skeletal muscle cell line. Trisk 95 protein synthesis has been amplified using a pcDNA3.1 plasmid-vector system. The stable overexpression of the protein in the selected clones was confirmed with immunocytochemistry at the myotube stage. Control and Trisk 95-transfected, differentiated (5 to 7 days old) C2C12 myotubes were used for functional experiments. First we examined whether the protein overexpression alters the Ca^{2+}-homeostasis of the myotubes. The resting intracellular [Ca^{2+}] was similar ($p>0.2$) in both groups (77.4±5.2 nM, n=48 in control and 71.3±4.8 nM, n=54 in Trisk 95 overexpressing cells). The presence of functional ryanodine receptors and the presence of releasable Ca^{2+} in the SR were investigated using 15 mM caffeine. No significant difference was observed in the amplitude of caffeine-induced transient elevation of intracellular [Ca^{2+}]. On the other hand Ca^{2+}-transients could also be evoked by depolarization using 120 mM KCl both in normal extracellular [Ca^{2+}] and in a Ca^{2+}-free bathing solution. The amplitude of the KCl-induced Ca^{2+}-transients in Ca^{2+}-free solution

was significantly reduced in Trisk 95 overexpressing myotubes (98±10 nM, n=42) as compared to control cells (170±14 nM, n=29).

Localized Ca^{2+}-release events in triadin overexpressing C2C12 cells

Control myotubes readily displayed both Ca^{2+} sparks (Ca^{2+}-release events with large amplitude and short duration) and embers (events with small amplitude and long duration). Although, myotubes following the transfection with the triadin-plasmid generated Ca^{2+} sparks and embers as control cells did, the characteristic parameters of these events were altered. After triadin overexpression the relative frequency of Ca^{2+} sparks with large amplitudes were reduced as compared to control myotubes. The distribution of the average amplitude of embers, on the other hand, was not altered in triadin overexpressing C2C12 cells. Similarly, the histograms of FWHM demonstrate that the overexpression of triadin shifted the distribution to the left for sparks, whereas that of the embers remained unaffected. In accordance with the above, the average amplitude of the Ca^{2+} sparks was significantly smaller in triadin overexpressing myotubes than in control cells (0.59±0.01 *vs*. 0.81±0.01; $p<0.001$), while the average amplitude for the embers was only slightly affected in Trisk 95 overexpressing myotubes (0.22±0.01 *vs*. 0.26±0.02; $p>0.01$). FWHM for Ca^{2+} sparks, but not for embers, was significantly smaller in triadin transfected myotubes (1.26±0.07 μm; $p<0.01$) than in control cells (1.86±0.06 μm). In addition, the frequency of the spontaneous Ca^{2+}-release events was also significantly ($p<0.05$) reduced in Trisk 95 overexpressing myotubes (1.04±0.14 Hz for sparks and 0.09±0.04 Hz for embers) as compared to the control group (1.85±0.21 Hz for sparks and 0.19±0.02 for embers).

Triadin overexpression in mouse skeletal muscle cells in primary culture

The overexpression of triadin in primary cultured skeletal muscle cells of mice was carried out using an adenoviral transfection. Both control and triadin

overexpressing myotubes generated Ca^{2+}-transients probably triggered by spontaneous action potentials. These Ca^{2+}-transients were visualised using laser scanning confocal microscopy in the line-scan mode. The analysis of these images revealed that both the amplitude and frequency of the Ca^{2+}-transients in triadin overexpressing myotubes was significantly reduced (0.56±0.03 and 0.26±0.01 Hz, n=71; $p<0.02$ and 0.001, respectively) as compared to controls (0.68±0.02 and 0.75±0.04 Hz, n=169, respectively. Ca^{2+}-transients were also elicited using external electrical stimulation. The observations also confirmed the significant ($p<0.05$) reduction in the amplitude following the overexpression of Trisk 95, from a control value of 0.64±0.09 (n=17, four cells) to 0.46±0.05 (n=45, nine cells).

Inhibition of endogenous triadin expression using specific shRNAs in rat primary cultured skeletal muscle cells

In contrast to the measurements on triadin overexpression, to study the effects of reduced Trisk 95 expression, a system where the frequency of ECRE is relatively low under control conditions – rat myotubes in primary culture – was selected. Differentiating myotubes were transfected with Rhodamine-red labelled Trisk 95 specific shRNA using a liposome-induced transfection protocol. Two days after transfection the shRNA positive cells were easily identified by their red fluorescence.

Ca^{2+}-transients in cultured rat skeletal muscle cells with suppressed triadin expression

30 mM caffeine to the bathing medium induced a transient elevation in intracellular [Ca^{2+}] both on control myotubes and on myotubes with suppressed Trisk 95 expression. The latter transients were always smaller than those in control suggestive of a reduced SR content. In line with this observation, the resting intracellular [Ca^{2+}] was found to be increased in myotubes with reduced

Trisk 95 expression if held in a calcium free extracellular solution (64.9±3.3 *vs.* 85.7±6.9 nM; n = 15 and 12; control myotubes and myotubes with reduced triadin expression, respectively). In addition, depolarisation, induced by the addition of 120 mM KCl in the absence of external Ca^{2+}, could readily induce Ca^{2+}-release from the SR, but in contrast to what was seen for triadin overexpressing cells, myotubes with reduced Trisk 95 expression had, in spite of the reduced SR Ca^{2+}-content, identical KCl-evoked Ca^{2+}-transients to those measured in control cells.

Ca^{2+}-release events in cells with suppressed Trisk 95 expression

Control myotubes readily produced spontaneous Ca^{2+}-transients which had an average amplitude of 0.62±0.05 (n=553 on 23 myotubes), and appeared with an average frequency of 1.01±0.22 Hz. On the other hand, ECRE were scarce on these cells. A large fraction of images (225 out of 279), were devoid of such events. Although both sparks and embers were occasionally observed their numbers were low, all together 143 sparks and 23 embers (in 54 images) were detected. The suppression of triadin expression in these myotubes had dramatic effects on the appearance of ECRE. Ca^{2+} sparks and embers became readily observable in both x-y and in line-scan images (n=649 and 117 for sparks and embers, respectively, from 5 myotubes, 218 out of 385 images). Not only ECRE but spontaneous Ca^{2+}-transients were also present on these cells. They appeared with approximately the same frequency (0.99±0.16 Hz; n=888 on 20 myotubes) but with a larger amplitude (1.39±0.08; $p<0.01$) than those in control (see above). Not only was the frequency of ECRE altered in myotubes with suppressed Trisk 95 expression but their characteristic parameters were also changed. The proportion of Ca^{2+} sparks with large amplitude was increased in myotubes with suppressed Trisk 95 expression. As a result, the average amplitude of all sparks was significantly increased, from 0.49±0.03 in control to 0.89±0.04 ($p<0.01$), in these cells. Not only the amplitude of sparks, but the

average amplitude of embers was also increased, from 0.27±0.05 to 0.49±0.03 ($p<0.01$). On the other hand, neither the average duration of embers (229±33 *vs*. 198±13 ms in control and in shRNA treated myotubes, nor that of sparks (39.7±4.3 *vs*. 44.0±2.5 ms) was altered significantly ($p>0.3$ and 0.07, respectively). In contrast, the spatial spread (from 1.67±0.16 to 1.38±0.07; $p<0.02$) was reduced, while the rise time (from 15.4±2.2 to 22.0±2.9 ms; $p<0.01$) of Ca^{2+} sparks was increased in myotubes with suppressed triadin expression.

Discussion

Primary, HD cultures

In vitro chondrogenesis is a dynamic, multistep process regulated by a variety of molecular processes, many of which involve activation and deactivation of protein kinases and phosphatases sensitive to changes of intracellular [Ca^{2+}]. In chicken HD mesenchymal cell cultures, chondrogenic mesenchymal cells differentiate into chondroblasts and then to chondrocytes during a 6-day-long culturing period. The majority of chondroblasts, characterised by the ability of production of a cartilage specific ECM, appear from culturing day 3. We have previously demonstrated that elevation of free cytosolic [Ca^{2+}] at the time of differentiation of chondroblasts was mainly due to a Ca^{2+}-influx and it was indispensable to cartilage formation in chicken HD mesenchymal cell cultures. In the present work we describe, that chondrogenic cells secreted ATP during the differentiation period. Administration of ATP to the culture medium evoked Ca^{2+}-transients exclusively in the presence of extracellular Ca^{2+} and mostly on day 3 of culturing, when the final commitment of chondroblasts occurs. Moreover, ATP caused elevated protein expression of the chondrogenic transcription factor Sox9 and ATP also stimulated cartilage matrix production. Administration of suramin, which blocks all P2X receptors but not $P2X_4$ did not impede the effects of ATP, furthermore, $P2X_4$ appeared in

the plasma membrane fraction and gave signals with immunocytochemistry only from day 3. Expressions of both ionotropic purinergic receptors $P2X_1$, $P2X_4$, $P2X_5$ and $P2X_7$ and metabotropic purinergic receptors $P2Y_1$, $P2Y_3$ and $P2Y_5$ at the mRNA level and $P2Y_1$, $P2Y_2$ and $P2Y_4$ at the protein level were detected. Among the agonists of the metabotropic receptors, ADP and UDP did not evoke any Ca^{2+}-transients in HDC and had no influence on cartilage formation. On the other hand administration of UTP resulted in transient elevation of cytosolic Ca^{2+} concentration in 3-day-old HDC but did not stimulate matrix production during the chondrogenesis. Based on these data, we suggest a role of $P2X_4$ in the generation of ATP-dependent Ca^{2+}-transients of differentiating chondroblasts.

Skeletal muscle cultures

The 95 kDa triadin (Trisk 95), an integral protein of the sarcoplasmic reticulum membrane in skeletal muscle, interacts with both the ryanodine receptor (RyR) and calsequestrin. While its role in the regulation of Ca^{2+}-homeostasis has been extensively studied, data is not available on whether the overexpression or the interference with the expression of Trisk 95 would affect Ca^{2+} sparks the localized, elementary Ca^{2+}-release events (ECRE). In the present study ECRE and Ca^{2+}-transients were studied using laser scanning confocal microscopy on C2C12 cells and on primary cultures of skeletal muscle. Liposome or adenovirus-mediated Trisk 95 overexpression and shRNA interference with triadin translation were used to modify the level of the protein. Stable overexpression in C2C12 cells significantly decreased the amplitude and frequency of Ca^{2+} sparks, and the frequency of embers. In line with these observations, depolarisation-evoked Ca^{2+}-transients were also suppressed. Similarly, adenoviral transfection of Trisk 95 into cultured mouse skeletal muscle cells significantly decreased both the frequency and amplitude of spontaneous global Ca^{2+}-transients. Inhibition of endogenous triadin expression by RNA interference caused opposite effects. Primary cultures of rat skeletal

muscle cells expressing endogenous Trisk 95 readily generated spontaneous Ca^{2+}-transients but rarely produced Ca^{2+} sparks. Their transfection with specific shRNA sequence significantly reduced the triadin-specific immunoreactivity. Functional experiments on these cells revealed that while caffeine-evoked Ca^{2+}-transients were reduced, ECRE appeared with higher frequency. These results suggest that Trisk 95 negatively regulates RyR function by suppressing localized Ca^{2+}-release events and global Ca^{2+}- signals in cultured muscle cells.

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