

# Trisk 32 regulates IP<sub>3</sub> receptors in rat skeletal myoblasts

Tamás Oláh<sup>1</sup>, János Fodor<sup>1</sup>, Sarah Oddoux<sup>2</sup>, Olga Ruzsnavszky<sup>1</sup>, Isabelle Marty<sup>2</sup>, László Csernoch<sup>1</sup>

<sup>1</sup> *University of Debrecen, Medical and Health Science Centre, Department of Physiology, Debrecen, Hungary*

<sup>2</sup> *Inserm U836, Grenoble Institut des Neurosciences; Université Joseph Fourier, Grenoble, France*

Address all correspondence to:

Dr. László Csernoch,  
Department of Physiology,  
University of Debrecen,  
Debrecen, Hungary H-4012,  
P.O.Box 22,

Tel.: +36-52-255-575,

Fax: +36-52-255-116,

E-mail: [csl@phys.dote.hu](mailto:csl@phys.dote.hu)

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## Abstract

To date four isoforms of triadins have been identified in rat skeletal muscle. While the function of the 95 kDa isoform in excitation-contraction coupling has been studied in detail, the role of the 32 kDa isoform (Trisk 32) remains elusive. Here Trisk 32 overexpression was carried out by stable transfection in L6.G8 myoblasts. Co-localization of Trisk 32 and IP<sub>3</sub> receptors (IP<sub>3</sub>R) was demonstrated by immunocytochemistry and their association was shown by co-immunoprecipitation. Functional effects of Trisk 32 on IP<sub>3</sub>-mediated Ca<sup>2+</sup> release were assessed by measuring changes in [Ca<sup>2+</sup>]<sub>i</sub> following the stimulation by bradykinin or vasopressin. The amplitude of the Ca<sup>2+</sup> transients evoked by 20 μM bradykinin was significantly higher in Trisk 32-overexpressing ( $p < 0.01$ ; 426±84 nM, n=27) as compared to control cells (76±12 nM, n=23). The difference remained significant ( $p < 0.02$ ; 217±41 nM, n=21 and 97±29 nM, n=31, respectively) in the absence of extracellular Ca<sup>2+</sup>. Similar observations were made when 0.1 μM vasopressin was used to initiate Ca<sup>2+</sup> release. Possible involvement of the ryanodine receptors (RyR) in these processes was excluded, after functional and biochemical experiments. Furthermore, Trisk 32 overexpression had no effect on store-operated Ca<sup>2+</sup>-entry, despite a decrease in the expression of STIM1. These results suggest that neither the increased activity of RyR, nor the amplification of SOCE are responsible for the differences observed in bradykinin- or vasopressin-evoked Ca<sup>2+</sup> transients, rather, they were due to the enhanced activity of IP<sub>3</sub>R. Thus Trisk 32 not only co-localizes with, but directly contributes to the regulation of Ca<sup>2+</sup> release via IP<sub>3</sub>R.

*Keywords: -Inositol 1,4,5-trisphosphate-, Skeletal muscle, Myoblasts, Calcium transient, Endoplasmic reticulum*

## Abbreviations

The abbreviations used are:

[Ca<sup>2+</sup>]<sub>e</sub>, extracellular calcium concentration; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular calcium concentration; AM, acetoxymethyl ester; AVP, arginine-vasopressin; CICR, Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release; DAG, diacylglycerol; DHPR, dihydropyridine receptor; ER, endoplasmic reticulum; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; IP<sub>3</sub>R, inositol 1,4,5-trisphosphate receptor; IP<sub>3</sub>R-III, IP<sub>3</sub>R type III; PIP<sub>2</sub>, phosphatidylinositol 4,5 bisphosphate; PLC, phospholipase C; RyR, ryanodine receptor; SERCA, sarco-endoplasmic reticulum calcium ATPase; SOCE, store-operated Ca<sup>2+</sup>-entry; SR, sarcoplasmic reticulum; STIM1, stromal interaction molecule 1; TG, thapsigargin; Trisk 32, 32 kDa isoform of skeletal muscle triadin; TRPC, transient receptor potential canonical;

## Introduction

In mammalian cells changes in intracellular calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>) control a wide variety of functions, including division, secretion, motility and contractility. To precisely regulate these changes in [Ca<sup>2+</sup>]<sub>i</sub> highly organized structures are present in adult skeletal muscle fibers. The 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<sup>2+</sup> is released from the sarcoplasmic reticulum (SR), the intracellular Ca<sup>2+</sup> store (for review see e.g. [11, 26]).

In the early stages of skeletal muscle differentiation - in myoblasts - this complex structure, however, is not at all functional, rather, inositol 1,4,5-trisphosphate receptors (IP<sub>3</sub>R) have a significant role in the Ca<sup>2+</sup> release from the endoplasmic reticulum (ER) (see e.g. [7]). In response to different external stimuli (as the activation of bradykinin or vasopressin receptors), phospholipase C (PLC) hydrolyses phosphatidylinositol 4,5 bisphosphate (PIP<sub>2</sub>) in the plasma membrane to produce IP<sub>3</sub> and diacylglycerol (DAG). While DAG activates protein kinase C (PKC), IP<sub>3</sub> binds to the Ca<sup>2+</sup> release channel of the ER. Molecular and physiological properties of the IP<sub>3</sub>R closely resemble those of the RyR [10]. It has at least four known isoforms, which are all present in rodent skeletal muscle together with the basic molecular machinery for a functioning IP<sub>3</sub> messenger system [22]. The binding of IP<sub>3</sub> to its receptor activates Ca<sup>2+</sup> release from the store to increase [Ca<sup>2+</sup>]<sub>i</sub> [34].

RyRs have been shown to interact with a number of different proteins present in the triadic junction including, among others, calsequestrin, junctate, junctin, JP45, mitsugumins, and triadin (for review see [31]). From this group of SR membrane proteins triadin was first to be identified in rabbit skeletal muscle in 1990 [5, 16] as a 95 kDa glycoprotein specifically located in the triads, and was later proved to decrease the extent of Ca<sup>2+</sup> release via RyRs [9, 24]. Since then several isoforms have been identified both in skeletal and cardiac muscle [13, 14, 19, 33].

The 32 kDa isoform of triadin (Trisk 32), smallest skeletal muscle triadin [33], is identical to CT1, the major cardiac triadin [17], and is the only triadin isoform expressed in both muscles. It derives from an alternative splicing after the eight first exons of the triadin gene and possesses a unique C-terminal end. Interestingly though, the expression of this isoform precedes that of RyR during muscle differentiation [ZZZ]. This unique timing of its expression raises several questions. First, why would a member of a family of proteins that were originally described as regulating RyR be expressed at times when RyR is not yet present and, second, could there be an alternative function of Trisk 32 in skeletal muscle?

Despite the structural and functional similarity to RyR, proteins that specifically interact with and modify the function of IP<sub>3</sub>R have not been characterized. In skeletal muscle, Trisk 32 has been shown to be localized within the longitudinal SR, co-localized with IP<sub>3</sub>R and the mitochondria, and to be associated with IP<sub>3</sub>R [33]. The existence of a non-triadic Ca<sup>2+</sup>-release complex centered around IP<sub>3</sub>R and involving Trisk 32 has been proposed, and in this context, the function of Trisk 32 could be the regulation of the Ca<sup>2+</sup> releases via IP<sub>3</sub>R [33].

To test this hypothesis Trisk 32 has been overexpressed in a rat skeletal muscle cell line that contains endogenously the components of the IP<sub>3</sub> signaling cascade. The protein was found to co-localize and co-immunoprecipitate with IP<sub>3</sub>R in transfected cells. The expression of this triadin isoform greatly increased Ca<sup>2+</sup> release from the ER following the stimulation of the cells by either bradykinin or vasopressin strengthening the idea that Trisk 32 is functionally associated with this calcium release channel.

## Experimental procedures

### Cell cultures and transfection

L6.G8 rat skeletal muscle myoblasts were obtained from European Collection of Cell Cultures (ECACC) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, Budapest, Hungary) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin and were incubated at 37 °C in a humidified incubator with 5% CO<sub>2</sub> and 95% O<sub>2</sub> (according to the instructions of the supplier). Cells were seeded at a density of 1000-2000 cells/cm<sup>2</sup> and were cultured for 4 days before the experiments.

Full length coding sequence of rat Trisk 32 (accession number AJ812276) was ligated into the EcoR1 restriction site of pcDNA 3.1 (Invitrogen, ZZZ) expression vector [33]. Stable transfection was performed in Opti-MEM reduced serum content medium using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) for 2.5 h at 37 °C. Cells were allowed to express the transfected 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 and experiments were carried out on those separated clones (ZZZ that particular clone) of Trisk 32 transfected cells that showed the highest Trisk 32 expression. To obtain a control for the transfection, cells were transfected by empty pcDNA 3.1 vector using the same protocol, and experiments were carried out on pools of transfected cells. The transfection with the empty or Trisk 32 containing vector did not affect cell

viability but significantly reduced, to the same extent, the proliferation of the myoblasts (see Online Resource 2A and 2B, respectively)

The efficiency of Trisk 32 overexpression was monitored at protein level by immunostaining and Western-blot using a specific anti-Trisk 32 antibody corresponding to the C-terminal end of the peptide [33]. Functional experiments were carried out on myoblasts.

## Immunostaining

Cultured cells were washed with ice-cold phosphate-buffered saline (PBS; 0.02 M NaH<sub>2</sub>PO<sub>4</sub>, 0.1 M NaCl), fixed with 4% paraformaldehyde or 100% methanol at -20°C for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and blocked with 1% BSA diluted in PBS (blocking solution) for 30 min at room temperature. The cells were then incubated overnight at 4 °C with the anti-Trisk 32 primary antibody (dilution was 1:500 in blocking solution) produced in rabbit or in Guinea pig [33]; the anti-IP<sub>3</sub>-receptor type I, II, III primary antibody (dilution was 1:50 in blocking solution), corresponding to C-terminal amino acid residues 2402-2701 of IP<sub>3</sub>R, produced in rabbit (Santa-Cruz Biotechnology Inc., Heidelberg, Germany); the anti-B2 bradykinin receptor primary antibody (dilution was 1:100 in blocking solution), corresponding to the C-terminal region of the protein, produced in rabbit (Sigma); the anti-arginine-vasopressin receptor V1a antibody (dilution was 1:50 in blocking solution), corresponding to C-terminal amino acid residues 349-418 of the protein, produced in rabbit (Santa-Cruz). The secondary antibody, anti-rabbit or anti-guinea pig IgG, labeled with fluorescein or Cy3 was incubated for 1 h at room temperature. The nuclei were stained with DAPI in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Images were acquired with LSM 510 META confocal microscope (Zeiss, Oberkochen, Germany). (For negative controls for immunocytochemical stainings see Online Resource 1C.)

## RT-PCR analysis

For RT-PCR analysis, total RNA was isolated from myoblasts using Trizol reagent (Invitrogen). The volume and purity of the isolated RNA were verified with Nano-Drop 1000 Spectrophotometer (Wilmington, Delaware, USA). The assay mixture (20 µl) for reverse transcriptase reaction (Omniscript, Qiagen) contained 500 ng RNA, 0.25 µl RNase inhibitor, 0.25 µl oligo (dT), 2 µl dNTP (200 µM), 1 µl M-MLV RT in 1×RT buffer. Amplifications of specific cDNA sequences were performed with specific primers (Integrated DNA Technologies, Coralville, IA, USA) that were designed based on published rat Trisk 32 nucleotide sequences (GenBank Accession No.: AJ812276, forward primer: 5'-TGAACGATGTTGTGGAGGAA-3', reverse primer: 5'-CTCTGGCTTTCGGTGTG-3'). PCR reactions were allowed to proceed in a final volume of 50 µl (containing 2 µl forward and reverse primers, 1 µl dNTP (200 µM), and 0,5 µl Promega GoTaq® DNA polymerase in 1×reaction buffer) in a Bio-Rad C1000 Thermal Cycler (Bio-Rad, Budapest, Hungary) with the following settings: 2 min at 95 C for initial denaturation followed by repeated cycles of denaturation at 94 C for 1 min, primer annealing for 60 s at an optimized temperature, and extension at 72 C for 90 s. After the final cycle, further

extension was allowed to proceed for another 10 min at 72 C. PCR products were analyzed using EZ-Vision Three (Amresco, Solon, USA) on agarose gel.

### **Preparation of cell extracts**

Cells in cultures were washed with ice-cold PBS, harvested in homogenization buffer (20 mM Tris-HCl, pH 7.4, 5 mM EGTA, 1 mM 4-(2-aminoethyl) benzenesulphonyl fluoride, 20  $\mu$ M leupeptin; all from Sigma) and disrupted by sonication on ice. Protein content of the samples was measured by a modified bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) using BSA as a standard. The rat light SR vesicle used as positive control in Western-blot experiments was a gift from I. Jona [1].

### **Western-blot analysis**

Total cell lysates were examined by Western-blot analysis. Samples for SDS-PAGE were prepared by the addition of 1/5 volume of 5-fold concentrated electrophoresis sample buffer (310 mM Tris-HCl, pH 6.8; 10 % SDS, 50 % glycerol, 100 mM DTT, 0.01 % bromophenol blue) to cell lysates and boiled for 5 min at 80 °C. About 100  $\mu$ g of protein was separated by 10% SDS-PAGE gel for immunological detection of Trisk 32, TRPC1, STIM1, RyR, IP<sub>3</sub>R-I, II, and III. Proteins were transferred electrophoretically to nitrocellulose membranes (Bio-Rad, Budapest, Hungary). After blocking with 5% non-fat dry milk in PBS, membranes were incubated with the following primary antibodies overnight at 4 °C: anti-Trisk 32 antibody (dilution: 1:5000), anti-TRPC1 antibody (dilution: 1:200), anti-STIM1 antibody (dilution: 1:500), anti-IP<sub>3</sub>R type III antibody (dilution: 1:200) corresponding to amino acid residues 22-230 of human IP<sub>3</sub>R-III, produced in mouse (BD Transduction Laboratories, Le Pont de Claix-Cedex, France), anti-RyR antibody (dilution: 1:100) corresponding to whole protein of pig RyR, produced in rabbit [18], anti-IP<sub>3</sub>R type I antibody (dilution: 1:200) corresponding to cytoplasmic amino acid residues 1894-1973 of human IP<sub>3</sub>R-I, produced in rabbit (Santa-Cruz), and anti-IP<sub>3</sub>R type II antibody (dilution: 1:200) corresponding to C-terminus of human IP<sub>3</sub>R-II, produced in goat (Santa-Cruz). After washing three times for 10 min with PBST (PBS supplemented with 0.1% Tween 20), membranes were incubated with a secondary antibody, peroxidase-conjugated goat anti-rabbit or anti-mouse, or rabbit anti-goat IgG (Bio-Rad) in 1:1000 dilution in PBS containing 5% non-fat dry milk for 1 h. Signals were detected by enhanced chemiluminescence (ECL) reaction (Pierce).

### **Whole cell calcium measurement**

Changes in  $[Ca^{2+}]_i$  were measured using the calcium sensitive fluorescent dye Fura-2 as reported earlier [27]. Myoblasts were placed on a coverslip and incubated with Fura-2 AM (acetoxy-methylester; 10  $\mu$ M) for 1 h (37 °C, 5% CO<sub>2</sub>) in DMEM supplemented with 10% FBS and neostigmin to inhibit acetylcholine-esterase. Thereafter cells were equilibrated in normal Tyrode's solution (in mM: 137 NaCl, 5.4 KCl, 0.5 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, 11.8 HEPES, 1 g/l glucose, pH 7.4, all from Sigma) for 30 min at room temperature. Coverslips with the Fura-2 AM loaded cells were then placed on the stage of an inverted fluorescent microscope (Diaphot; Nikon, Tokyo, Japan). Measurements were performed in normal or

calcium-free Tyrode's solution. To obtain a calcium-free Tyrode's solution no  $\text{CaCl}_2$  was added in the presence of 5 mM EGTA (Sigma). Bradykinin (Sigma) was used at a final concentration of 20  $\mu\text{M}$  in normal and calcium-free Tyrode's solution. 0.1  $\mu\text{M}$  arginine-vasopressin (AVP; from Sigma) was used in calcium-free Tyrode's solution. The SERCA inhibitor thapsigargin (Sigma) was used at a final concentration of 2  $\mu\text{M}$  in calcium-free Tyrode's solution. The  $\text{IP}_3\text{R}$  agonist thimerosal (Sigma) was used at a final concentration of 50  $\mu\text{M}$  in calcium-free Tyrode's solution. Caffeine (Sigma), and ryanodine (Sigma) were used in 30 mM and 10  $\mu\text{M}$  concentrations respectively, in normal Tyrode's solution. Xestospongine C (Merck, Budapest, Hungary) was used as a pre-treatment in 50  $\mu\text{M}$  concentration in normal Tyrode's solution for 1 h in room temperature. Cells were continuously washed by Tyrode's solution using a background perfusion system. Test solutions were directly applied to the cells through a perfusion capillary tube (Perfusion Pencil<sup>TM</sup>; AutoMate Scientific, San Francisco, CA, USA) with an internal diameter of 250  $\mu\text{m}$  at a 0.35 ml/min rate, using a local perfusion system (Valve Bank<sup>TM</sup> 8 version 2.0, AutoMate Scientific). All measurements were performed at room temperature. Excitation wavelength was alternated between 340 and 380 nm by a dual wavelength monochromator (Deltascan, Photon Technology International, New Brunswick, NJ, USA), while the emission was monitored at 510 nm using a photomultiplier.  $[\text{Ca}^{2+}]_i$  was calculated from the ratio of fluorescence intensities ( $R = F_{340}/F_{380}$ ) using an in vivo calibration ( $R_{\min} = 0.2045$ ,  $R_{\max} = 8.315$ ,  $K_d \beta = 1183$ ) as described [12]. Data were statistically analyzed by Student's *t*-test. The data are presented as mean  $\pm$ SEM.

## Confocal measurements

Calcium transients were also monitored with the LSM 510 META confocal laser scanning microscope (Zeiss, Oberkochen, Germany) as described earlier [28]. L6 myoblasts were incubated with 10  $\mu\text{M}$  Fluo-4-AM for 1 h at 37 °C. Calcium imaging was performed in calcium-free Tyrode's solution. Line-scan images were used to monitor the fluorescence intensity, and were recorded at 24 ms per line and 512 pixels per line using a 63 $\times$  water immersion objective. Fluo-4 was excited with an argon ion laser. Cells were continuously washed by  $\text{Ca}^{2+}$ -free Tyrode's solution using a background perfusion system. 0.1  $\mu\text{M}$  AVP solution dissolved in  $\text{Ca}^{2+}$ -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 [28], developed in the Department of Physiology, University of Debrecen, Hungary. Fluorescence of the images (*F*) was normalized to baseline fluorescence ( $F_0$ ) and amplitudes of the transients were determined as  $\Delta F/F_0$ . The data are presented as mean  $\pm$ SEM.

## Results

## Cell culturing and transfection

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 (referred to as Trisk 32 or T32 in the figures hereon), immunocytochemistry, RT-PCR and Western-blot experiments were performed and the clone showing the highest expression of the protein (Fig. 1A right panel, Fig. 1B and Fig. 1C) was used for further experiments. The endogenous mRNA expression of Trisk 32 was slightly detectable in control cells (Fig. 1B). On the other hand the endogenous protein expression of Trisk 32 in control cells or in cells transfected by the pcDNA 3.1 vector alone, could not be shown with Western-blot (Fig. 1A left panel and Fig. 1C). Note that the protein is detected as a double band on the Western-blot as described earlier [ZZZ].

## Effect of Trisk 32 overexpression on the function of IP<sub>3</sub> receptors

The presence of IP<sub>3</sub>R-III (Online Resource 1E, Lanes 1 and 2), I and II (Online Resource 1F) was shown by Western-blot both in control and Trisk 32-overexpressing myoblasts. The amount of IP<sub>3</sub>R-I and III was similar in control and transfected cells, while IP<sub>3</sub>R-II was undetectable. Based on these measurements we concluded that the major isoform in L6.G8 myoblasts was IP<sub>3</sub>R-III. The presence of Trisk 32 protein could only be shown clearly in Trisk 32-overexpressing cells (Online Resource 1E, Lane 2). Co-localization of Trisk 32 and IP<sub>3</sub>R was confirmed by immunocytochemistry, using double-fluorescent labeling of the two proteins (Online Resource 1D). To prove that they are not only partially co-localized but also associated, co-immunoprecipitation experiments were performed (Online Resource 1E, Lanes 3 and 4).

ZZZ Supp info-ba After immunoprecipitation with anti-Trisk 32 antibody, the immune complexes were analyzed in Western-blot with anti-IP<sub>3</sub>R-III and anti-Trisk 32 antibodies. In control cells neither Trisk 32, nor the IP<sub>3</sub>R were present in the immunoprecipitated samples. In the samples of Trisk 32-overexpressing cells the immunoprecipitation of Trisk 32 leads to the co-immunoprecipitation with IP<sub>3</sub>R-III (Online Resource 1E, Lane 4). ZZZ

To show the functional effects of Trisk 32 overexpression on the regulation of IP<sub>3</sub>R and the Ca<sup>2+</sup> homeostasis of the cells, line-scan images were taken by confocal microscopy in the absence of extracellular calcium concentration ([Ca<sup>2+</sup>]<sub>e</sub>). The IP<sub>3</sub> pathway was stimulated by the local application of 0.1 μM AVP (the presence of V1a AVP receptors is shown in Online Resource 1B). The amplitude of the Ca<sup>2+</sup> transients evoked by AVP (Fig. 2) were significantly higher ( $p < 0.01$ ) in Trisk 32-overexpressing cells ( $3.25 \pm 0.23$ ,  $n = 34$ , expressed as  $F/F_0$ ; Fig. 2C) as compared to control ( $1.45 \pm 0.17$ ,  $n = 21$ ; Fig. 2A) and empty pcDNA3.1 vector transfected ( $2.02 \pm 0.23$ ,  $n = 25$ ; Fig. 2B) myoblasts. The amplitude of the transients did not differ significantly ( $p > 0.05$ ) between control and empty pcDNA 3.1 vector transfected cells (Fig. 2D). From these experiments and from those presented below (see Fig. 4), as well as from our earlier results [9] we concluded that the transfection with the empty vector does not affect the IP<sub>3</sub> pathway. To examine the effect of the inhibition of IP<sub>3</sub>Rs, Trisk 32 overexpressing cells were pre-treated with 50 μM Xestospongine C for 1 h, and then fluorescent [Ca<sup>2+</sup>]<sub>i</sub> measurements were performed with the local application of 0.1 μM AVP in the absence of [Ca<sup>2+</sup>]<sub>e</sub>. The application of the inhibitor significantly decreased the amplitudes of the AVP-induced Ca<sup>2+</sup> transients

compared to the untreated Trisk 32 overexpressing cells ( $106\pm 31$  nM,  $n=9$  and  $179\pm 14$  nM,  $n=9$ , respectively,  $p<0.05$ , Fig. 2E).

ZZZ Representative records of the measurements are shown in Fig. 3A. In this case  $20\ \mu\text{M}$  bradykinin was used to activate the  $\text{IP}_3$  pathway in control and transfected myoblasts (the presence of B2 bradykinin receptors on these cells is shown in Online Resource 1A). In some of the experiments  $1.8\ \text{mM}$   $[\text{Ca}^{2+}]_e$  was applied to achieve a more physiological condition, in other experiments  $0\ \text{mM}$   $[\text{Ca}^{2+}]_e$  was used to study clearly the  $\text{Ca}^{2+}$  released from the SR without the entry of extracellular  $\text{Ca}^{2+}$ . In normal Tyrode's solution with  $1.8\ \text{mM}$   $[\text{Ca}^{2+}]_e$ , significant difference could be observed between control and transfected cells both in the amplitude ( $76\pm 12$  nM,  $n=23$  and  $426\pm 84$  nM,  $n=27$  respectively,  $p<0.01$ , Fig. 3B) and in the maximal rate of rise ( $3.3\pm 1.1$  nM/s and  $35.1\pm 9.6$  nM/s respectively,  $p<0.01$ , Fig. 3C) of the bradykinin-evoked  $\text{Ca}^{2+}$  transients. In the absence of extracellular  $\text{Ca}^{2+}$  the significant difference between the two cell types remained in the amplitude ( $97\pm 29$  nM,  $n=31$  and  $217\pm 41$  nM,  $n=21$  respectively,  $p<0.02$ , Fig. 3B), however the difference in the maximal rate of rise of the transients did not prove to be significant ( $8.7\pm 3.5$  nM/s and  $18.9\pm 4.5$  nM/s, respectively,  $p=0.08$ , Fig. 3C). It should be noted that these differences were not due to an increase in the resting  $[\text{Ca}^{2+}]_i$  ( $[\text{Ca}^{2+}]_{\text{rest}}$ ) since it was essentially identical in control and Trisk 32 overexpressing cells; ( $73\pm 2$  nM,  $n=31$  and  $74\pm 2$  nM,  $n=21$  respectively,  $p>0.9$ ) in the absence of  $[\text{Ca}^{2+}]_e$ . Note also that the amplitude of the bradykinin- and the AVP-evoked  $\text{Ca}^{2+}$  transients in the absence of  $[\text{Ca}^{2+}]_e$  was similar in Trisk 32 overexpressing cells ( $217\pm 41$  nM,  $n=21$  and  $179\pm 14$  nM,  $n=9$ , respectively,  $p>0.5$ ).

The observed augmentation of agonist-evoked  $\text{Ca}^{2+}$  transients observed in Trisk 32-overexpressing cells could be explained either by altered  $\text{IP}_3\text{R}$  function – increased channel conductance or increased  $\text{IP}_3$  sensitivity – or by modified SOCE that accompanied store depletion. To explore the possibility that the increased conductance of  $\text{IP}_3\text{R}$  would be the explanation for the increased elevation in  $[\text{Ca}^{2+}]_i$  following agonist application,  $\text{IP}_3\text{Rs}$  were directly activated by the addition of  $50\ \mu\text{M}$  thimerosal to the culture medium. The drug induced a slow rise in  $[\text{Ca}^{2+}]_i$  that appeared with a considerable latency in both control, mock-transfected, and Trisk 32-overexpressing cells (Fig. 4A). Pooled data proved that these calcium transients are essentially identical ( $p>0.8$ ) both in their amplitude ( $84.2\pm 5.4$ ,  $82.7\pm 7.4$ , and  $83.1\pm 4.8$  nM; Fig. 4B) and in their rate of rise ( $0.26\pm 0.04$ ,  $0.25\pm 0.01$ , and  $0.27\pm 0.04$  nM/s in control, in empty pcDNA3.1 vector-transfected, and in Trisk 32-overexpressing cells, respectively; Fig. 4C).

### Effect of Trisk 32 overexpression on SOCE

To examine whether the overexpression of Trisk 32 alters the SOCE mechanism of the myoblasts, the expression level of the key molecules of SOCE was tested by Western-blot. The expression of STIM1 (Fig. 5A) decreased significantly, to  $48\pm 7\%$  of the control in the transfected myoblasts (Fig. 5C), while the expression of TRPC1 did not change (Fig. 5B and C).

Measurements of intracellular calcium concentration were performed to assess the functional consequences of Trisk 32 overexpression and the decreased expression of STIM1 on SOCE. Internal calcium-stores were thus emptied by the application of  $2\ \mu\text{M}$  thapsigargin (TG) in the absence of  $[\text{Ca}^{2+}]_e$ . The changes in  $[\text{Ca}^{2+}]_i$  evoked by re-establishing the normal  $1.8\ \text{mM}$  extracellular calcium concentration ( $[\text{Ca}^{2+}]_e$ ) were then measured in control (Fig. 5D upper panel) and



in Trisk 32-overexpressing L6 myoblasts (Fig. 5D bottom panel) [4]. Neither the amplitude ( $105\pm 18$  nM,  $n=22$  and  $126\pm 22$  nM,  $n=21$ , respectively  $p>0.4$ , Fig. 5E), nor the maximal rate of rise ( $0.8\pm 0.2$  nM/s and  $1.4\pm 0.4$  nM/s, respectively  $p>0.2$ , Fig. 5F) of SOCE differed significantly in the two cell types.

To assess whether a change in ER  $\text{Ca}^{2+}$  content could underlie some of the observed alterations, the parameters of the thapsigargin-evoked  $\text{Ca}^{2+}$  transients were compared for control and Trisk 32 overexpressing cells. Neither the integral ( $27.0\pm 3.1$  mM·s,  $n=7$  and  $28.7\pm 4.5$  mM·s,  $n=15$ , respectively,  $p>0.8$ ) nor the amplitude ( $146\pm 19$  nM, and  $199\pm 25$  nM, respectively,  $p>0.1$ ) of these signals were altered significantly by the transfection (Fig. 5G).

### Effect of Trisk 32 overexpression on the function of RyRs

To exclude the possible involvement of RyR in the changes described above,  $\text{Ca}^{2+}$  release via ryanodine receptors was examined by applying 30 mM caffeine on control and Trisk 32-overexpressing myoblasts (Fig. 6A). Only two thirds of the cells responded to caffeine (19 from 31, and 20 from 30 in control and Trisk 32 overexpressing cells, respectively) and the expression of RyR could not be shown by Western-blot (Fig. 6C), which means that only a minimal amount of the receptor was present and functional in these cells. The amplitude of the  $\text{Ca}^{2+}$  transients – where such transients were observed at all – did not differ significantly ( $61\pm 7$  nM,  $n=19$  and  $53\pm 6$  nM,  $n=20$  respectively,  $p>0.3$ ; Fig. 6B). To examine the contribution of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release via RyRs in the bradykinin-evoked  $\text{Ca}^{2+}$  transients, RyRs were blocked by 10  $\mu\text{M}$  ryanodine then 20  $\mu\text{M}$  bradykinin was applied to activate the  $\text{IP}_3$  pathway in Trisk 32-overexpressing cells. Although the amplitude of the transients were lower in the presence of ryanodine, this difference did not prove to be significant if compared to those obtained in the absence of the drug ( $275\pm 26$  nM,  $n=21$  and  $426\pm 84$  nM,  $n=27$ , respectively,  $p>0.1$ ; Fig. 6D). Similarly when RyRs were blocked by 10  $\mu\text{M}$  ryanodine then the  $\text{IP}_3$  pathway was activated by 0.1  $\mu\text{M}$  AVP in the absence of  $[\text{Ca}^{2+}]_e$  in Trisk 32-overexpressing cells, the difference in the amplitude of the transients was not significant ( $179\pm 14$  nM,  $n=9$  in the absence and  $132\pm 23$  nM,  $n=9$  in the presence of the drug,  $p>0.1$ ).

## Discussion

Triadin was described in skeletal muscle two decades ago [5, 16], since then several isoforms were discovered, their localization described, and tissue distribution revealed. A study of Vassilopoulos and co-workers [33] proved the existence, among others, of a 32 kDa isoform of skeletal muscle triadin. They showed that unlike the other isoforms it localizes outside of the triad, in the longitudinal SR, and co-localizes with  $\text{IP}_3\text{R}$ -s and mitochondria. The possibility was raised that the function of Trisk 32 could either be connected to  $\text{Ca}^{2+}$  release via  $\text{IP}_3\text{R}$ , to the maintenance of sarcomere structure during contraction, or to the  $\text{Ca}^{2+}$ -storage in the mitochondria.

To clarify whether or not Trisk 32 plays any role in the regulation of  $\text{IP}_3\text{R}$ , in a way analogous to the interaction between Trisk 95 and RyR, Trisk 32 was stably overexpressed in L6.G8 skeletal muscle myoblasts. The co-localization and

the physical association of Trisk 32 and IP<sub>3</sub>R was shown in transfected cells, and their functional association was also demonstrated. To stimulate the IP<sub>3</sub> pathway either bradykinin or arginin-vasopressin was used. B<sub>2</sub> bradykinin receptors were shown to be present on the L6 cell line [20] and their coupling to the IP<sub>3</sub> pathway was also demonstrated in skeletal muscle [23]. Similarly, the presence of the IP<sub>3</sub> pathway-coupled V1 vasopressin receptors was described on the L6 cell line [29]. These results were confirmed here by showing the presence of these receptors using immunocytochemical labeling (see Online Resource 1A and 1B).

It should be noted that the cells – L6.G8 myoblasts – used in these experiments represent the earliest stage of development. Previous studies have revealed that triadin expression parallels differentiation [33], that is, triadin expression is very low at the myoblast stage and increases as muscle cells differentiate. L6.G8 myoblasts should, therefore, provide an ideal system for studying Trisk 32 overexpression since any interaction between IP<sub>3</sub>R and triadins – if present – should be minimal in control cells, while in Trisk 32 overexpressing cells it should be close to maximal.

The major finding of this work is that the overexpression of Trisk 32 resulted in a significantly higher amplitude and maximal rate of rise of the Ca<sup>2+</sup> transients evoked either by bradykinin (20 μM) or vasopressin (0.1 μM) via the IP<sub>3</sub> pathway. This observation could have at least four independent but functionally related explanations. First, Trisk 32 overexpression might alter the coupling of the surface membrane receptors to the production of IP<sub>3</sub>, resulting in an increased amount of IP<sub>3</sub> in transfected cells. Second, through direct protein-protein interaction Trisk 32 could enhance the opening of IP<sub>3</sub>R in the presence of its agonist. Third, the overexpression of Trisk 32 could lead to an increased ER Ca<sup>2+</sup> content and, consequently, to an increased amount of Ca<sup>2+</sup> released from the store. Finally, Trisk 32 might modulate the coupling of the emptying of the intracellular Ca<sup>2+</sup> store to surface membrane Ca<sup>2+</sup> influx resulting in an increased SOCE. It should also be noted that an interaction, either direct coupling or via the released Ca<sup>2+</sup> (Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release; CICR), to RyR-mediated Ca<sup>2+</sup> release cannot, in theory, be ruled out.

We present a number of observations against the first of the above mentioned possibilities. First of all, the stimulation of two independent pathways – B<sub>2</sub> and V1 receptors – resulted in a similar enhancement of the Ca<sup>2+</sup> signals, clearly arguing against this possibility, since it would require that Trisk 32 should at the same time interact with both B<sub>2</sub> bradykinin and V1 vasopressin receptors. Although an effect on PLC cannot simply be ruled out using only the functional – Ca<sup>2+</sup> transients – evidence, immunocytochemical staining does not favor a localization of the Trisk 32 protein that could easily be reconciled with such an interaction, that is, an interaction with a protein found in the plasma membrane.

We also present evidence that IP<sub>3</sub> receptor activation *per se* is unaltered if the receptors are stimulated directly. Namely, the extent of activation of IP<sub>3</sub>R by thimerosal – a widely used IP<sub>3</sub>R activator ([30, 32]; for review see eg. [8]) – was identical in control, in empty pcDNA3.1 vector transfected, and in Trisk 32-overexpressing cells as assessed by the amplitude and rate of rise of the Ca<sup>2+</sup> transients following thimerosal treatment. It should, however, be noted that thimerosal has been reported to interact with type I but not with type III IP<sub>3</sub>R [6]. The above mentioned observation could thus be interpreted as an indication that in Trisk 32-overexpressing cells the conductance of IP<sub>3</sub>R is unaltered (note that independent evidence suggests that the content of the Ca<sup>2+</sup> store was identical in

these cells, see below) or that the Trisk 32 protein interacts only with type III IP<sub>3</sub>R.

Importantly though, both functional and co-localization/co-immunoprecipitation experiments are strongly in favor of a direct interaction between IP<sub>3</sub>R (possibly type III, see above) and Trisk 32. In this framework the presence and direct protein-protein interaction of Trisk 32 and the calcium channel would result in an increased amount of Ca<sup>2+</sup> released from the ER. This was observed as an enhancement of the Ca<sup>2+</sup> transients in the absence of external Ca<sup>2+</sup>. Note that in the presence of normal external Ca<sup>2+</sup> the difference between transfected and non-transfected cells was even greater suggesting that the increased release and, therefore, the more pronounced depletion of Ca<sup>2+</sup> in the ER resulted in an enhanced SOCE in Trisk 32 overexpressing cells.

On the other hand, we provide evidence that the overexpression of Trisk 32 does not directly affect the mechanism of SOCE. To examine the possibility, the ER Ca<sup>2+</sup> store was emptied by the application of the SERCA pump inhibitor thapsigargin in a Ca<sup>2+</sup>-free extracellular milieu, and then the re-addition of extracellular Ca<sup>2+</sup> triggered the Ca<sup>2+</sup>-influx via the plasma-membrane [4]. Neither the amplitude, nor the maximal rate of rise of SOCE differed significantly in Trisk 32-overexpressing cells, as compared to control. However, as examined by Western-blot, the expression of TRPC1 – an important store-operated Ca<sup>2+</sup> channel of the plasma-membrane [2, 3] – did not change in Trisk 32-overexpressing cells, but the expression of STIM1 – the Ca<sup>2+</sup> sensor of the ER [15, 25] – decreased significantly, despite the fact that its functional consequence was not detectable by the [Ca<sup>2+</sup>]<sub>i</sub> measurements (similar observation – decreased STIM1 expression with no parallel decrease in SOCE – was made on transfected C2C12 cells, too, [21]). Taken together, these results suggest that the main effect of Trisk 32 overexpression is not the alteration of the functionality of SOCE.

In addition, these experiments provided means to tackle the filling of the intracellular Ca<sup>2+</sup> stores. By measuring the amplitude and the integral of the thapsigargin-evoked Ca<sup>2+</sup> transients and finding no significant difference in either when comparing control and transfected cells, suggest that the overexpression of Trisk 32 does not affect the Ca<sup>2+</sup> content of the ER Ca<sup>2+</sup> store.

The 95 kDa isoform of triadin is known to regulate Ca<sup>2+</sup> release via RyRs [9, 24]. To exclude the possibility that such an interaction from Trisk 32 or an enhanced CICR was responsible for the increased Ca<sup>2+</sup> transients evoked by bradykinin or AVP, functional [Ca<sup>2+</sup>]<sub>i</sub> measurements were performed. Neither the application of 30 mM caffeine an agonist of RyR alone nor the presence of 10 μM ryanodine when bradykinin was applied initiated significant alterations in the Ca<sup>2+</sup> signals. That is, the caffeine-evoked responses were essentially identical in control and transfected cells, while the amplitude of the bradykinin-evoked transients were not significantly lower with or without ryanodine, and, furthermore, they did not decrease to the level of control cells. These results indicate that the effects of the overexpression of Trisk 32 cannot be accounted for solely by an altered RyR function. It should, nevertheless, be noted that the slight reduction in the amplitude of the bradykinin-evoked Ca<sup>2+</sup> transients in the presence of ryanodine suggests that CICR might be involved, albeit to a lesser extent, in the Ca<sup>2+</sup> signals of L6 cells.

In conclusion, the significant enhancement of the bradykinin- and vasopressin-evoked Ca<sup>2+</sup> transients in Trisk 32 overexpressing cells is due to the increased Ca<sup>2+</sup> release from the intracellular Ca<sup>2+</sup> store resulting from a direct interaction of Trisk 32 and IP<sub>3</sub>R.

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## Conflict of interest

The authors declare that they have no conflict of interest.

## Ethical standards

The authors declare that the experiments comply with the current laws of the country in which they were performed.

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## Figure Captions

### Fig. 1

Overexpression of Trisk 32 in L6.G8 myoblasts. **(A)** Immunocytochemical staining of control and Trisk 32-transfected L6 cell cultures demonstrating the presence of Trisk 32 protein. The rat isoform was expressed from the plasmid used for stable transfection. Images were recorded from 2  $\mu\text{m}$  thick optical slices. Original magnification was 40 $\times$ . **(B)** mRNA expression of Trisk 32 was detected by RT-PCR. Samples from control and transfected cells were compared. GAPDH was used as a control. **(C)** Western-blot analysis of Trisk 32 was performed to analyze the overexpression at protein level. (SR – rat light SR fraction; C – control L6 myoblasts;  $\emptyset$  – L6 myoblasts transfected with empty pcDNA 3.1 vector; T32 – Trisk 32-overexpressing L6 myoblasts)

### Fig. 2

Examination of the functional association of Trisk 32 and IP<sub>3</sub>R. Confocal microscopy line-scan images and time courses of Fluo-4 fluorescence in control myoblasts **(A)**, in myoblasts transfected with empty pcDNA 3.1 vector **(B)**, and in Trisk 32-overexpressing myoblasts **(C)** showing the transients evoked by the application of 0.1  $\mu\text{M}$  AVP in the absence of  $[\text{Ca}^{2+}]_e$ . Time courses were calculated from the lines marked by arrows on the line-scan images. Horizontal and vertical calibrations are the same for all traces in A, B and C. **(D)** Pooled data from the amplitude of the transients. **(E)** Effect of pre-treatment with 50  $\mu\text{M}$  Xestospongine C for 1 h on the amplitude of 0.1  $\mu\text{M}$  AVP-induced  $\text{Ca}^{2+}$  transients in Trisk 32-overexpressing cells in the absence of  $[\text{Ca}^{2+}]_e$ .  $[\text{Ca}^{2+}]_i$  of the cells was measured with PTI Delta Scan system. Here, and in all subsequent figures asterisks (\*) mark significant difference between control and Trisk 32 overexpressing cells and data represent mean  $\pm$  standard error (SE) of the mean. Paragraph signs (§) mark significant difference compared to empty pcDNA 3.1 vector transfected cells (shown as  $\emptyset$ -vector in the bar graphs). Hashmark (#) indicates significant ( $p < 0.05$ ) change between data obtained in the absence and presence of Xestospongine C. Numbers in parentheses give the number of cells measured.

### Fig. 3

**(A)** Representative records of 20  $\mu\text{M}$  bradykinin-evoked  $\text{Ca}^{2+}$  transients in the presence (upper panels) and absence (bottom panels) of extracellular calcium ( $[\text{Ca}^{2+}]_e$ ) in control and Trisk 32-overexpressing cells. Pooled data for the amplitude **(B)** and the maximal rate of rise **(C)** of the transients. Hashmark (#) indicates significant ( $p < 0.05$ ) change between data obtained in the absence and presence of  $[\text{Ca}^{2+}]_e$ . Numbers in parentheses give the number of cells measured.

### Fig. 4

Direct activation of IP<sub>3</sub>Rs with thimerosal. **(A)** Representative records of  $\text{Ca}^{2+}$  transients evoked by 50  $\mu\text{M}$  thimerosal measured in the absence of extracellular calcium in control, empty pcDNA3.1 vector transfected and Trisk 32-overexpressing cells. Pooled data for the amplitude **(B)** and the maximal rate of rise **(C)** of the transients. Numbers in parentheses give the number of cells studied.

### Fig. 5

The effect of Trisk 32 overexpression on store-operated  $\text{Ca}^{2+}$ -entry (SOCE). By using Western-blot analysis, the expression of STIM1 **(A)** and TRPC1 **(B)** proteins were shown in control (C) and

Trisk 32-overexpressing (T32) cells. 100-100  $\mu\text{g}$  protein samples were used in each lane for the Western-blot experiments. **(C)** Quantitative analysis of Western-blot results (mean  $\pm$  SE) obtained from control and transfected myoblasts. **(D)** Changes in  $[\text{Ca}^{2+}]_i$  demonstrating SOCE were recorded following the re-establishment of the normal (1.8 mM)  $[\text{Ca}^{2+}]_e$  in control and in Trisk 32-overexpressing cells. The internal calcium-stores were emptied by 2  $\mu\text{M}$  thapsigargin (TG) in the absence of  $[\text{Ca}^{2+}]_e$ . Representative records of 4-4 independent cultures. Mean values of the amplitude **(E)** and the maximal rate of rise **(F)** of SOCE after the re-administration of external calcium. **(G)** Integral and amplitude of thapsigargin evoked  $\text{Ca}^{2+}$  transients. Numbers in parentheses indicate the number of cells measured.

## Fig. 6

Functional effects of Trisk 32 overexpression on  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$ -release (CICR) via ryanodine receptors (RyR). **(A)** Representative records of calcium transients evoked by the RyR agonist 30 mM caffeine in control and in Trisk 32-overexpressing L6 cells. **(B)** Amplitudes of caffeine-evoked  $\text{Ca}^{2+}$  transients in both cell types. **(C)** Western-blot analysis showing the expression of RyR in rat skeletal muscle quadriceps microsome, used as positive control (Lane 1), control L6 cells (Lane 2) and Trisk 32-overexpressing L6 cells (Lane 3). **(D)** Amplitudes of 20  $\mu\text{M}$  bradykinin-evoked  $\text{Ca}^{2+}$  transients in the absence and presence of the RyR antagonist 10  $\mu\text{M}$  ryanodine, in Trisk 32-overexpressing myoblasts, at normal 1.8 mM  $[\text{Ca}^{2+}]_e$ . Numbers in parentheses indicate the number of cells measured.