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ORIGINAL ARTICLE

Expression study of calsequestrin in rabbit soleus muscles

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ABSTRACT

Calsequestrin (CASQ), a high-capacity calcium-binding protein localized in the sarcoplasmic reticulum (SR), was first identified in rabbit skeletal muscle in 1971. Initially considered a passive calcium buffer, subsequent studies over the past three decades have elucidated the multifunctional nature of the skeletal muscle isoform, CASQ1. In addition to maintaining intraluminal SR calcium concentrations essential for muscle contraction and relaxation, CASQ1 acts as a dynamic calcium sensor, modulating calcium release during excitation-contraction coupling. It also contributes structurally to the organization and stabilization of the terminal cisternae of the SR.

Recent evidence suggests CASQ1 may influence reverse-directional calcium influx from the extracellular milieu, implicating it in broader calcium homeostasis mechanisms. Dysregulation or mutation in the CASQ1 gene has been associated with various skeletal muscle pathologies, emphasizing its clinical relevance. In this study, we investigated the expression pattern of the CASQ1 gene in the soleus muscle of the rabbit, a muscle predominantly composed of slow-twitch (Type I) fibres critical for postural stability. Our analysis adds to the growing body of evidence characterizing fibre-type-specific expression of calcium-regulatory proteins.

This study is limited to gene expression profiling in a single muscle type and species. Functional characterization at the protein level and cross-species comparisons were not within the scope of this investigation.

The objective of this study was to elucidate the expression profile of CASQ1 in rabbit soleus muscle, thereby contributing to the understanding of its role in calcium signalling and muscle fibre specialization. These findings may facilitate future research on CASQ1-related myopathies.

KEY WORDS

Calsequestrin (CASQ1); Rabbit soleus muscle; Skeletal muscle; Calcium homeostasis; Sarcoplasmic reticulum; RT-PCR; Gene expression

ARTICLE HISTORY

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Introduction

Skeletal muscle movement and posture maintenance rely on fluctuations in calcium ion (Ca^{2+}) levels within the cytosol of myotubes, the cells that make up skeletal muscle tissue. A brief increase in intracellular calcium (Ca^{2+}) serves as an essential mediator between electrical signals moving through the transverse (T)-tubule membrane and the triggering of muscle contraction, termed as excitation-contraction coupling.

The dihydropyridine receptor (DHPR), found on the transverse (t)-tubule membrane, acts as a voltage sensor during skeletal muscle excitation-contraction (EC) coupling. DHPR changes its structure when it detects an action potential that was triggered by acetylcholine at the neuromuscular junction [1]. Through a mechanical coupling mechanism, this structural alteration directly activates the ryanodine receptor type 1 (RyR1), which is located on the sarcoplasmic reticulum (SR) membrane. After it is activated, RyR1 helps the SR release calcium ions into the cytoplasm. Muscle fiber contraction results from the activation of the contractile machinery caused by the increase in cytosolic calcium concentration.

In skeletal muscle, the sarcoplasmic reticulum (SR) is the main intracellular calcium reservoir. Mitochondria also play a role in calcium control and storage. Calcium ions in the cytosol are actively returned to the SR during muscle relaxation. The sarcoplasmic/endoplasmic reticulum Ca2+-ATPase 1a (SERCA1a), an ATP-dependent calcium pump present in the SR membrane, mediates this reuptake.

The precise organization of these proteins within the triad junction—formed by a central segment of the sarcoplasmic reticulum flanked by two transverse tubules—is essential for the temporary increase and subsequent clearance of calcium ions in the cytoplasm, processes that drive skeletal muscle contraction and relaxation.

Calcium ions required for skeletal muscle contraction originate from both the sarcoplasmic reticulum (SR) and the extracellular environment. Store-operated calcium entry (SOCE) is one important way that calcium enters the cell from the outside. Orai1, a calcium channel on the t-tubule membrane, and Stromal Interaction Molecule 1 (STIM1), a calcium sensor in the SR membrane are two proteins essential to this process. Functional clusters called puncta are formed when STIM1 physically connects with Orai1 in response to a decrease in calcium levels within the SR. Calcium can move from the extracellular space into the cytosol as a result of these puncta opening Orai1 channels [2].

Besides STIM1 and Orai1, other proteins also contribute to the regulation of SOCE in skeletal muscle. Transient receptor potential canonical (TRPC) channels are among those involved, supporting calcium influx through this pathway. STIM2, a protein closely related to STIM1, also plays a role—albeit less clearly defined—in SOCE and in the later stages of skeletal muscle development. However, the specific functions of STIM2 in skeletal muscle remain relatively underexplored in current research.

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Calsequestrin 1 (CASQ1), predominantly found in adult fast-twitch skeletal muscles, is highly concentrated within the sarcoplasmic reticulum (SR). It serves as a low-affinity, high-capacity calcium-binding protein, capable of associating with approximately 40 to 80 calcium ions per molecule [3]. This characteristic is critical for ensuring a rapid release of calcium from the SR during muscle contraction and for facilitating efficient calcium reuptake during relaxation, all while maintaining osmotic stability despite the high calcium concentration within the SR. Beyond its role in calcium storage, CASQ1 also functions as a calcium sensor. It detects changes in calcium levels within the SR and influences the activity of ryanodine receptor 1 (RyR1), controlling calcium release into the cytosol [4]. This regulation depends on CASQ1's structural state, particularly its ability to polymerize in response to calcium depletion (Figure 1).

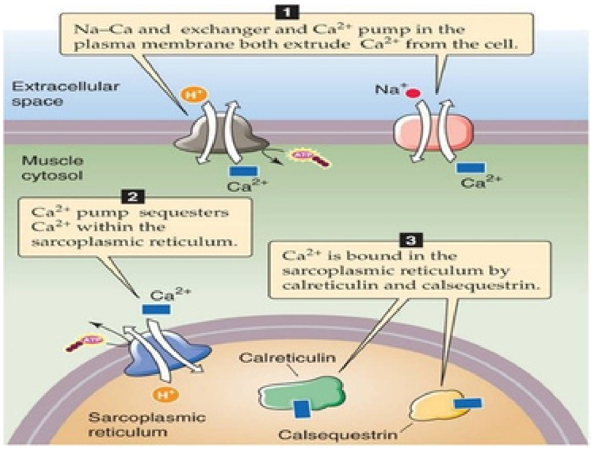

Figure 1. Physiological anatomy of skeletal muscles.

Materials and Methods:

Tissue extraction

The extensor digitorum longus muscle of the rabbit (*Oryctolagus cuniculus*) was collected from Dr. Naresh Bal, Department of Biotechnology, KIIT, Bhubaneswar, and stored at -121°C. This muscle extends from the area just below the knee to the heel and plays a key role in movements such as standing and walking.

Homogenization procedure

TRIzol Reagent (Invitrogen), is commonly used for the isolation of tRNA from cells and tissues. Prior to homogenization, the centrifuge rotor should be prechilled to 4°C. Approximately 50 mg of frozen tissue is weighed and transferred into an Eppendorf tube containing a stainless-steel bead. TRIzol reagent is then added to the sample, and tissue homogenization is carried out. Following homogenization, chloroform is added to initiate phase separation for RNA extraction.

RNA extraction

The homogenized tissue was centrifuged at 5400 rpm, and the clear upper aqueous layer was gently transferred to a new Eppendorf tube. RNA precipitation was carried out by adding isopropanol, and the mixture was centrifuged again to obtain the RNA pellet. The pellet was washed twice using chilled 70% ethanol at 4°C . Following the washes, it was air-dried and then dissolved in RNase-free water . RNA concentration was measured with a NanoDrop spectrophotometer, and its integrity was evaluated through agarose gel electrophoresis.

Complementary DNA (cDNA) synthesis:

cDNA synthesis was carried out using a two-step method with random primers (TaKaRa) producing quality cDNA libraries.

Materials

The following reagents were used for cDNA synthesis:

- Oligonucleotides/Random primers
- Messenger RNA (mRNA)
- Nuclease-free water
- 5× First-Strand (FS) Buffer
- M Dithiothreitol (DTT)
- dNTP Mix (Deoxynucleotide Triphosphates)
- RNaseOUT™ (RNase Inhibitor)
- SuperScript II Reverse Transcriptase (SSII RT)

Method

cDNA synthesis

First-strand cDNA was generated from tRNA through two-step reverse transcription method with Random 6-mer primers (TaKaRa). In this process, reverse transcriptase enzymes use the RNA template along with a complementary primer to synthesize cDNA, which can then serve as a template for subsequent PCR amplification.

Step 1: Primer annealing and RNA denaturation

A reaction mixture was prepared by combining template RNA, Random 6-mer primers, dNTP mix, and water in a sterile microcentrifuge tube free of RNase. To denature secondary RNA structures and promote effective primer binding, the mixture was incubated for five minutes at 65°C.

Step 2: Reverse transcription reaction

Following denaturation, the reverse transcription reaction was initiated by adding PrimeScript Buffer (5×), RNase inhibitor, PrimeScript RTase, and RNase-free water to the annealed RNA-primer mixture. The final reaction mixture was incubated under the following thermal conditions:

- 30°C for 10 minutes (primer extension initiation)
- 42–50°C for 30–60 minutes (cDNA synthesis)
- 95°C for 5 minutes (enzyme inactivation)

cDNA verification and storage

To verify successful cDNA synthesis, the reaction products were subjected to 2% agarose gel electrophoresis. Samples were loaded alongside a DNA ladder, and electrophoresis was performed under standard conditions. The gel was visualized using a UV transilluminator. Synthesized cDNA was stored at −20°C until further use.

Real-time quantitative PCR (RT-qpcr)

RT-qPCR was executed using Power SYBR Green PCR Master Mix (Applied Biosystems) to quantify the expression of the target gene CASQ1, with 18S rRNA serving as the endogenous reference gene. All reactions were carried out in triplicate for each sample.

The following primer sequences were used:

- **CASQ1 Forward:** GCAGTGGCCATGAAACTTGG
- **CASQ1 Reverse:** CCGATCACCCACAATCTGTT
- **18S rRNA Forward:** AACAGATACGGTCGTACTTC
- **18S rRNA Reverse:** TACCGTGAATTCCTATAAG

The amplification reaction was monitored in real time using SYBR Green fluorescence, which correlates with the accumulation of double-stranded DNA during the PCR cycles. The relative expression of CASQ1 was calculated based on the fluorescence data derived from the amplification plots, normalized to 18S rRNA expression levels. All reactions were performed following standard RT-qPCR protocols [5,6].

RT-qPCR enables both quantitative and semi-quantitative analysis of gene expression, as it allows detection and measurement of DNA amplification during the exponential phase of the PCR, unlike conventional endpoint PCR. The technique depends on the enzymatic replication of DNA with a thermostable DNA polymerase and sequence-specific primers [7].

Polymerase chain reaction (PCR)

PCR is a fundamental technique in molecular biology used to amplify targeted DNA regions. It uses a heat-resistant DNA polymerase, and involves cycles of temperature changes to achieve exponential replication of the desired DNA segment.

PCR Steps:

PCR consists of three main cyclic steps:

- **Denaturation:** To denature the DNA, the reaction mixture was first heated to 94°C for 30 to 2 minutes. Hydrogen bonds are broken creating single-stranded templates for the following stages of synthesis.
- **Annealing:** To allow the primers to bind to their particular complementary sequences on the single-stranded DNA, the temperature was then decreased to between 54 and 60°C for 20 to 40 seconds. DNA replication is initiated by primers, which are brief single-stranded oligonucleotides that range in length from 20 to 30 bases. A forward primer and a reverse primer, which bind to opposing strands in opposite directions, were used in each reaction [8].
- **Extension (Elongation):** The temperature was raised to 72-80°C, which is required for Taq polymerase activity. At this point, cDNA strands are created in the 5′ to 3′ direction by the enzyme adding nucleotides to the 3′ ends of the primers. Taq polymerase can elongate to 1,000 base pairs each minute under ideal situations [9].

These three steps were repeated for 40 cycles to achieve substantial amplification of the target DNA fragment. PCR amplification was carried out using a thermocycler, an automated instrument that precisely controls the temperature changes required for the reaction.

Agarose gel electrophoresis

Materials

- 1× TAE (Tris-Acetate-EDTA) Buffer
- Agarose
- Ethidium Bromide (EtBr)
- Distilled water (dH_2O)
- 50 kb DNA Ladder
- 6× DNA Loading Dye
- Microwave or hot air oven
- Electrophoresis chamber
- Gel casting tray and comb
- Cellophane tape (for sealing)
- UV Transilluminator

Method

To achieve the final concentration of 1× TAE, 600 mg of agarose, 30 mL of distilled water, and 600 µL of 50× TAE buffer were combined to create a 2% agarose gel. A microwave or hot air oven was used to heat the mixture until the agarose was completely dissolved and the mixture became transparent. 1 µL of ethidium bromide was added for nucleic acid staining after the mixture had cooled to about 60°C.

Cellophane tape was used to fix the gel casting tray, and a comb was placed in the appropriate location. After that, the tray was filled with the molten agarose, which was then allowed to solidify at room temperature. The gel was moved to the electrophoresis chamber after polymerization, where it was completely submerged in 1× TAE buffer to run the samples.

After being isolated from rabbit tissue, nucleic acid samples were mixed with 6× loading dye and carefully put onto the agarose gel's wells. As a molecular size marker, a 50 kb DNA ladder was positioned in a reference lane, also known as lane 8. Until the tracking dye had moved through the gel a sufficient distance, the electrophoresis was run at 100 volts.

Following electrophoresis, the gel was placed on a UV transilluminator, where the DNA bands were observed and recorded under ultraviolet illumination.

Results and Discussion

RNA quantification

In the soleus muscle tissue, RNA was successfully extracted and quantified, yielding 261 ng/µl. This concentration was considered sufficient for further downstream processes such as cDNA synthesis and RT-PCR analysis, indicating efficient RNA isolation [10] (Figure 2).

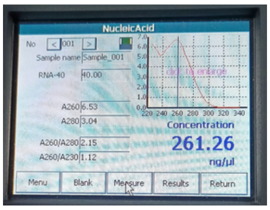
NucleicAcid
No 001
Sample name Sample_001
RNA-40 40.00
A260 6.53
A280 3.04
A260/A280 2.15
A260/A230 1.12
Concentration
261.26
ng/µl

Figure 2. Qualitative and quantitative analysis of RNA.

Agarose gel electrophoresis of RNA

Agarose gel electrophoresis was conducted to assess RNA integrity. The RNA bands observed on the gel appeared sharp and intact, suggesting high-quality RNA. This confirmation allowed the continuation of the workflow toward cDNA synthesis without risk of degradation affecting the results [11] (Figure 3).

Figure 3. Quality of RNA assessed by agarose gel electrophoresis.

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cDNA Figure

Reverse transcription was performed on the purified RNA. The synthesized cDNA was validated through agarose gel electrophoresis, showing distinct bands corresponding to expected sizes. This confirmed that the RNA was effectively reverse transcribed and ready for qPCR analysis [12] (Figure 4).

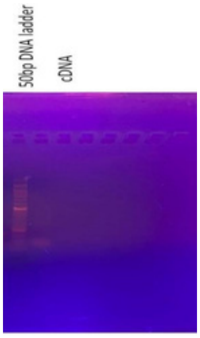

Figure 4. Visualization of cDNA using agarose gel electrophoresis.

Amplification and melt curves of CASQ1 in soleus muscle

Quantitative PCR was used to evaluate CASQ1 gene expression in the soleus muscle. The amplification curves confirmed the presence of CASQ1 mRNA, while the melt curve showed a single, sharp peak. This indicated high specificity of the primers and the absence of non-specific amplification or primer-dimer formation [13]. The internal control gene 18s also showed strong amplification, validating the assay quality (Figure 5 & Figure 6).

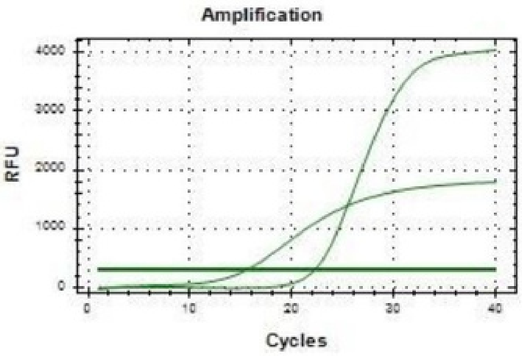

Figure 5. Amplification curve of CASQ1 gene in rabbit soleus muscle.

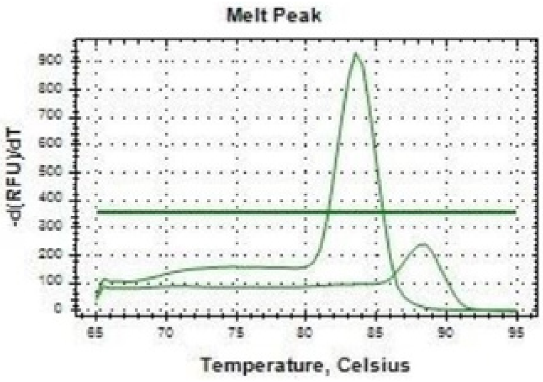

Figure 6. Melt curve peak of the soleus muscle.

Gene expression study

The gene expression profile revealed successful amplification of CASQ1 in the experimental samples, while no signal was observed in the negative control. The target gene appeared at the expected position in the amplification plot, confirming its expression in rabbit soleus muscle tissue [14] (Figure 7).

Figure 7. Amplification plot of gene expression generated through qPCR analysis

Discussion

Calsequestrin is the primary protein responsible for binding calcium within the sarcoplasmic reticulum and is essential for maintaining calcium balance and regulation. In mammals, it occurs in two forms: CASQ1, which is present in both fast- and slow-twitch skeletal muscles, and CASQ2, mainly found in the heart and in slow-twitch muscle fibers. Extensive studies have explored its structural features, subcellular localization, and functional significance in both skeletal and cardiac muscles, highlighting key differences relevant to their physiology. Calsequestrin not only serves as a calcium buffer but also functions as a dynamic sensor and modulator of calcium release, and has been implicated in various muscular pathologies. Despite numerous studies on skeletal muscle, the cardiac context remains comparatively underexplored. This study highlights the expression of calsequestrin (CASQ1) in the rabbit soleus muscle, offering valuable insights into its role within slow-twitch muscle fibers. The findings enhance our understanding of calcium regulation and signaling in muscle function and physiology [15].

Conclusions

Calsequestrin serves as a key regulator of calcium (Ca^{2+}) homeostasis in muscle physiology, functioning as the primary Ca^{2+}-binding protein within the sarcoplasmic reticulum. It exists in two isoforms: CASQ1, predominantly expressed in fast- and slow-twitch skeletal muscles, and CASQ2, mainly found in cardiac tissue and slow-twitch muscle fibers. The role of calsequestrin extends beyond calcium buffering, contributing to excitation-contraction coupling and intracellular signaling. In this study, we successfully demonstrated the expression of CASQ1 in the rabbit soleus muscle, a slow-twitch muscle type. The results confirm that calsequestrin is actively transcribed in this tissue, supporting its established role in calcium handling and storage in slow-contracting muscle fibers. These findings provide a basis for future investigations into the functional significance of calsequestrin in skeletal muscle physiology and its potential implications in muscle-related disorders involving calcium dysregulation.

Disclosure Statement

No potential conflict of interest was reported by the author.

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