CALSEQUESTRIN DISTRIBUTION, STRUCTURE AND FUNCTION, ITS ROLE IN NORMAL AND PATHOLOGICAL SITUATIONS AND THE EFFECT OF THYROID HORMONES. A REVIEW. Petr Novák and Tomáš Soukup, Institute of Physiology, Academy of Sciences of the Czech Republic, (v. v. i.), Vídeňská 1083, 14220 Prague 4, Czech Republic.

Abbreviations used:

CaBP-calcium binding protein

CBB- Coomassie Brilliant Blue

cCSQ-cardiac calsequestrin

DHPR-dihydropyridine receptor

ECC-excitation-contraction coupling

GAPDH- glyceraldehyde 3-phosphate dehydrogenase

NCX-Na^+/Ca^{2+} exchanger

RyR-ryanodine receptor

sCSQ-skeletal calsequestrin

SERCA-sarco/endoplasmic reticulum Ca^{2+}-ATPase

SR-sarcoplasmic reticulum

TT-transverse tubule

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Summary

Calsequestrin is the main calcium binding protein of the sarcoplasmic reticulum, serving as an important regulator of Ca^{2+}. In mammalian muscles, it exists as a skeletal isoform found in fast- and slow-twitch skeletal muscles and a cardiac isoform expressed in hearts and slow-twitch muscles. Recently, many excellent reviews that summarised in great detail various aspects of the calsequestrin structure, localisation or function both in skeletal and cardiac muscle have appeared. The present review focuses on skeletal muscle: an information on cardiac tissue is given, where differences between both tissues are functionally important. The article reviews the known multiple roles of calsequestrin including pathology in order to introduce this topic to the broader scientific community and to stimulate an interest in this protein. Newly we describe our results on the effect of thyroid hormones on skeletal and cardiac calsequestrin expression and discuss them in the context of available literature data on this topic.

Key words: skeletal and heart muscle – calcium binding proteins – calsequestrin – muscle pathology – thyroid hormones

Introduction

Ca^{2+} is one of the most important signalling molecules involved in various cellular processes (for review see Berchtold *et al.* 2000, Carafoli 2002, Berridge *et al.* 2003, Clapham 2007). It is regulated by many CaBPs functioning as Ca^{2+} effectors, sensors or buffers. About 200 CaBPs are encoded by the human genome (Carafoli *et al.* 2001) and CSQ is the most abundant CaBP in the SR of skeletal and cardiac muscle (MacLennan and Wong 1971, for review see Beard *et al.* 2004). It is an acidic protein that binds Ca^{2+} with moderate affinity, but with high capacity. In skeletal muscles, calsequestrin provides a large pool of Ca^{2+} releasable from the SR and at the same time it maintains the concentrations of free Ca^{2+} at sufficiently low

levels, which is important for easier and more efficient inward transport of the released Ca^{2+} by SERCA pumps as well as for the control of the RyR activity itself.

CSQ-like proteins in other tissues and organisms

Beside skeletal and cardiac CSQ isoforms of higher vertebrates with known amino acid sequence, many proteins reacting with antibodies against the above isoforms and sharing often significant similarity in their primary structures were described in other tissues and in various other organisms. In higher vertebrates, such proteins were detected in rat smooth muscles from the aorta and stomach (Volpe *et al.* 1994) and in the guinea pig urinary bladder (Moore *et al.* 2004). Besides muscle tissue, high levels of CSQ-like proteins were detected in Purkinje cells of chicken cerebellum (Volpe *et al.* 1991) and in the rat liver endoplasmic reticulum (Damiani *et al.* 1988). In invertebrates, a CSQ-like protein was found in body-wall muscle cells of *Caenorhabditis elegans* that shows moderate similarity (50% similarity, 30% identity) to rabbit sCSQ and is not essential for body-wall muscle formation and contraction (Cho *et al.* 2000). Another CSQ-like protein was purified from sea urchin (*Strongylocentrotus droebachiensis* and *Arbacia punctulata*) eggs, antigenically related to cCSQ, capable of binding anti-cCSQ antibody (Oberdorf *et al.* 1988). Furthermore, CSQ-like proteins were also discovered in some plant cells. A form of "CSQ" was detected in cultured *Streptanthus tortuosus* cells and spinach leaves recognised by antibodies against canine cCSQ (in *S. tortuosus*) and sCSQ (in spinach) (Krause *et al.* 1989). Additionally, a 42-kDa CSQ-like CaBP was found in calcium-accumulating cells of water lettuce (*Pistia stratiotes*) (Franceschi *et al.* 1993). Such widespread occurrence may suggest multiple functions of the "CSQ -like family" in various cells and tissues of diverse organisms. Their function in animal non-muscle cells and in plant cells remains, however, still in the realm of speculation.

Ultrastructural localisation of CSQ

As CSQ functions as an important part of ECC in muscle tissues (see further), it is important to know its ultrastructural localisation *in situ*. It was first described using indirect immunoferritin labelling of ultrathin frozen sections of rat skeletal muscle (gracilis). Most of the CSQ was found in the lumen of the terminal cisternae of the SR (Jorgensen *et al.* 1983). Corresponding localisation in the lumen of the peripheral and the interior junctional SR as well as in the lumen of the corbular SR was shown by indirect immunocolloidal gold labelling of ultrathin frozen sections in adult rat atrial and ventricular muscle cells (Jorgensen *et al.* 1985).

The architecture of the specialised junctional domain of the SR connecting it with the membrane of the TT system as revealed by electron microscopy was recently reviewed in great detail by Franzini-Armstrong

(2009). She showed that the site facing the TT is occupied by a complicated molecular complex consisting of RyRs, two membrane-spanning proteins junctin and triadin (which mediate interactions of RyRs and CSQ) and CSQ, the main luminal protein (Fig. 1). She found that under the conditions prevailing within the SR lumen (physiological ionic strength, mostly due to K^+ and Ca^{2+} ions), CSQ forms long linear polymers and the fixed protein gel is clearly visible in the electron microscope. CSQ was found in this domain in all analysed muscles in a variety of vertebrate species (Damiani *et al.* 1986). In skeletal muscles, CSQ has a mostly diffuse configuration, appearing as a network of thin lines, while the considerably narrower junctional SR cisternae of cardiac muscles are filled with condensed CSQ (Franzini-Armstrong 2009). Based on comparison of experiments with overexpression of CSQ (~10-fold) targeted to cardiac muscle and null mutations of all three junctional domain proteins, Franzini-Armstrong (2009) suggests that the apparent difference between the skeletal and cardiac CSQ disposition and junctional domain appearance is not due to the difference in the presence of sCSQ versus cCSQ isoforms, but more likely due to the ratio of either CSQ isoform to triadin and junctin. These experiments, however, do not suggest if there are differences in the total amount of these proteins between skeletal and cardiac muscle. None the less, she concludes that a specific interaction of CSQ with triadin and junctin assures clustering of CSQ at the junctional domain and defines its specific architecture.

Structure and Ca^{2+}-binding characteristics of CSQ

Rabbit sCSQ is encoded by a relatively small and uncomplicated gene that is only 8.6 kb long and contains 11 exons. The cloning and characterisation of the gene was described by Zarain-Herzberg *et al.* (1988), who also demonstrated the unlikeliness of alternative splicing of this gene. The molecule of rabbit sCSQ consists of three domains that form disk-like shapes and fold into α/β structures with a five-stranded mixed β-sheet in the core, flanked by four α-helices. The domains are connected by short connecting loops (Wang *et al.* 1998).

The complete amino acid sequence of canine cCSQ was deduced by cDNA cloning. The following comparison showed that the nascent forms of adult canine cCSQ and adult rabbit sCSQ contain 63 % identical residues (Scott *et al.* 1988). The cCSQ crystal structure is nearly superimposable on sCSQ which also applies for their front-to-front-type dimers (Park *et al.* 2004). Besides, Kim *et al.* (2007) reported the crystal structure of human cCSQ that is very similar to canine cCSQ and rabbit sCSQ.

Generally the CSQ molecule exists as either a monomer or a wide range of high molecular mass clusters, depending on the ionic environment (Kim *et al.* 2007). As proposed by Park *et al.* (2003), Ca^{2+} regulation by CSQ involves an interplay among protein folding, Ca^{2+} binding, and CSQ polymerisation. Formation of ribbon-like linear polymers is permitted by front-to-front and back-to-back contacts between the CSQ monomers and is promoted by Ca^{2+} and inhibited by K^{+} that induces CSQ folding. In the presence of 1 mM Ca^{2+}, cCSQ is mostly monomer/dimer, while sCSQ is mostly polymerised (Wei *et al.* 2009).

The highly extended structure of CSQ becomes much more compact upon binding Ca^{2+} (Cozens and Reithmeier 1984). Binding Ca^{2+} is associated with a loss in protein hydrophobicity (the burying of hydrophobic amino acid side chains) and it prevents CSQ from interactions with other proteins. It also protects CSQ from proteolytic digestion. Canine cCSQ binds 800-900 nmol of Ca^{2+}/mg of protein (35-40 mol of Ca^{2+}/mol of CSQ) (Mitchell *et al.* 1988). A comparison of rabbit sCSQ and sheep cCSQ showed that Ca^{2+} binding to cCSQ is 50% lower than to sCSQ in spite of the fact that cCSQ contains an extended C-terminal tail (a part of the molecule binding Ca^{2+}) composed of >70% acidic residues (Wei *et al.* 2009).

The structure of CSQ appears to be a key to elucidating its interactions with other molecules and thus its function (for review and pictures demonstrating the CSQ structure and binding see e.g., Park *et al.* 2003, Beard *et al.* 2004, 2009).

CSQ function and interaction within the membrane complex

Both in skeletal and cardiac muscles, CSQ forms a quaternary complex with RyRs, junctin and triadin (Zhang *et al.* 1997, for review see Beard *et al.* 2009) that is involved in ECC, the process linking sarcolemma and T-tubule membrane depolarisation to Ca^{2+} release from the SR via the RyRs (for review see Sandow 1965, Murray *et al.* 1998, Dulhunty 2006, Beard *et al.* 2005, 2009, Mackrill 2010). CSQ in skeletal muscles thus localises calcium near the junctional face of SR terminal cisternae from which it can be released into the cytosol as a consequence of muscle stimulation by neuronal activity. Depolarisation following nerve impulses and spreading into deep infoldings of TTs activates DHPR calcium channels coupled with the RyR calcium

release channels resident on the adjacent SR membrane. The DHPRs are supposed to induce Ca^{2+} release in skeletal muscles via direct physical interactions with the RyRs (Block *et al.* 1988, Franzini-Armstrong and Protasi 1997). Released calcium then binds to troponin C which relieves tropomyosin`s block of myosin/actin binding and starts a chain of reactions leading to muscle contraction (ECC). In vertebrate adult skeletal muscle fibres, contraction depends almost exclusively on Ca^{2+} released from the SR and not on the influx of extracellular Ca^{2+} (Melzer *et al.* 1995). In cardiac muscle, the RyRs are activated by Ca^{2+} that passes through the DHPRs from the extracellular space (for review see Beard *et al.* 2004, 2009, Dulhunty 2006, Chen *et al.* 2010). CSQ is thus a part of the highly specialised system controlling release and re-uptake of calcium and linking two well organised membrane systems, the exterior sarcolemma with its transverse tubules and the internal SR membranes, together. CSQ plays its role as the main CaBP in accord with and dependent on its contact with triadin and junctin linking it to the RyRs. Together this complex can sense the Ca^{2+} concentration in cytoplasmic and SR lumen compartments, which enables appropriate regulation of Ca^{2+} store load and Ca^{2+} release via the RyRs.

Triadin binds specifically to the RyR/Ca^{2+} release channel and CSQ (Guo and Campbell 1995) and its binding to the C-terminal luminal loop of the RyR1 seems important for ensuring rapid Ca^{2+} release during ECC in skeletal muscle (Goonasekera *et al.* 2007). However, its role is not essential, as pan-triadin null mice have no obvious contractile dysfunction (Shen *et al.* 2007). Junctin got its name, because it is an integral component of the junctional SR membrane in both cardiac and skeletal muscle. A short N-terminal region of this 26-kDa protein is located in the cytoplasm, and the bulk of the molecule projects into the SR lumen (Jones *et al.* 1995, for review see Dulhunty *et al.* 2009). Experiments on RyR1 terminal loop mutants revealed that junctin and triadin bind to different sites on RyR, thus elucidating the formation of the whole complex (Goonasekera *et al.* 2007).

Experiments on canine cardiac RyRs suggest that CSQ serves as a luminal Ca sensor that inhibits the RyR at low luminal calcium concentration and triadin 1 (the predominant triadin isoform in cardiac muscle) and/or junctin may be required to mediate interactions of CSQ with RyR (Györke *et al.* 2004). It was reported that the control of the RyR channels by CSQ *in vitro* is phosphorylation-dependent and that only dephosphorylated CSQ induces Ca^{2+} release from the SR (Szegedi *et al.* 1999). Later experiments showed, though, that under 1 mM luminal calcium concentration and in the presence of triadin and junctin, phosphorylation of CSQ does not alter its ability to inhibit native RyR activity (Beard *et al.* 2005).

The ability of CSQ to regulate skeletal RyRs also depends on its polymerisation. It was shown that CSQ readily forms a wide range of high molecular mass clusters (Maguire *et al.* 1997, Kim *et al.* 2007) and that triadin is implicated in this process (Guo and Campbell 1995). Under physiological conditions CSQ is polymerised and regulates the activity of the RyRs. Lowering of luminal Ca^{2+} from 1 mM to 100 μM for several minutes leads to sCSQ depolymerisation and dissociation of 65–75% of CSQ from the junctional face membrane resulting in a loss of CSQ regulation of the skeletal RyRs (Wei *et al.* 2006).

Calcium concentration, CSQ structure, formation of quarternary RyR-triadin-junctin-CSQ complexes and RyR activity itself are tightly connected, which is a prerequisite for the fine tuning of the muscle contraction. Furthermore, similarly to the other channels, the complexes are strongly targeted by many drugs, opening prospects for pharmacological regulation of many pathological conditions and diseases including malignant hyperthermia, skeletal muscle myopathies, cardiac arrhythmias, epilepsy, neurodegeneration, pain and cancer (for review see Mackrill 2010).

CSQ in fast and slow skeletal muscles and in the heart

CSQ is produced in a skeletal and a cardiac isoform, which are products of two different genes (Scott *et al.* 1988, Fliegel *et al.* 1990). The proportion of both isoforms varies according to the muscle and species, the precise value is likely dependent on the proportion of fast type 2 and slow type 1 fibres in the given muscle (Murphy *et al.* 2009). The skeletal isoform (sCSQ, CSQ1) is the only isoform found in fast-twitch and is the major isoform in slow-twitch muscles, while the cardiac isoform (cCSQ, CSQ2) is the only transcript in cardiac muscle and a minor transcript in adult slow-twitch muscle (Fliegel *et al.* 1987-fast, Scott *et al.* 1988-cardiac, Biral *et al.* 1992, for review see Beard *et al.* 2004). The sCSQ:cCSQ ratio in the rabbit soleus muscle was reported to be ~3:1 (Damiani *et al.* 1990) or higher (Fliegel *et al.* 1989). The sCSQ levels are much higher in fast- compared to slow-twitch muscles, as the amount of released calcium that must be taken up at a given moment is much higher in fast than in slow muscles. The content of sCSQ in homogenates of rat fast EDL and slow soleus was determined as 1.4±0.1 and 0.43±0.3 mg/g muscle wet weight, respectively (Murphy

et al. 2009). Maximal Ca^{2+} binding capacity was estimated as ~80 mol/mol of sCSQ and ~60 mol/mol of cCSQ (Park *et al.* 2004).

The general properties of sCSQ and cCSQ are similar (Beard *et al.* 2004). Both of them fulfil their main function as SR luminal Ca^{2+} sensors for the skeletal RyR1 (sCSQ) or cardiac RyR2 (cCSQ) isoform (Györke *et al.* 2004, Wei *et al.* 2006, Qin *et al.* 2008). Structurally, the cCSQ isoforms differ mainly in having a highly extended acidic C-terminal tail. Most of our knowledge about differences of molecular interactions between sCSQ and cCSQ comes from studies of isolated systems. Experiments with the incorporation of microsomal vesicles (formed from terminal cisternae of the SR) into artificial lipid bilayers enabled the formation of a model of the sCSQ structure and regulation of RyR1 by Ca^{2+} concentration changes (Wei *et al.* 2006, 2009). Similar experiments on the heart showed that cCSQ binding to RyR2 under physiological conditions can be quite different (Wei *et al.* 2009). When sCSQ is added back to the luminal solution bathing lipid bilayers at a physiological 1 mM Ca^{2+} concentration it inhibits RyR1 (Beard *et al.* 2002), while cCSQ activates RyR2 under the same conditions (Wei *et al.* 2009). Based on their extensive studies, Beard and colleagues concluded that the role of cCSQ in the heart can be quite different from that of sCSQ in skeletal muscles (Wei *et al.* 2009), although much less is known about molecular interactions of cCSQ and RyR2 (Beard *et al.* 2009). Similarly, they suggest the existence of differences in regulation of skeletal RyR1 by sCSQ in fast twitch and cCSQ in slow-twitch muscles (Beard *et al.* 2009).

CSQ changes during the postnatal development

As already mentioned, skeletal and heart muscles differ in the mechanism of their activation by Ca^{2+}. In the heart, contraction is initiated by membrane ionic current and spontaneous SR Ca^{2+} release, while skeletal muscles derive all calcium required for contraction from their internal stores. In developing skeletal muscles, ECC is supposed to be similar to the "cardiac-like" mechanism and it becomes the "skeletal type" after

maturation of the whole calcium handling system, including DHPRs, RyRs, SERCA and CSQ (Flucher and Franzini-Armstrong 1996, for review see Franzini-Armstrong 2009). It was shown in rabbits that during the development the cardiac/slow isoforms of these Ca^{2+} handling proteins are down-regulated and gradually replaced by their fast skeletal counterparts (Froemming and Ohlendieck 1998). Interestingly, the relation between both CSQ isoforms can be changed by 30 days of chronic low frequency electrostimulation that induces a slight down-regulation of "fast" sCSQ and increase of the relative density of "slow" cCSQ in microsomal preparations (Froemming *et al.* 2000). The cCSQ is the only isoform expressed in the heart at all developmental stages. On the other hand, skeletal muscles express both CSQ isoforms, although the expression ratio between cCSQ and sCSQ isoforms is developmentally regulated and varies from muscle to muscle. During the development it is cCSQ that is the most abundant isoform in foetal and neonatal slow-twitch skeletal muscles, while in adults it is co-expressed in slow muscles. sCSQ is also already expressed in embryonic muscles, its levels are higher in presumptive fast-twitch muscles than in slow ones and they steeply increase before birth and reach adult values in both fast and slow muscles of the rabbit 4 days after birth (Leberer *et al.* 1986). In contrast to slow muscles, cCSQ completely disappears from fast muscles between two and four weeks postnatally and the sCSQ is supposed to be the only isoform in the adult animals (Leberer *et al.* 1986, Sacchetto *et al.* 1993).

Pathological changes

Much about CSQ function in skeletal and heart muscle can be learnt from knockouts and mutations expressing different levels of CSQ isoforms. In contrast to molecular interactions, where sCSQ in isolated systems has been mostly studied, the majority of the transgenic experiments were focused on cardiac myocytes. The mutations can strike at different loci and thus affect various CSQ functions including CSQ-

RyR interactions and the Ca^{2+} buffering capacity. Furthermore, due to the differences in ECC even similar mutations in skeletal and cardiac isoforms can lead to different consequences.

Knockout mice lacking sCSQ are viable and fertile, capable of muscle contraction, although their skeletal muscles appear slightly atrophic. The preserved contraction is apparently achieved through adaptive changes in the muscle structure and protein composition (Paolini *et al.* 2007). Functional changes that are much more evident in fast-twitch muscles (EDL) with a great majority of fibres expressing only sCSQ than in slow-twitch muscles (soleus) expressing both skeletal and cardiac CSQ isoforms include prolonged rise and decay of tension, impaired Ca^{2+} release from the SR and decline in the amplitude of the Ca^{2+} transient, striking proliferation of SR junctional domains, large decrease in size of the junctional SR cisternae, higher density of mitochondria and increased density of Ca^{2+}-release channels. It can be concluded that sCSQ is essential for the normal development of the SR and its calcium release units and for the storage and release of appropriate amounts of SR Ca^{2+}. Also, cCSQ deletion mice are viable and maintain relatively normal Ca^{2+} release and contractile function, although they exhibit increase in SR volume and near absence of triadin-1 and junctin (Knollmann *et al.* 2006, 2009). It appears that the main role of cCSQ is to protect the heart against premature Ca^{2+} release and triggered arrhythmias and together with triadin it seems important for the structural organisation of the SR.

Transgenic mice exhibiting 10-fold higher levels of cCSQ in myocardium survived into adulthood, but had severe cardiac hypertrophy with 1.9-fold increase in heart mass (Jones *et al.* 1998). Their hearts adapted to CSQ overexpression by altering expression of other proteins. RyR, junctin, and triadin were down regulated by 50% or more, while Ca^{2+}-ATPase and phospholamban were unchanged or slightly increased (Jones *et al.* 1998).

Transgenic mice (DBA/C57BL/6, 8 weeks old) with cardiac-specific overexpression of cCSQ developed chronic heart failure, cachexia, and exercise intolerance. Despite the latter effect, there was no significant

change in skeletal muscle fibre-type composition, although fast glycolytic tibialis anterior and plantaris muscles underwent atrophy, whereas slow oxidative soleus muscle maintained muscle mass (Li *et al.* 2007). This demonstrates that the observed changes in the ability of skeletal muscle to withstand increased physiological demands resulted from heart failure and not from cCSQ overexpression per se. This course can suggest that the changes of CSQ buffering occur first and are followed by the CSQ-RyR activity regulation (Jones *et al.* 1998). Altogether, the described changes of cCSQ resulting in severe deterioration of the heart performance showed only mild consequences in skeletal muscles (Li *et al.* 2007).

It seems that both up- and down-regulation of CSQ expression are compatible with life, although changes of calcium handling have particular effects on the structure and function especially of cardiac muscle. Interestingly, in the models of severe muscle dystrophies like dystrophic mdx mice lacking the dystrophin isoform Dp427, there are no changes in expression of CSQ, DHPRs, RyRs and SERCA. They, however, show drastic reduction of CSQ-like proteins of 150-220 kDa in dystrophic microsomes (Culligan *et al.* 2002).

Changes in CSQ expression also result in many pathological changes in humans. A new type of surplus protein myopathy was described in four patients from four different families. It had benign evolution and was characterised by inclusions composed of an excess of SERCA1 (a SERCA isoform typical for fast-twitch skeletal muscles) and sCSQ (Tomelleri *et al.* 2006).

A significant association between sCSQ gene polymorphism and type 2 diabetes was found in the Old Order Amish, a strictly endogamous ethnoreligious group with a limited number of ancestors. This gene is encoded on chromosome 1q21, a region that has been linked to type 2 diabetes in diverse populations (Fu *et al.* 2004). Increased CSQ expression and calcium binding were reported in streptozotocin-induced diabetic rat skeletal muscle (entire hind limb skeletal muscles were investigated using anti-sCSQ antibody on Western blots) though not in their cardiac muscle (Howarth *et al.* 2002).

Various cCSQ (both missense and nonsense, Lahat *et al.* 2001, Postma *et al.* 2002) and RyR mutations (Marks *et al.* 2002, Györke 2009) cause the most significant CSQ related disease-catecholaminergic polymorphic ventricular tachycardia (CPVT). It is a familiar arrhythmogenic disorder manifested at a young age and characterised by syncopal events, seizures and sudden cardiac death during physical or emotional stress in absence of gross structural changes of the myocardium (Lahat *et al.* 2001, Postma *et al.* 2002, Terentyev *et al.* 2006, 2008). The CPVT-related cCSQ missense mutations lead to significant reduction in Ca^{2+}-binding capacity and differences in Ca^{2+}-dependent monomer-polymer transitions (Kim *et al*. 2007).

The aforementioned examples show that CSQ research is of great importance for biomedicine as it can be of great help in our understanding of various pathological conditions which can have practical implications.

Another pathological condition related to CaBPs is the alteration of the thyroid status. Antibodies against CSQ were found in 40% of patients with active thyroid-associated ophthalmopathy (a progressive orbital disorder associated with Graves' hyperthyroidism and, less often, Hashimoto's thyroiditis), but in 0% of normal subjects (Gunji *et al.* 1998). Recently it was announced that in patients with Graves` disease, both cCSQ and sCSQ genes were highly upregulated (Gopinath *et al.* 2009).

CSQ expression in experimentally altered thyroid states

Experimental studies analysing effects of thyroid hormone levels on CaBPs expression focused mainly on cardiac muscle apparently due to the profound impact of thyroid hormones on the heart function mediated by regulating the transcription of genes for calcium transporter proteins of the sarcolemma and the SR and for specific myofibrillar proteins (for review see Dillmann 1990). Measurements of SERCA2, NCX and phospholamban in the rat heart showed that atria exhibit a greater change in the protein content than ventricles in response to triiodothyronine exposure (Shenoy *et al.* 2001). Much less is known about regulation of Ca^{2+} transport systems due to altered concentrations of thyroid hormones in skeletal muscle (e. g. Simonides and

van Hardeveld 1985, Connelly *et al.* 1994). It was found that the hyperthyroid status increases and the hypothyroid status decreases protein and mRNA levels of RyR and SERCA after acute 4- or 8-day treatment in rabbits (Arai *et al.* 1991) and mRNA of RyR1 and 2, NCX and type 2 inositol-1, 4, 5-triphosphate receptors after chronic thyroid status alteration in rats (Hudecová *et al.* 2004). Studies related to CSQ changes in animals with the altered thyroid status are exceptional. Arai *et al.* (1991) found that the acute changes of the thyroid status for 4 and 8 days in the rabbit soleus might suggest that hyperthyroidism increased expression of sCSQ compared to cCSQ, while hypothyroidism had rather the opposite effect. The same authors reported that cCSQ expression in rabbit ventricles was only slightly increased after both treatments and their results also implied possible differences in cCSQ expression between ventricles and atria.

In order to better understand, the chronic effects of thyroid hormone alterations we investigated expression of sCSQ and cCSQ and the fibre type composition in fast and slow skeletal muscles and the former also in the heart of euthyroid, hypothyroid and hyperthyroid adult inbred female Lewis strain rats at mRNA and/or protein levels. The HY status was induced with 0.05 % solution of methimazole (2-mercapto-1-methylimidazole, Sigma) in drinking water, the TH status by intraperitoneal injections of 3, 3', 5-triiodo-L-thyronine (Sigma, sodium salt, T3, 150 mg/kg body weight) 3 times a week (cf. Soukup *et al.* 2001). CSQ isoforms were determined by SDS-PAGE followed by western blot analysis (expression was normalised to GAPDH and CBB) and gene expression was assessed using reverse transcription and subsequent real-time polymerase chain reaction. Our pilot experiments (Novák *et al.* 2008, 2010a) in agreement with previous findings in the rat (Murphy *et al.* 2009) show that the protein and transcript levels for sCSQ are the highest in the fast extensor digitorum longus (EDL), intermediate in the soleus (Fig. 2) and hardly detectable in the heart (not shown). The hypothyroid status decreases and the hyperthyroid status increases the already high protein levels of sCSQ in the fast EDL (Fig. 2). Neither the hypothyroid nor hyperthyroid status, however, has a significant effect on the "intermediate" levels of sCSQ found in the soleus muscle (Fig. 2) and on the

practically non-detectable levels in the heart (not shown). The same proportion of sCSQ expression under all thyroid states was received when the values were expressed either to GAPDH (Fig. 2) or CBB (not shown). The mRNA levels for cCSQ are the highest in the heart, intermediate in the soleus and the lowest in the EDL muscle and in the latter they appear slightly increased in the hypothyroid status (Novák *et al.* 2008, 2010a).

It is worth to remember here the difference between heart and skeletal muscles exposed to increased levels of thyroid hormones. While in hearts hyperthyroidism leads to cardiac hypertrophy due to the increased haemodynamic load, skeletal muscles are not subjected to this change imposed on the hearts. On the other hand, skeletal muscles react to thyroid hormone alteration by modification of their fibre type composition and MyHC content (d`Albis and Butler-Browne 1993, Larsson *et al.* 1994, Vadászová *et al*. 2004, 2006a, b, Vadászová-Soukup and Soukup 2007, Soukup *et al.* 2009, for review see Soukup and Jirmanová 2000).

Skeletal muscle fibre types can be recognised by immunostaining with specific monoclonal antibodies against each MyHC isoform (Smerdu and Soukup 2008, Soukup *et al.* 2002, 2009) or by their mATPase activity (Soukup *et al.* 2009, Novák *et al*. 2010b). Using the latter for the rat, the fibres that are stained positively after acid preincubations at pH 4.3 and 4.5 of the mATPase reaction are classified as type I fibres, while the fibres that are stained positively after the alkaline preincubation at pH 10.3 and remain unstained after both acid preincubations at pH 4.3 and 4.5 are type 2A fibres and the fibres characterised by high mATPase activity after preincubation at pH 10.3 and by moderate staining after preincubation at pH 4.5 are 2B fibres. Classification by mATPase reaction thus allows determination of type 1, 2A and 2B fibres (containing most of 2X/D fibres), while the more modern immunohistochemical approach reveals four types: type 1, 2A, 2X/D and 2B fibres (for review see e.g. Hämäläinen and Pette 1993, Schiaffino and Reggiani 1996, Soukup and Jirmanová 2000, Pette and Staron 2000, 2001, Pette 2002, Schiaffino 2010).

The EDL muscle of Lewis strain rats contains a low number of slow type 1 fibres (about 5%), higher number of 2A fibres (about 15%) and prevailing percentage (almost 80%) of the fastest 2B fibres (Table 1 EDL, Soukup *et al.* 2009, Novák *et al*. 2010b). On the other hand, the soleus muscle of the Lewis strain rats is composed of a great majority of slow-twitch or type 1 fibres (about 95 to 99%) complemented by a variable number of fast-twitch 2A fibres (Table 1 SOL, Soukup *et al.* 2009, Novák *et al*. 2010b). Our preliminary comparison of fibre type and CSQ level changes indicates that the observed increase of the sCSQ level can be caused by the increase of the fastest 2B fibres in the hyperthyroid status (where they form more than 80% of

all the fibres), while the decreased level of sCSQ in the hypothyroid rats can result from a decline in proportion of these fibres (Table 1 EDL). No changes of the sCSQ levels in the soleus can be explained by the lack of the fastest 2B fibres and the significant increase of 2A fibres in the hyperthyroid soleus has surprisingly only small and non-significant effect on the increase of the sCSQ level. Similarly, Murphy *et al.* (2009) found more than a 3x higher content of sCSQ in EDL type 2 fibres compared to soleus type 1 fibres and they also presumed the existence of differences in sCSQ levels between subgroups of fast type 2 fibres. Our results thus suggest that the observed changes in expression of sCSQ in rat muscles resulting from chronic alteration of the thyroid hormone levels are more likely a part of complex fibre type changes induced by thyroid hormones and revealed by the switch of MyHC isoforms and muscle fibre types. The twitch profile is to a large extent dictated by SR properties and apparently the appearance of the SR reflects the CSQ content. Schiaffino *et al.* 1970 found that most fibres in the EDL muscle have a richly developed SR, which is consistent with a rapid calcium release and uptake during the contraction-relaxation cycle, opposite to the major type 1 slow fibres of the soleus muscle with a relatively poorly developed SR. Normalisation of CSQ to GAPDH, which is a cytosolic housekeeping protein, may reflect changes in the content of the SR, which are likely to be affected by thyroid hormone changes in accordance with fibre type changes. Because we received in both muscles in different thyroid states the same proportion of sCSQ expression adjusted to CBB as to GAPDH and because all fast EDL fibres posses rich SR, we believe that the observed changes might reflect the relative abundance of CSQ within the SR connected with different sCSQ levels in different fibre types.

The effect of triiodothyronine levels on CSQ gene transcription would be thus regulated co-ordinately with other proteins rebuilt during muscle fibre type transformation and the expression of sCSQ apparently proceeds in a fibre type-specific manner. The final answer can be, however, obtained only by a single fibre analysis of MyHC isoforms and the sCSQ content.

Conclusions

Mammalian skeletal and cardiac CSQ isoform genes as well as the structure of respective protein are relatively simple, but the protein is well suited to bind both Ca^{2+} and other proteins like RyRs, junctin and triadin, which strongly enhances CSQ`s ability to regulate calcium movements. Because of the central role of CSQ in Ca^{2+} regulation, it is not surprising that mutations of its gene and disturbances of its structure have various pathological consequences. Experiments relating to thyroid hormone levels suggest that SR calcium storage capacity (CSQ) is less affected than the calcium release (RyRs) and uptake (SERCA, NCX) and that minor changes observed in muscles of rats with altered hormone levels are probably related to complex changes taking part during muscle fibre type transformation. The involvement of CSQ in thyroid hormone-related alterations of the calcium homeostasis thus remains an open question. Further studies are thus needed to reveal more about CSQ structural and functional modifications and their physiological role in the regulation of Ca^{2+} homeostasis.

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Table 1

THYROID STATUS	**HY**	**EU**	**TH**
EDL	*n=13*	*n=20*	*n=10*
1	9.7±5.3*	6.2±3.7	2.2±2.0***
2A	23.6±7.9**	15.8±3.8	15.9±4.6
2B	66.7±10.4***	78.0±5.2	82.0±5.3
THYROID STATUS	**HY**	**EU**	**TH**
SOL	*n=13*	*n=24*	*n=8*
1	99.6±1.2	99.2±1.2	65.7±7.2***
2A	0.4±1.2	0.8±1.2	34.3±7.2***

Text to tables

Table 1. Fibre type composition of the extensor digitorum longus (EDL) and soleus (SOL) muscles as determined by mATPase reaction at pH 4.5 from 9- to 11-month-old euthyroid (EU), hypothyroid (HY, treated with methimazole) and hyperthyroid (TH, treated with triiodothyronine) female inbred Lewis strain rats (the same set as analysed for the CSQ in Fig.2). The numerical proportions (%) of muscle fibre types were assessed by 2-D stereological methods using the principles of an unbiased counting frame and point counting by the C.A.S.T. Grid System (Olympus, Albertslund, Denmark) (Zacharova and Kubinova 1995, Zacharova *et al.* 1997, 1999, 2005). n = number of muscles analysed. $^{*}p\leq0.05$, $^{**}p\leq0.01$, $^{***}p\leq0.001$ compared to the EU rats. The significance of the differences was evaluated by the Student`s t-test and/or Mann-Whitney test.

Text to figures

Fig.1. A schematic representation of the SR junctional domain complex of the skeletal muscle connecting TT and SR membranes, demonstrating organization of DHPRs, RyRs, triadin, junctin and CSQ.

Fig. 2 (A). An illustrative Western blot of skeletal CSQ in the fast extensor digitorum longus (EDL) and slow soleus (SOL) skeletal muscles of euthyroid (EU), hyperthyroid (TH, treated with triiodothyronine) and hypothyroid (HY, treated with methimazole) 9- to 11-month-old inbred female Lewis strain rats. After separation of muscle proteins by SDS-PAGE, CSQ was revealed on western blots by specific monoclonal antibody. Corresponding amounts of GAPDH are shown in the bottom panel as loading control. **(B**) The mean content of skeletal CSQ compared relatively to the content in the EDL of EU rats. Results are expressed in arbitrary units adjusted to GAPDH expression. The data represent average values (means ± S.D.) from 5 to 9 animals in each group; each value from an individual animal is based on 3 to 4 measurements (gels). The significance of the differences was evaluated by the Student`s t-test and/or Mann-Whitney test. $^{*}p\leq0.05$, $^{**}p\leq0.01$, $^{***}p\leq0.001$ compared to the EU rats.

Fig.1

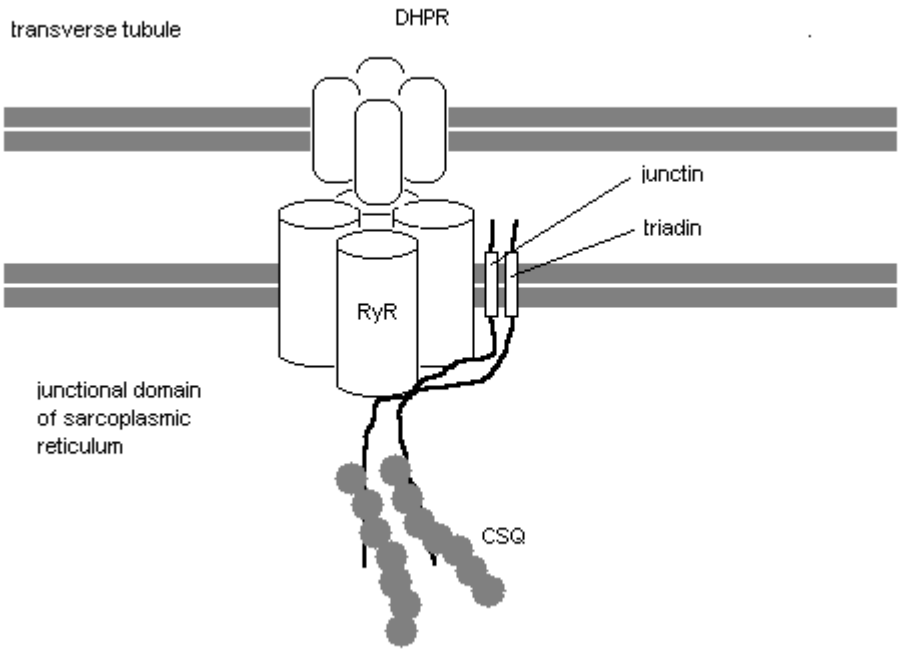
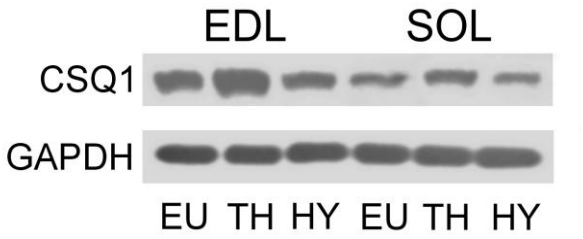
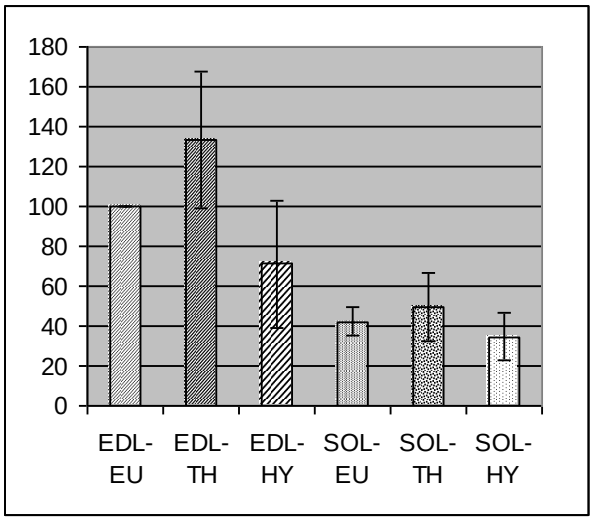

transverse tubule
DHPR
junctin
triadin
RyR
junctional domain
of sarcoplasmic
reticulum
CSQ

Fig. 2

A


EDL
SOL
CSQ1
GAPDH
EU TH HY EU TH HY

B


180
160
140
120
100
80
60
40
20
0
EDL-EU
EDL-TH
EDL-HY
SOL-EU
SOL-TH
SOL-HY