

Supramolecular calsequestrin complex

Protein–protein interactions in chronic low-frequency stimulated muscle, postnatal development and ageing

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As recently demonstrated by overlay assays using calsequestrin-peroxidase conjugates, the major 63 kDa Ca²⁺-binding protein of the sarcoplasmic reticulum forms complexes with itself, and with junctin (26 kDa), triadin (94 kDa) and the ryanodine receptor (560 kDa) [Glover, L., Culligan, K., Cala, S., Mulvey, C. & Ohlendieck, K. (2001) *Biochim. Biophys. Acta* **1515**, 120–132]. Here, we show that variations in the relative abundance of these four central elements of excitation–contraction coupling in different fiber types, and during chronic electrostimulation-induced fiber type transitions, are reflected by distinct alterations in the calsequestrin overlay binding patterns. Comparative immunoblotting with antibodies to markers of the junctional sarcoplasmic reticulum, in combination with the calsequestrin overlay binding patterns, confirmed a lower ryanodine receptor expression in slow soleus muscle compared to fast fibers, and revealed a drastic reduction of the RyR1 isoform in chronic low-frequency stimulated tibialis anterior muscle. The fast-to-slow transition process included a distinct reduction in

fast calsequestrin and triadin and a concomitant reduction in calsequestrin binding to these sarcoplasmic reticulum elements. The calsequestrin-binding protein junctin was not affected by the muscle transformation process. The increase in calsequestrin and decrease in junctin expression during postnatal development resulted in similar changes in the intensity of binding of the calsequestrin conjugate to these sarcoplasmic reticulum components. Aged skeletal muscle fibers tended towards reduced protein interactions within the calsequestrin complex. This agrees with the physiological concept that the key regulators of Ca²⁺ homeostasis exist in a supramolecular membrane assembly and that protein–protein interactions are affected by isoform shifting underlying the finely tuned adaptation of muscle fibers to changed functional demands.

Keywords: calsequestrin; calcium homeostasis; chronic low-frequency stimulation; excitation–contraction coupling; ryanodine receptor.

The physiological importance of direct protein–protein interactions being involved in Ca²⁺-regulatory processes is exemplified by a supramolecular triad membrane complex mediating between sarcolemmal excitation and muscular contraction [1]. It is well established that physical coupling between the voltage-sensing dihydropyridine receptor and the Ca²⁺-release channel provides the signal transduction mechanism between the transverse tubules and the junctional sarcoplasmic reticulum (SR) in mature skeletal muscle fibers [2]. Conversely, it has not yet been determined how many SR elements are involved in the regulation of the contraction-inducing efflux of Ca²⁺-ions from the SR lumen through the ryanodine receptor (RyR) complex, and which components prevent passive disintegration of these large heterogeneous SR membrane assemblies. Previous studies on excitation–contraction coupling have established

that the RyR1 isoform of the Ca²⁺-release channel exists in a close neighborhood relationship with various potential regulators, such as triadin (TRI), junctin (JUN), JP-45, JP-90, the histidine-rich Ca²⁺-binding protein, calsequestrin (CSQ) and CSQ-like proteins [3].

Domain binding experiments [4], differential coimmuno precipitation studies [5] and chemical crosslinking analysis [6] indicate that the RyR of 560 kDa, TRI of 94 kDa, JUN of 28 kDa and CSQ of 63 kDa form a tightly associated complex in skeletal muscle membranes. TRI and CSQ appear to function as endogenous regulators of the Ca²⁺-release channel [7]. Thus, the high-capacity, low-affinity Ca²⁺-binding element CSQ [8] and its larger isoforms of 150–220 kDa, termed CSQ-like proteins (CLPs) [9], do not only represent the major Ca²⁺-reservoir complex within the terminal cisternae region [10], but are also directly involved in regulating ion fluxes [11]. The existence of a subpopulation of CSQ within supramolecular SR complexes from mature skeletal muscle fibers has recently been shown using an optimized overlay technique [5]. Peroxidase-conjugated CSQ clearly labelled itself [12] and its binding-protein JUN, TRI and the RyR [5]. Protein–protein coupling between CSQ and the other junctional elements could be modified by detergent treatment, changes in Ca²⁺ concentration, antibody adsorption and purified CSQ binding [5]. Based on these findings showing a tightly associated junctional SR complex providing the physiological basis of regulating

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Abbreviations: CSQ, calsequestrin; CLP, calsequestrin-like protein;

IB, immuno blot; JUN, junctin; mAb, monoclonal antibody;

POD, peroxidase; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; TRI, triadin.

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excitation–contraction coupling, we extended our investigation of CSQ complex formation on muscle tissues under varying physiological conditions. A high degree of adaptability to changed functional demands and a large regenerative capacity are intrinsic properties of differentiated skeletal muscle fibers [13]. In addition, muscle fibers undergo major molecular changes during development [14] and ageing [15]. Major alterations in the relative abundance and/or isoform expression pattern of Ca^{2+} -regulatory membrane proteins involved in excitation–contraction coupling are associated with these cell biological changes. We therefore applied the CSQ overlay technique to study complex formation in developing, transforming and ageing skeletal muscle fibers.

Blot overlays are a technically challenging approach to studying complex protein–protein interactions between different elements of a heterogeneous membrane assembly. Recently, we enhanced the sensitivity of detection [5,12], which overcame the main obstacle of a previously unsuccessful approach to determining high-molecular-mass CSQ-binding elements [16]. However, due to the many steps involved in this analytical procedure, the visualization of SR proteins via a peroxidase (POD)-CSQ conjugate does not achieve the same degree of linear signaling achieved by Western blotting for example. At the current state of optimization, the blot overlay technique represents a semiquantitative tool, similar to immuno precipitation analysis. Nevertheless, this does not limit the range of potential biochemical applications of the blot overlay method in determining protein linkage. Its greatest advantage is the direct visualization of protein–protein interactions under controlled conditions. In contrast, other established protein biochemical methods for the analysis of large membrane complexes such as chemical crosslinking analysis might introduce artifacts by random protein linkage. Although gel filtration chromatography, domain binding studies with recombinant or isolated peptide domains, differential coimmuno precipitation or analytical ultra centrifugation supply sophisticated data, they do not directly illustrate protein interactions within supramolecular complexes. In this regard, the analyses using the optimized CSQ-POD overlay procedure presented in this study are an excellent example of applying a direct decoration method to studying heterogeneous membrane assemblies under differing biological conditions.

EXPERIMENTAL PROCEDURES

Animals

Skeletal muscle from young, adult and ageing New Zealand white rabbits were obtained from the Biomedical Facility, National University of Ireland, Dublin. The relevant ages of the animals used were (d, days; y, years): 14d, 21d, 28d, 41d, 44d, 1.0y, and 2.4y after birth, whereby the last age group represents the oldest rabbits commercially available in Ireland. For evaluating potential variations in CSQ complex formation in different skeletal muscle fiber types, psoas, gastrocnemius and soleus muscles were dissected and separately prepared for the isolation of microsomal membranes [17]. Chronic low-frequency stimulated muscles were produced by tele-stimulation for 0, 5 and 78 days through the peroneal nerve of the left hind

limb of adult male rabbits in the Animal Facility of the University of Konstanz [18].

Materials

Protease inhibitors, peroxidase-conjugated secondary antibodies, and acrylamide stock solutions were obtained from Boehringer Mannheim (Lewis, East Sussex, UK). Primary antibodies were purchased from Affinity Bioreagents, Golden, CO, USA (mAb VIIIID1₂ to fast calsequestrin; pAb to slow calsequestrin; mAb IIIH11 to the fast SERCA1 isoform of the Ca^{2+} -ATPase; mAb IID8 against the slow SERCA2 isoform of the Ca^{2+} -ATPase, and mAb IIG12 to muscle triadin) and Upstate Biotechnology, Lake Placid, NY, USA (pAb to the RyR1 isoform of the ryanodine receptor Ca^{2+} -release channel). Immobilon-P nitrocellulose membranes were from Millipore Corporation (Bedford, MA, USA). An affinity-purified polyclonal antibody to junctin was a generous gift from Steve Cala (Wayne State University, Detroit, MI, USA). The EZ-Link-Plus activated peroxidase kits, Slide-A-Lyzer dialysis cassettes and chemiluminescence substrates were purchased from Perbio Science UK Ltd. (Tattenhall, Cheshire, UK). All other chemicals used were of analytical grade and purchased from Sigma Chemical Company (Poole, Dorset, UK).

Membrane preparation

Microsomal membrane vesicles were isolated from rabbit skeletal muscle homogenates by an established protocol at 0–4 °C in the presence of a protease inhibitor cocktail (0.2 mM Pefabloc, 1.4 μM pepstatin A, 0.3 μM E-64, 1 μM leupeptin, 1 mM EDTA, and 0.5 μM soybean trypsin inhibitor) [19]. Using bovine serum albumin as a standard, the protein concentration of isolated membrane vesicles was determined by the method of Bradford [20]. Following isolation, membrane vesicles were immediately used for electrophoretic separation, blot overlay assays and immunoblot analysis.

Gel electrophoresis and immunoblot analysis

SDS/PAGE under reducing conditions was carried out by standard methodology [21] using 7% gels and 20 μg protein per lane [22]. Protein band patterns were visualized by Coomassie Brilliant Blue or Silver staining. For blotting experiments, separated microsomal muscle proteins were electrophoretically transferred for 1 h at 100 V onto nitrocellulose membranes by the method of Towbin *et al.* [23]. Membrane blocking, incubation with primary antibodies, washing steps, incubation with peroxidase-conjugated secondary antibodies, visualization of immuno-decorated protein bands and densitometric scanning of developed immunoblots was carried out as described previously [24].

Calsequestrin blot overlay

Recently established optimum conditions were used for CSQ blot overlay assays [5,12]. Skeletal muscle CSQ was purified to homogeneity by Phenyl-Sepharose chromatography as described by Cala & Jones [25]. The purified SR protein was conjugated to an amine-reactive marker enzyme as described in the manufacturer's instructions of the

EZ-Link-Plus activated peroxidase kit. A Pierce Slide-A-Lyzer dialysis cassette system was employed to remove contaminants from the CSQ-POD conjugate. As previously documented [5], homogeneity of the CSQ preparation and successful POD conjugation was evaluated by silver staining and immunoblotting of electrophoretically separated proteins. Nitrocellulose replicas of protein gels were incubated with the CSQ-POD complex overnight at room temperature. After several washes, decorated protein bands were visualized by the enhanced chemiluminescence technique. Densitometric scanning of developed overlay blots was performed on a Molecular Dynamics 300S computing densitometer (Sunnyvale, CA, USA) with IMAGE QUANT v3.0 software [22]. Protein identification by mass spectroscopy was performed with trypsin-digested protein samples by J. Coffey (Micromass UK Ltd, Manchester, UK) using a Q-ToF Ultima API/CapLC system and sequence similarities determined with the EXPASY-PEPTIDENT program. N-Terminal sequencing for protein band identification was carried out by J. Fox, Alta Bioscience (School of Biosciences, University of Birmingham, Edgbaston, UK) and sequence similarities determined with the BLAST-P program.

RESULTS

To complement previous domain binding studies and chemical crosslinking analyses of triadic protein-protein interactions, we present here the analysis of CSQ interactions with electrophoretically separated microsomal proteins derived from developing, transforming and ageing skeletal muscle fibers. Because CSQ represents the luminal protein backbone regulating SR Ca^{2+} buffering during the excitation-contraction-relaxation cycle, a POD-conjugated

CSQ probe was employed to determine potential differences in the overlay pattern under varying physiological conditions. Following the identification of the purified SR protein of apparent 63 kDa as calsequestrin (Fig. 1), the results from our CSQ overlay analysis are presented with respect to fiber types (Fig. 2), chronic low-frequency stimulation-induced fiber transitions (Fig. 3), developing (Fig. 4) and ageing skeletal muscles (Fig. 5).

Identification of purified calsequestrin

To determine the CSQ-binding proteins, the purified status of CSQ for usage as a POD-conjugated probe had to be properly established. To unequivocally identify the protein species purified by Phenyl-Sepharose chromatography from the alkaline extracted microsomal fraction (Fig. 1A), three independent methods were employed, i.e. immunoblotting with monoclonal antibody VIIIID₁₂ to fast CSQ (Fig. 1B), N-terminal sequencing (Fig. 1C) and mass spectroscopy of tryptic fragments (Fig. 1C). All three methods clearly identified the 63 kDa SR protein species used for POD-conjugation as rabbit fast skeletal muscle CSQ. Immunoblotting with a polyclonal antibody to the slow CSQ isoform did not reveal a signal above background labeling (Fig. 1B) demonstrating that the purified protein species represents almost exclusively the fast isoform. Mass spectroscopic analysis revealed that the sequence of 11 trypsin fragments of the protein band of approximately 63 kDa matched 29% of the entire CSQ sequence (SwissProt P07221) (Fig. 1C). Using N-terminal sequencing, this finding was confirmed by a match of a 20 amino acid stretch of sequence (EGLDFPEYDGVDRVINVNA) with the primary structure of CSQ [26] (Fig. 1C). Successful

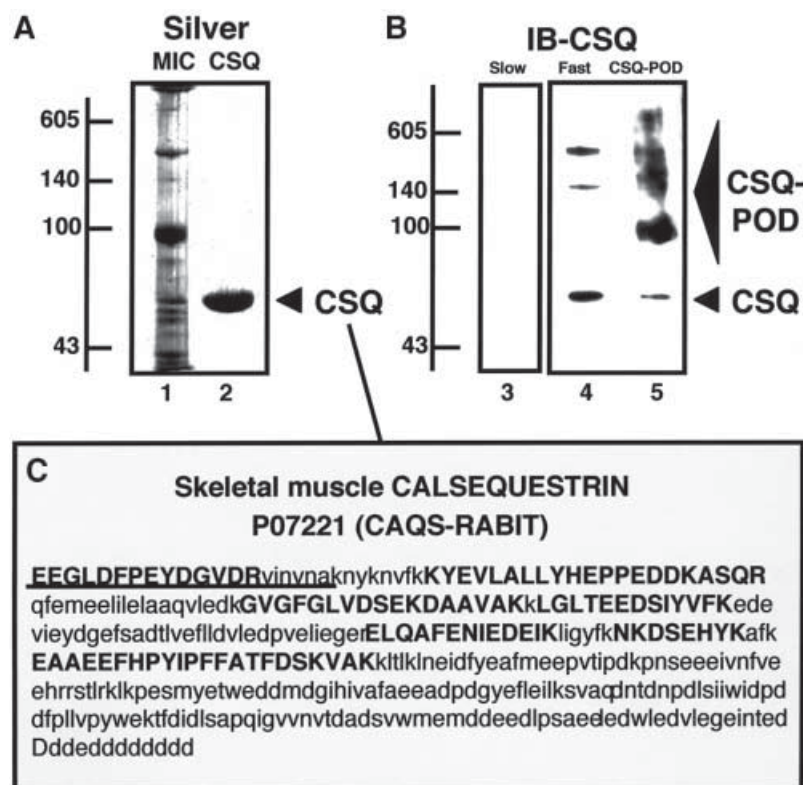


Fig. 1. Identification and conjugation of purified calsequestrin from rabbit skeletal muscle. Shown is a silver-stained gel (A) of microsomes (MIC) (lane 1) and purified calsequestrin (CSQ) (lane 2), and an immunoblot (B) of purified CSQ prior (lanes 3 and 4) and after (lane 5) conjugation to a peroxidase (POD) marker. The blots have been immunodecorated with a polyclonal antibody to slow CSQ (lane 3) and mAb VIIIID₁₂ to the fast isoform of CSQ (lanes 4 and 5). The relative positions of CSQ and CSQ-POD are marked by arrow heads. Molecular mass standards (in kDa) are indicated on the left. In (C) is shown the primary sequence of CSQ with capital letters marking the sequence determined by mass spectroscopy and the underlined sequence showing the peptide domain determined by N-terminal sequencing.

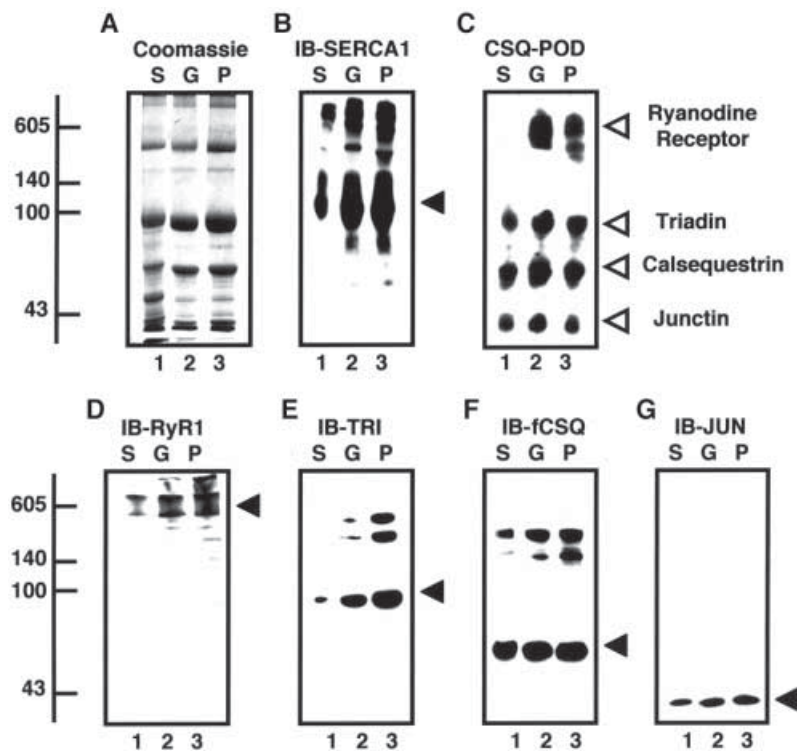


Fig. 2. Calsequestrin complex formation in fast and slow muscle fibers. Shown is a Coomassie-stained gel (A) of microsomal preparations and identical blots (B–G) labeled with antibodies to the fast SERCA1 isoform of the Ca^{2+} -ATPase (B), the RyR1 isoform of the Ca^{2+} -release channel (D), triadin (TRI) (E), fast calsequestrin (fCSQ) (F) and junctin (JUN) (G). In (C) is shown a blot overlay using a CSQ-POD probe. Lanes 1–3 represent membrane vesicles derived from soleus (S), gastrocnemius (G) and psoas (P) muscle homogenates, respectively. The relative positions of immuno-decorated proteins are marked by closed arrow heads and the protein species recognized by the CSQ-POD overlay technique are indicated by open arrow heads. Molecular mass standards (in kDa) are indicated on the left.

conjugation of purified CSQ to the POD-marker was demonstrated by a shift to a higher relative molecular mass, as illustrated by the immunoblot analysis in Fig. 1B. Sequence information by peptide sequencing or mass spectroscopy for bands recognized by blot overlay did not reveal sufficient data for proper databank searches (not shown). Therefore, the identification of CSQ-decorated bands described below was performed by immunoblotting with established antibodies to triad markers.

Calsequestrin complex formation in fast and slow muscle fibers

In order to determine potential difference in CSQ complex formation in slow vs. fast skeletal muscle fibers, the electrophoretically separated protein complement of the microsomal fraction derived from soleus, gastrocnemius and psoas muscle homogenates was analysed by blot overlay. Prior to comparative immunoblotting with junctional SR markers and CSQ-POD binding, the fiber type-specific differences of the preparations were established. Although the Coomassie-stained gel representing the three different muscles did not show any major differences in the overall protein band pattern (with the exception of a low-molecular-mass species in soleus) (Fig. 2A), immuno-decoration with mAb IIIH11 to the fast SERCA1 isoform of the SR Ca^{2+} -ATPase demonstrated the well established difference in slow vs. fast fiber distribution in soleus vs. gastrocnemius and psoas muscles (Fig. 2B). The CSQ overlay binding pattern showed a highly specific binding pattern to four major protein species of ≈ 28 , 63, 94 and 560 kDa in predominantly fast-twitching muscle (Fig. 2C). The specificity of our newly developed CSQ-POD overlay assay has been documented previously [5]. Incubation with antibodies to CSQ, the ionic detergent SDS or the nonionic

detergent Triton X-100 eliminates these interactions (not shown). Interestingly, the CSQ-POD probe exhibited only very weak labeling of the 560 kDa band in soleus muscle microsomes (Fig. 2C). This agrees with the reduced expression of the RyR1 isoform in slow-twitching muscle as illustrated in the immunoblot analysis of Fig. 2D.

Due to the heterogeneous self-aggregation of triadin [27], the 94 kDa band of the fast isoform is often accompanied by high-molecular-mass bands in fast-twitch muscle (Fig. 2E). The decreased relative density of fast triadin in soleus muscle preparations (Fig. 2E) is partially reflected by a reduced CSQ overlay signal (Fig. 2C). This result shows both the strength and limitations of the overlay technique. On the one hand, the CSQ-POD probe clearly labels the main triad components forming the SR Ca^{2+} -binding and -release complex, but on the other hand changes in protein concentration are only semiquantitatively revealed. Immunoblotting of fast CSQ and its binding-protein JUN showed relatively similar levels in microsomal preparations derived from predominantly fast- and slow-twitching muscle fibers (Fig. 2F,G) and this is also reflected by the CSQ-POD overlay pattern of these two SR proteins (Fig. 2C).

Calsequestrin complex formation in chronic low-frequency stimulated muscle fibers

The isoform-specific expression of many SR proteins is affected during fast-to-slow fiber transitions, including CSQ [28]. We therefore studied the complex formation of this terminal cisternae Ca^{2+} -binding protein in chronic low-frequency stimulated muscle fibers. During the fast-to-slow transition process, a drastic decrease in the 110 kDa protein band region was illustrated by Coomassie staining of the electrophoretically separated microsomal fraction (Fig. 3A). This protein species mostly represents the fast

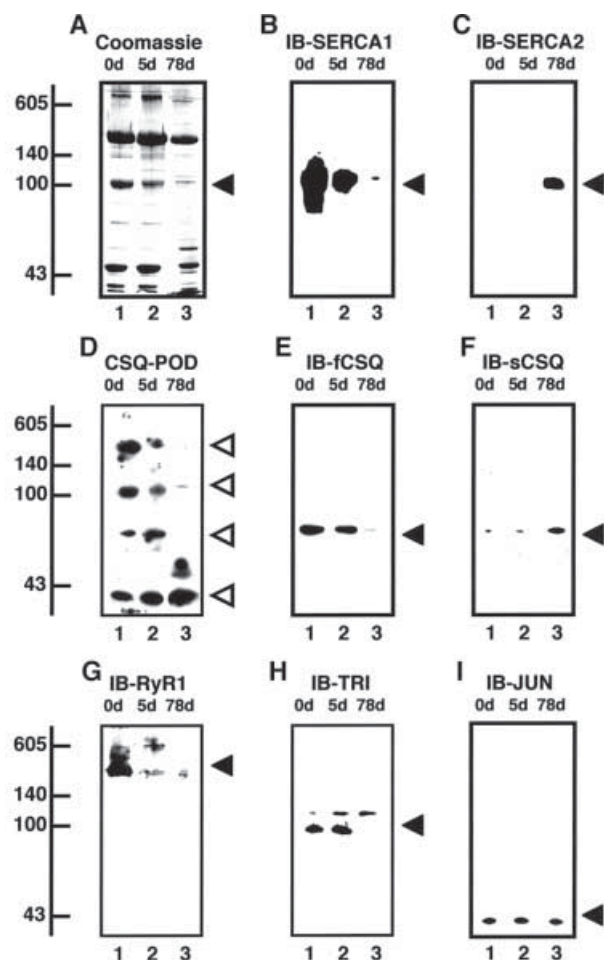


Fig. 3. Calsequestrin complex formation in chronic low-frequency stimulated muscle fibers. Shown is a Coomassie-stained gel (A) of microsomal preparations and identical blots (B–I) labeled with antibodies to the fast SERCA1 isoform of the Ca^{2+} -ATPase (B), the slow SERCA2 isoform of the Ca^{2+} -ATPase (C), the fast CSQ isoform (E), the slow/cardiac CSQ isoform (F), the RyR1 isoform of the Ca^{2+} -release channel (G), triadin (TRI) (H), and junctin (JUN) (I). In (D) is shown a blot overlay using a CSQ-POD probe. Lanes 1–3 represent membrane vesicles derived from unstimulated control, 5 day and 78 day chronic low-frequency (10 Hz) stimulated muscle, respectively. The relative positions of a 110 kDa Coomassie-stained band (A) and immuno-decorated proteins (B–I) are marked by closed arrow heads and the protein species recognized by the CSQ-POD overlay technique are indicated by open arrow heads. Molecular mass standards (in kDa) are indicated on the left.

SERCA1 isoform of the SR Ca^{2+} -ATPase as revealed by the drastic reduction of this fast-twitch marker following chronic electro-stimulation (Fig. 3B). The switch between the fast SERCA1 and slow SERCA2 (Fig. 3B,C), and the exchange of the fast CSQ with the slow CSQ isoform (Fig. 3E,F) agrees with previous studies [28] and clearly documents a successful fiber transition. The CSQ-POD overlay pattern showed some changes in the labeling intensity of the apparent 94 and 560 kDa bands after 5 days of electro-stimulation, and a drastic decrease in the decoration of the 63, 94 and 560 kDa bands after 78 days of chronic low-frequency stimulation (Fig. 3D). The latter

finding agrees with the stimulation-induced reduction in the fast isoforms of CSQ, TRI and the RyR1 (Fig. 3E,G,H). The disproportionate weakening of immuno labeling of the RyR1 band in stimulated muscle fibers is probably due to a combination of factors, i.e. the existence of proteolytic degradation products, heterogeneous aggregates and/or an electrophoretic separation artifact often seen with very large membrane proteins such as the Ca^{2+} -release channel. At high abundance the antibody to the RyR1 recognizes all separated RyR species (Fig. 3G, lane 1). However, at reduced density, major RyR bands are recognized (Fig. 3G, lane 2), but molecular species of lower relative concentration are covered by other SR proteins with a similar electrophoretic mobility and are thus not properly recognized by the antibody. The appearance of a double band pattern of immuno decorated TRI (Fig. 3H) is probably due to the tight aggregation of this triadic component. As has been previously documented [27], native triadin exists as a disulfide-linked polymer and even under reducing conditions these complexes do not completely disintegrate. In Fig. 3H, the major protein band of apparent 94 kDa represents the monomeric TRI unit and this molecule exhibits a dramatic reduction in its relative density following electro-stimulation. In contrast, both the CSQ binding to JUN and the relative concentration of JUN did not decrease after 78 days of muscle fiber transformation (Fig. 3D,I). A very interesting observation was the apparent lack of interaction between the fast CSQ-containing overlay probe and the slow CSQ band in 78 day stimulated tibialis anterior microsomes (Fig. 3D,F). Possibly, fast and slow CSQ isoforms exhibit different degrees of self-aggregation and heterogeneous protein–protein interactions. Slow CSQ might be involved in a more indirect type of physiological coupling process in transformed fibers, while fast CSQ appears to be a directly interacting endogenous regulator of the Ca^{2+} -release and Ca^{2+} -cycling process in fast muscle.

Calsequestrin complex formation in developing and ageing muscle fibers

Because many Ca^{2+} -regulatory proteins exhibit changes in their isoform expression pattern and/or relative abundance during postnatal myogenesis [29], we performed CSQ blot overlay of 14- to 41-day-old-muscle preparations. Due to the limited degree of differentiation during early myogenesis it was not possible to prepare fiber-type specific microsomal vesicles from developing muscle specimens. These analyses were performed with mixed fiber populations. Blotting of electrophoretically separated microsomes from 1, 3 and 7-day-old-rabbits did not reveal a sufficient signal-to-noise ratio for proper comparative overlay and immunoblot analysis (not shown). Shown are the data obtained with muscle preparations from young animals before (14 day old) and after (41 day old) maturation of the excitation–contraction coupling mechanism [29]. Although the overall protein band pattern is relatively similar during postnatal development (Fig. 4A), immunoblotting clearly showed an increase in the relative expression of RyR1, fast CSQ and the fast SERCA1 isoform of the Ca^{2+} -pump (Fig. 4C,E,F). The double band pattern of the RyR1 protein species (Fig. 4C) is probably due to the proteolytic degradation of the Ca^{2+} -release channel during membrane preparation.

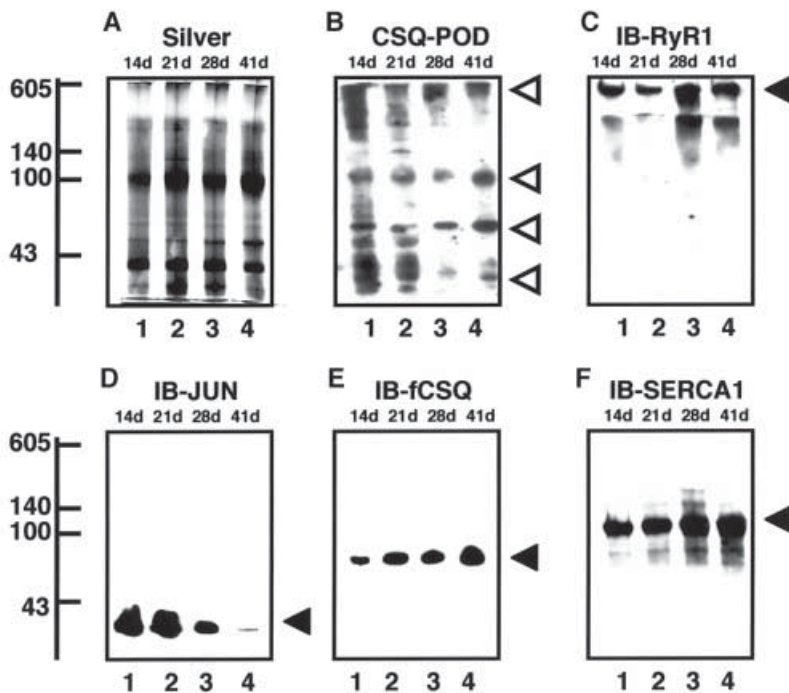


Fig. 4. Calsequestrin complex formation in developing muscle fibers. Shown is a silver-stained gel (A) of microsomal preparations and identical blots (B–F) labeled with antibodies to the RyR1 isoform of the Ca^{2+} -release channel (C), junctin (JUN) (D), fast calsequestrin (fCSQ) (E), and the fast SERCA1 isoform of the Ca^{2+} -ATPase (F). In (B) is shown a blot overlay using a CSQ-POD probe. Lanes 1–4 represent membrane vesicles derived from 14-, 21-, 28- and 41-day-old postnatal muscle, respectively. The relative positions of immuno-decorated proteins are marked by closed arrow heads and the protein species recognized by the CSQ-POD overlay technique are indicated by open arrow heads. Molecular mass standards (in kDa) are indicated on the left.

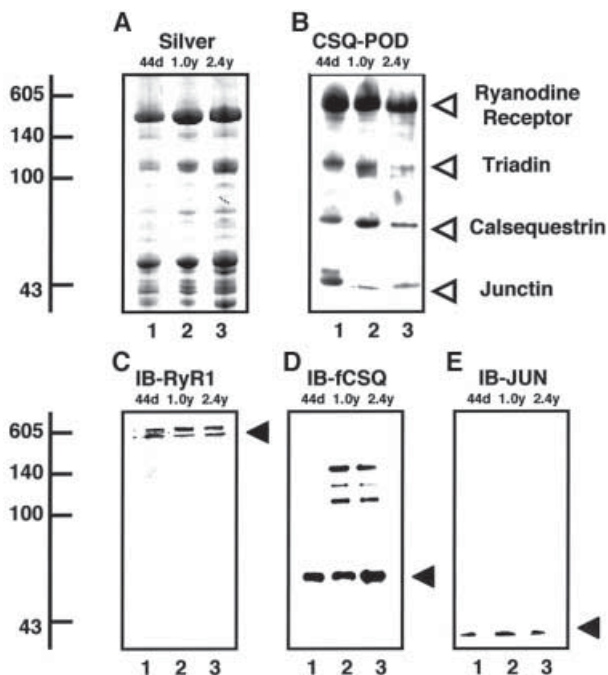


Fig. 5. Calsequestrin complex formation in ageing muscle fibers. Shown is a silver-stained gel (A) of microsomal preparations and identical blots (B–E) labeled with antibodies to the RyR1 isoform of the Ca^{2+} -release channel (C), fast calsequestrin (fCSQ) (D), and junctin (JUN) (E). In (B) is shown a blot overlay using a CSQ-POD probe. Lanes 1–3 represent membrane vesicles derived from 44-day-, 1-year- and 2.4-year-old-muscle, respectively. The relative positions of immuno-decorated proteins are marked by closed arrow heads and the protein species recognized by the CSQ-POD overlay technique are indicated by open arrow heads. Molecular mass standards (in kDa) are indicated on the left.

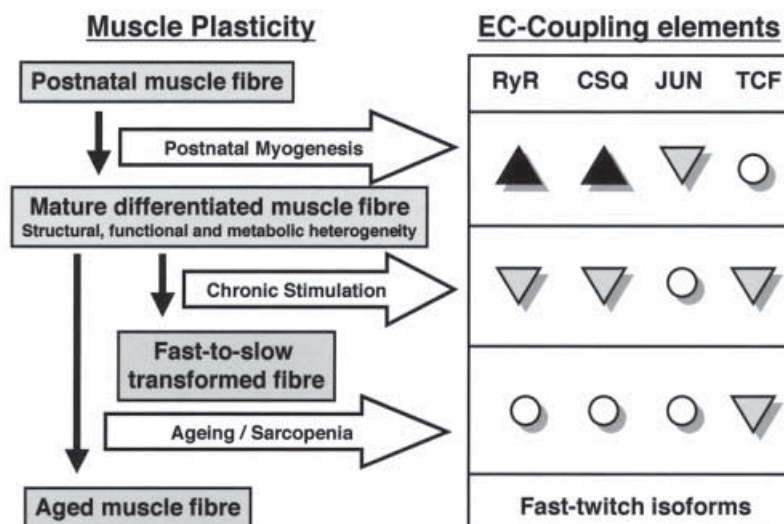
Even in the presence of a protease inhibitor cocktail, a certain degree of degradation occurs with large proteins, probably because of the high Ca^{2+} levels in muscle homogenates. In contrast to the other triad markers, the expression of JUN was greatly reduced during myogenesis (Fig. 4D). The changes in the relative density of the four SR elements studied were reflected by a modified CSQ-POD overlay pattern, which is especially striking for the reduced interactions between JUN and CSQ (Fig. 4B).

One of the key elements of excitation–contraction coupling, the voltage-sensing dihydropyridine receptor, is believed to play a key pathophysiological role in sarcopenia, the age-related functional decline of skeletal muscle [30]. It was therefore of interest to determine whether CSQ complex formation is modified during pathophysiological downstream events of muscle ageing. Silver staining of electrophoretically separated microsomal proteins from aged muscle showed an increase of the 110 kDa SR protein band (Fig. 5A), but otherwise exhibited no major changes in the protein band pattern. Although the immunoblot analysis of the RyR1, CSQ and JUN did not reveal drastic changes in their expression during ageing (Fig. 5C–E), the CSQ-POD overlay showed a tendency of reduced linkage of CSQ to TRI, JUN and the RyR1 in senescent fibers (Fig. 5B). Immunoblotting of TRI in both ageing and developing microsomes showed weak and broad labeling patterns (not shown), probably due to high-molecular-mass isoforms [27], and the analysis of this triad marker could thus not be further pursued.

DISCUSSION

CSQ of apparent 63 kDa and its isoforms of higher relative molecular mass play a central role in Ca^{2+} -cycling through the SR lumen [10]. The results of our CSQ overlay analysis of microsomal membrane proteins isolated from varying

Fig. 6. Calsequestrin complex formation during postnatal myogenesis, fiber transitions and ageing. Summarized are the findings of the comparative immunoblot and CSQ-POD overlay analysis presented in this study. A change in ryanodine receptor (RyR), calsequestrin (CSQ) and junctin (JUN) expression, and triad complex formation (TCF) is indicated by the following symbols: ▲, increase; ▼, decrease; ○, no major change. Listed are modifications of the CSQ-containing supra-molecular triad complex during postnatal myogenesis, stimulation-induced fiber transitions and the ageing process.



fiber types, developing muscle, transforming fibers and ageing muscle (as summarized in Fig. 6) agrees with the concept that this ion-buffering SR element exists in a supramolecular complex. Clusters of negatively charged residues in the carboxy-terminal region of CSQ represent Ca^{2+} -binding domains [8,26], whereby CSQ oligomerization is associated with positive co-operativity with respect to high capacity Ca^{2+} -binding [31]. CSQ aggregation and solubilization cycles seem to be intrinsically linked to the Ca^{2+} -uptake and -release mechanism of the skeletal muscle SR [32]. The results presented here suggest that protein-protein interactions between CSQ and the RyR, TRI, JUN and itself are important for regulating overall SR Ca^{2+} -handling. A similar complex has previously been described to exist in cardiac muscle fibers [33].

CSQ functions as the major Ca^{2+} -reservoir element of the SR lumen, but also acts as an endogenous regulator of the RyR Ca^{2+} -release units [11]. Many luminal proteins are retained in the SR by expressing the carboxy-terminal retrieval signal KDEL, but CSQ remains associated with the terminal cisternae region without this mechanism [34]. Interestingly, deletion of its carboxy-terminal domain, phosphorylation sites or post-translational glycosylation does not affect the proper targeting of CSQ [35–37]. Thus self-aggregation and tight anchoring to other SR elements, as demonstrated in this study by blot overlay analysis, possibly prevent a high degree of heterogeneous CSQ distribution and mechanisms other than the KDEL signal are responsible for continuous recycling from the Golgi complex [34].

That mature motor units retain a high capacity of plasticity and that the neuron-specific impulse pattern exerts a critical phenotypic influence on fibers are generally accepted concepts of modern muscle biology [38]. Adult skeletal muscle fibers are not static entities with inalterable contractile properties, but represent extremely versatile biological entities with a high capacity to transform into faster or slower twitching units. Terminally differentiated skeletal muscle fibers may undergo fast-to-slow transitions induced by changes in mechanical loading, neuromuscular activity or hormonal influence. Especially well established are changes in elements of the contractile apparatus such as

troponin isoforms, and myosin light and heavy chains [38]. However, the enormous functional, metabolic and structural diversity of muscle fibers is not only reflected on the molecular level by the diversity in myosin isoforms, but also encompasses many ion-regulatory proteins.

Because fiber type-specific isoform expression patterns exist for key Ca^{2+} -regulatory proteins [39], it is not surprising that changes in fiber type composition also influences the abundance and/or isoform expression of excitation-contraction coupling elements as demonstrated in this study. With respect to understanding the molecular changes associated with muscle transition, the finding that SR complex formation is drastically reduced after chronic low-frequency stimulation is extremely interesting. Compared to the CSQ-POD overlay pattern in soleus microsomes, the long-term electro-stimulated muscle preparations exhibited a much more pronounced decrease in coupling between CSQ and TRI. This agrees with the physiological concept that chronic electro-stimulation induces major adaptive responses of Ca^{2+} -handling proteins in muscle fibers undergoing phenotypic changes and suggests that transformed fibers might exhibit a more cardiac-like Ca^{2+} -induced Ca^{2+} -release mechanism [40].

Numerous muscle proteins proceed through isoform transitions during myogenesis. Ca^{2+} -regulatory membrane proteins are detectable relatively early in prenatal myogenesis [41]. Probably the same myogenic differentiation program that controls the up-regulation of contractile proteins [42] is also responsible for the initiation of the expression of voltage sensors, Ca^{2+} -reservoir elements, Ca^{2+} -release units and Ca^{2+} -uptake pumps in developing fibers [43]. During the first weeks after birth, the functional maturation of the elements regulating the excitation-contraction-relaxation cycle occurs whereby the transverse tubular dihydropyridine receptor complex and the SR RyR units show temporal differences in their developmental induction during myogenesis [44]. Our immunoblot analysis of developing fibers agrees with this concept and showed that the expression of fast isoforms of the Ca^{2+} -release and -reservoir complex clearly increase at later stages of postnatal myogenesis. Previous biochemical studies on potential changes in triad components during postnatal

myogenesis demonstrated increased expression of fast isoforms of CSQ, sarcalumenin, the Ca^{2+} -ATPase and the α_1 -dihydropyridine receptor and showed a greater tendency of Ca^{2+} -regulatory proteins to oligomerize in adult muscle fibers as compared to early postnatal stages [29]. Hence, during postnatal development protein–protein interactions within triad junctions become more complex and oligomerization appears to be an essential prerequisite for proper physiological functioning of key membrane proteins in matured skeletal muscle fibers.

Takekura *et al.* [45] suggest that the induction process for the molecular differentiation and structural organization of the triad junction can be divided into three main events. After membrane docking between the transverse tubular membrane system and the SR, the RyR Ca^{2+} -release units are incorporated into the junctions and membrane couplings are positioned at the I-A band interface, and the process is completed by the transverse orientation of dihydropyridine receptor-containing membrane domains [45]. The CSQ-POD overlay analysis presented in this study indicates that within 6 weeks of postnatal development the proper physical coupling within the supramolecular SR Ca^{2+} -release complex units has occurred. Especially interesting is the apparent lack of coupling between the CSQ-POD probe and slow CSQ after chronic electro-stimulation. Perhaps cardiac/slow CSQ does not form as tightly a terminal cisternae aggregate for Ca^{2+} -binding in the SR lumen as is apparently present in fast-twitching fibers.

With the advancement of age, skeletal muscle fibers undergo many structural and functional changes. Prominent biological features of cellular decline are abnormal metabolism, impaired bioenergetics and ion homeostasis, and a loss of muscle mass due to fiber atrophy [46]. Pathophysiological alterations in the capacity to maintain normal Ca^{2+} -homeostasis and the functional impairment of excitation–contraction coupling appear to be major factors triggering senescent muscle fiber weakness. Both, pharmacological binding studies and immunoblotting have clearly shown a drastic decline in the voltage-sensing α_1 -subunit of the DHPR complex [30,47]. Here, we can show that aged muscle fibers also exhibit a tendency towards reduced SR complex formation. Thus, uncoupling between the voltage sensor and Ca^{2+} -release channel units, in conjunction with altered turnover of key Ca^{2+} -regulatory SR membrane proteins [48] and reduced protein coupling, might play an important role in sarcopenia. Abnormal voltage-sensing leads to a drastic reduction of the amount of Ca^{2+} -ions available for initiating mechanical responses in aging fibers and therefore results in a reduced Ca^{2+} -peak transient [49]. As shown by our CSQ-POD overlay analysis of senescent fibers, changes in protein interactions between other SR Ca^{2+} -regulatory proteins might also be involved in triggering impaired triadic signal transduction resulting in a progressive functional decline of skeletal muscles.

In conclusion, the four central elements of the signal transduction mechanism at the junctional SR, the Ca^{2+} -binding protein CSQ, the RyR Ca^{2+} -release channel, the auxiliary triad element TRI and the CSQ-binding element JUN, show decreased protein–protein interactions during fiber type shifting and the ageing process. Reduced protein coupling between the major elements regulating Ca^{2+} -homeostasis in long-term stimulated tibialis anterior fibers is considerably more pronounced than in slow-twitch soleus

muscle. This supports the biochemical concept that the Ca^{2+} -mediated signal transduction process underlying excitation–contraction coupling is regulated by tight direct protein–protein interactions in fast fibers and via a more cardiac-like Ca^{2+} -induced Ca^{2+} -release mechanism in transformed fibers. Molecular interactions between triad components are probably both of structural and functional importance. This involves the initial formation of junctional couplings and the maintenance of peripheral triad structures by preventing passive disintegration of the Ca^{2+} -release complex. The major physiological function of the triad complex is in mediating signal transduction at the triad contact zones and regulating ion flux mechanism from the SR lumen to the cytosol. It is not known whether only one molecular hierarchy of successive protein coupling exists during triad assembly and re-organizing, and whether only two sets of factors act as positive and negative regulators of the junctional Ca^{2+} -release process. The results from the blot overlay study presented in this study suggest a molecular scenario of interdependence between the major excitation–contraction coupling elements from skeletal muscle. The initial triggering factor could be a change in cytosolic Ca^{2+} -levels. It has previously been established that enhanced neuronal stimulation leads to a higher free Ca^{2+} -concentration in slower contracting fibers and that a calcineurin-dependent transcriptional pathway controls fiber type-specific expression patterns [50]. Changes in the relative abundance of one particular triad marker, such as TRI, might then result in reduced stabilization of the interactions between the RyR1 isoform and auxiliary or regulatory elements. This in turn may cause the disintegration of a tight triad complex and introduce the establishment of a Ca^{2+} -induced Ca^{2+} -release mechanism lacking direct physical coupling between the major excitation–contraction coupling elements.

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