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Calsequestrin binds to monomeric and complexed forms of key calcium-handling proteins in native sarcoplasmic reticulum membranes from rabbit skeletal muscle

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Abstract

Ca^{2+}-handling proteins are important regulators of the excitation–contraction–relaxation cycle in skeletal muscle fibres. Although domain binding studies suggest protein coupling between various Ca^{2+}-regulatory elements of triad junctions, no direct biochemical evidence exists demonstrating high-molecular-mass complex formation in native microsomal membranes. Calsequestrin represents the protein backbone of the luminal Ca^{2+} reservoir and thereby occupies a central position in Ca^{2+} homeostasis; we therefore used calsequestrin blot overlay assays in order to determine complex formation between sarcoplasmic reticulum components. Peroxidase-conjugated calsequestrin clearly labelled four major protein bands in one-dimensional (1D) and 2D electrophoretically separated membrane preparations from adult skeletal muscle. Immunoblotting identified the calsequestrin-binding proteins of approximately 26, 63, 94 and 560 kDa as junctin, calsequestrin itself, triadin and the ryanodine receptor, respectively. Protein–protein coupling could be modified by ionic detergents, non-ionic detergents, changes in Ca^{2+} concentration, as well as antibody and purified calsequestrin binding. Importantly, complex formation as determined by blot overlay assays was confirmed by differential co-immunoprecipitation experiments and chemical crosslinking analysis. Hence, the key Ca^{2+}-regulatory membrane components of skeletal muscle form a supramolecular membrane assembly. The formation of this tightly associated junctional sarcoplasmic reticulum complex seems to underlie the physiological regulation of skeletal muscle contraction and relaxation, which supports the biochemical concept that Ca^{2+} homeostasis is regulated by direct protein–protein interactions.

Keywords: Calsequestrin; Ryanodine receptor; Triadin; Junctin; Sarcoplasmic reticulum

1. Introduction

In mature skeletal muscle fibres, contact zones between the transverse tubules and the terminal cisternae region of the sarcoplasmic reticulum (SR) play a key role in the regulation of excitation–contraction coupling [1]. The triadic signal transduction process

Abbreviations: CSQ, calsequestrin; DSP, dithiobis-succinimidyl-propionate; JN, junctin; POD, peroxidase; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; TRI, triadin

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mediates the coupling between surface membrane depolarization and Ca^{2+}-induced muscle fibre contraction [2]. Following sarcolemmal depolarization, direct physical interactions between the voltage-sensing α_1-subunit of the dihydropyridine receptor and the junctional ryanodine receptor are believed to initiate opening of the Ca^{2+}-release channel [3,4]. The prevention of passive disintegration of SR receptor complexes is proposed to be mediated by junctional proteins, such as triadin, JP-45 and JP-90. Triadin of apparent 94 kDa forms disulfide-bonded high-molecular-mass clusters under native conditions [5] and seems to provide physiological coupling between the luminal Ca^{2+}-binding complex and ryanodine receptor (RyR) units [6]. The exact role of the more recently identified junctional components JP-45 and JP-90 is not clear [7,8]. During muscle relaxation, the re-uptake of Ca^{2+} ions into the SR lumen is provided by an energy-dependent process mediated by Ca^{2+}-ATPases [9]. As a terminal cisternae Ca^{2+} reservoir, calsequestrin (CSQ) of apparent 63 kDa [10] mediates between uptake and release cycles during excitation–contraction coupling and muscle relaxation [11,12]. Besides CSQ, other luminal Ca^{2+}-binding proteins are represented by sarcalumenin, calreticulin and several CSQ-like proteins [13,14].

CSQ exists as a fast and slow isoform. In slow-twitching muscle fibres the cardiac/slow CSQ isoform accounts for only 25% of the total CSQ content in adult rabbit [15], we therefore focused on the major fast CSQ isoform in this study. Negatively charged residues are clustered in the carboxy-terminal region of CSQ representing the ion binding domains [16]. Interestingly, deletion of the carboxy-terminal domain or phosphorylation sites does not affect the segregation of CSQ to the junctional SR [17,18]. Upon ion binding, Ca^{2+}-induced conformational changes cause CSQ to fold into a more compact structure burying hydrophobic side chains [19,20]. Recently, the crystal structural analysis of CSQ revealed that this Ca^{2+}-binding protein contains three very negatively charged thioredoxin folds that condense to form an acidic platform for the clustering of Ca^{2+} ions [21]. CSQ oligomers show positive co-operativity with respect to high-capacity Ca^{2+} binding [22], suggesting that protein–protein interactions are an important integral part of regulating the overall SR Ca^{2+} concentration [23]. The luminal Ca^{2+} concentration influences the probability of the Ca^{2+} release channel opening which in turn dictates the overall Ca^{2+} flux rates from the SR lumen [24], hence CSQ might directly act as an endogenous regulator of the RyR complex. Although CSQ is a relatively low-affinity Ca^{2+}-binding protein with a dissociation constant of approximately 1 mM, its sequestration capability of 40–50 Ca^{2+} ions per protein monomer puts it into the class of high-capacity ion-binding proteins [11,12]. A junctional 26 kDa SR protein was identified as a CSQ-binding protein. This protein named junctin is postulated to have adhesive properties within the CSQ-mediated Ca^{2+} reservoir of the terminal cisternae region [25].To establish the quaternary structure of Ca^{2+}-regulatory SR complexes [26], previous domain binding studies have mainly used recombinant fragments and peptides [6,27–30]. In contrast, here we have employed native membrane vesicles in order to determine direct protein–protein interactions between key Ca^{2+}-handling proteins. The combination of CSQ blot overlay assays under varying conditions with comparative immunoblotting, differential co-immunoprecipitation and chemical crosslinking analysis clearly establishes the tight association between junctin and CSQ and suggests that CSQ forms self-aggregates. In addition, CSQ also appears to be closely associated with the Ca^{2+}-release channel complex and its auxiliary junctional components, which agrees with the idea that CSQ is an endogenous triadic regulator of the RyR [31]. This study shows for the first time that CSQ binds to the full range of expected target receptors and thereby fully supports the physiological concept of protein coupling during Ca^{2+} sequestration and the signal transduction process underlying excitation–contraction coupling in skeletal muscle fibres.

2. Materials and methods

2.1. Materials

Conjugated secondary antibodies, protease inhibitors and acrylamide stock solutions were purchased from Boehringer Mannheim (Lewes, East Sussex, UK). Primary antibodies were obtained from Affinity Bioreagents (Golden, CO, USA), Upstate Biotechnology (Lake Placid, NY, USA), Novocastra Labo-

ratories (Newcastle upon Tyne, UK) and Sigma (Poole, Dorset, UK). Immobilon-P nitrocellulose membranes were from Millipore Corp. (Bedford, MA, USA). Chemiluminescence substrates, EZ-Link-Plus activated peroxidase kits, Slide-A-Lyzer dialysis cassettes and chemical crosslinkers were obtained from Perbio Science UK (Tattenhall, Cheshire). All other chemicals used for protein solubilization, subcellular fractionation procedures, protein purification, immunoprecipitation and electrophoretic separation were of analytical grade and purchased from Sigma.

2.2. Isolation of membrane vesicles

Muscle tissues from adult male New Zealand white rabbits were obtained from the Biomedical Facility, National University of Ireland, Dublin. Crude microsomal membrane vesicles were isolated from rabbit skeletal muscle homogenates by an established protocol [32] 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) [33]. Subcellular fractions enriched in longitudinal SR tubules, free transverse tubules and triad junctions were prepared as previously described in detail [34]. Using bovine serum albumin as a standard, the protein concentration of isolated membrane vesicles was determined by the method of Bradford [35]. Following isolation, membrane vesicles were immediately used for electrophoretic separation, immunoprecipitation experiments or chemical crosslinking analysis.

2.3. Protein conjugation and blot overlay assay

CSQ was purified to homogeneity by phenyl–Sepharose chromatography from the alkaline-extracted supernatant of adult rabbit skeletal muscle microsomes [19]. To visualize CSQ interactions in blot overlay assays, the purified protein was conjugated to the amine reactive peroxidase (POD) marker enzyme as described in the manufacturer's instructions of the Pierce EZ-Link-Plus activated peroxidase kit (Perbio Science UK, Tattenhall, Cheshire). Contaminates were removed from the CSQ–POD conjugate by a Slide-A-Lyzer dialysis cassette system (Perbio Science UK). Protein purification and POD conjugation was evaluated by Coomassie blue staining and immunoblotting of electrophoretically separated proteins (see Fig. 1). Following the establishment of the most suitable dilution of the CSQ–POD complex, nitrocellulose replicas of protein gels were blocked for 2 h and then incubated for 1 h at room temperature. The blocking and incubation buffer consisted of 0.05% Tween 20, 0.15 M NaCl and 50 mM Tris–Cl, pH 7.5. Visualization of decorated protein bands was accomplished by enhanced chemiluminescence [36]. Densitometric scanning of developed overlay blots was performed on a Molecular Dynamics 300S computing densitometer (Sunnyvale, CA, USA) with Image Quant V3.0 software. For protein band identification, N-terminal sequencing was carried out by Alta Bioscience, University of Birmingham (Edgbaston, UK) and sequence similarities determined with the BLAST-P program.

2.4. Gel electrophoresis

Standard one-dimensional (1D) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to Laemmli [37]. Non-reducing and denaturing buffer consisted of 125 mM Tris–Cl (pH 6.8), 20% (v/v) glycerol, 20% (w/v) sucrose, 0.002% (w/v) bromophenol blue and 3% (w/v) SDS. Reducing sample buffer was complemented with 75 mM dithiothreitol. Resolving gels with a 7% (w/v) polyacrylamide concentration or a 3–12% (w/v) gradient were used in combination with a 5% (w/v) and a 3% (w/v) stacking gel system, respectively. For the 1D separation of microsomes, light SR, free transverse tubules and triads, 20 μg of membrane protein was electrophoresed for 200 Vh employing a Mini-Protean II electrophoresis system from Bio-Rad Laboratories (Hemel Hempstead, Herts, UK). For the diagonal non-reducing/reducing 2D separation of membrane complexes, 20 μg of protein was separated in the first dimension using non-reducing conditions, followed by the incubation of these gels for 30 min with SDS-containing sample buffer. First-dimension gels were then mounted on top of 7% (w/v) 2D SDS–PAGE gels, sealed with 1% (w/v) agarose solution and electrophoresed for 200 Vh under reducing conditions. Extracted rat muscle myofibrils served as a source of very high-molecular-mass protein standards, whereby the highest molecular mass

band was represented by the muscle protein titin of apparent 2800 kDa [33].

2.5. Immunoblotting

Following 1D or 2D separation, muscle proteins were electrophoretically transferred for 1 h at 100 V onto nitrocellulose membranes by the method of Towbin et al. [38] using a Mini-Protean II blotting system from Bio-Rad Laboratories. Nitrocellulose sheets were blocked and incubated with 1:1000 diluted primary and secondary antibodies by standard methodology [33,34]. A polyclonal antibody to junctin was produced against a peptide representing amino acids 195–210 (SKHTHSAKGNNQKRKN-COOH) of canine junctin [25]. The peptide was synthesized with cysteine added at the N-terminus and coupled to thyroglobulin using *m*-maleimido-benzoyl-sulfosuccinimide [39]. Peptide antiserum was produced in rabbits. Unfractionated antiserum was used for immunoblotting experiments. Affinity-purified antibodies used for immunoprecipitation experiments were prepared by fractionating on peptide coupled to ω-aminobutyl agarose [40]. All other primary antibodies were obtained from commercial sources: monoclonal antibody (mAb) VIIID1_2 to calsequestrin, mAb IIG12 to triadin, mAb IIH11 to the fast SERCA1 isoform of the SR Ca^{2+}-ATPase and mAb 20A to the α_2-subunit of the dihydropyriine receptor from Affinity Bioreagents; mAb 34C to the ryanodine receptor and mAb MY32 to the fast myosin heavy chain (Sigma); mAb NCL-43 to β-dystroglycan from Novocastra Laboratories; and a pAb against the ryanodine receptor from Upstate Biotechnology. Following antibody incubation, nitrocellulose sheets were washed several times and then immunodecorated protein bands visualized by enhanced chemiluminescence [36]. Densitometric scanning of developed immunoblots was performed on a Molecular Dynamics 300S computing densitometer with Image Quant V3.0 software.

2.6. Immunoprecipitation

Membrane proteins were solubilized by incubating microsomes (10 mg protein/ml) in 50 mM Tris–Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM PMSF containing 2% (v/v) Tween 20 detergent for 30 min on ice with regular agitation. Suitability of solubilizing detergents was evaluated by immunoblotting. Following an initial pre-incubation step with unconjugated Protein-A Sepharose (Sigma), the supernatant fraction with CSQ-containing complexes was incubated for 2 h at 4°C with antibody-immobilized matrixes on an end-over-end mixture in the above buffer with 0.1% (v/v) Tween 20 using established methodology [41]. Antibody–antigen complexes were then removed by Protein-A binding [41]. Differential co-immunoprecipitation experiments were performed with antibodies against calsequestrin, the ryanodine receptor, triadin and junctin. The matrix was then separated from the supernatant by centrifugation, washed three times with the above buffer and proteins eluted by boiling for 5 min in SDS-containing sample buffer [37]. The composition of immunoprecipitated complexes was immediately analysed by gel electrophoretic separation and immunoblotting. For control experiments, non-specific IgG was purchased from Sigma and used as a non-specific agent in immunoprecipitation studies. In addition, to test the specificity of the immunoprecipitation procedure, complexes precipitated with antibodies to calsequestrin were also tested for the presence of the abundant muscle marker proteins Ca^{2+}-ATPase, α_2-dihydropyridine receptor and β-dystroglycan.

2.7. Chemical crosslinking analysis

Membranes were incubated with chemical crosslinker probes in order to stabilize native SR complex formations containing CSQ. Freshly prepared vesicles were incubated with the hydrophobic 1.2 nm crosslinker dithiobis-succinimidyl-propionate (DSP). Using previously established crosslinking protocols [42], microsomes were treated at room temperature in 50 mM HEPES (pH 8.0), 0.9% (w/v) NaCl for 30 min with 10–200 μg crosslinker per mg membrane protein. Termination of the crosslinking reaction was achieved by the addition of 50 μl of 1 M ammonium acetate per ml reaction medium. The crosslinker-stabilized complex was then solubilized in 4% (w/v) SDS-containing buffer lacking reducing agents [37] and electrophoretically separated in order to compare DSP-induced shifts in the relative electrophoretic mobility of SR components.

3. Results

Since CSQ is considered to play a central role in mediating between excitation–contraction coupling and muscle relaxation within the Ca^{2+}-regulatory apparatus of the internal membrane systems of skeletal muscle fibres, we have determined complex formation between this Ca^{2+}-binding protein and other SR components using native membrane vesicles. CSQ was purified to homogeneity and conjugated to the marker enzyme POD (Fig. 1) and then used in 1D and 2D blot overlay assays under varying biochemical conditions (Figs. 2–4). For comparative purposes, immunoprecipitation experiments (Figs. 5 and 6) and chemical crosslinking analyses (Fig. 7) were performed.

3.1. Purification and conjugation of skeletal muscle CSQ

Using the established isolation procedure of Cala and Jones [19], the terminal cisternae protein CSQ was purified to homogeneity. In the absence of Ca^{2+} ions, CSQ bound with high affinity to the hydrophobic matrix of a phenyl–Sepharose column and could be specifically eluted with 10 mM $CaCl_2$, as evidenced by Coomassie blue staining and immunoblotting of the purified protein (Fig. 1). Following incubation with the marker enzyme POD, the relative molecular mass of the CSQ-containing complex shifted to a position of lower electrophoretic mobility (Fig. 1) demonstrating successful protein conjugation.

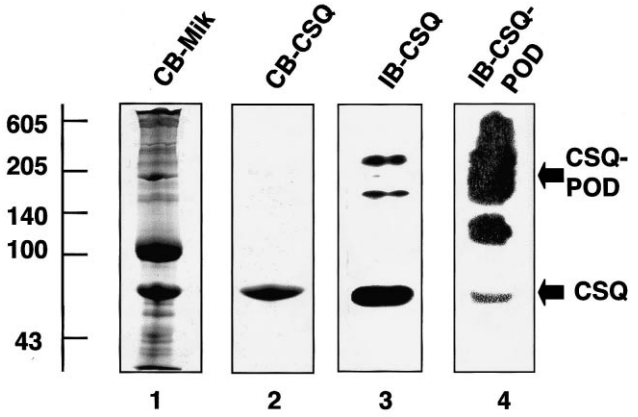

Fig. 1. Purification and conjugation of rabbit skeletal muscle calsequestrin. Shown are Coomassie blue (CB) stained gels of microsomes (Mic) (lane 1) and purified calsequestrin (CSQ) (lane 2) from rabbit skeletal muscle, as well as immunoblots (IB) of purified calsequestrin (CSQ) (lane 3) and peroxidase-conjugated calsequestrin (CSQ–POD) (lane 4), labelled with mAb VIIID1$_2$ against the fast-twitch isoform of calsequestrin. The position of immunodecorated protein bands is marked by arrows. Sizes of molecular mass standards (in kDa) are indicated on the left.

3.2. CSQ blot overlay assay using 1D electrophoresis

In order to directly visualize potential interactions between CSQ and other microsomal proteins, the CSQ–POD conjugate was employed in blot overlay assays. Native membrane preparations enriched in the SR were electrophoretically separated, transferred to nitrocellulose sheets and then incubated under varying conditions with CSQ aggregates. Under both reducing and non-reducing conditions, the isolated terminal cisternae component formed stable interactions with several protein bands (Fig. 2a). Comparative immunoblotting identified these sarcoplasmic reticulum components as the RyR Ca^{2+}-release channel of apparent 565 kDa, triadin monomers of 94 kDa, CSQ of approximately 63 kDa and junctin of apparent 26 kDa (Fig. 2b). Under non-reducing conditions, high-molecular-mass bands representing disulfide-bonded triadin oligomers and CSQ-like proteins are probably included in the broadly labelled band between 100 and 600 kDa (Fig. 2a). Thus, the major Ca^{2+}-reservoir protein CSQ and its binding-protein junctin form a relatively stable linkage with the Ca^{2+}-release channel complex and the auxiliary junctional component triadin in native muscle membranes. Control immunoblotting with antibodies to relatively abundant microsomal proteins, such as the Ca^{2+}-ATPase SERCA1 isoform or the α_2-subunit of the dihydropyridine receptor, did not reveal a significant overlap with the protein bands labelled by the CSQ–POD conjugate (not shown). This clearly demonstrated the specificity of the CSQ overlay procedure employed.

3.3. CSQ blot overlay assay using 2D electrophoresis

Diagonal non-reducing/reducing 2D gel electrophoresis techniques are generally accepted as having

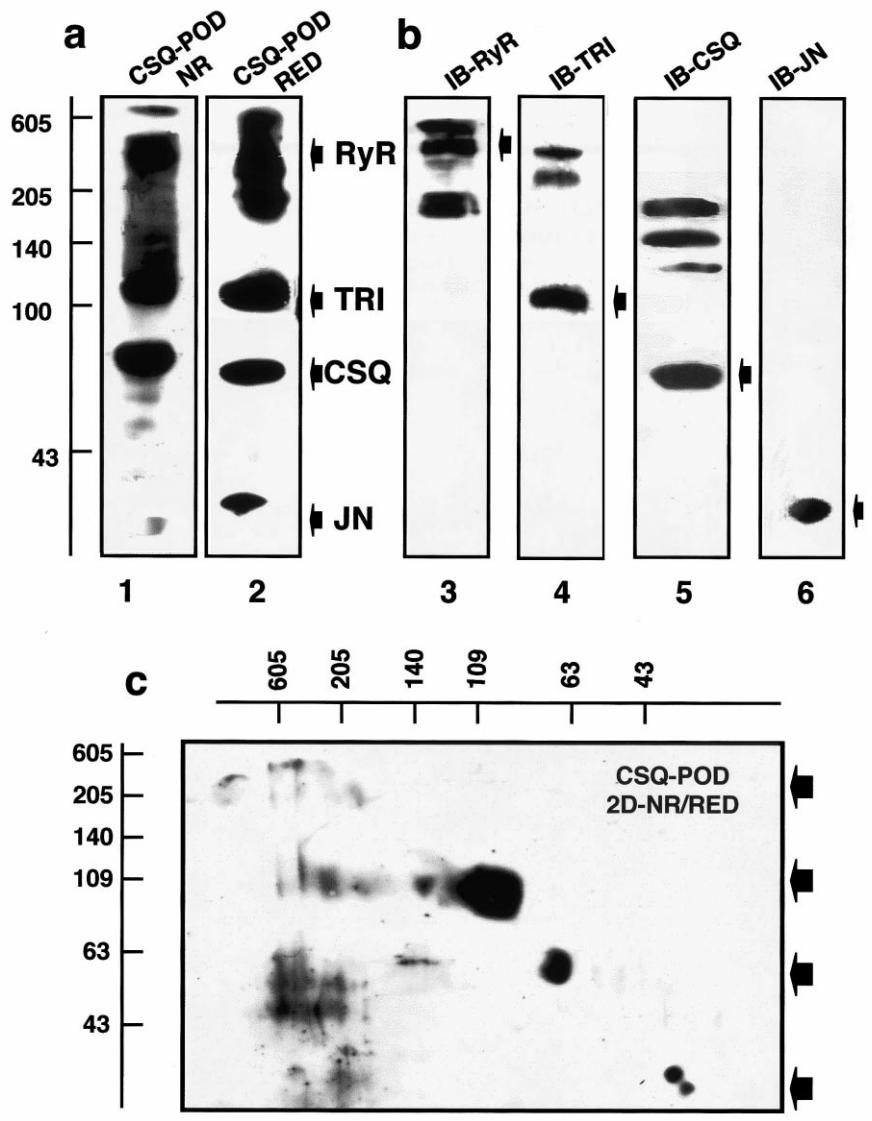

Fig. 2. Calsequestrin blot overlay of sarcoplasmic reticulum using one- and two-dimensional gel electrophoresis. Shown are nitrocellulose replicas of electrophoretically separated sarcoplasmic reticulum preparations using non-reducing (NR) (lane 1) or reducing (RED) (lane 2) conditions, labelled with calsequestrin–peroxidase conjugates (CSQ–POD) (a). Lanes 3–6 represent identical immunoblots (IB) of the sarcoplasmic reticulum labelled with antibodies to the ryanodine receptor (RyR), triadin (TRI), calsequestrin (CSQ) and junctin (JN), respectively (b). Gels in lanes 3–6 have been run under reducing conditions. The lower molecular mass band in lane 3 represents a degradation product of the Ca^{2+}-release channel, while the higher molecular mass bands in lanes 4 and 5 are disulfide-bonded oligomers of triadin and CSQ-like proteins, respectively. (c) A nitrocellulose replica of an electrophoretically separated sarcoplasmic reticulum preparation using non-reducing conditions in the first dimension and reducing conditions in the second dimension (2D-NR/RED), labelled with a calsequestrin–peroxidase conjugate (CSQ–POD). The relative positions of the ryanodine receptor (RyR), triadin (TRI), calsequestrin (CSQ) and junctin (JN) are marked by arrows. The position of protein bands marked by the overlay assay or immunodecoration is indicated by arrows. Sizes of molecular mass standards (in kDa) are shown on the left.

a higher resolution power as compared to standard 1D gels. This method makes more distinctions in the separation of proteins exhibiting a different relative electrophoretic mobility under reducing conditions as compared to non-reducing conditions. In order to exploit these technical advantages in our analysis of protein–protein interactions between key ion-regulatory proteins of the SR, we employed this technique in order to determine whether CSQ-containing complexes are partially preserved following membrane

homogenization, subcellular fractionation and electrophoretic separation. As illustrated in Fig. 2c, the three lower bands recognized by the CSQ overlay technique showed a characteristic shift off the diagonal. Although relatively diffuse, the shifted bands were positioned below the high-molecular-mass band containing the RyR.

3.4. CSQ overlay of subcellular fractions

The specificity of protein interactions was further illustrated by blot overlays using different subcellular fractions from skeletal muscle fibres. Labelling was performed with non-reduced membrane fractions enriched in the longitudinal tubules of the SR, triad couplings formed between the junctional SR and transverse tubules, and free transverse tubular membranes. As shown above for heavy SR vesicles (Fig. 2), triad membranes also exhibited staining of four main regions at the relative positions corresponding to the RyR Ca^{2+}-release channel, triadin, CSQ and junctin (Fig. 3a). The protein band of approximately 150 kDa in triads is presumably a degradation product of the RyR since it is not seen in the other fractions. Overlay of electrophoretically separated membranes enriched in longitudinal tubules and transverse tubules with the CSQ–POD conjugate revealed strong interactions only with the triadin and CSQ protein bands (Fig. 3a). Both components can be considered contaminating proteins in these vesicular fractions. Since triadin exists under non-reducing conditions as a broad mixture of oligomeric complexes [5], fainter banding at higher molecular mass probably represents disulfide-bonded structures of triadin in all three subcellular preparations. It is surprising that this pattern is more pronounced in the non-triad fractions, but possibly the higher density of very large proteins in the junctional couplings partially suppresses the proper labelling of triadin aggregates. N-terminal sequencing identified the upper

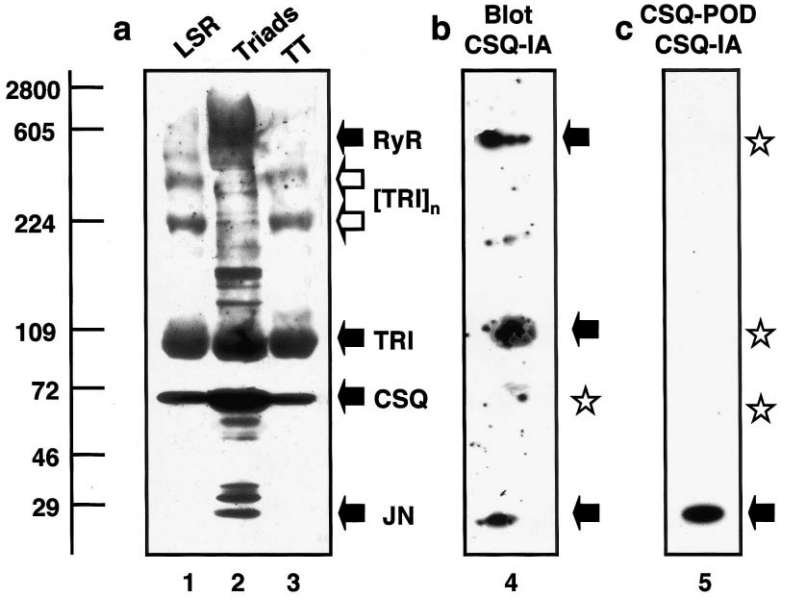

Fig. 3. Calsequestrin blot overlay of subcellular fractions from mature rabbit skeletal muscle. Shown are nitrocellulose replicas of electrophoretically separated membrane preparations using non-reducing conditions, labelled with calsequestrin–peroxidase conjugates (CSQ–POD). Lanes 1–3 represent subcellular fractions enriched in longitudinal tubules of the sarcoplasmic reticulum (LSR), triads and free transverse tubules (TT), respectively. The relative position of the ryanodine receptor (RyR), triadin (TRI), calsequestrin (CSQ) and junctin (JN), is marked by closed arrows. High-molecular-mass forms of triadin are indicated by open arrows. Lanes 4 and 5 show control immunoadsorption (IA) experiments using triad membrane preparations. In b, nitrocellulose replicas were pre-incubated mAb VIIID1_2 against the fast-twitch isoform of calsequestrin prior to the overlay assay (Blot CSQ–IA). In c, the calsequestrin–peroxidase conjugate was pre-incubated with the antibody to calsequestrin prior to the overlay assay (CSQ–POD CSQ–IA). The position of protein bands marked by the overlay assay are indicated by arrows. The relative position of protein bands not labelled due to immunoadsorption are marked by star signs. Sizes of molecular mass standards (in kDa) are shown on the left.

band of the two lowest protein bands labelled in triads as a proteolytic product of CSQ. The identified sequence EEGLDFPETD showed a 90% identity with the published sequence of rabbit skeletal muscle CSQ [16]. Thus the band of approximately 26 kDa represents junctin and the band of apparent 32 kDa is a fragment of the Ca^{2+}-binding protein (Fig. 3a).

3.5. Inhibition of CSQ interactions by antibodies

Incubation of microsomal blots with antibodies to CSQ specifically eliminated the labelling of the 63 kDa protein band, which represents CSQ monomers, but did not alter the staining pattern of the other three SR components (Fig. 3b). On the other hand, pre-treatment of CSQ–POD conjugates with monoclonal antibody VIIID1$_2$ to CSQ eliminated labelling of the three high-molecular-mass protein bands and only showed staining of the 26 kDa junctin protein (Fig. 3c). Consequently, antibody binding to CSQ–POD conjugates probably caused enough steric hindrance to eliminate proper interactions between the Ca^{2+}-binding protein and the RyR/triadin complex. However, the coupling between CSQ and its binding-protein junctin did not seem to be affected under these experimental conditions.

3.6. Specificity of CSQ overlay assays

To investigate the significance of the labelling pattern, blot overlay assays were performed in the presence of increasing concentrations of ionic or non-ionic detergent, Ca^{2+} ions and purified CSQ. Low concentrations of ionic detergent disrupted the interactions between CSQ conjugates and junctin, and 1% sodium dodecyl sulfate completely abolished all protein–protein interactions (Fig. 4a). While 0.1% Triton X-100 affected the labelling of the RyR and junctin, even at a 1% concentration of this non-ionic detergent the triadin band of approximately 94 kDa was still labelled (Fig. 4b). At 1 mM $CaCl_2$, interactions between junctin and CSQ were severely affected and at higher Ca^{2+} concentrations only the triadin band was labelled in CSQ overlay assays (Fig. 4c). In the presence of unconjugated CSQ, labelling of the RyR and CSQ was eliminated (Fig. 4d). However, triadin coupling to CSQ–POD conjugates was comparable to staining levels in control samples and the labelling intensity of the triadin band of apparent 94 kDa was slightly increased (Fig. 4d).

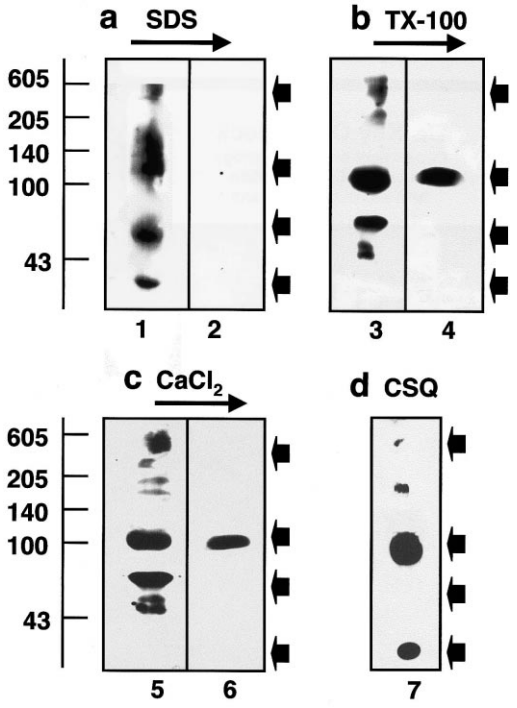

Fig. 4. Calsequestrin blot overlay of sarcoplasmic reticulum under various biochemical conditions. Shown are nitrocellulose replicas of electrophoretically separated sarcoplasmic reticulum preparations using reducing conditions, labelled with calsequestrin–peroxidase conjugates. Blot overlay assays were performed in the presence of: (a) 0.1% of the ionic detergent sodium dodecyl sulfate (SDS) (lane 1), 1% SDS (lane 2); (b) 0.1% of the non-ionic detergent Triton X-100 (TX-100) (lane 3), 1% TX-100 (lane 4); (c) 1 mM $CaCl_2$ (lane 5), 100 mM $CaCl_2$ (lane 6); and (d) 1 μg/ml purified calsequestrin (CSQ) (lane 7). The relative position of the ryanodine receptor (RyR), triadin (TRI), calsequestrin (CSQ) and junctin (JN) is marked by arrows. Sizes of molecular mass standards (in kDa) are shown on the left.

3.7. Immunoprecipitation of heterogeneous CSQ complexes

In order to confirm the biochemical findings determined by CSQ blot overlay, co-immunoprecipitation and chemical crosslinking was performed. Analytical results from both established procedures agreed with the filter overlay findings. Prior to our immunoprecipitation analysis, we determined the suitability of detergents to solubilize the muscle membrane proteins of interest. The successful solubilization using

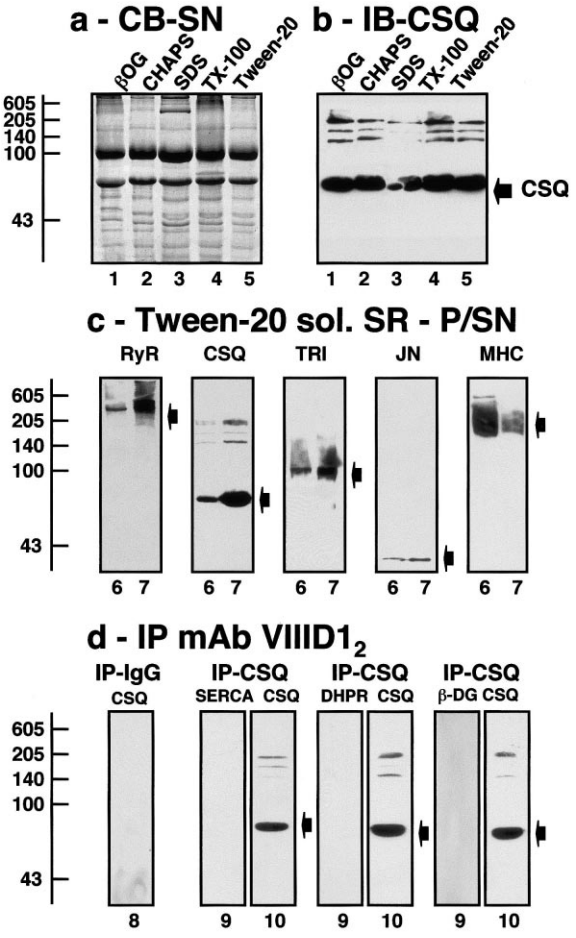

Fig. 5. Detergent solubilization and immunoprecipitation of calsequestrin from the sarcoplasmic reticulum of rabbit skeletal muscle. Shown is a Coomassie-stained gel (a) and identical anti-CSQ immunoblot (b) of the detergent-solubilized supernatant membrane fraction. Lanes 1–5 represent solubilization with 2% β-octylglucoside, CHAPS, SDS, Triton X-100 and Tween 20, respectively. Following solubilization (sol.) with Tween 20, the pellet (P) (lane 6) and supernatant (SN) (lane 7) fraction was immunoblotted with antibodies to the ryanodine receptor (RyR), calsequestrin (CSQ), triadin (TRI), junctin (JN), and the fast myosin heavy chain (MHC). (d) A control immunoblot of CSQ following immunoprecipitation with non-specific IgG (lane 8). Lanes 9 and 10 represent fractions immunoprecipitated with mAb VIIID1$_2$ to CSQ and then immunoblotted with antibodies to abundant membrane markers, such as the fast SR Ca^{2+}-ATPase (SERCA), the transverse tubular α_2-dihydropyridine receptor (DHPR) and the surface β-dystroglycan (β-DG), or calsequestrin (CSQ), respectively. The relative position of immunodecorated bands is marked by arrows. Sizes of molecular mass standards (in kDa) are indicated on the left.

various standard detergents (β-octylglucoside, CHAPS, SDS, Triton X-100, Tween 20) is shown by the Coomassie-stained gel and CSQ immunoblot analysis of the microsomal supernatant (Fig. 5a,b). Fig. 5c documents the more detailed immunoblot analysis of the pellet and supernatant fraction following treatment of microsomes with 2% (v/v) Tween 20-containing buffer. This detergent was used in this study for the initial solubilization prior to immunoprecipitation. The results of this analysis revealed that the RyR, CSQ, triadin and junctin are all solubilized by the described detergent. The majority of these four triad proteins was found in the supernatant fraction, while the mostly insoluble protein myosin heavy chain remained in the pellet-associated fraction. In Fig. 5d we show important control experiments demonstrating the specificity of the immunoprecipitation analysis performed with monoclonal antibody VIIID1$_2$. Non-specific IgG was shown not to immunoprecipitate CSQ. In addition, matrix-immobilized antibodies to CSQ did not precipitate abundant muscle marker proteins of the longitudinal SR tubules (SERCA), transverse tubules (α_2-dihydropyridine receptor) and the surface (β-dystroglycan). However, the monoclonal antibody to CSQ used in this study reproducibly precipitated the 63 kDa band representing CSQ.

Immobilized monoclonal antibody VIIID1$_2$ against CSQ precipitated besides CSQ also the RyR, triadin and junctin, as detected by immunoblotting (Fig. 6a). Thus the four main protein species recognized by CSQ–POD overlays form a stable enough complex to be precipitated by an affinity matrix specific only for CSQ. More importantly, differential co-immunoprecipitation experiments also showed that immobilized antibodies to the RyR, triadin and junctin also precipitated CSQ and the other three SR proteins (Fig. 6b–d). Besides clearly detecting the main CSQ band of apparent 63 kDa, CSQ-like proteins were also observed in the immunoprecipitated fractions using antibodies to the RyR, triadin and junctin (Fig. 6b–d). These results agree with the idea that SR Ca^{2+}-handling proteins form a relatively tightly associated membrane complex.

3.8. Chemical crosslinking of SR complexes

Chemical crosslinking with a 1.2 nm probe showed

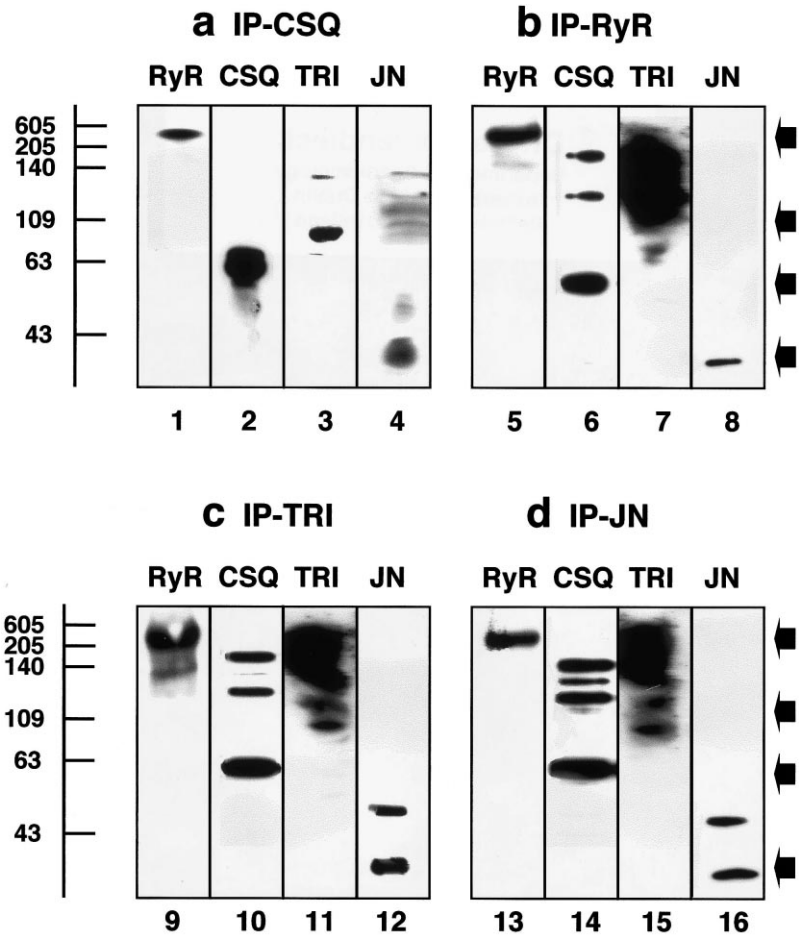

Fig. 6. Differential co-immunoprecipitation of calsequestrin-containing complexes in the sarcoplasmic reticulum from rabbit skeletal muscle. Shown are immunoblots of membrane fractions immunoprecipitated (IP) with antibodies to calsequestrin (CSQ) (a), the ryanodine receptor (RyR) (b), triadin (TRI) (c) and junctin (JN) (d). Lanes 1, 5, 9 and 13 were labeled with antibodies to the RyR, lanes 2, 6, 10 and 14 with an antibody to CSQ, lanes 3, 7, 11 and 15 with an antibody to TRI, and lanes 4, 8, 12 and 16 with an antibody to JN. The relative position of the four main immunodecorated bands representing the RyR, TRI, CSQ and JN is marked by arrows. Sizes of molecular mass standards (in kDa) are indicated on the left.

distinct shifts of CSQ and the RyR to positions of drastically reduced electrophoretic mobility (Fig. 7a,b). Since the high-molecular-mass bands representing both protein species exhibited a potential overlap, this also indicates that the major elements of the SR form a tightly associated membrane assembly. Immunoblotting with antibodies to triadin and junctin following chemical crosslinking did not result in specific enough labelling for proper identification of distinct band shifts (not shown). However, incubation of crosslinked membranes with the CSQ–POD conjugate clearly demonstrated a shift to high-molecular-mass complexes including triadin and junctin (Fig. 7c). Increasing concentrations of crosslinker caused a decrease in the labelling of the 26 kDa junctin and the 94 kDa triadin band, while the band of lowest electrophoretic mobility clearly intensified in labelling. Crosslinking-induced conformational changes probably weakened the interactions between the CSQ–POD conjugate and the electrophoresed CSQ band. The increased band intensity at 63 kDa at the highest crosslinker concentration used might represent sub-complexes between CSQ and its binding-protein junctin (Fig. 7c).

4. Discussion

An extremely steep concentration gradient exists for Ca^{2+} ions between the SR lumen and the cytosol

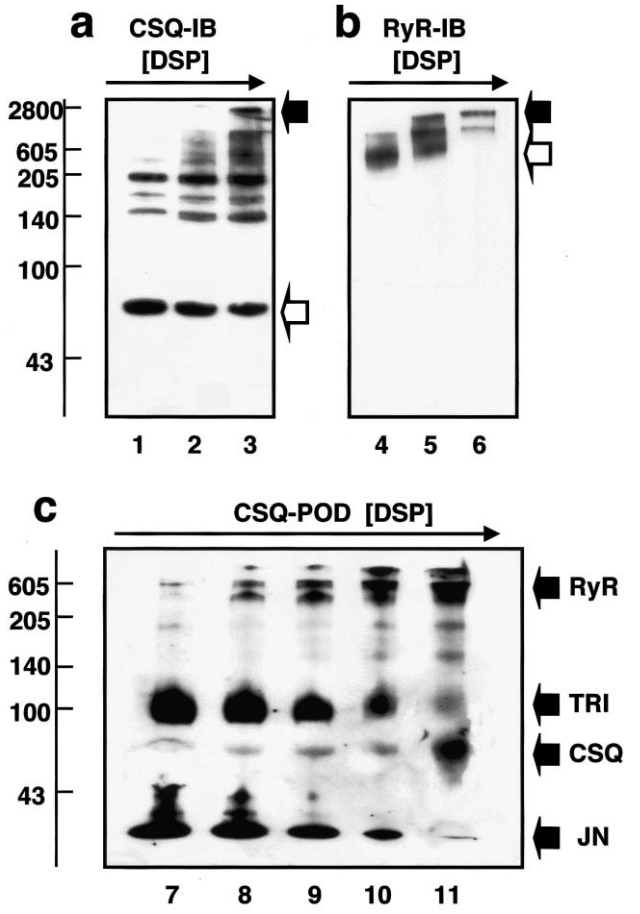

Fig. 7. Chemical crosslinking analysis of calsequestrin-containing complexes in the sarcoplasmic reticulum from rabbit skeletal muscle. (a,b) Nitrocellulose replicas of an electrophoretically separated sarcoplasmic reticulum preparation using non-reducing conditions following crosslinking with 0 (lanes 1, 4), 50 (lanes 2, 5), and 100 (lanes 3, 6) μg dithiobis-succinimidyl-propionate (DSP) per mg protein, respectively. Blots were immunodecorated (IB) with antibodies to calsequestrin (CSQ) (a) and the ryanodine receptor (RyR) (b). The relative position of monomers and crosslinker-stabilized complexes are marked by open and closed arrows, respectively. (c) A nitrocellulose replica of electrophoretically separated sarcoplasmic reticulum preparation using non-reducing conditions following chemical crosslinking with 0 (lane 7), 10 (lane 8), 50 (lane 9), 100 (lane 10), and 200 (lane 11) μg dithiobis-succinimidyl-propionate (DSP) per mg protein, respectively, labelled with a calsequestrin–peroxidase conjugate (CSQ–POD). The relative position of the ryanodine receptor (RyR), triadin (TRI), calsequestrin (CSQ) and junctin (JN) is marked by arrows. Sizes of molecular mass standards (in kDa) are indicated on the left.

in skeletal muscle fibres [2]. In order to partially relieve the ion-pumping activity of the Ca^{2+}-ATPase units in the longitudinal tubules, a large percentage of luminal Ca^{2+} is bound to CSQ aggregates within the terminal cisternae [11,12]. However, CSQ does not only represent a Ca^{2+}-buffering system [43], but can be considered an endogenous regulator of the junctional RyR whose transient openings cause a rapid flux of Ca^{2+} ions into the cytosol [31]. Consequently Ca^{2+} cycling via terminal cisternae CSQ units plays a central role in the regulation of the excitation–contraction–relaxation cycle [1,2]. Although the key elements of Ca^{2+} handling have been characterized and protein coupling is thought to play an important role in co-operative kinetics, protection against proteolytic degradation and ion flux regulation [26], no direct biochemical evidence exists showing that interactions exist between ion-regulatory components. In contrast to previous reports on domain binding studies with recombinant proteins [6,27–30], this investigation employed purified proteins and native membrane structures. Here we show that CSQ, the RyR and the junctional proteins triadin and junctin form high-molecular-mass complexes. Thus the formation of a tightly associated junctional SR complex appears to underlie the physiological regulation of contraction and relaxation, which supports the cell biological concept that Ca^{2+} homeostasis is provided by supramolecular complexes in skeletal muscle.

Using CSQ overlay assays under various biochemical conditions, co-immunoprecipitation techniques and chemical crosslinking analysis, we present evidence that the major Ca^{2+}-reservoir component of the terminal cisternae region forms direct protein–protein linkages with the Ca^{2+}-release channel units and its auxiliary proteins. The lack of labelling of the RyR and junctin in the light SR and free transverse tubules fraction by the CSQ–POD conjugate agrees with previous studies, which have shown that these two proteins are highly enriched in the junctional SR region [5,44]. In addition, the finding that the overlay assay displayed a stronger CSQ band of apparent 63 kDa in the triad fraction as compared to longitudinal or transverse tubules is also supported by reports on the localization of this Ca^{2+}-binding protein in the junctional terminal cisternae [45]. The overlay data from the diagonal non-reducing/reducing 2D gel electrophoretic analysis illustrate within one filter assay both CSQ binding to immobilized monomers, as well as interactions between purified CSQ and preserved

SR protein aggregates. These findings suggest that a sub-population of the SR complex appears to exhibit a relatively tight association, but that the majority of the non-covalent bonds are broken between the four ion-regulatory components during the isolation procedure.

The results from the blot overlay assays in the presence of detergents or Ca^{2+} ions indicate that the coupling between CSQ and the junctional component triadin are relatively strong and not easily disrupted by changes in the ionic or lipid environment. Interactions between CSQ and the other SR components appear to be more sensitive. The luminal domains of triadin and junctin contain 'KEKE'-association motifs proposed to be involved in protein–protein coupling [6,30,46]. Peptide domains with alternating negatively and positively charged amino acid residues might be directly involved in the junctin/triadin-mediated linkage between the major luminal Ca^{2+}-binding complex and junctional Ca^{2+}-release channel units [30,46]. Our data show that protein complex formation via triadin clusters is relatively stable suggesting a central role for this junctional protein in the functional coupling between Ca^{2+} storage and Ca^{2+} efflux. Self-aggregation of CSQ was disrupted by interactions with both detergents and Ca^{2+} ions. This suggests a complex scenario for conformational changes in the luminal Ca^{2+}-sequestration apparatus during ion-cycling. Homogeneous protein–protein coupling involved in ion buffering seems to be a highly dynamic mechanism probably involving both hydrophobic and hydrophilic peptide domains. Ca^{2+}-induced changes in heterogeneous membrane complex formation indicate that the free ion concentration within the SR lumen is an important parameter, as suggested by previous physiological studies [24]. CSQ can be considered a positive regulator of the RyR [31] and the ionic micro environment of CSQ clusters dictates co-operative ion binding [20].

Competitive blot overlay assays with purified CSQ indicate that unconjugated CSQ blocked certain interaction sites and/or temporarily triggered conformational changes in Ca^{2+}-binding protein clusters that interfered with the proper interactions between conjugated CSQ and electrophoretically separated CSQ and the RyR. Wang et al. [21] suggested a structure-based mechanism for high-capacity Ca^{2+} binding. Amino-terminal arm exchange, helix–helix contacts and Ca^{2+} cross-bridges are postulated to be involved in CSQ polymerization. The collapse of specific CSQ domains, usually Ca^{2+}-induced, might also be triggered by peptide interactions. On the other hand, variations in the exposure of critical binding domains did not seem to occur in stable CSQ–POD conjugates. With respect to binding patterns to auxiliary triad components, CSQ appeared to have formed a matrix for interactions between the conjugate and triadin/junctin protein bands at high concentrations. This indicates, as already demonstrated by overlay assays in the presence of varying salt and detergent concentrations, that relatively strong interactions exist between triadin and CSQ.

These findings provide the first direct biochemical evidence that the key Ca^{2+}-regulatory components of the skeletal muscle SR form a supramolecular membrane assembly under native conditions. In analogy to junctional cardiac SR membranes [46], protein coupling seems to also be important for the physiological fine regulation of skeletal muscle Ca^{2+} homeostasis. Our studies confirm recent reports on CSQ-binding domains in triadin monomers [6,27–30]. Those approaches employed mostly recombinant proteins and peptide probes in an indirect way and fully agree with our findings on native protein configurations containing CSQ. On the other hand, a previous attempt with CSQ overlays failed to identify high-molecular-mass interactions between SR proteins [47]. Possibly the advance in detection methodology, i.e., enhanced chemiluminescence, or more importantly the careful isolation of membrane vesicles in the presence of a protease inhibitor cocktail (preventing the degradation of large muscle proteins) have overcome these technical problems. CSQ was recently shown by high-resolution X-ray crystallography to form homer–dimer units [21]. The analysis of native skeletal muscle microsomes by blot overlay assays, co-immunoprecipitation and chemical cross-linking, as presented here, strongly indicate that besides dimerization, this Ca^{2+}-binding protein also forms heterogeneous complexes. Direct protein–protein interactions appear to play an essential physiological role in the sequestration and release of Ca^{2+} ions, the overriding second messenger system in the fine regulation of the excitation–contraction–relaxation cycle.

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