# Complex formation between calsequestrin and the ryanodine receptor in fast- and slow-twitch rabbit skeletal muscle

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**Abstract Linkage between the high-capacity $Ca^{2+}$-binding protein calsequestrin and the ryanodine receptor is proposed to be essential for proper $Ca^{2+}$-release during skeletal muscle excitation-contraction coupling. However, no direct biochemical evidence exists showing a connection between these high-molecular-mass complexes in native skeletal muscle membranes. Here, using immunoblot analysis of chemically crosslinked membrane vesicles enriched in triad junctions, we have demonstrated that a very close neighborhood relationship exists between calsequestrin and the ryanodine receptor in both main fiber types. Hence, the luminal $Ca^{2}$+-reservoir complex appears to be directly coupled to the membrane $Ca^{2+}$-release complex and oligomerization seems to be of functional importance.**



*Key words:* Calsequestrin; Ryanodine receptor; Excitation-contraction coupling; Triad; Skeletal muscle

## 1. Introduction

The high-capacity, medium-affinity $Ca^{2+}$-binding protein calsequestrin (CSQ) represents the major $Ca^{2+}$-storage site of the sarcoplasmic reticulum (SR) in skeletal muscle fibers [1–5]. This luminal component of the terminal cisternae exhibits a highly acidic amino acid composition whereby most of its negatively charged residues cluster in the carboxy-terminal region, possibly representing the ion binding domains [6–8]. CSQ aggregates exhibit positive co-operative with respect to high capacity $Ca^{2+}$-binding [9] pointing towards an important physiological role for protein-protein interactions within CSQ clusters. Numerous investigations on $Ca^{2+}$-induced conformational changes suggest that upon ion binding, CSQ increases its α-helical content and folds into a more compact structure burying hydrophobic side chains [10–13]. CSQ is proposed to be directly involved in the $Ca^{2+}$-release process during excitation-contraction (EC) coupling, providing a large $Ca^{2+}$-reservoir available for ion release by the ryanodine receptor (RyR) $Ca^{2+}$-release channel [14]. It is now clearly established that changes in luminal $Ca^{2+}$-concentration influence the channel opening probability of the RyR and $Ca^{2+}$-release rates from the SR [15]. CSQ is proposed to participate in these regulatory processes and might act as an endogenous regulator of the RyR possibly through the junctional component triadin [16].

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*Abbreviations:* CSQ, calsequestrin; DHPR, dihydropyridine receptor; DSP, dithiobis-succinimidyl propionate; EC, excitation-contraction; mAb, monoclonal antibody; RyR, ryanodine receptor; SR, sarcoplasmic reticulum

With respect to triad complexes, direct physical interactions between the transverse tubular dihydropyridine receptor (DHPR) [17] and the RyR $Ca^{2+}$-release channel of the SR [18] are hypothesized to be involved in a highly specialized signal transduction mechanism in mature skeletal muscle fibers [19–21]. Receptor co-localization as determined by electron microscopy [22,23], various physiological findings [24–27], differential co-immunoprecipitation experiments [28] and receptor domain binding studies [29] strongly argues in favor of this model of direct protein-protein interactions [30]. Recently, crosslinking analysis of native triad membranes demonstrated that the junctional $\alpha_1$-DHPR forms distinct high molecular weight complexes with RyR tetramers [31]. Thus, both receptors appear to exist in close proximity and voltage-sensing of the $\alpha_1$-DHPR might directly trigger $Ca^{2+}$ release from the SR via activation of the RyR complex [19–21,30]. With respect to CSQ coupling to triad receptor complexes, electron microscopical analyses demonstrated a periodic attachment of CSQ at the junctional SR via elongated structures [5,23]. Several different SR proteins are implicated to be directly or indirectly involved in providing this structural and functional integrity of CSQ clusters and its link to the $Ca^{2+}$-release complex.

Proteins potentially involved in the avoidance of passive disintegration of the junctional signal transduction complex, the stabilization of overall triad architecture, the regulation of $Ca^{2+}$-homeostasis and/or the physical linkage of CSQ aggregates to the junctional face membrane are triadin, junctin and the 90-kDa JRS-protein [32–38]. Disulfide-bonded clusters of 94-kDa triadin are implicated to mediate interactions between CSQ and the $Ca^{2+}$-release complex [37], whereby the protein junctin appears to be an adhesive component of the high-capacity, medium affinity $Ca^{2+}$-binding units [38]. Guo and Campbell [37] reported that interactions between CSQ and the luminal domain of triadin are $Ca^{2+}$-dependent and that the cytoplasmic domain of triadin binds to the RyR but not to the DHPR. On the other hand, studies by Fan et al. [39] indicate that a cytoplasmic domain of triadin binds to a domain of the DHPR which is considered critical for signal transduction during EC-coupling.

To test the above hypothesis that CSQ aggregates are linked to the triadic membrane complex between key components of the excitation-contraction-relaxation cycle, chemical crosslinking was employed. Since skeletal muscle fibers feature an enormous molecular diversity as reflected by marked histochemical, physiological and biochemical differences between fast- and slow-twitch fibers [40], we investigated both main subtypes of skeletal muscles. This is important because different fiber types exhibit not only specific isoform expression patterns for muscle proteins involved in the contractile apparatus, but they also retain cell biological differences between


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mature slow- and fast-twitch fibers with respect to excitation-concentration (EC) coupling and the $Ca^{2+}$-regulatory system [23,41,42].

## 2. Materials and methods

### 2.1. Materials

Peroxide-conjugated secondary antibodies, protease inhibitors and chemicals for enhanced chemiluminescence were purchased from Boehringer-Mannheim (Lewis, UK). [$^3$H]Ryanodine and $^{45}Ca^{2+}$ were from Amersham (Little Chalfont, UK). Dithiobis-succinimidyl propionate was obtained from Pierce and Warriner Limited (Chester, UK) and Immobilon NC nitrocellulose membranes were from Millipore Corporation (Bedford, MA, USA). All other chemicals were of analytical grade and purchased from Sigma Chemical Company (Dorset, UK).

### 2.2. Antibodies

Production, purification and characterization of primary antibodies to muscle membrane proteins used in this study were previously described in detail [31,37,43,44]. Monoclonal antibodies VIIID1$_2$, IIG12, IIID5, IIH11 and IID8 are directed against epitopes in CSQ triadin, the $\alpha_1$-subunit of the DHPR, as well as the fast and the slow isoforms of the SR $Ca^{2+}$-ATPase, respectively. Polyclonal antisera Rb-53, Rb-55 and Rb-48 to peptides representing the last 15 amino acids of the carboxy-termini of β-DHPR, γ-DHPR and the RyR [31], respectively, were produced by Research Genetics (Huntington, AL, USA).

### 2.3. Chemical crosslinking analysis of triad membranes

Standard subcellular fractionation procedures using sucrose density gradient centrifugation were performed for the isolation of rabbit skeletal muscle membranes enriched in triad junctions [31,34]. Protein concentration was determined according to Bradford [45] using myofibrillar proteins as a standard. Triad membranes were crosslinked at room temperature for 30 min at pH 8 under optimized conditions [46] using the homo-bifunctional 12-Å probe DSP [47] at a concentration range of 25–100 μg DSP per mg membrane protein as previously described in detail [31,48]. Following termination of the crosslinking reaction by the addition of ammonium acetate [31], membrane protein complexes were solubilized in SDS sample buffer under non-reducing conditions [49]. Binding of [$^3$H]ryanodine and $^{45}Ca^{2+}$ to untreated muscle membranes and chemically crosslinked membranes was performed by standard procedures [50,51].

### 2.4. Gel electrophoresis and immunoblot analysis

Electrophoretic separation of muscle proteins on large standard gel was carried out under reducing conditions using 5–15% (w/v) gradient SDS-polyacrylamide gels. Bio-Rad Protean II xi cells (Bio-Rad Laboratories, Hemel Hempstead, UK) were run for 5000 Vh at constant voltage with 60 μg protein per lane [49]. Since gel electrophoretic resolution under non-reducing conditions and transfer efficiency of large crosslinked membrane complexes was previously established to be better using mini-gels as compared to standard large gels [31], a Bio-Rad Mini-Protean II gel system (Bio-Rad Laboratories, Hemel Hempstead, UK) was employed for analyzing crosslinked protein complexes. SDS-polyacrylamide mini-gel electrophoresis was performed with 7% (w/v) separation gels and 5% (w/v) stacking gels or 5% (w/v) resolving gels without a stacking gel system at a constant voltage for 440 Vh and 280 Vh, respectively. Following electrophoresis of crosslinked complexes under non-reducing conditions with 20 μg protein per lane, gel bands containing high-molecular-mass complexes were excised, and then incubated for 10 min at 50°C in SDS sample buffer [49] supplemented with 75 mM DTT, pH 6.8. Reduced gel slices were placed on top of a second 7% (w/v) slab gel using 1% (w/v) agarose, pH 6.8 for proper positioning and run under reducing conditions. Following electrophoretic separation, proteins were transferred to Immobilon NC membranes for 1 h at 100 V on ice [52]. In the case of large gels, a Hoefer Transfor Cell TE-52X (Hoefer Scientific Instruments, San Francisco, CA, USA) was used and in the case of mini-gels a Bio-Rad Mini-Protean II transfer system (Bio-Rad Laboratories, Hemel Hemstead, UK) was employed. Both primary and secondary antibodies were used at a dilution of 1:1000 and 5% (w/v) fat-free milk powder dissolved in Tris-buffered saline was utilized as blocking and washing buffer as described previously [31,43]. Immunoreactivity of blots was determined using the enhanced chemiluminescence method.

## 3. Results and discussion

Rabbit psoas and soleus muscles are established model systems of predominantly fast- and slow-twitch skeletal muscles, respectively [40]. Based on these findings, we performed a comparative analysis of complex formation between key components of EC-coupling in membrane vesicles isolated from

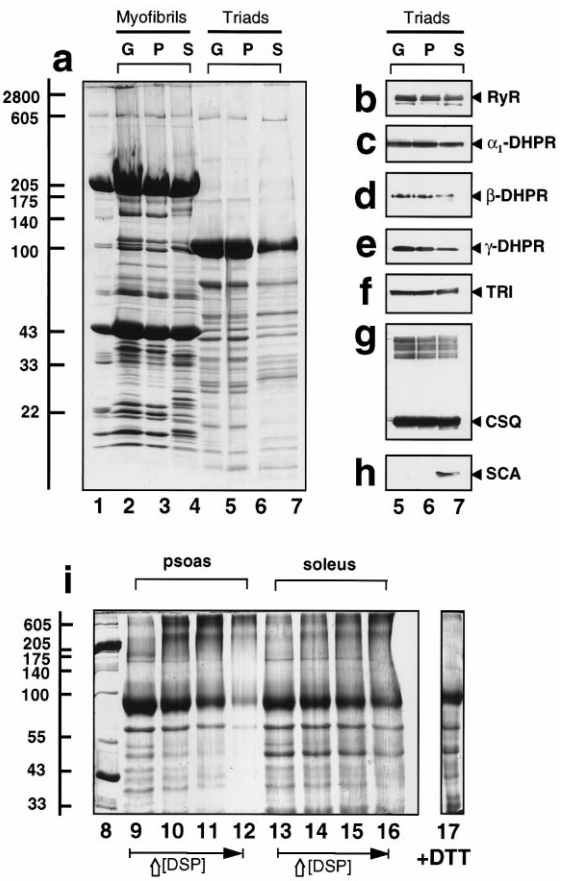


Fig. 1. Fiber-type specific distribution of EC-coupling components and protein profile of triads following chemical crosslinking. Shown is a reduced Coomassie-stained 5–15% (w/v) gradient gel (a) of the crude myofibril fraction (lanes 2–4) and membranes enriched in triads (lanes 5–7) isolated from rabbit gastrocnemius (G) (lanes 2,5), psoas (P) (lanes 3,6) and soleus (S) (lanes 4,7) muscles. Identical immunoblots of triad preparations from the different types of skeletal muscle were labeled with polyclonal antiserum Rb-48 to the ryanodine receptor (RyR) (b), Rb-53 to the β-subunit of the dihydropyridine receptor (β-DHPR) (d) and Rb-55 to the γ-subunit of the DHPR (γ-DHPR) (e), as well as with mAb IIID5 to the $\alpha_1$-DHPR (c), mAb IIG12 to triadin (TRI) (f), mAb VIIID $1_2$ to calsequestrin (CSQ) (g) and mAb IID8 to the slow-twitch isoform SERCA2 of the SR $Ca^{2+}$-ATPase (SCA) (h). In (i) is shown a Coomassie-stained 7% (w/v) polyacrylamide gel of membranes enriched in triads isolated from psoas (lanes 9–12) and soleus (lanes 13–17) muscles. Membranes were treated with 0 (lanes 9,13), 25 (lanes 10,14), 50 (lanes 11,15), and 100 (lanes 12,16,17) μg dithiobis-succinimidyl propionate (DSP) per mg membrane protein, respectively. Lanes 8–16 were electrophoresed under non-reducing conditions, while the sample in lane 17 was chemically reduced with dithiothreithol (DTT) prior to application to the gel. Molecular mass markers ($\times 10^{-3}$), as deduced from rat myofibrillar proteins (lanes 1,8), are indicated on the left.

these two well-defined subtypes of muscle. Although no major overall differences in the protein band pattern of junctional couplings isolated from gastrocnemius, psoas and soleus muscle were observed, specific differences in the abundance of marker components of slow- and fast-twitch skeletal muscle could be seen in the crude myofibril preparations from the three different muscles. Fig. 1a illustrates the well established fiber-type specific differences in proteins of the contractile apparatus [40] such as the myosin light chain (16–25 kDa) and tropomyosin (32–36 kDa) regions. In addition, immunoblotting of triad membranes using mAb IID8 to the slow-twitch isoform of the SR $Ca^{2+}$-ATPase revealed strong staining only in the soleus fraction (Fig. 1h). Hence, triads isolated from these muscles are derived from fibers with defined twitch characteristics. With respect to abundance in the triad fraction, CSQ in gastrocnemius, psoas and soleus did not show marked differences (Fig. 1g). Triadin, RyR, $\alpha_1$-DHPR and β-DHPR were found to be slightly reduced in soleus, while the expression of the γ-DHPR was significantly lower in slow-twitch muscle (Fig. 1b–f).

It was previously established that 12.5–50 μg DSP per mg membrane protein does not induce general clustering of triad and SR proteins from rabbit skeletal muscle preparations [31]. As illustrated in Fig. 1i, at a concentration ratio of 25 or 50 μg hydrophobic crosslinker per mg membrane protein, a relatively comparable overall protein band pattern was observed in triads from both psoas and soleus muscles. However, a tendency towards reduction in the electrophoretic mobility of distinct proteins was evident with increasing amounts of DSP. This is especially conspicuous for the 100-kDa band representing mostly the SR $Ca^{2+}$-ATPase isoforms (Fig. 1i). We therefore used 25 and 50 μg crosslinker per mg protein for our analysis of complex formation between CSQ and other EC-coupling components in triad preparations. Coomassie-stained protein band patterns at a higher crosslinker concentration, i.e. 100 μg DSP per mg protein, is shown for comparative purposes. Distinguishing between non-reducing and reducing conditions, it can be seen that protein bands representing crosslinked high-molecular-mass complexes under non-reducing conditions regain the faster electrophoretic mobility of their apparent monomers following chemical reduction (Fig. 1i).

Since the relative molecular masses of the monomers of CSQ, RyR and DHPR and that of the crosslinked membrane complexes differed widely, two gel systems were employed in our immunoblot analysis. Both 5% (w/v) separating gels lacking a stacking gel system and 7% (w/v) gels containing a stacking gel were used. Hence, identical nitrocellulose transfers could be immunologically labeled for a large range of relative molecular masses. Representative immunoblots in Fig. 2 display an immunoreactive overlap which exists between protein bands representing CSQ aggregates and the RyR. This was observed following crosslinking with both 25 and 50 μg DSP per mg protein. Under non-crosslinked conditions, CSQ exhibited not only a monomer band of apparent 63 kDa but also three additional higher molecular mass bands ranging from approximately 140 kDa to 200 kDa (Fig. 2a,d). These distinct protein bands recognized by mAb $VIIID1_2$ are either biologically crosslinked CSQ species which are inert to chemical reduction or represent CSQ-like proteins as already discussed in earlier studies [31,44,53]. For comparative purposes, the immunoblot analysis of uncrosslinked RyR with

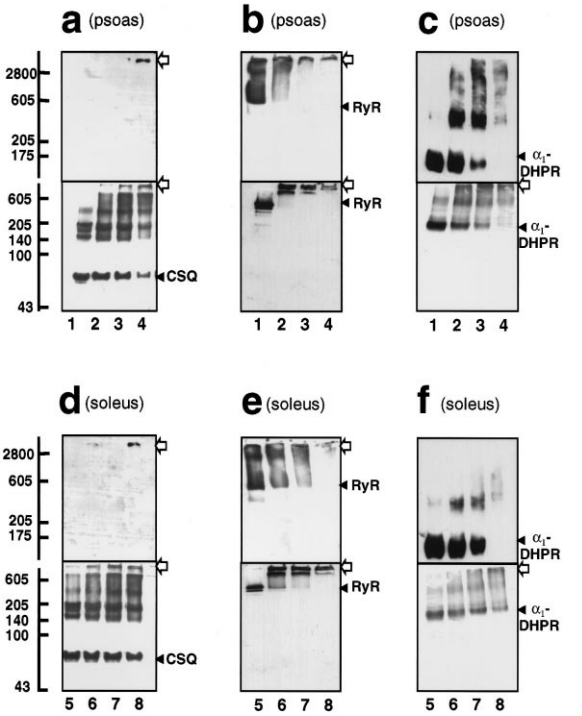


Fig. 2. Chemical crosslinking analysis of key components of EC-coupling in triad membranes from fast- and slow-twitch rabbit skeletal muscles. Shown are immunoblots of triad couplings isolated from rabbit psoas (a–c, lanes 1–4) and soleus muscles (d–f, lanes 5–8) stained with mAb $VIIID1_2$ to calsequestrin (CSQ) (a,d), polyclonal antiserum Rb-48 to the ryanodine receptor (RyR) (b,e) and mAb IIID5 to the $\alpha_1$-subunit of the dihydropyridine receptor ($\alpha_1$-DHPR) (c,f). Proteins were separated on 5% (w/v) resolving gels run without a stacking gel (upper part of blots) and also on 7% (w/v) separating gels with a 5% (w/v) stacking gel (lower part of blots). Muscle membranes were treated with 0 (lanes 1,5), 25 (lanes 2,6), 50 (lanes 3,7), and 100 (lanes 4,8) μg DSP per mg membrane protein, respectively. In (b) and (e), lane 1 and lane 5 were electrophoresed under non-reducing conditions in the upper part of blots, while samples were chemically reduced prior to application to the gel in the lower part of the blots. The position of monomers (arrowheads) and crosslinking-induced oligomeric complexes (open arrows) is indicated. Molecular mass markers ($\times 10^{-3}$) are indicated on the left.

and without chemical reduction is shown using a 5% (w/v) resolving gel lacking a stacking gel and a 7% (w/v) stacking gel system, respectively (Fig. 2b,e). In contrast to CSQ and $\alpha_1$-DHPR (not shown), the RyR exhibited a rigid but chemically reducible high-molecular-mass complex formation even prior to chemical crosslinking. Incubation with DSP shifted all immunoreactive RyR species to the complex of extremely low electrophoretic mobility (Fig. 2b,e). The determination of potential changes in relative molecular mass of these complexes following crosslinking was deterred by the very large size of oligomerized triad complexes. Since these complexes of more than 3000 kDa just barely enter the resolving gel, SDS-polyacrylamide gel electrophoresis does not exhibit a linear relationship between that range of electrophoretic mobility and relative molecular masses of proteins [49].

Antibodies to the $\alpha_1$-DHPR also showed staining of this complex (Fig. 2c). However, immunostaining of this subunit within the high-molecular-mass triad complex, although convincing in fast-twitch muscle membranes, was relatively weak in soleus preparations (Fig. 2f). Thus, crosslinking-induced

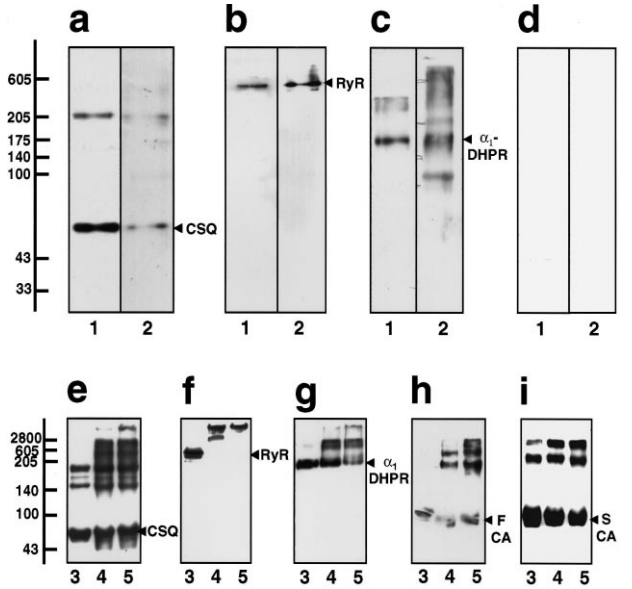


Fig. 3. Re-electrophoresis of key components of EC-coupling eluted from crosslinked triad complexes. Shown are immunoblots of triad proteins which had been excised, chemically reduced and then re-electrophoresed on a second 7% (w/v) slab gel following crosslinking and electrophoretic separation under non-reducing conditions in the first dimension as illustrated in Fig. 2. The position of apparent monomers of calsequestrin (CSQ) (a), the ryanodine receptor (RyR) (b) and the $\alpha_1$-subunit of the dihydropyridine receptor ($\alpha_1$-DHPR) (c), as recognized by immunolabeling with antibodies VIIID$1_2$, Rb-48, and IIID5, respectively, is indicated by arrowheads. Staining with mAb IIG12 did not reveal detectable levels of triadin (d). Lanes 1 and 2 represent samples from psoas and soleus muscles, respectively. In lanes e–i are shown comparative immunoblots illustrating that no immunodecorative overlap exists between the CSQ-RyR-DHPR complex (e: CSQ; f: RyR; g: $\alpha_1$-DHPR) and the fast (FCA) (h) and slow (SCA) (i) SR $Ca^{2+}$-ATPase isoform complexes. Muscle membranes were treated with 0 (lane 3), 25 (lane 4) and 50 (lane 5) μg DSP per mg membrane protein, respectively. Molecular mass markers ($\times 10^{-3}$) are indicated on the left.

complex formation between triad receptors appears to be at least partially dependent on skeletal muscle fiber type. The β- and γ-subunits of the DHPR did not shift to the same high-molecular-mass position following incubation with crosslinker (not shown). Possibly crosslinking-induced alterations of epitopes in auxiliary DHPR subunits account for this lack of immunolabeling and/or steric hindrance within this gigantic complex prevented a proper binding of individual DHPR subunits via a hydrophobic 12-Å probe such as DSP. Immunoblotting with antibodies to triadin demonstrated crosslinking-induced shifts of this triad component to very high-molecular-mass complexes (not shown) but no distinct labeling of the CSQ-RyR-DHPR complex could be documented.

Following chemical crosslinking, detergent-solubilized membrane complexes were incubated with immobilized antibodies to the RyR, $\alpha_1$-DHPR or CSQ in order to immunoprecipitate triad complexes (not shown). However, differential immunoblotting did not reveal strong enough labeling to unequivocally determine the presence of individual members of the complexes previously detected by overlap of immunodecoration (Fig. 2). Therefore, in order to substantiate the above described oligomerization, we employed an alternative approach to immunoprecipitation. High-molecular-mass triad complexes were excised, then chemically reduced and re-electrophoresed under reducing conditions (Fig. 3a–d). In a non-reducing environment, DSP-crosslinked proteins exhibit a slower electrophoretic mobility and move to the oligomer position as illustrated in Fig. 2. However, chemical reduction breaks the covalent, DSP-induced disulfide bonds between crosslinked membrane proteins and causes oligomeric complexes to disintegrate into monomers. Thus, separation on a second slab gel under reducing conditions causes previously complexed proteins to move again with a higher electrophoretic mobility (Fig. 3a–c). Immunoblotting of the re-electrophoresed high-molecular-mass complex from both psoas and soleus triads clearly exposed the presence of CSQ (Fig. 3a), the RyR (Fig. 3) and the $\alpha_1$-subunit of the DHPR (Fig. 3c) in the excised gel bands. Besides the appearance of apparent 63-kDa CSQ, 565-kDa RyR and 170-kDa $\alpha_1$-DHPR monomers, labeled protein bands higher than the monomers were also observed. Possibly, chemical reduction cannot fully reverse all crosslinking-induced alterations during the complex formation of triad proteins. Triadin was not found in detectable amounts in the re-electrophoresed samples (Fig. 3d).

To illustrate the specificity and triad complex oligomerization, chemical crosslinking of the slow and fast SR $Ca^{2+}$-ATPase isoforms is shown in Fig. 3h,i. In contrast to the overlapping immunodecoration of junctional triad components, the crosslinked $Ca^{2+}$-ATPase bands representing the highest molecular mass species of these two SR components clearly do not overlap with CSQ and the RyR (Fig. 3e–i). Thus, apparent dimers, tetramers and octamers of the highly abundant $Ca^{2+}$-ATPases, which are present in both the longitudinal tubules and terminal cisternae [54], are not an integral part of CSQ-RyR-DHPR complexes. In functional control studies, no fundamental difference in $^{45}Ca^{2+}$-binding was found in untreated vs. crosslinked vesicles, while specific [$^3$H]ryanodine binding to crosslinked microsomes was 3–4-fold increased in comparison with control vesicles (not shown). Therefore, $Ca^{2+}$-binding to the major SR $Ca^{2+}$-binding proteins, including CSQ, is not affected by DSP. On the other hand, crosslinking-induced stabilization of RyR oligomerization and/or CSQ-RyR-DHPR complex formation changes the affinity of the RyR for the plant alkaloid investigated.

Hence, the two receptor complexes established to be of crucial importance for proper signal transduction during EC-coupling [19–21] not only exist in close vicinity to each other [28,31], but CSQ clusters also appear to be directly linked to the $Ca^{2+}$-release complex. This agrees with electron microscopic investigations of junctional SR membranes which showed a periodic attachment of CSQ via elongated structures [5,23]. In addition, the SR $Ca^{2+}$-release complex consisting of RyR tetramers [18] and the voltage-sensing $\alpha_1$-DHPR [17] can be chemically crosslinked as confirmed in this study. However, vesicular structures derived from triad junctions from predominantly slow-twitch soleus muscles exhibited a much less pronounced apparent linkage between the $\alpha_1$-DHPR and RyR than preparations from fast-twitch psoas muscles. Thus, formation of junctional triad receptor complexes could be at least partially dependent on fiber type. This agrees with the idea that differences exist between the regulation of EC-coupling between the two main skeletal muscle fiber types and that slow-twitch muscles may be more cardiac-like in their signal transduction processes, i.e. increased sensitivity within $Ca^{2+}$-induced $Ca^{2+}$-release mechanisms [19–21]. On the other

hand, DHPR components other than the $\alpha_1$-subunit were not found in the RyR-coupled DHPR receptor complex. A subpopulation of junctional DHPR which directly interacts with the RyR and other EC-coupling components might maintain this linkage exclusively via the $\alpha_1$-subunit without a close contact to auxiliary $Ca^{2+}$-channel subunits. With respect to regulation of both receptors, a reciprocal signaling appears to exist. While the DHPR controls the $Ca^{2+}$-release activity of the skeletal muscle RyR-1 isoform, the RyR controls the $Ca^{2+}$-channel activity of the DHPR [55]. The physical closeness of the key components involved in this $Ca^{2+}$-regulatory system within junctional couplings is in agreement with our results. Recently, Zhang et al. [56] could show by CSQ-affinity chromatography, immunoprecipitation experiments and filter overlay assays that CSQ forms a quaternary complex with junctin, triadin and the RyR in cardiac junctional SR. Hence, the cardiac $Ca^{2+}$-regulatory SR membrane system appears to be also based on oligomeric complexes involving the $Ca^{2+}$-release and $Ca^{2+}$-storage units.

Previous attempts at crosslinking skeletal muscle membranes with probes such as glutaraldehyde, NHS-ASA, DFDNB, DFNPS, DMS, EDC and EGS did not result in the formation of high-molecular-mass complexes between the RyR, CSQ, triadin and the DHPR [34,57]. While studying the effect of RyR oligomerization on the low- and high-affinity binding sites for ryanodine, dimers and tetramers of the 565-kDa RyR subunit were observed, but crosslinking to other SR components was not detected [57]. Using the *N*-hydroxysuccinimide ester MBS, no crosslinking was noted between CSQ and other SR components such as the SERCA $Ca^{2+}$-ATPase isoforms or the 53-kDa SR glycoprotein [58]. Therefore successful crosslinking of membrane complexes between the SR and triad components appears to be restricted to hydrophobic 12-Å probes such as DSP, as well as water-soluble 11.4-Å probes such a $BS^3$ [31]. Other crosslinking approaches might have failed because of steric hindrance within junctional coupling complexes. In conclusion, the electron microscopic observations that regular junctional structures exist in skeletal muscle triads [5,22,23] are confirmed by crosslinking of native muscle membranes. CSQ and the RyR, as well as the $\alpha_1$-DHPR appear to exist in a gigantic complex mediating highly specialized signal transduction during EC-coupling in skeletal muscles. Although differences exist in the fine tuning of EC-coupling between fast and slow muscles, both main fiber types exhibit the oligomerization of proteins involved in $Ca^{2+}$-handling as an intrinsic property.

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