CMLS, Cell. Mol. Life Sci. 58 (2001) 312–320
1420-682X/01/020312-09 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Research Article

Native skeletal muscle dihydropyridine receptor exists as a supramolecular triad complex

G. R. Froemming and K. Ohlendieck*

Department of Pharmacology, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4 (Ireland), Fax + 353 1 2692749, e-mail: kay.ohlendieck.ucd.ie

Received 10 October 2000; received after revision 28 November 2000; accepted 4 January 2001

Abstract. One of the central elements of excitation-contraction coupling, the voltage-sensing dihydropyridine receptor, is believed to exist as a high-molecular-mass complex in the triad junction. Although freeze-fracture electron microscopical analysis suggests a tetrad complex, no direct biochemical evidence exists demonstrating the actual size of the native membrane complex. Using a combination of various two-dimensional gel electrophoresis techniques, we show here that the principal α_1-subunit of the dihydropyridine receptor and its auxiliary α_2-subunit form a triad complex of approximately 2800 kDa under native conditions. Established Ca^{2+}-ATPase tetramers and calsequestrin monomers were employed for the internal standardization of the gel systems used. Thus, the large voltage-sensing complex appears to be tightly associated, since it does not disintegrate during subcellular fractionation and native electrophoresis procedures. Our findings support the cell biological hypothesis that native dihydropyridine receptor units form a tetrad structure within the transverse tubules.

Key words. Dihydropyridine receptor; Ca^{2+}-ATPase; calsequestrin; two-dimensional electrophoresis; triad; skeletal muscle.

Coupling between motorneuron-induced surface membrane depolarization and Ca^{2+}-mediated activation of the contractile apparatus is provided by two large Ca^{2+}-channel complexes in skeletal muscle fibers [1]. The central elements of excitation-contraction coupling involved in voltage-sensing and Ca^{2+} release are usually referred to as the dihydropyridine receptor [2] and the ryanodine receptor Ca^{2+}-release channel [3], respectively. In skeletal muscle, both receptors are postulated to interact by direct physical means during signal transduction at the triad junction [4]. Previous electron microscopical freeze-fracture studies have suggested that the dihydropyridine receptor forms supramolecular structures [5–8]. A single population of large intramembrane particles has been found in the junctional domains of the transverse tubular membrane system [9]. The characteristic clustering of groups of four particles forming regular tetrad structures coincides with a high abundance of the dihydropyridine receptor in junctional membranes [10]. The idea that skeletal muscle tetrads represent the dihydropyridine receptor is strongly supported by the fact that tetrad structures are missing in dihydropyridine receptor-deficient dysgenic muscle fibers [11] and that genetic restoration of the receptor in mutant cells causes the reappearance of tetrads [12]. However, no direct biochemical data are available demonstrating the actual size of the native voltage-sensing membrane complex. We therefore performed a variety of two-dimensional gel electrophoresis techniques in order to confirm the quaternary structure

* Corresponding author.

of the transverse tubular complex by biochemical methodology.

The receptor for 1,4-dihydropyridine represents an L-type Ca^{2+} channel which is highly enriched in the surface membrane invaginations of skeletal muscle fibers [2]. Within the transverse tubular membrane system, the receptor complex exists in a non-junctional form in the free transverse tubules and as a junctional form in the triad structure formed by contact zones between the terminal cisternae of the sarcoplasmic reticulum and the neighboring transverse tubular regions [9]. Besides its function as a voltage-sensor triggering Ca^{2+} release via the junctional ryanodine receptor, this protein can also act as a slowly activating Ca^{2+} channel and has therefore presumably a dual function in skeletal muscle cells [1]. The receptor consists of five subunits, the physiologically and pharmacologically principal α_{1S}-subunit which contains the voltage-sensing domains, the pore-forming structures and various binding sites for agonists and antagonists, and the auxiliary subunits α_2-δ, β, and γ which have important regulatory and receptor targeting functions [2, 13, 14].

To standardize the two-dimensional native gel electrophoresis system employed in this study, we analyzed the sarcoplasmic reticulum components Ca^{2+}-ATPase and calsequestrin in parallel to the dihydropyridine receptor. Both proteins represent important Ca^{2+}-regulatory elements involved in the excitation-contraction-relaxation cycle and are biochemically well characterized [15, 16]. The Ca^{2+}-ATPase units of the longitudinal tubules and terminal cisternae are responsible for the rapid energy-dependent re-uptake of Ca^{2+} ions during the relaxation step following muscle contraction [15]. Based on electron microscopical and biochemical investigations, the SERCA1 isoform of the native Ca^{2+}-ATPase complex is postulated to form a tightly associated homo-tetrameric structure [17, 18]. Calsequestrin is a high-capacity, low-affinity Ca^{2+}-binding protein located in the terminal cisternae region [16] and was shown to be an important endogenous regulator of the Ca^{2+}-release channel [19]. Although calsequestrin is also believed to exist in large aggregates, these protein-protein interactions appear to be weaker than those of the Ca^{2+}-pump complex. If calsequestrin complexes are not stabilized by chemical cross-linking, they disintegrate during routine subcellular fractionation protocols [20]. We were therefore able to use Ca^{2+}-ATPase tetramers and calsequestrin monomers as positive and negative controls in our oligomerization analysis of muscle membrane complexes, and provide direct biochemical evidence that the principal α_1-subunit of the dihydropyridine receptor and its auxiliary α_2-subunit exist natively in a supramolecular triad complex.

Materials and methods

Materials. Protease inhibitors, peroxidase-conjugated secondary antibodies, and acrylamide stock solution were purchased from Boehringer Mannheim (Lewes, UK). Immobilon-P nitrocellulose membranes were from Millipore (Bedford, Mass). Chemiluminescence substrates and Tris-(2-carboxyethyl)phosphine hydrochloride were obtained from Pierce and Warriner (Chester, UK). IPG-strips pH 3–10 and IPG buffer pH 3–10 were from Amersham Pharmacia Biotech (Little Chalfont, UK). All other chemicals used for protein solubilization, subcellular fractionation procedures, and electrophoretic separation were of analytical grade and purchased from Sigma (Poole, UK).

Antibodies. Antibodies were produced and characterized as previously described in detail [18, 20]. Monoclonal antibodies IIID5 against the α_{1S}-subunit of the dihydropyridine receptor [21] and $VIIID1_2$ against the fast-twitch isoform of calsequestrin [20] were a generous gift from Dr. Kevin P. Campbell (Howard Hughes Medical Institute, University of Iowa, Iowa City, Iowa). Monoclonal antibodies IIH11 against the fast-twitch isoform SERCA1 of the sarcoplasmic reticulum Ca^{2+}-ATPase [21] and 20A against the α_2-subunit of the dihydropyridine receptor [22] were purchased from Affinity Bioreagents (Golden, Colo).

Skeletal muscle membrane preparation. Bulk skeletal muscles were dissected from the back and hind legs of New Zealand white rabbits obtained from the Biomedical Facility, University College Dublin. All preparative steps were carried out at 4 °C in the presence of a protease inhibitor cocktail (0.2 mM pefabloc, 1.4 μM pepstatin, 0.15 μM aprotinin, 0.3 μM E-64, 1 μM leupetin, 0.5 mM soybean trypsin inhibitor, and 1 mM EDTA) [22]. Crude microsomes, as well as subcellular fractions enriched in sarcoplasmic reticulum or triads were prepared by standard methodology [20–23]. Membrane vesicles were resuspended in storage buffer (10% w/v sucrose, 25 mM HEPES, pH 7.5, 3 mM $MgCl_2$, 2 mM EGTA), quick-frozen in liquid nitrogen and then stored at −70 °C. The protein concentration of membrane samples was determined according to Bradford [24] using bovine serum albumin as a standard.

Isoelectric focusing. Prior to isoelectric focusing, triad samples were dialyzed overnight against deionized water at 4 °C and then solubilized and electrophoresed [25]. In accordance with the manufacturer's instructions for the optimum usage of the IPGphor isoelectric focusing system (Amersham Pharmacia Biotech), triad membranes were solubilized in lysis buffer {5 M urea, 2 M thiourea, 2% w/v SB 3-10 (N-decyl-N,N-dimethyl-3-ammonio-1-propane-sulfonate), 40 mM Tris, 2 mM TCEP-HCl (Tris-[2-carboxyethylphosphine] hydrochloride), 2% w/v CHAPS} and then diluted in IPG strip rehydration buffer (8 M urea, 2% w/v CHAPS, 0.5% w/v

IPG-buffer pH 3–10, 2 mM TCEP-HCl) to achieve a final protein concentration of 25 μg protein for immunoblotting experiments. Individual 7-cm-long IPG strips (pH 3–10) were rehydrated for 13 h in porcelain IPG strip holders with 125 μl of the above protein solution. To avoid protein degradation, a freshly prepared protease inhibitor cocktail was added prior to rehydration. Isoelectric focusing was carried out with 60 μA per strip using the IPGphor isoelectric focusing system. The running conditions were a 200-V step for 1 h, 400 V for 1 h, a 2000-V gradient step for 1 h, a 5000-V gradient step for 2 h, an 8000-V gradient step for 4 h, and a final 8000-V step for 10 h [25]. Following isoelectric focusing, the cover fluid was poured off and the strips were stored at −70 °C until further use. Prior to separation in a second-dimension gel system, focused strips were equilibrated for 1 h in a solution of 30% w/v glycerol, 20% w/v sucrose, 3% w/v SDS, 6 M urea, 50 mM Tris-HCl, pH 8.8, and 5 mM TCEP-HCl. After equilibration, the strips were embedded in an agarose solution (1% w/v agarose, 6% w/v glycerol, 0.1% w/v SDS, 198 mM glycine, 25 mM Tris-HCl, pH 8.8) on top of an 8% w/v separation polyacrylamide gel, pH 8.8, and then electrophoresed for 280 Vh [26].

One- and two-dimensional gel electrophoresis. Native and non-native polyacrylamide gel electrophoresis were carried out according to Chang and Hosey [27] and Laemmli [28], respectively, as previously described in detail [29]. Non-denaturing sample buffer contained 50 mM Tris-Cl, pH 6.8, 20% v/v glycerol, 20% w/v sucrose, and 0.1% w/v digitonin. Non-reducing and denaturing buffer consisted of 125 mM Tris-Cl, pH 6.8, 20% v/v glycerol, 20% w/v sucrose, and 3% w/v sodium dodecyl sulfate. Reducing and denaturing sample buffer were complemented with 75 mM dithiothreitol. All electrophoretic sample buffers also contained 0.002% w/v bromophenol blue as a dye marker. In non-denaturating gel electrophoresis, 0.1% w/v digitonin but no sodium dodecyl sulfate was present in the resolving and stacking gel systems [27]. Non-reducing and reducing electrophoresis in the presence of ionic detergent was performed by standard methodology [28]. Resolving gels with a 7% w/v polyacrylamide concentration were used in combination with a 5% w/v stacking gel system. For the one-dimensional separation of crude microsomes, sarcoplasmic reticulum, and triads, 10 μg of membrane protein was electrophoresed under reducing or non-reducing conditions for 280 Vh employing a Mini-Protean II electrophoresis system from Bio-Rad (Hempel Hempstead, UK). For the two-dimensional separation of triads, 30 μg of membrane protein was separated in the first dimension using native or non-reducing conditions, followed by the incubation of these gels for 1 h with SDS-containing sample buffer [29]. First-dimension gels were mounted on top of the 7% w/v second-dimension polacrylamide gels, sealed with 1% w/v agarose solution and subsequently electrophoresed for 280 Vh under reducing conditions. Myofibrils, prepared from rat skeletal muscle homogenates, served as a source of high-molecular-mass protein standards [20]. The highest-molecular-mass band was represented by the muscle protein titin of apparent 2800 kDa.

Immunoblotting. Muscle proteins separated on one- or two-dimensional gels were transferred for 1 h at 100 V onto nitrocellulose membranes by the method of Towbin et al. [30] using a Bio-Rad Mini-Protean II blotting system. Nitrocellulose sheets were blocked with blocking solution (5% w/v fat-free milk, 0.15 M NaCl, 10 mM Tris-HCl, pH 7.4, 0.1% w/v Tween 20) and then incubated with 1:1000-diluted primary antibodies against the α_{1S}-dihydropyridine receptor, the α_2-dihydropyridine receptor, the SERCA1 isoform of the Ca^{2+}-ATPase, or fast calsequestrin, followed by two washing steps and a final incubation with 1:1000-diluted peroxidase-conjugated secondary antibody [20]. After four washing steps, immunodecorated protein bands were visualized by enhanced chemiluminescence. Densitometric scanning of developed immunoblots was performed on a Molecular Dynamics 300S computing densitometer (Sunnyvale, Calif.) with Image Quant V3.0 software.

Results

Two-dimensional electrophoretic analysis of the native α_1-dihydropyridine receptor subunit. To determine whether the native dihydropyridine receptor exists as a supramolecular structure, various two-dimenisonal gel electrophoresis techniques were used. As illustrated in figure 1a, immunoblotting with monoclonal antibody IIID5 to the α_{1S}-subunit of the skeletal muscle dihydropyridine receptor revealed labeling of a distinct band of apparent 170 kDa in one-dimensional gels. No other bands were immunodecorated in microsomes and vesicle populations enriched in the sarcoplasmic reticulum and triads under either reducing or non-reducing conditions, making this antibody highly suitable for the proper identification of dihydropyridine receptor-containing membrane complexes in two-dimensional gel systems. Three different two-dimensional gel techniques were employed, i.e., a non-reducing (but detergent-containing) first dimension (fig. 1b), an isoelectric focusing first dimension (fig. 1c), and a native first dimension (fig. 1d), each combined with a reducing and denaturing second-dimension gel electrophoretic step. The non-reducing/reducing two-dimensional gel showed that the majority of the approximately 170-kDa protein band representing the α_{1S}-dihydropyridine receptor stays on the diagonal of this gel system and that the immunodecorated band is only stretched in the horizontal direction

(fig. 1b). Thus, the presence of detergent disrupts sensitive protein-protein interactions within this membrane complex and the α_{1S}-subunit appears to run mostly as a monomeric structure under these conditions. Isoelectric focusing confirmed that only one major species of this receptor subunit appears to exist in skeletal muscle with respect to pI values of approximately 5–6 (fig. 1c). The native gel analysis presented in figure 1d clearly illustrates the shift off the diagonal between the native first dimension and the chemically reducing and denaturing second dimension. In contrast to figure 1b, the labeled spot representing the α_{1S}-dihydropyridine receptor in figure 1d is positioned at an approximate molecular mass of 2800 kDa, when compared to rat myofibrillar marker proteins such as titin. Since the entire dihydropyridine receptor complex, if present at an approximate one-to-one ratio between its individual α_1-α_2-δ-β-γ subunits, should not run during native electrophoretic separation at a relative molecular mass of above 450–500 kDa, this result strongly indicates that this transverse tubular complex exists in a supramolecular triad membrane structure.

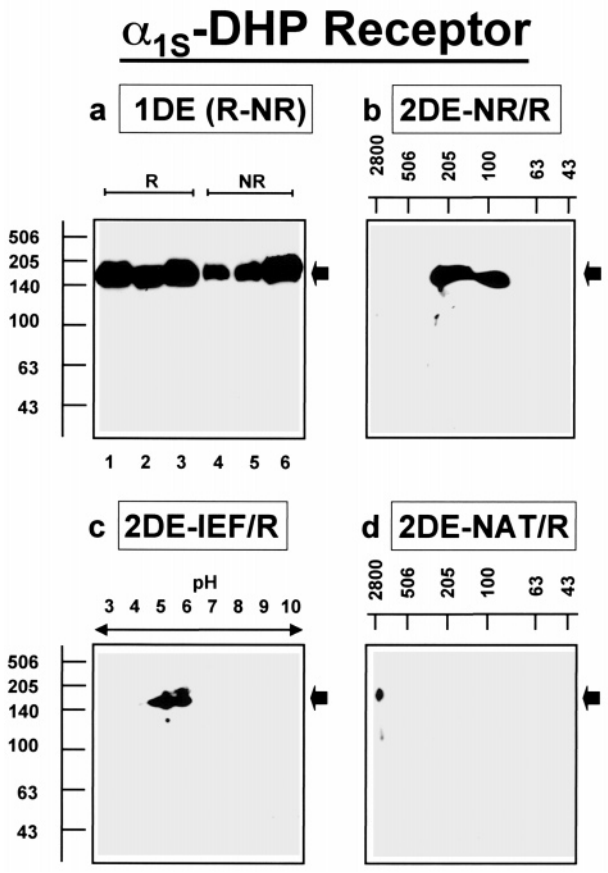

Figure 1. Two-dimensional electrophoretic analysis of the native α_{1S}-dihydropyridine receptor subunit. Shown are immunoblots of skeletal muscle membrane preparations labeled with monoclonal antibody IIID5 to the α_{1S}-subunit of the dihydropyridine (DHP) receptor. Immunodecorated bands are marked by arrows. The relative sizes of molecular-mass standards (in kDa), as deduced from rat myofibril marker proteins, are indicated on the left and at the top of two-dimensional gels. (*a*) Lanes 1 and 4, 2 and 5, and 3 and 6 represent crude microsomes, sarcoplasmic reticulum membranes, and triad membranes, respectively. Samples were electrophoresed in lanes 1–3 under reducing conditions (R) and in lanes 4–6 under non-reducing conditions (NR) using standard one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1DE). (*b*) Triad membranes were first electrophoretically separated under non-reducing (NR) conditions and then electrophoresed in the second dimension under reducing (R) conditions (2DE-NR/R). (*c*) In the first dimension, triads underwent isoelectric focusing (IEF), followed by a second-dimension electrophoretic separation under reducing (R) conditions (2DE-IEF/R). (*d*) Triads were first electrophoretically separated under native (NAT) conditions and then electrophoresed in the second dimension under reducing (R) conditions (2DE-NAT/R).

Two-dimensional electrophoretic analysis of the native α_2-dihydropyridine receptor subunit. The auxiliary α_2-subunit of the dihydropyridine receptor was investigated in order to confirm the above findings. In analogy to figure 1, similar results were obtained when native immunoblots were labeled with monoclonal antibody 20A (fig. 2). One-dimensional gel electrophoresis confirmed that this antibody exclusively recognized a protein of apparent 175 kDa. A slight increase in electrophoretic mobility was observed between the reduced versus the non-reduced form indicating the loss of the δ-subunits from the main α_2-subunit following chemical reduction (fig. 2a). The difference between the non-reduced and reduced α_2-dihydropyridine receptor was more convincingly illustrated by two-dimensional electrophoresis. In contrast to the horizontal α_{1S}-dihydropyridine receptor band (fig. 1b), in diagonal non-reducing/reducing two-dimensional gel electrophoresis, the protein band representing the α_2-subunit formed a vertical immunolabel. The upper part of this band represents the α_2-δ complex and the lower part presumably the α_2-subunit without its usually disulfide-bonded δ-subunits. This agrees with the previously published structural relationship between the auxiliary α_2-subunit and the three smaller δ-subunits of the dihydropyridine receptor [31]. Results from isolectric focusing experiments suggested, as had been shown for the other dihydropyridine receptor subunit (fig. 1c), that the auxiliary subunit appears to also exist as only one molecular species with respect to its isoelectric point (fig. 2c). Most importantly, the immunoblot analysis of the α_2-subunit following native two-dimensional gel electrophoresis agreed with the results obtained with the principal receptor subunit. As shown in figure 2d, the main spot immunodecorated by monoclonal antibody 20A shifts off the diagonal. The difference between the position of the α_2-dihydropyridine receptor at approximately 175 kDa in figure 2b and approximately 2800

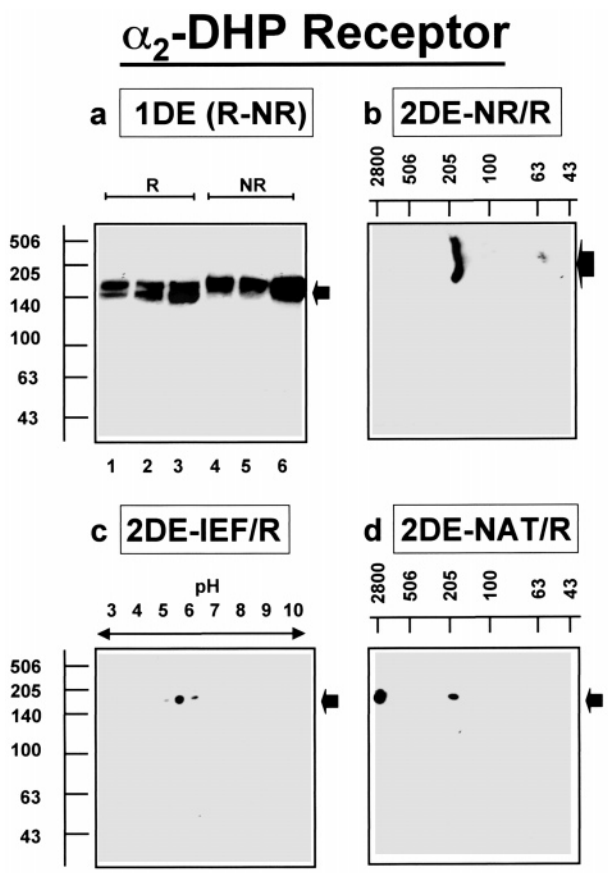

Figure 2. Two-dimensional electrophoretic analysis of the native α_2-dihydropyridine receptor subunit. Shown are immunoblots of skeletal muscle membrane preparations labeled with monoclonal antibody 20A to the α_2-subunit of the dihydropyridine (DHP) receptor. Immunodecorated bands are marked by arrows. The relative sizes of molecular-mass standards (in kDa), as deduced from rat myofibril marker proteins, are indicated on the left and at the top of two-dimensional gels. (*a*) Lanes 1 and 4, 2 and 5, and 3 and 6 represent crude microsomes, sarcoplasmic reticulum membranes, and triad membranes, respectively. Samples were electrophoresed in lanes 1–3 under reducing conditions (R) and in lanes 4–6 under non-reducing conditions (NR) using standard one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1DE). (*b*) Triad membranes were first electrophoretically separated under non-reducing (NR) conditions and then electrophoresed in the second dimension under reducing (R) conditions (2DE-NR/R). (*c*) In the first dimension, triads underwent isoelectric focusing (IEF), followed by a second-dimension electrophoretic separation under reducing (R) conditions (2DE-IEF/R). (*d*) Triads were first electrophoretically separated under native (NAT) conditions and then electrophoresed in the second dimension under reducing (R) conditions (2DE-NAT/R).

kDa in figure 2d clearly demonstrated the change in relative electrophoretic mobility between the denatured and the native receptor complex. The minor spot in figure 2d represents monomeric receptor subunits which possibly disintegrated from the large native complex during subcellular fractionation procedures and/or electrophoretic separation.

Immunoblotting with antibodies to the β- and γ-subunits of the dihydropyridine receptor did not produce sufficiently good labeling for proper evaluation of their relative position in the various gel electrophoresis systems employed (not shown). Nevertheless, we could clearly show that both large dihydropyridine receptor subunits exist under native conditions in a supramolecular triad complex.

Determination of Ca^{2+}-ATPase complex formation as an internal standard of two-dimensional gel electrophoresis. The fast-twitch SERCA1 isoform of the sarcoplasmic reticulum Ca^{2+}-ATPase was previously shown to exist as an oligomeric structure, estimated to be a tetramer. This abundant muscle membrane protein is thus an ideal candidate for a postive control in the standardization of the various gel electrophoretic techniques employed in this study. As shown in figure 3a, monoclonal antibody IIH11 recognizes exclusively the SERCA1 band of apparent 110 kDa under reducing conditions. This protein band broadened considerably under non-reducing conditions and higher bands representing tetrameric Ca^{2+}-pump units became visible. In diagonal non-reducing/reducing two-dimensional gel electrophoresis, a distinct shift of the immunoreactive signal off the diagonal from apparent 110 kDa to approximately 500 kDa was visible (fig. 3b). This agrees with previous studies on the oligomeric structure of the Ca^{2+}-ATPase [17, 18] and suggests that the homo-tetrameric structure of this ion pump is very tightly associated. Isoelectric focusing suggested that probably two distinct SERCA1 complexes exist with respect to their isoelectric point, i.e., one with a pI value of approximately 6 and another with a pI value of approximately 7 (fig. 3c). The oligomeric structure of the physiologically active Ca^{2+}-pump units was confirmed by native two-dimensional gel electrophoresis. Monoclonal antibody IIH11 recognized a large Ca^{2+}-ATPase complex possibly representing an even higher number of monomer copies, as suggested by non-reducing gel electrophoresis (fig. 3d). Overall, these findings with a well-established oligomeric muscle protein could be used as a positive control in our immunoblot analysis and demonstrate the suitability of the two-dimensional gel electrophoresis methods employed in the characterization of protein-protein interactions.

Determination of calsequestrin monomers as a negative control in two-dimensional gel electrophoresis. Although calsequestrin is believed to exist in native muscle membranes as an aggregate and the physiologically active units are proposed to form dimers, these complexes are highly sensitive and disintegrate during muscle membrane homogenization and subcellular fractionation procedures. This sarcoplasmic reticulum component is therefore suitable as a negative control in two-dimen-

sional electrophoresis techniques used to study complex formation of membrane proteins. As can be seen in figure 4a, monoclonal antibody $VIIID1_2$ recognized three protein bands of higher relative molecular mass, the so-called calsequestrin-like proteins [20], besides the 63-kDa calsequestrin monomer. No major difference was detected between reducing and non-reducing conditions in one-dimensional gels. This was confirmed by the immunoblotting of diagonal non-reducing/reducing two-dimensional gels which showed that calsequestrin

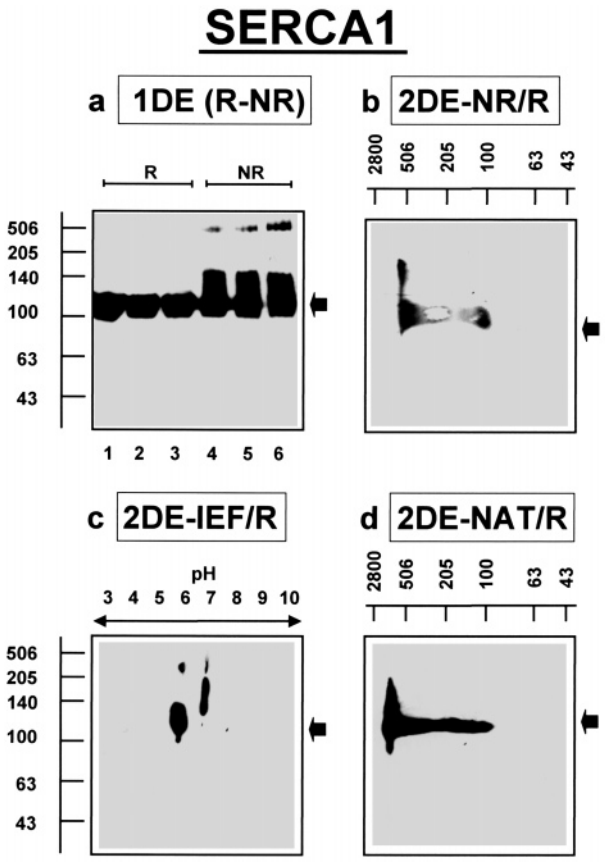

Figure 3. Determination of Ca^{2+}-ATPase complex formation by two-dimensional gel electrophoresis. Shown are immunoblots of skeletal muscle membrane preparations labeled with monoclonal antibody IIH11 to the fast-twitch isoform of the sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPase (SERCA1). Immunodecorated bands are marked by arrows. The relative sizes of molecular-mass standards (in kDa), as deduced from rat myofibril marker proteins, are indicated on the left and at the top of two-dimensional gels. (*a*) Lanes 1 and 4, 2 and 5, and 3 and 6 represent crude microsomes, sarcoplasmic reticulum membranes, and triad membranes, respectively. Samples were electrophoresed in lanes 1–3 under reducing conditions (R) and in lanes 4–6 under non-reducing conditions (NR) using standard one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1DE). (*b*) Triad membranes were first electrophoretically separated under non-reducing (NR) conditions and then electrophoresed in the second dimension under reducing (R) conditions (2DE-NR/R). (*c*) In the first dimension, samples underwent isoelectric focusing (IEF), followed by a second-dimension electrophoretic separation under reducing (R) conditions (2DE-IEF/R). (*d*) Triads were first electrophoretically separated under native (NAT) conditions and then electrophoresed in the second dimension under reducing (R) conditions (2DE-NAT/R).

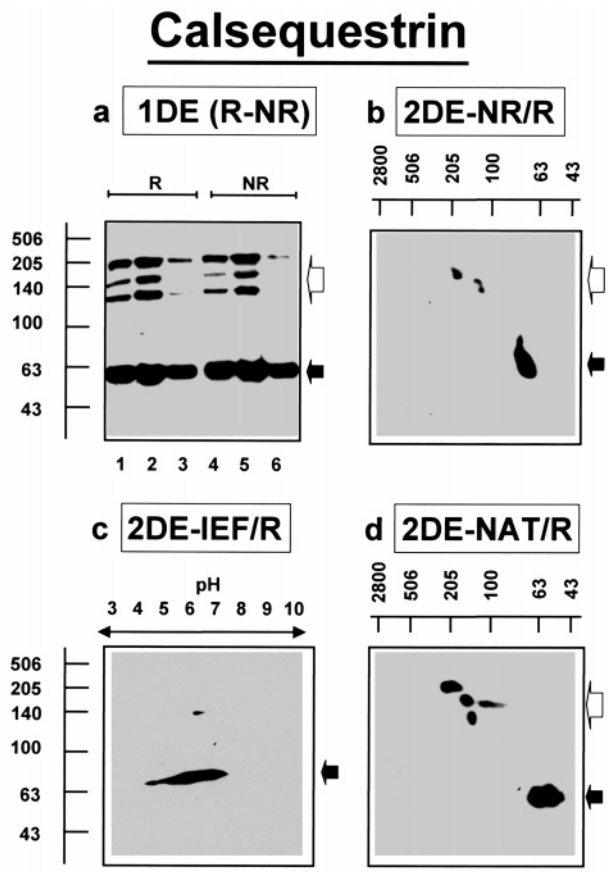

Figure 4. Immunoblot analysis of calsequestrin following two-dimensional gel electrophoresis. Shown are immunoblots of skeletal muscle membrane preparations labeled with monoclonal antibody $VIIID1_2$ to the fast-twitch isoform of the terminal cisternae Ca^{2+}-binding protein calsequestrin. The relative sizes of molecular-mass standards (in kDa), as deduced from rat myofibril marker proteins, are indicated on the left and at the top of two-dimensional gels. (*a*) Lanes 1 and 4, 2 and 5, and 3 and 6 represent crude microsomes, sarcoplasmic reticulum membranes and triad membranes, respectively. Samples were electrophoresed in lanes 1–3 under reducing conditions (R) and in lanes 4–6 under non-reducing conditions (NR) using standard one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1DE). (*b*) Triad membranes were first electrophoretically separated under non-reducing (NR) conditions and then electrophoresed in the second dimension under reducing (R) conditions (2DE-NR/R). (*c*) In the first dimension, samples underwent isoelectric focusing (IEF), followed by a second-dimension electrophoretic separation under reducing (R) conditions (2DE-IEF/R). (*d*) Triads were first electrophoretically separated under native (NAT) conditions and then electrophoresed in the second dimension under reducing (R) conditions (2DE-NAT/R). Immunodecorated bands are marked by arrows.

and calsequestrin-like proteins did not shift off the diagonal. Following isoelectric focusing, the calsequestrin monomer of apparent 63 kDa exhibited a broad-spectrum pH range with pI values ranging from 4 to 8. This probably reflects the different phosphorylation status of subsets of calsequestrin pools within the terminal cisternae lumen. In native two-dimensional gels, none of the calsequestrin species shifted off the diagonal between the native first dimension and the denaturing second dimension (fig. 4d). Thus, in stark contrast to the other three muscle membrane proteins investigated in this study, calsequestrin was represented by monomers in this gel system and this finding could be used as a suitable control in our oligomerization analysis.

Discussion

The excitation-contraction-relaxation cycle of skeletal muscle fibers is mediated by a variety of membrane protein complexes [32]. Of central importance is the voltage-sensing dihydropyridine receptor of the transverse tubular membrane system [2]. The physical coupling hypothesis of excitation-contraction coupling suggests a direct interaction between the α_{1S}-subunit of the dihydropyridine receptor and the ryanodine receptor Ca^{2+}-release channel complex [4]. A previous study by Chang and Hosey [27] suggested that these complexes exist in vivo as relatively tightly associated supramolecular structures. Isolating them in their native configuration should therefore be possible. Here, we employed a variety of two-dimensional gel electrophoresis techniques to estimate the relative size of the native muscle dihydropyridine receptor complex. Assuming an approximate one-to-one ratio between the individual subunits in the physiologically active dihydropyridine receptor, a junctional complex consisting of the main α_{1S}-subunit (170 kDa) and its auxiliary subunits α_2-δ (175 kDa), β (52 kDa), and γ (32 kDa) should not run in its native form at a gel electrophoretic position much above 450–500 kDa [2, 13, 14]. However, immunoblotting of both the α_1- and the α_2-subunit following two-dimensional gel electrophoresis with a native first dimension revealed a relative electrophoretic mobility of the dihydropyridine receptor comparable to the gigantic muscle protein titin of approximately 2800 kDa. The established sizes of sarcoplasmic reticulum Ca^{2+}-ATPase tetramers and calsequestrin monomers were used for the internal standardization of the gel systems employed in this study. The difference in molecular mass between a complex (containing presumably one copy of each individual subunit) and the actual native complex suggests that the transverse tubular voltage-sensor exists in the native triad membrane within a supramolecular complex. Since salt washes clearly disrupt the proposed native tetrad configuration [33], relatively weak non-covalent forces between receptor domains are probably responsible for the formation of larger membrane complexes.

Our findings support the concept that tetrad structures, as detectable by electron microscopical freeze-fracture analysis [5–12], represent dihydropyridine receptor complexes forming a large membrane assembly. Native dihydropyridine receptors might be clustered in tightly associated junctional units containing four copies of individual subunits, thereby increasing the overall size of the complex, as diagrammatically represented in figure 5. In addition, direct and/or indirect interactions between the dihydropyridine receptor and other junctional sarcoplasmic reticulum proteins, such as the ryanodine receptor, triadin, junctin, calsequestrin, and/or the 90-kDa junctional face protein [34] might at least partially induce the great increase in relative molecular mass seen in native gels [32]. Based on results from chemical cross-linking studies [33], these high-molecular-mass structures are possibly more heterogeneous and contain other sarcoplasmic reticulum components besides the dihydropyridine receptor. Immunoblotting with antibodies to various other microsomal proteins did not result in satisfactory labeling using two-dimensional native gel systems. We were therefore unable to investigate further the exact composition of this high-molecular-mass triad complex. However, the tight association between the voltage-sensor tetrad structures and other Ca^{2+}-regulatory excitation-contraction coupling components is also likely to play a part in the drastic increase in the relative molecular mass of the native dihydropyridine receptor.

This report agrees with the current molecular concept of excitation-contraction coupling which holds that large Ca^{2+}-regulatory membrane complexes are involved in the signal transduction process at the triad junction in mature skeletal muscle fibers [32]. It is well established that the α_{1S}-dihydropyridine receptor acts as the voltage-sensor of the skeletal muscle cell periphery and that its auxiliary α_2/δ-β-γ subunits modulate the ion channel physiology [1, 2, 13, 14]. Following voltage-sensing via the α_{1S}-subunit, direct linkage between small stretches of primary sequence in the II-III loop domain of the α_{1S}-dihydropyridine receptor and the cytoplasmic domain of the Ca^{2+}-release channel trigger the release of Ca^{2+} ions from the sarcoplasmic reticulum [4]. It remains to be determined whether different molecular mechanisms underlie purely structural or functional receptor coupling processes and how many triad membrane proteins are actually involved in preventing a passive disintegration of junctional membrane complexes. The junctional component triadin appears to form disulfide-bonded clusters which form a link be-

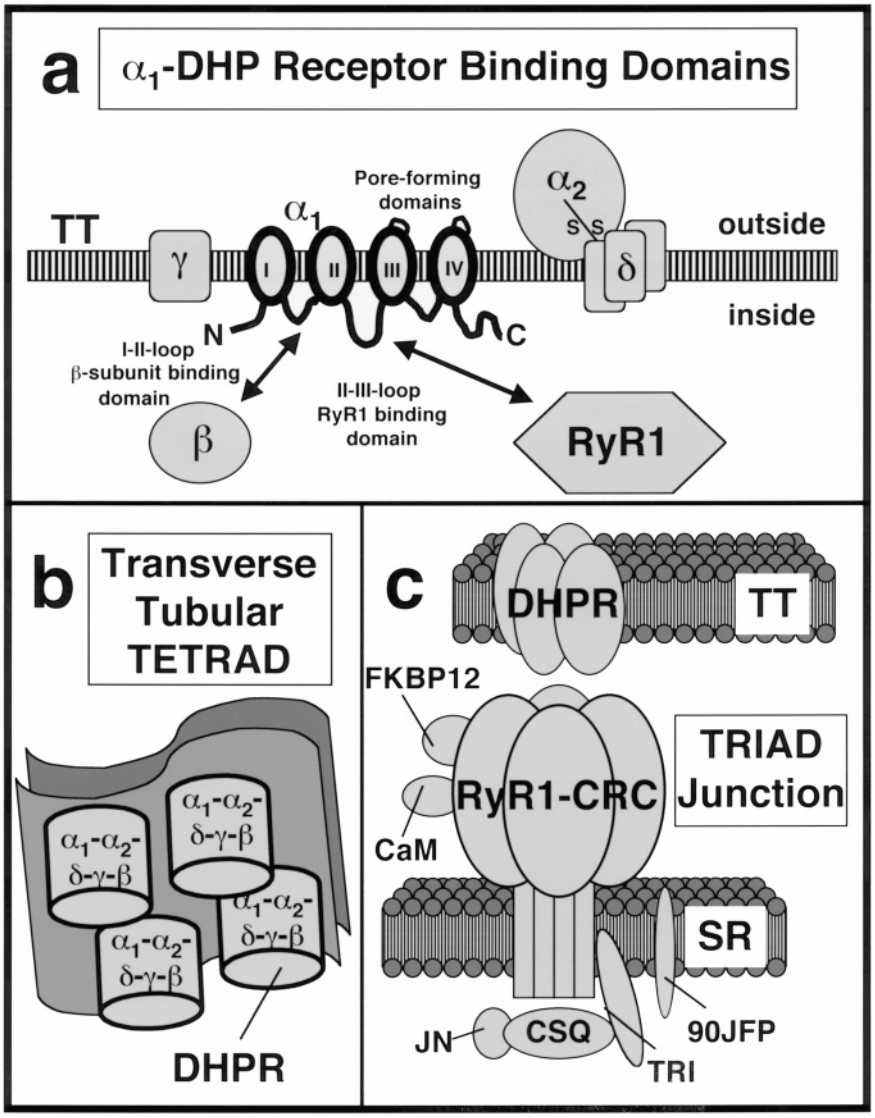

Figure 5. Model of the supramolecular dihydropyridine receptor (DHPR) complex. Shown are the molecular arrangement of the skeletal muscle DHPR consisting of the principal α_1-subunit with its distinct I-II loop and II-III loop, binding domains for its β-subunit and the ryanodine receptor RyR1 isoform, respectively, the auxiliary α_2-subunit and its disulfide-bridged connection with the three δ-subunits, and the β- and γ-subunits (*a*). Freeze-fracture electron microscopical studies have suggested that the native DHPR exists in a tetrad structure (*b*) and this report supports the existence of such a high-molecular-mass complex. During excitation-contraction coupling, the DHPR complex of the transverse tubules (TT) forms a direct physical linkage with the junctional ryanodine receptor Ca^{2+}-release channel units (RyR1-CRC) of the sarcoplasmic reticulum (SR). Within the triad junctions of adult skeletal muscle fibers, other ion-regulatory proteins are proposed to be linked to this receptor assembly, i.e., triadin (TRI), the junctional face protein of apparent 90 kDa (90JFP), calsequestrin (CSQ), junctin (JN), calmodulin (CaM), and the regukatory FKBP12 subunit of the ryanodine receptor (*c*).

tween the ryanodine receptor and the terminal cisternae Ca^{2+}-binding protein calsequestrin [35, 36]. A quaternary complex consisting of triadin, the ryanodine receptor, calsequestrin, and the calsequestrin-binding protein junctin has been described for heart sarcoplasmic reticulum [37]. Thus, a junctional complex of similar composition probably exists in skeletal muscle in very close proximity to the dihydropyridine receptor tetrads, thereby forming a gigantic triad membrane structure. The high-molecular-mass complex consisting of dihydropyridine receptor units appears to be the structural basis of voltage-sensing during the excitation-contraction coupling process, a unique cell biological phenomena involving ion-regulatory muscle membrane proteins.

Acknowledgements. Research in the authors' laboratory was supported by a Training and Mobility of Researchers grant from the European Community (FMRX-CT960032). We would like to thank Ms. Alison Murphy (Conway Institute, University College Dublin) for expert technical assistance and Kevin P. Campbell (University of Iowa) for providing our laboratory with monoclonal antibodies to muscle proteins.

1 Melzer W., Herrmann-Frank A. and Lüttgau H. C. (1995) The role of Ca^{2+} ions in excitation-contraction coupling of skeletal muscle fibres. Biochim. Biophys. Acta **1241:** 59–116
2 Catterall W. A. (1995) Structure and function of voltage-gated ion channels. Annu. Rev. Biochem. **64:** 493–532
3 Franzini-Armstrong C. and Protasi F. (1997) Ryanodine receptors of striated muscles: a complex channel capable of multiple interactions. Physiol. Rev. **77:** 699–729
4 Leong P. and MacLennan D. H. (1998) Complex interactions between skeletal muscle ryanodine receptor and dihydropyridine receptor proteins. Biochem. Cell Biol. **76:** 681–694
5 Block B. A., Imagawa T., Campbell K. P. and Franzini-Armstrong C. (1988) Structural evidence for direct interaction between the molecular components of the transverse tubules/sarcoplasmic reticulum junction in skeletal muscle. J. Cell Biol. **107:** 2587–2600
6 Franzini-Armstrong C. and Nunzi G. (1983) Junctional feet and particles in the triads of a fast-twitch muscle fibre. J. Muscle Res. Cell Motil. **4:** 233–252
7 Osame M., Engel A. G., Rebouche C. J. and Scott R. E. (1981) Freeze-fracture electron microscopic analysis of plasma membranes of cultured muscle cells in Duchenne dystrophy. Neurology **31:** 972–979
8 Franzini-Armstrong C. (1984) Freeze-fracture of frog slow tonic fibres: structure of surface and internal membranes. Tissue Cell **16:** 146–166
9 Franzini-Armstrong C. and Jorgensen A. O. (1994) Structure and development of E-C coupling units in skeletal muscle. Annu. Rev. Physiol. **56:** 509–534
10 Flucher B. E. and Franzini-Armstrong C. (1996) Formation of junctions involved in excitation-contraction coupling in skeletal and cardiac muscle. Proc. Natl. Acad. Sci. USA **93:** 8101–8106
11 Franzini-Armstrong C., Pincon-Raymond M. and Rieger F. (1991) Muscle fibres from dysgenic mouse in vivo lack a surface component of peripheral couplings. Dev. Biol. **146:** 364–376
12 Takekura H., Bennett L., Tanabe T., Beam K. G. and Franzini-Armstrong C. (1994) Restoration of junctional tetrads in dysgenic myotubes by dihydropyridine receptor cDNA. Biophys. J. **67:** 793–803
13 Isom L. L., DeJong K. S. and Caterall W. A. (1994) Auxilliary subunits of voltage-gated ion channels. Neuron **12:** 1183–1194
14 Gurnett C. A. and Campbell K. P. (1996) Transmembrane auxiliary subunits of voltage-dependent ion channels. J. Biol. Chem. **271:** 27975–27978
15 MacLennan D. H., Rice W. J. and Green N. M. (1997) The mechanism of Ca^{2+} transport by sarco(endo)plasmic reticulum Ca^{2+}-ATPases. J. Biol. Chem. **272:** 28815–28818
16 Yano K. and Zarain-Herzberg A. (1994) Sarcoplasmic reticulum calsequestrins: structural and functional properties. Mol. Cell Biochem. **135:** 61–70
17 Martonosi A. N. (1995) The structure and interactions of Ca^{2+}-ATPase. Biosci. Rep. **15:** 263–282
18 Maguire P. B. and Ohlendieck K. (1996) Oligomerization of sarcoplasmic reticulum Ca^{2+}-ATPase from rabbit skeletal muscle. FEBS Lett. **396:** 115–118
19 Ohkura M., Furukawa K., Fujimori H., Kuruma A., Kawano S., Hiraoka M. et al. (1998) Dual regulation of the skeletal muscle ryanodine receptor by triadin and calsequestrin. Biochemistry **37:** 12987–12993
20 Maguire P. B., Briggs F. N., Lennon N. J. and Ohlendieck K. (1997) Oligomerization is an intrinsic property of calsequestrin in normal and transformed skeletal muscle. Biochem. Biophys. Res. Commun. **240:** 721–727
21 Ohlendieck K., Briggs F. N., Lee K. F., Wechsler A. W. and Campbell K. P. (1991) Analysis of excitation-contraction coupling components in chronically stimulated canine skeletal muscle. Eur. J. Biochem. **202:** 739–747
22 Froemming G. R. and Ohlendieck K. (1999) Isoform-specific interactions between halothane and the ryanodine receptor Ca^{2+}-release channel: implications for malignant hyperthermia and the protein theory of anesthetic action. Naturwissenschaften **86:** 584–587
23 Froemming G. R., Murray B. E. and Ohlendieck K. (1999) Self-aggregation of triadin in the sarcoplasmic reticulum of rabbit skeletal muscle. Biochim. Biophys. Acta **1418:** 197–205
24 Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248–254
25 O'Farrell P. Z., Goodman H. M. and O'Farrell P. H. (1977) High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell **12:** 1133–1141
26 Molloy M. P. (2000) Two-dimensional electrophoresis of membrane proteins using immobilized pH gradients. Anal. Biochem. **280:** 1–10
27 Chang C. F. and Hosey M. M. (1990) A combined non-denaturating and denaturating gel electrophoretic analysis of the subunit composition of a membrane protein: the skeletal muscle L-type calcium channel. Biochem. Biophys. Res. Commun. **172:** 751–758
28 Laemmli U. K. (1970) Cleavage of bacteriophage T7 early RNAs and proteins on slab gels. Nature **227:** 680–685
29 Froemming G. R. and Ohlendieck K. (1999) Complex formation of skeletal muscle Ca^{2+}-regulatory membrane proteins by halothane. Eur. J. Pharmacol. **365:** 91–102
30 Towbin H., Staehelin T. and Gordon J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:** 4350–4354
31 Jay S. D., Sharp A. H., Kahl S. D., Vedvick T. S., Harpold M. M. and Campbell K. P. (1991) Structural characterization of the dihydropyridine-sensitive calcium channel alpha 2-subunit and the associated delta peptides. J. Biol. Chem. **266:** 3287–3293
32 Murray B. E., Froemming G. R., Maguire P. B. and Ohlendieck K. (1998) Excitation-contraction-relaxation cycle: role of the Ca^{2+}-regulatory membrane proteins in normal, stimulated and pathological skeletal muscle. Int. J. Mol. Med. **1:** 677–687
33 Murray B. E. and Ohlendieck K. (1997) Cross-linking analysis of the ryanodine receptor and α_1-dihydropyridine receptor in rabbit skeletal muscle. Biochem. J. **324:** 689–696
34 Froemming G. R., Pette D. and Ohlendieck K. (1999) The 90 kDa junctional sarcoplasmic reticulum protein forms an integral part of a supramolecular triad complex in skeletal muscle. Biochem. Biophys. Res. Commun. **261:** 603–609
35 Guo W. and Campbell K. P. (1995) Association of triadin with the ryanodine receptor and calsequestrin in the lumen of the sarcoplasmic reticulum. J. Biol. Chem. **270:** 9027–9030
36 Guo W., Jorgensen A. O. and Campbell K. P. (1996) Triadin, a linker for calsequestrin and the ryanodine receptor. Soc. Gen. Physiol. Ser. **51:** 19–28
37 Zhang L., Kelley J., Schmeisser G., Kobayashi Y. M. and Jones L. R. (1997) Complex formation between junctin, triadin, calsequestrin and the ryanodine receptor: proteins of the cardiac junctional sarcoplasmic reticulum membrane. J. Biol. Chem. **272:** 23389–23397