J. Membrane Biol. 131, 219–228 (1993)

The Journal of
Membrane Biology

Detection and Localization of Triadin in Rat Ventricular Muscle

Neil R. Brandt†, Anthony H. Caswell†, Stephanie A. Lewis Carl‡, Donald G. Ferguson‡, Tara Brandt†, Jean-Pierre Brunschwig†, and Arthur L. Bassett†

†Department of Molecular and Cellular Pharmacology, University of Miami School of Medicine, Miami, Florida, and ‡Department of Physiology and Biophysics, University of Cincinnati, College of Medicine, Cincinnati, Ohio

Summary. Dyads (transverse tubule—junctional sarcoplasmic reticulum complexes) were enriched from rat ventricle microsomes by continuous sucrose gradients. The major vesicle peak at 36% sucrose contained up to 90% of those membranes which possessed dihydropyridine (DHP) binding sites (markers for transverse tubules) and all membranes which possessed ryanodine receptors and the putative junctional foot protein (markers for junctional sarcoplasmic reticulum). In addition, the 36% sucrose peak contained half of the vesicles with muscarine receptors. Vesicles derived from the nonjunctional plasma membrane as defined by a low content of dihydropyridine binding sites per muscarine receptor and from the free sarcoplasmic reticulum as defined by the M_r 102K Ca^{2+} ATPase were associated with a diffuse protein band (22–30% sucrose) in the lighter region of the gradient. These organelles were recovered in low yield. Putative dyads were not broken by French press treatment at 8,000 psi and only partially disrupted at 14,000 psi. The monoclonal antibody GE4.90 against skeletal muscle triadin, a protein which links the DHP receptor to the junctional foot protein in skeletal muscle triad junctions, cross-reacted with a protein in rat dyads of the same M_r as triadin. Western blots of muscle microsomes from preparations which had been treated with 100 mM iodoacetamide throughout the isolation procedure showed that cardiac triadin consisted predominantly of a band of M_r 95 kD. Higher molecular weight polymers were detectable but low in content, in contrast with the ladder of oligomeric forms in rat psoas muscle microsomes. Cardiac triadin was not dissolved from the microsomes by hypertonic salt or Triton X-100, indicating that it, as well as skeletal muscle triadin, was an integral protein of the junctional SR. The cardiac epitope was localized to the junctional SR by comparison of its distribution with that of organelle markers in both total microsome and in French press disrupted dyad preparations. Immunofluorescence localization of triadin using mAb GE4.90 revealed that intact rat ventricular muscle tissue was stained following a well-defined pattern of bands every sarcomere. This spacing of bands was consistent with the interpretation that triadin was present in the dyadic junctional regions.

Key Words triadin · rat dyads · excitation-contraction coupling · transverse tubule · junctional sarcoplasmic reticulum

Introduction

Most current concepts for the organization and composition of the cardiac dyad junction are based upon analogies with the skeletal muscle triad. The triad junction, where the terminal cisterna of the sarcoplasmic reticulum is held in regular apposition to the transverse (T-) tubule invagination of the sarcolemma, has been shown to be the site of excitation-contraction coupling (Gage & Eisenberg, 1969). The morphological similarities between skeletal muscle and heart suggest that the dyad may contain similar proteins. The junctional SR's of both tissues contain a ryanodine-sensitive Ca^{2+} release channel which is most likely the junctional foot protein (JFP); these two proteins, however, are coded on different genes (Otsu et al., 1990). Similarly, both types of T-tubules contain dihydropyridine (DHP) sensitive Ca^{2+} channels although the functions appear to be different.

Despite the strong resemblance of cardiac dyads to skeletal muscle triads, there is strong evidence that the mechanism of excitation-contraction coupling is different. There is general recognition that the major mechanisms of transmission in the heart is through the cytoplasmic transmitter Ca^{2+} (Fabiato, 1989) while in skeletal muscle Ca^{2+} is not thought to serve as the primary control of Ca^{2+} release (Rios, Ma & Gonzalez, 1991). There is considerable evidence that the DHP receptor acts as the membrane voltage sensor in skeletal muscle without the necessity of Ca^{2+} flow (Rios & Brum, 1987). In cardiac muscle, where Ca^{2+}-induced Ca^{2+} release is the likely mechanism, the DHP receptor acts as a true channel (Cleemann & Morad, 1991). The differences between the two muscle types are reflected in differences in the sequence of the two DHP receptor isoforms (Tanabe et al., 1990).

Since differences in the composition of skeletal muscle and cardiac muscle junctional constituents may reflect and illuminate the differences in those physiological properties, we have derived preparations of dyads from rat ventricular muscle. Dyads have been identified in the ventricular muscle microsomes from several species (Brandt, 1985; Brandt & Bassett, 1986; Doyle et al., 1986) following the

principles developed in the isolation of skeletal muscle triads (Caswell et al., 1988). Markers for the T-tubule and the junctional SR codistribute on isopycnic sucrose gradients unless the junction is disrupted by mechanical or chemical treatments. Surprisingly, attempts to identify and isolate dyads from rat ventricular muscle microsomes have not been reported even though the rat has been most extensively used in physiological and pathophysiological studies. We report here the identification and initial characterization of dyads in rat ventricular muscle microsomes. These dyads, although they have several properties in common with those from other species, show distinct species specific characteristics.

We have recently identified a new protein in the skeletal muscle triad junction called triadin (Brandt et al., 1990; Kim et al., 1990*b*; Caswell et al., 1991). This protein maintains a linkage between the DHP-receptor and the JFP and may be involved in transduction of the E-C coupling signal (Brandt et al., 1992*a*). We show here that the monoclonal antibody against triadin cross-reacts with a protein of similar M_r in rat cardiac dyads and co-distributes with junctional markers when cardiac microsomes are centrifuged on isopycnic gradients. Immunofluorescence microscopy with the mAb against triadin shows high reactivity against presumptive junctional regions of ventricular cells which are disposed in a regular sarcomeric pattern.

Materials and Methods

Membrane Preparations

Sprague-Dawley male rats (3–5 months old, 300 to 350 gm) were employed for most studies. Hearts were excised from heparinized animals under surgical anesthesia and cleared of blood in oxygenated and heparinized Ringers/HCO_3^- by rhythmical clamping of the aortic stump. Microsomes were prepared from total ventricular muscle by the protocol previously reported for cat tissue (Brandt & Bassett, 1986). Ventricles from 3–4 rats were homogenized in 20 ml of buffered sucrose (250 mM sucrose, 3 mM histidine, 2 mM dithiothreitol, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, pH 7.3) for 30 sec in a Waring blender minicup. The homogenate was sedimented at 10,000 × *g* for 15 min and the supernatant decanted through eight layers of cheesecloth. The supernatant was centrifuged for 35 min at 140,000 × *g* and the pellet washed once with buffered sucrose which contained 25 mM Na pyrophosphate pH 7.4. The microsomes were then centrifuged on linear (15–50%, w/w) sucrose gradients in the Beckman SW 41 rotor at 20,000 rpm overnight. In experiments comparing rat ventricle and rat psoas muscle, microsomes from both muscles were isolated in a similar sucrose medium except that the dithiothreitol was replaced by 100 mM iodoacetamide. After removal of blood, the tissues were soaked in the medium for 10 min prior to homogenization. Centrifugation protocols were as described above. Terminal cisternae/triads were prepared for rabbit back muscle as described by Caswell, Lau and Brunschwig (1976).

Biochemical and Pharmacological Assays

Muscarine receptors and dihydropyridine (DHP) binding sites were assayed by Millipore filtration employing 0.5 nM [^{3}H]quinuclidinyl benzilate (QNB) and 0.5 nM [^{3}H]PN 200–110, respectively, as ligands and Whatman GF/C filters as described in an earlier communication (Brandt & Bassett, 1986). Ligand concentrations were saturating. Ryanodine binding was determined with 3.4 nM [^{3}H] ryanodine in 600 mM NaCl, 1.0 mM $CaCl_2$, 20 mM Tris MOPS, pH 7.1 as described by Seifert and Cassida (1986). At this ligand concentration, which is below the K_D (~6 nM), the nonspecific binding is at filter background. Membrane proteins were phosphorylated with the catalytic subunit of protein kinase A and [^{32}P]γATP as described by Dombradi et al. (1984). Labeled proteins were detected by autoradiography after sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Electrophoresis and Immunology

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 5% Laemmli slab gels unless otherwise noted in the figure legends. All samples were prepared in normal solubilizing medium (2.5% 2-mercaptoethanol, 1% SDS, 0.03 M TrisCl, pH 6.8) except when described as nonreduced samples (no 2-mercaptoethanol). Gels were either stained with Coomassie blue or Stains All (Campbell, MacLennan & Jorgensen, 1983) or were Western blotted onto nitrocellulose using the Genie (Ideal Scientific) apparatus with a 12-volt battery charger (Sears) for 90 min. Blots were blocked with 3% bovine serum albumin (BSA) in Tris buffered saline (TBS: 200 mM NaCl, 20 mM TrisCl, pH 7.4) and then incubated with the monoclonal antibody GE4.90 against triadin in TBS, 1% BSA as characterized by Caswell et al. (1992). Development was carried out by successively incubating the blots with biotinylated anti-mouse IgG, peroxidase-streptavidin and then 4-chloronaphthol and H_2O_2 as substrates. ELISA reactions were carried out on gradient fractions using anti-triadin mAB GE4.90 as primary antibody and the biotin streptavidin system described above except that 2,2′ azinobis (3-ethylbenzothiazoline-sulfonate) in 0.1 M Na citrate, pH 4.5 was used as the final substrate. For some experiments, $\log_2$ (concentration) *vs.* log (OD_{405}) plots were constructed with constant slopes and ELISA values read from a common intercept. ELISA OD_{405} values were also used directly from a single dilution when material was limited. The distribution curves obtained by the two methods were identical when the maximal response was not saturated.

Data Analysis

The distribution patterns for protein and ligand binding assays when ventricular muscle microsomes were centrifuged on linear sucrose gradients were analyzed using the nonlinear curve fitting program of SigmaPlot (Jandel Scientific) software. For each function of each gradient assayed, the maximum value was set to 1.0 and all other values normalized. The normalized values and the corresponding sucrose densities were then compiled and data fitted to a double Gaussian distribution modeled by the equation:

$$f = a_1*\exp(-0.5*((x - c_1)/w_1)\hat{}2))) + a_2*\exp(-0.5*((x - c_2)/w_2)\hat{}2)))$$

where a, c and w refer to the peak amplitude, center and width. Iteration was allowed to proceed to the default tolerance value (0.001). The model curve was then plotted using small increments of x (sucrose density).

Cryosectioning and Immunofluorescent Labeling

Strips of ventricular muscle tissue were pinned at rest length in a relaxing solution containing 4 mM EGTA for 10 min, mounted on cork specimen holders using gum tragacanth, and flash frozen in liquid-nitrogen-cooled isopentane. Sections, 5 μM thick, were obtained using a Micron cryostat (Carl Zeiss), collected on gelatin-coated glass slides and immunostained immediately. Prior to staining, sections were fixed to the slides using freshly prepared 4% paraformaldehyde for 15 min and washed in phosphate buffered saline (PBS) (3 changes of 5 min each). Sections were then incubated for 15 min in 0.5% Triton X-100 and washed three times in PBS. Slides were incubated for 60 min in unconjugated goat anti-rat and goat anti-mouse IgG (H + L) diluted 1:30 in PBS containing 1% BSA, to block nonspecific binding sites. Sections were incubated overnight at 4°C in the triadin monoclonal antibody GE 4.90. Three PBS/BSA washes of 10 min each were used to remove unbound primary antibody. Sections were subsequently stained using goat anti-mouse IgG antibody conjugated to rhodamine for 1 hr. Excess secondary antibody was washed off using three 10 min changes of PBS/BSA and coverslips were mounted using a 70% glycerol : PBS mixture containing 4% 1,4 Diaza bicyclo-[2,2,2]octane. In control experiments, sections were incubated with PBS/BSA followed by secondary antibody.

Materials

All radioactive compounds were purchased from New England Nuclear. Ryanodine was a gift from S.P. Pennick Company. All other chemicals were of at least reagent grade.

Results

Identification of Organelle Constituents of Rat Ventricular Muscle Microsomes

Microsomes from rabbit (Brandt, 1985), cat (Brandt & Bassett, 1986), cow and sheep (Doyle et al., 1986) ventricular muscle centrifuge as a single broad band (mean buoyant density 30% sucrose) on isopycnic gradients. Organelle specific marker scans reveal that this is a heterogenous population comprised of free plasma membrane (24% sucrose), T-tubule—junctional SR dyads (33% sucrose) and longitudinal reticulum or free SR as the major component (30% sucrose). In contrast, rat microsomes separate on continuous sucrose gradients into two visually distinct populations: a diffuse white band between 20–30% sucrose and a sharp, yellow and partially coagulated band between 34 and 37% sucrose.

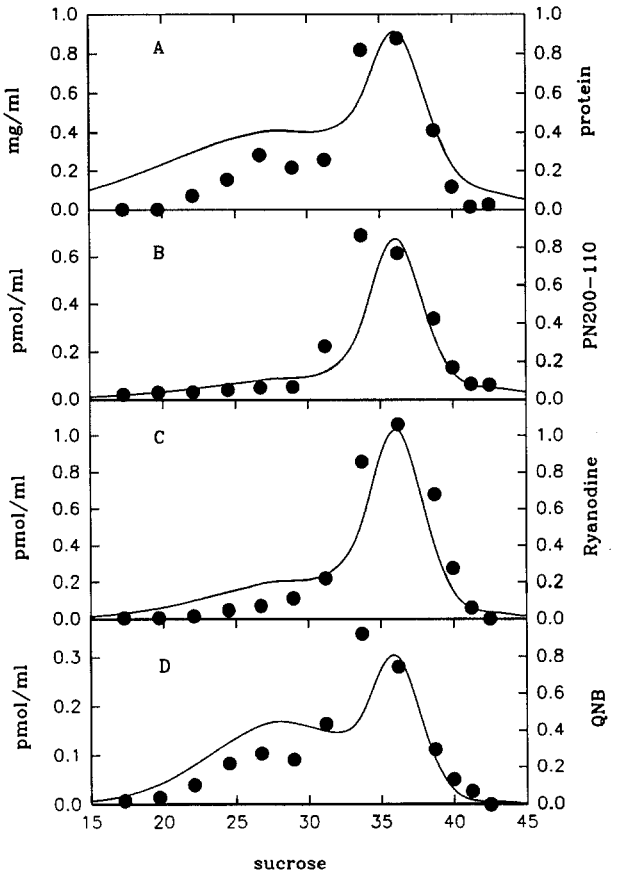

Fig. 1. Distribution of membrane marker activities after centrifugation of rat cardiac microsomes on continuous sucrose gradients. Microsomes were prepared from rat ventricular muscle as described in Materials and Methods and centrifuged on linear sucrose (15–50%) gradients to equilibrium. The gradient was scanned for protein (Panel *A*) and ryanodine binding as an assay for junctional SR (Panel *B*), PN 200-110 binding to DHP receptors (Panel *C*) and QNB binding as an assay for muscarine acetylcholinergic receptors (Panel *D*). The data points are from a single preparation; the solid lines were generated from the best fit to a double Gaussian distribution using the normalized data (scale 0 to 1.0 on right vertical axis) from 10 microsomal preparations.

Figure 1 shows the protein and organelle marker distribution patterns for a single rat ventricle microsome preparation (data points) and the fitted curve (solid line) for 10 preparations centrifuged on linear 15–50% sucrose gradients. The positions of the two visualized vesicle populations is reflected in the protein distribution pattern (Fig. 1*A*). The broad lighter peak is centered at 28% sucrose while the sharp peak appears at 36% sucrose.

Dihydropyridine receptors are exclusively found in transverse tubules of skeletal muscle (Fosset et al., 1982; Brandt, Kawamoto & Caswell,

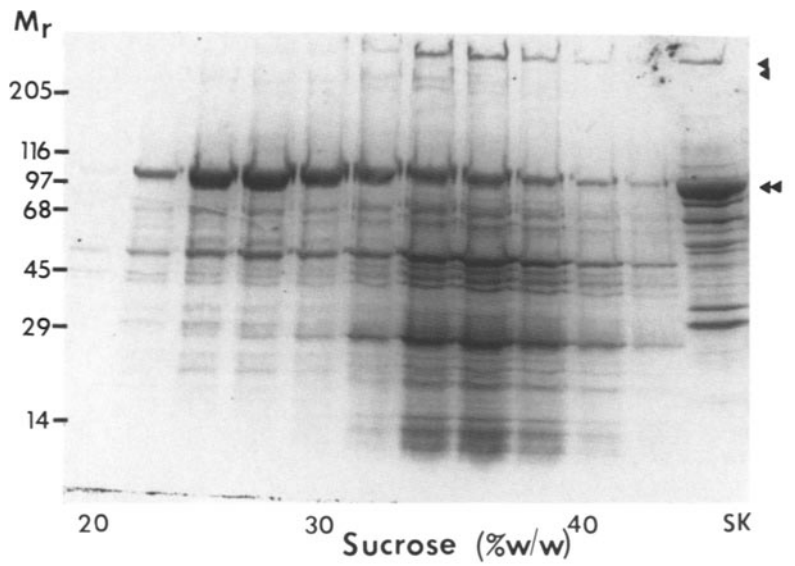

Fig. 2. SDS-PAGE of rat microsome fractions. Equal volume aliquots (30 μl) from the gradient shown in Fig. 1 (data points) electrophoresed on a 5–15% gradient slab gel stained with Coomassie blue. The far right lane (SK) was loaded with 20 μg skeletal muscle TC/triads. The skeletal muscle JFP (M_r 565K) and its first calpain degradation product (M_r 415K) are indicated by single arrowheads. The M_r 102K SR Ca^{2+} ATPase is marked by the double arrowhead. A combination of high and low M_r markers from Sigma were used to calibrate the gel.

1985) and are highly enriched relative to other plasma membrane markers in putative T-tubule fraction of cardiac microsomes (Brandt, 1985). Figure 1*B* shows that the bulk of the DHP receptors of rat cardiac microsomes appear in the sharp peak at 36% sucrose (average specific activity 0.86 pmol/mg for 10 preparations) with a secondary peak at 32% sucrose. A low content of DHP receptors was found in the lighter region of the gradient (20–28% sucrose, average specific activity = 0.1 pmol/mg), the gradient region where nonjunctional plasma membrane from other species has been reported to band (Brandt, 1985).

The distribution pattern for ryanodine receptors (Fig. 1*C*) closely matched that for DHP receptors in position and shape with a dominant peak at 36% sucrose. Under the assay conditions for the gradient scan (3.4 nM ryanodine), the average specific activity was 1.4 pmol/mg. Scatchard analysis of the ryanodine binding curve for the pooled heavy microsome fraction revealed a B_{max} of 6 pmol/mg with a K_D of 5 nM. These fractions are enriched in a high M_r protein (Fig. 2) electrophoresing slightly faster than the skeletal muscle junctional foot protein (M_r 565K) but slower than its first calpain degradation product (M_r 415K) (Brandt et al., 1992*b*). A protein of similar mobility has been identified as the ryanodine-sensitive Ca^{2+} channel in canine cardiac junctional SR (Seiler et al, 1984; Inui, Saito & Fleischer, 1987) and it is therefore probable that the rat cardiac high M_r protein is the JFP of the rat ventricular dyad. Those same gradient fractions were also shown to be enriched in a Stains All blue stained protein of M_r 55K (*not shown*), which is presumable calsequestrin, another marker for junctional SR (Campbell, MacLennan & Jorgensen, 1982). The comigration of the T-tubule (DHP receptor) and junctional SR markers strongly suggests the presence of dyads in the heavy microsome peak fraction. This does not exclude the possibility, however, that unattached junctional SR may also be present in the preparation.

In contrast to the almost monomodal patterns for DHP and ryanodine receptors, two distinct peaks were found in the muscarine acetylcholine receptor pattern as detected by QNB binding (Fig. 1*D*). The relative content in the two peaks (28 and 36% sucrose) was variable among preparations but in most cases 60% of the receptors were associated with the heavy microsome peak. Muscarine receptors have been previously detected in both nonjunctional sarcolemma and T-tubules and the distinction between those two domains of the cell membrane was made upon the relative content of muscarine and DHP receptors. For the rat preparation, the specific activity in the 36% sucrose peak was 0.26 pmol/mg. That specific activity and the content relative to the DHP receptor content is not dissimilar to the values reported for the dyad fractions from other species (Brandt, 1985). On the other hand, the average specific activity (0.23 pmol/mg) in the lighter gradient region is about five-fold lower than that reported for other mammalian cardiac microsome preparations.

The major protein of free SR in ventricular muscle microsome preparations from other species is the Ca^{2+} ATPase, M_r 102K (Jones et al., 1979; Brandt, 1985; Brandt & Bassett, 1986; Inui et al., 1986; Doyle et al., 1986). A band of that mobility which co-electrophoresed with the SR Ca^{2+} ATPase of skeletal muscle was found in all fractions of the rat microsome gradient but predominated in the lighter

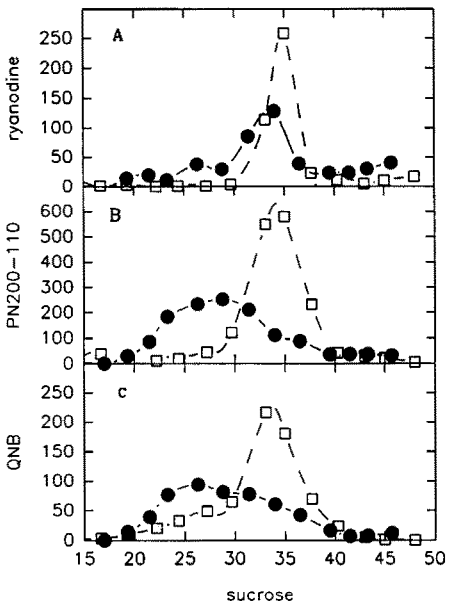

Fig. 3. Distribution of organelle markers after French press treatment of rat dyads. The heavy microsomes containing putative dyads were isolated by centrifugation of rat ventricular muscle microsomes on continuous sucrose gradients. These vesicles were then centrifuged directly (open squares) or after French press extrusion at 13,000 psi (filled circles) on continuous sucrose gradients. After reaching equilibrium, the gradients were scanned for ryanodine binding (Panel *A*), PN200-110 binding (Panel *B*) and QNB binding (Panel *C*) as described in Materials and Methods. All data expressed fmol ligand bound/ml gradient fraction.

sucrose fractions (Fig. 2). Since this SDS-PAGE gel was loaded with equal volume aliquots from the sucrose gradient (Fig. 1*A*, data points for related protein content), it underemphasizes the enrichment of the M_r 102K band in the lower density region. Those gradient fractions enriched in the M_r 102K protein were also enriched in low M_r proteins phosphorylatable by protein kinase A including phospholamban (Manalan & Jones, 1982).

Disruption of Putative Rat Dyads

The co-migration of DHP and ryanodine receptors could indicate that (i) the receptors are on the same membrane, (ii) that the isopycnic points of the T-tubule and junctional SR are identical or (iii) that these two organelles with different intrinsic isopycnic points are held together by bridging structures. Figure 3 shows the distribution of ryanodine (*A*), DHP (*B*) and muscarine (*C*) receptors when the putative dyad fraction was recentrifuged directly (open squares) or after French press extrusion at 13,000 psi (filled circles). The isopycnic point of the ryanodine receptors (Fig. 3*A*) was not changed by the disruptive treatment, indicating that this is the intrinsic buoyant density of the junctional SR. On the other hand, about 2/3 of the DHP receptors were shifted to lighter gradient fractions (Fig. 3*B*), implying a lower isopycnic point for the T-tubules. The distribution patterns for the muscarine receptors on both the untreated and French pressed membranes (Fig. 3*C*) matched the respective distribution patterns for the DHP receptors. The fact that muscarine receptors were not found in lighter gradient fractions when the untreated dyad fraction was recentrifuged suggests that the muscarine receptors are on the T-tubule membrane.

The rat dyads appeared relatively resistant to French press disruption since release of DHP receptors was not detected at 8,000 psi, the pressure which almost completely breaks skeletal muscle triads (Caswell et al., 1988) and cat ventricular muscle dyads (Brandt & Bassett, 1986). Furthermore, breakage did not exceed 70% at pressures up to 14,000 psi; greater pressures considerably reduced all ligand binding activity and smeared the distribution patterns. The DHP receptor distribution pattern for five preparations extruded between 10,000 and 14,000 psi could be modeled as a double Gaussian curve with centers at 30 and 36% sucrose, identical to the minor and major peaks found in the modeled DHP receptor pattern for the total microsome preparation (Fig. 1*B*, solid line). The 30% sucrose peak most likely represents the intrinsic isopycnic point of the T-tubule as it does in the cat (Brandt & Bassett, 1986). The higher French press pressures caused more extensive disruption of the dyads but also caused the appearance of DHP receptor activity at 25 to 30% sucrose.

Detection of a Cardiac Protein Cross-Reacting with mAb GE4.90 Anti-Skeletal Muscle Triadin

Triadin is an intrinsic protein of the junctional face of the terminal cisternae of rabbit skeletal muscle triad junction which structurally links the ryanodine receptor to the DHP receptor of the T-tubule (Kim et al., 1990*b*). When triads were solubilized for Western blotting in normal (5% 2-mercaptoethanol) Laemmli medium, triadin appeared as a band at M_r 95K. On the other hand, when the SH reducing agent was eliminated from the solubilizing medium, the antibody recognized a ladder of bands which were multiples of 95 kD (Caswell et al., 1991).

To test for the native state of triadin in skeletal muscle and for the presence of triadin in ventricular muscle, microsomes were prepared from rat psoas and rat ventricle in the presence of 100 mM iodoacetamide to inhibit intermolecular crosslinking through

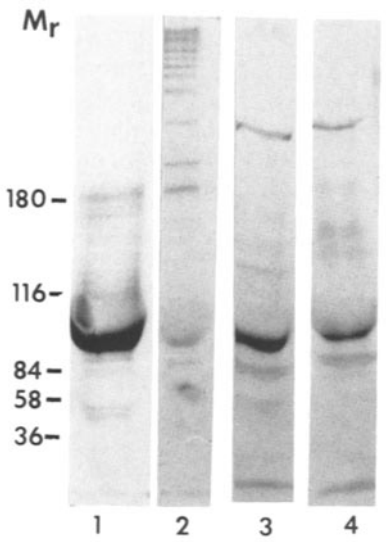

Fig. 4. Cross-reaction of anti-triadin mAb GE 4.90 with rat microsomes. Microsomes were prepared from rat psoas and ventricle as described in Materials and Methods with the substitution of 100 mM iodacetamide for dithiothreitol for all buffers. Psoas microsomes (lanes *1* and *2*) and cardiac microsomes (*3* and *4*) were pelleted and redissolved in Laemmli sample buffer at 10 μg and 50 μl, respectively, in the presence (lanes *1* and *3*) and absence (*2* and *4*) of 5% mercaptoethanol. The samples were electrophoresed on 5% slab gels, Western blotted onto nitrocellulose, incubated with skeletal muscle anti-triadin mAb GE 4.90 and developed as described in Materials and Methods. The mobility of standard proteins was determined with the prestained standards from Sigma.

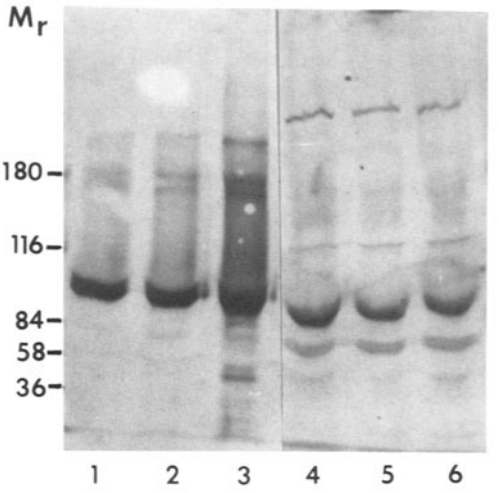

Fig. 5. Effects of 1 M NaCl and Triton X-100 on triadin. Rat psoas muscle (lanes *1–3*) and ventricular muscle microsomes (lanes *4–6*) (100 μg) were pelleted from sucrose buffer (lanes *1*, *4*) or sucrose buffer made 1 M in NaCl (lanes *2*, *5*) or 2 mg Triton X-100 per mg protein in the Beckman Airfuge (10 psi, 15 min). The drained pellets were resuspended in 100 μl standard (reducing) Laemmli buffer and electrophoresed on a 5% slab gel. Triadin epitopes were detected by blotting and development with mAb GE4.90.

disulfide bridge formation. The pelleted membranes were then solubilized in the normal Laemmli medium (5% 2-mercaptoethanol = 0.7 M) or in the absence of the mercaptan and Western blotted (Fig. 4). The blots were stained with the monoclonal antibody GE4.90 made against rabbit skeletal muscle triadin. Under reducing conditions, the antibody recognizes a band at M_r 95K in rat psoas muscle (lane *1*). Some of the fainter faster electrophoresing bands may be proteolytic breakdown products of triadin but at least two bands co-electrophoresed with rat serum proteins which reacted with the anti-mouse antibody of the detection system. The electrophoretic pattern for unreduced psoas microsomes (lane *2*) shows a ladder of higher M_r oligomeric forms of triadin. This observation thus supports the contention that the native state of triadin in skeletal muscle is a population of oligomers (Caswell et al., 1991). Lane *3* shows that the monoclonal antibody against skeletal muscle triadin clearly cross-reacted with a protein of similar M_r in the rat ventricle microsomes electrophoresed under reducing conditions with fainter bands at higher M_r's. Under nonreducing conditions (lane *4*), however, the major antigenic band of the ventricular membranes remained that at M_r 95K, implying that the native state of triadin is the monomer in the heart. Western blotting patterns identical to those shown in Fig. 4 were obtained for psoas and ventricle preparations made in the presence of 100 mM N-ethylmaleimide which may have a different selectivity for cysteines than iodoacetamide.

Rabbit skeletal muscle triadin was identified as an intrinsic protein in that it was not released from the membrane by 1 M salt; it was localized to the junctional domain by the fact that it resisted dissolution by Triton X-100 (Brandt et al., 1990). Figure 5 shows that the triadin-related protein in both rat psoas muscle (lanes *1, 2, 3*) and rat ventricle (lanes *4, 5, 6*) is also resistant to 1 M salt (lanes *2* and *5*) and Triton X-100 (lanes *3* and *6*).

Localization of the Cardiac mAB GE4.90 Epitope

The subcellular location of the cardiac triadin-like protein was identified using the protocols and organelle markers described in Figs. 1 and 3. Figure 6*A* shows the distribution patterns for the triadin epitope assayed by ELISA (filled circles) and DHP receptors (open squares) when rat ventricle microsomes were centrifuged to equilibrium. Both activi-

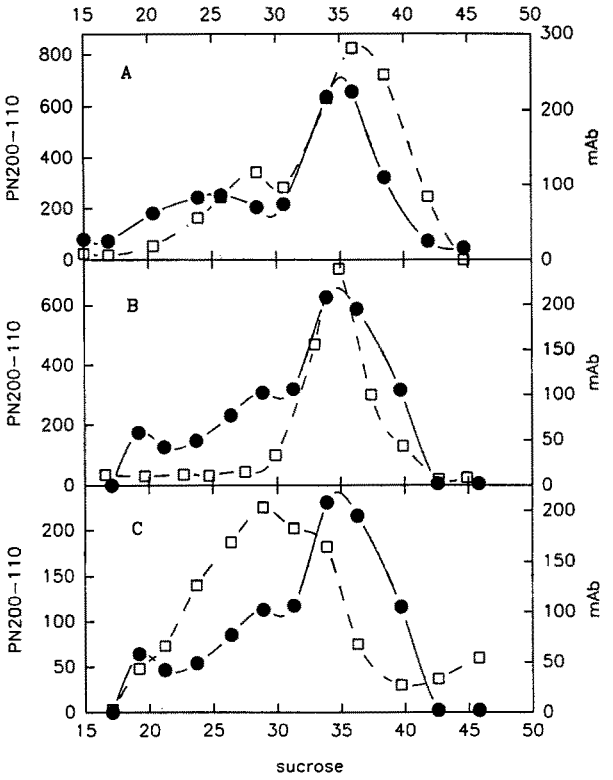

Fig. 6. Subcellular localization of the triadin epitope in rat ventricular muscle. Rat ventricular muscle microsomes (Panel *A*) were centrifuged on isopycnic sucrose gradients which were subsequently scanned for mAb GE4.90 crossreactivity (filled circles) and PN200-110 binding (open squares) expressed as fmol bound/ml gradient fraction. The bulk of the heavy microsomes were then recentrifuged directly (Panel *B*) or after French press extrusion at 13,000 psi (Panel *C*). The data in each panel represent the averaged value for two preparations.

ties are associated predominantly with the higher density region of the gradient in which junctional dyads are present. The ELISA pattern was confirmed by Western blotting. The epitope and ligand distributions, however, cannot be quantitatively compared because the immunoresponse to cardiac material appears to saturate at much lower levels than with skeletal muscle triadin. Figure 6*B* shows the distribution patterns for triadin and DHP receptors upon recentrifugation of the heavy microsomes. The ELISA pattern is similar to that in Fig. 6*A* and matches the DHP receptor pattern in position and shape in the heavier gradient fractions. These data then support the contention that the triadin epitope is most likely on a dyad component rather than on some other organelle. Figure 6*C* shows the distribution patterns after extrusion of the dyad fraction from the French press at 13,000 psi. The DHP receptors are now partially released to the position of the free T-tubules (as in Fig. 3*B*) but the triadin epitope distribution remained centered at 36% sucrose as in the untreated dyads. This is the position of the

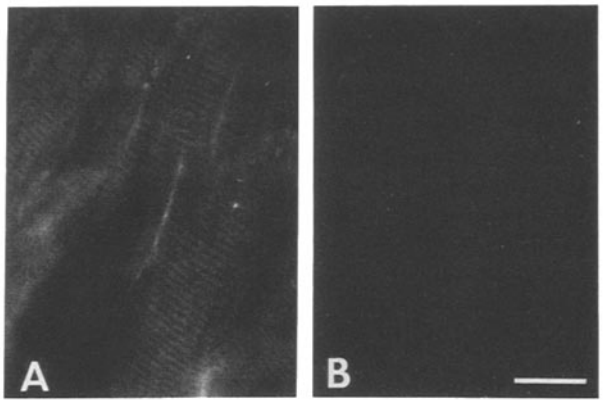

Fig. 7. Immunofluorescence localization of reactive site against mAb GE 4.90 in rat ventricular muscle. Rat ventricular muscle strips were cryosectioned and incubated with mAb GE 4.90 against triadin followed by rhodamine-conjugated secondary antibody (Panel *A*) or in secondary antibody alone (Panel *B*) as described in Materials and Methods. Bar = 20μ.

junctional SR after dyad disruption as defined by ryanodine binding (*see* Fig. 3*A*).

Immunofluorescence Localization of the Mab GE4.90 Epitope

To test whether or not the *in situ* location of the cardiac antigen was consistent with the biochemical *in vitro* localization, cryosections of rat papillary muscle were incubated with mAb GE 4.90 and then visualized using rhodamine-tagged secondary antibody (Fig. 7*A*). In this obliquely cut longitudinal section, the staining is observed to form a characteristic striated pattern. This suggests that triadin is located in a regularly disposed subcellular compartment. This is presumably the junctional surface of the SR, which is arranged into dyadic units lying at the Z-lines. In control sections treated only with secondary antibody (Fig. 7*B*), no staining was detected.

Discussion

Although rats have been used extensively for both physiological and biochemical studies of normal and pathological hearts, few experiments have been performed on fractionated microsomes (Bers, 1979; Mansier et al., 1983; Wibo, Bravo & Godfraind, 1991). Rat ventricle microsomes have been used as both sarcolemma (Janis et al., 1982; Murphy & Snyder, 1982; Nayler et al., 1985) and SR (Wei, Janis & Daniels, 1976; Heilmann et al., 1980; Limas, Spier & Kahlon, 1980; Wientzek & Katz, 1991) preparations. Nevertheless, ventricular muscle microsomes

from other species are known to contain nonjunctional sarcolemma, free SR, T-tubules, junctional SR and dyadic complexes of the latter two organelles.

Cardiac dyads have been defined by the comigration of T-tubule and junctional SR markers when the microsomes are fractionated on isopycnic gradients (Brandt, 1985; Brandt & Bassett, 1986). The T-tubules are enriched in DHP-receptors relative to other (i.e., muscarine, β-adrenergic or digitalis) receptors while the junctional SR is enriched in the JFP and calsequestrin and low in SR Ca^{2+} pump and phospholamban. The fact that the vesicle fraction contained dyadic complexes (as opposed to all markers being present on a single membrane) was established by the observation that the T-tubule markers appeared at a new isopycnic point upon French press treatment of the putative dyadic fraction (Brandt & Bassett, 1986). DHP receptors (Ashraf et al., 1986) and calsequestrin (Jorgensen et al., 1983) have been established as predominantly T-tubule and junctional SR proteins, respectively, in the rat heart by morphological techniques.

We show here that the rat ventricular microsomes can be separated into two populations on isopycnic gradients. The light microsomes contain nonjunctional sarcolemma as defined by a low DHP receptor content and free SR as identified by the M_r 102K protein corresponding to the SR Ca^{2+} ATPase. The heavy microsomes consist of junctional SR (ryanodine receptors) and T-tubules (high DHP receptor content relative to muscarine receptors). These two organelles appear to be associated as dyads since the markers comigrate on recentrifugation unless treated by French press extrusion. Free T-tubules and probably unliked junctional SR are also to be found in this membrane population.

The clear separation between the light and heavy microsome populations most likely is a consequence of a low yield of free SR which is the major species in ventricular microsomes from other species. The rat preparation also appeared low in nonjunctional plasma membrane; the light microsomes had a specific activity of 0.23 pmol muscarine receptor/mg protein which is 1/4 that found for other species. The specific activity of muscarine receptors in the heavy microsome fraction and the content relative to DHP receptors is almost identical to that reported for cat and rabbit (Brandt, 1985). The fact that the muscarine receptor distribution pattern matches that for DHP receptors when the heavy microsomes are recentrifuged with and without French press treatment confirms that the muscarine receptors are present in T-tubules. This observation was previously reported for cat ventricular muscle preparations (Brandt & Bassett, 1986). An additional factor for the separation of the rat dyads from nonjunctional membranes is the intrinsic isopycnic point of the junctional SR (36% sucrose) which is higher than that of cat (33% sucrose). The basis for this increased buoyant density is not yet clear.

The rat dyads are more resistant to mechanical disruption (60% broken at 14,000 psi by the French press) than those in the cat (100% broken at 8,000 psi (Brandt & Bassett, 1986)). In skeletal muscle, triadic vesicles may be subdivided into "strong" and "weak" on the basis of their susceptibility to cleavage by French press, hypertonic salt and calpain (Kim et al., 1990*a*). Using the criteria of susceptibility to French press, the rat heart dyad represents a "strong" dyad while the cardiac dyad of cat is of the "weak" type. It is possible that the two forms of junction are held together by different protein associations although the identity of these has not yet been established.

A mAb against triadin in skeletal muscle cross-reacts with a protein in isolated cardiac microsomes and in ventricular muscle cryosections. The cross-reactivity could be to an identical protein, to a homologous form in the heart or to a functionally different protein with a common epitope. The last possibility is unlikely in view of the similar distribution among organelles and within the sarcomere, the similar M_r and the resistance to separation from the membrane. We have shown here that the cardiac form is most likely an intrinsic protein of the junctional face of the junctional SR, the location of triadin in skeletal muscle. In addition, we have evaluated a second mAb AE8.91 which responds to a different epitope of skeletal muscle triadin. This mAB also reacts with rat cardiac triadin.

The content of triadin in ventricular muscle as estimated by staining of Western blots is considerably below that of skeletal muscle. This is also true of DHP receptors which are 10-fold lower in the heart than skeletal muscle and ryanodine receptors which are 2- to 3-fold lower. The fact that triadin can be detected by Western blotting in total microsomes, however, indicates that rat ventricle is a rich source of the protein. The antigenic response was not seen in cat or rabbit ventricular muscle membranes without prior enrichment of the dyad fraction. This yield of triadin in rat ventricle is consistent with the relatively high content of DHP and ryanodine receptors compared to other species.

The significant difference between the skeletal muscle and cardiac forms of triadin is in their oligomeric forms. Under nonreducing conditions, the skeletal forms appear predominantly in a ladder of oligomers. While higher M_r forms are seen for the cardiac preparation, the predominant form under nonreducing conditions is the monomer. Although

the differences in content of polymeric states in the two muscles might indicate the presence of isotypes, it is equally possible that they could occur as a consequence of differences in post-translational processing. We have shown that triadin is an essential architectural component of the skeletal muscle triad junction and proposed that it may play a physiological role in the excitation-contraction coupling. At this time we have yet to test whether cardiac triadin binds to cardiac JFP or cardiac DHP receptor and hence the architectural role of cardiac triadin is unknown. However, the close morphological resemblance between skeletal and cardiac junctions suggests that triadin could be mediating the apposition of the two organelles in both muscle types. On the other hand, cardiac triadin may not be actively involved in excitation-contraction coupling if Ca^{2+} induced Ca^{2+} release (Fabiato, 1989) is the physiological mechanism.

This work was supported by a Project Grant from the American Heart, Florida Affiliate and Sun Coast Chapter and grants from the American Heart Association, National Society, Florida Affiliate and Ohio Affiliate SW 91-08. Additional support came from grants NIH HL 19044 and NIH HL 34779. The secretarial skills of Mrs. N. Lowery, Ms. Norma Jacobs and Ms. Gerry Trebilcock are gratefully acknowledged. We also wish to thank Mr. Alan Mandveno for advice on computer modelling.

References

Ashraf, M., Park, W.H., Grupp, I., Schwartz, A. 1986. Distribution of ^3H nitrendipine in the isolated perfused rat heart as revealed by electron microscopic autoradiography. *J. Mol. Cell Cardiol.* **18:**265–272

Bers, D.M. 1979. Isolation and characterization of cardiac sarcolemma. *Biochim. Biophys. Acta* **55:**131–146

Brandt, N. 1985. Identification of two populations of cardiac microsomes with nitrendipine receptors: Correlation of the distribution of dihydropyridine receptors with organelle specific markers. *Arch. Biochem. Biophys.* **242:**306–319

Brandt, N.R., Kawamoto, R.M., Caswell, A.H. 1985. Dihydropyridine binding sites on transverse tubules isolated from triads of rabbit skeletal muscles. *J. Receptor Res.* **5:**155–170

Brandt, N., Bassett, A. 1986. Separation of dihydropyridine binding sites from cardiac junctional sarcoplasmic reticulum. *Arch. Biochem. Biophys.* **244:**872–875

Brandt, N.R., Caswell, A.H., Wen, S.-R., Talvenheimo, J.A. 1990. Molecular interactions of the junctional foot protein and dihydropyridine receptor in skeletal muscle triads. *J. Membrane Biol.* **113:**237–251

Brandt, N.R., Caswell, A.H., Brunschwig, J.-P., Kang, J.-J., Antoniu, B., Ikemoto, N. 1992*a*. Effects of anti-triadin antibody on Ca^{2+} release from sarcoplasmic reticulum. *FEBS Lett.* **299:**57–59

Brandt, N.R., Caswell, A.H., Brandt, T., Brew, K., Mellgren, R.L. 1992*b*. Mapping of the calpain proteolyses products of the junctional foot protein of the skeletal muscle triad junction. *J. Membrane Biol.* **127:**35–47

Campbell, K.P., MacLennan, D.H., Jorgensen, A.O. 1982. Staining of the Ca^{2+}-binding proteins, calsequestrin, calmodulin, troponin C, and S-100, with the cationic carbocyanine dye "Stains-All". *J. Biol. Chem.* **258:**11267–11273

Caswell, A.H., Lau, Y.H., Brunschwig, J.-P. 1976. Ouabain-binding vesicles from skeletal muscle. *Arch. Biochem. Biophys.* **176:**417–430

Caswell, A.H., Brandt, N.R., Brunschwig, J.-P., Kawamoto, R.M. 1988. Isolation of transverse tubule membranes from skeletal muscle: Ion transport activity, reformation of triad junctions and isolation of junctional spanning proteins of triads. *Methods in Enzymol.* **157:**68–84

Caswell, A.H., Brandt, N.R., Brunschwig, J.-P., Purkerson, S. 1991. Localization and partial characterization of the oligomeric disulfide-linked molecular weight 95,000 protein (triadin) which binds the ryanodine and dihydropyridine receptors in skeletal muscle triadic vesicles. *Biochem.* **30:**7507–7513

Cleemann, L., Morad, M. 1991. Role of Ca^{2+} channel in cardiac excitation-contraction coupling in the rat: Evidence from Ca^{2+} transients and contraction. *J. Physiol.* **432:**283–312

Dombradi, V.K., Silberman, S.R., Lee, E.Y.C., Caswell, A.H., Brandt, N.R. 1984. The association of phosphorylatase kinase with rabbit muscle T-tubules. *Arch. Biochem. Biophys.* **230(2):**615–630

Doyle, D.A., Kamp, T.J., Palfrey, H.C., Miller, R.J., Page, E. 1986. Separation of cardiac plasmalemma into cell surface and T-tubular components. *J. Biol. Chem.* **261:**6556–6564

Fabiato, A. 1989. Appraisal of the physiological relevance of two hypotheses for the mechanism of calcium release from mammalian cardiac sarcoplasmic reticulum: calcium-induced release versus charge-coupled release. *Mol. Cell. Biochem.* **89:**135–140

Fosset, M., Jaimovich, B., Delpont, E., Lazdunski, M. 1982. [^3H]nitrendipine receptors in skeletal muscle: Properties and preferential localization in transverse tubules. *J. Biol. Chem.* **258:**6080–6092

Gage, P.W., Eisenberg, R.S. 1969. Action potentials after potentials and excitation-contraction coupling in frog sartorias muscle without transverse tubules. *J. Gen. Physiol.* **53:**298–310

Heilmann, C., Lindt, T., Müller, W., Pette, D. 1980. Characterization of cardiac microsome from spontaneously hypertonic rats. *Basic Res. Cardiol.* **75:**92–96

Inui, M., Saito, A., Fleischer, S. 1987. Isolation of the ryanodine receptor from cardiac sarcoplasmic reticulum and identity with the feet structures. *J. Biol. Chem.* **262:**15637–15642

Inui, M., Wang, S., Saito, A., Fleischer, S. 1986. Characterization of junctional and longitudinal sarcoplasmic reticulum from heart muscle. *J. Biol. Chem.* **263:**10843–10859

Janis, R.A., Maurer, S.C., Sarmiento, J.G., Bolger, G.T., Triggle, D.J. 1982. Binding of [^3H]Nimodipine to cardiac and smooth muscle membranes. *Eur. J. Pharmacol.* **82:**191–194

Jones, L.R., Besch, H.R., Jr., Fleming, J.W., McConnaughey, M.M., Watanabe, A.M. 1979. Separation of vesicles of cardiac sarcolemma from vesicles of cardiac sarcoplasmic reticulum. *J. Biol. Chem.* **254:**530–539

Jorgensen, A.O., Shen, A.C.-Y., Denney, D.H., Jr., Campbell, K.P. 1983. Immunoelectron microscopical localization of calsequestrin in rat ventricular muscle. *J. Cell Biol.* **97:**260*a*

Kim, K.C., Caswell, A.H., Brunschwig, J.-P., Brandt, N.R. 1990*a*. Identification of a new subpopulation of triad junctions isolated from skeletal muscle; morphological correlations with intact muscle. *J. Membrane Biol.* **113:**221–235

Kim, K.C., Caswell, A.H., Talvenheimo, J.A., Brandt, N.R.

1990*b*. Isolation of a terminal cisterna protein which may link the dihydropyridine receptor to the junctional foot protein in skeletal muscle. *Biochemistry* **29:**9281–9289

Limas, C.J., Spier, S.S., Kahlon, J. 1980. Enhanced calcium transport by sarcoplasmic reticulum in mild cardiac hypertrophy. *J. Molec. Cell Cardiol.* **12:**1103–1116

Manalan, A.S., Jones, L.R. 1982. Characterization of the intrinsic cAMP-dependent protein kinase activity and endogenous substrates in highly purified cardiac sarcolemma vesicles. *J. Biol. Chem.* **257:**10052–10062

Mansier, P, Charlemange, D., Rossi, B., Pretesoille, M., Swynghedauw, B., Lelievre, L. 1983. Isolation of impermeable inside-out vesicles from an enriched sarcolemma fraction of rat heart. *J. Biol. Chem.* **208:**6628–6635

Murphy, K.M.M., Snyder, S.H. 1982. Calcium antagonist receptor binding sites labelled with [^{3}H]Nitrendipine. *Eur. J. Pharmacol.* **77:**201–202

Nayler, W.G., Dillon, J.S., Elz, J.S., McKelvie, M. 1985. An effect of ischemia on myocardial dihydropyridine binding sites. *Eur. J. Pharmacol.* **115:**81–89

Otsu, K., Willard, H.F., Khanna, V.K., Zorzato, F., Green, N.M., MacLennan, D.H. 1991. Molecular cloning of cDNA encoding the Ca^{2+} release channel (ryanodine receptor) of rabbit cardiac muscle sarcoplasmic reticulum. *J. Biol. Chem.* **265:**13472–13483

Rios, E., Brum, G. 1987. Involvement of dihydropyridine receptors in excitation-contraction coupling in skeletal muscle. *Nature* **325:**717–720

Rios, E., Ma, J., Gonzalez, A. 1991. The mechanical hypothesis of excitation-contraction (EC) coupling in skeletal muscle. *J. Muscle Res. & Cell Motil.* **12:**127–135

Schneider, M.F., Chandler, W.K. 1973. Voltage dependent charge movement in skeletal muscles: a possible step in excitation contraction coupling. *Nature* **242:**244–246

Seifert, J., Cassida, J.E. 1986. Ca^{2+}-dependent ryanodine binding site: Soluble preparation from rabbit cardiac sarcoplasmic reticulum. *Biochim. Biophys. Acta* **861:**399–405

Seiler, S., Wegener, A.D., Whang, D.D., Hathaway, D.R., Jones, L.R. 1984. High molecular weight proteins in cardiac and skeletal muscle. Junctional sarcoplasmic reticulum vesicles bind calmodulin are phosphorylated, and are degraded by Ca^{2+}-activated protease. *J. Biol. Chem.* **259:**8550–8557

Tanabe, T., Beam, K.G., Adams, B.A., Niidome, T., Numa, S. 1990. Regions of the skeletal muscle dihydropyridine receptor critical for excitation-contraction coupling. *Nature* **346:** 567–572

Wei, J.W., Janis, R.A., Daniel, E.E. 1976. Calcium accumulation and enzymatic activities of subcellular fractions from aortas and ventricles of genetically hypertensive rats. *Circ. Res.* **39:**133–140

Wibo, M., Bravo, G., Godfraind, T. 1991. Postnatal maturation of excitation-contraction coupling in rat ventricle in relation to the subcellular density of 1,4-dihydropyridine and ryanodine receptors. *Circ. Res.* **68:**662–673

Wientzek, M., Katz, S. 1991. Isolation and characterization of purified sarcoplasmic reticulum membranes from isolated adult rat ventricular myocytes. *J. Mol. Cell Cardiol.* **23:**1149–1163

Received 24 April 1992; revised 25 September 1992