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Identification of a New Subpopulation of Triad Junctions Isolated from Skeletal Muscle; Morphological Correlations with Intact Muscle

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Summary. It has been previously recognized that a number of protocols may cause breakage of the triad junction and separation of the constituent organelles of skeletal muscle. We now describe a fraction of triad junctions which is refractory to the known protocols for disruption. Triads were passed through a French press and the dissociated organelles were separated on a sucrose density gradient, which was assayed for PN200-110, ouabain and ryanodine binding. Ryanodine binding showed a single peak at the density of heavy terminal cisternae. On the other hand, the PN200-110 and ouabain, which are external membrane ligands, bound in two peaks: one at the free transverse tubule region and the other at the light terminal cisternae. Similarly, a two peak pattern of PN200-110 and ouabain binding was observed when triad junctions were broken by the Ca^{2+}-dependent protease, calpain, which selectively hydrolyzes the junctional foot protein. The light terminal cisternae vesicles were subjected to three different procedures of junctional breakage: French press, hypertonic salt treatment, and protease digestion using calpain or trypsin. The treated membranes were then centrifuged on density gradients. Only extensive trypsin digestion caused a partial shift of ouabain activity into the free transverse tubule region. These observations suggest that the triads are a composite mixture of breakage susceptible, "weak," and breakage resistant, "strong," triads. Scatchard analysis of PN200-110 suggests that the transverse tubules of strong triads contain a relatively high number of dihydropyridine receptors compared to those of weak triads. Thin section electron microscopic images of the strong triads comparable to those of intact muscle are presented.

Key Words excitation-contraction coupling · triad junctions · dihydropyridine receptor · ryanodine receptor · strong triads

Introduction

Excitation-contraction coupling (EC coupling) in skeletal muscle is believed to take place in the triad junction where the transverse tubule (T-tubule) and two closely apposing terminal cisternae (TC) of sarcoplasmic reticulum (SR) are held together by the electron dense bridges called feet (Franzini-Armstrong, 1970). The mechanism of the signal transfer from the T-tubule to TC has been extensively investigated over three decades employing a wide variety of experimental approaches. However, thus far no single major hypothesis that fully describes EC coupling has been established. Recent progress on the understanding of the triadic architecture at a molecular level began with the identification of the foot protein as a high molecular weight (M_r) protein (Cadwell & Caswell, 1982) and its subsequent localization in the triadic gap of intact muscle fibers using the immunogold technique (Kawamoto et al., 1986). Several studies have now indicated that the foot protein is the ryanodine receptor (Campbell et al., 1987; Lai et al., 1987; Inui, Saito & Fleisher, 1987) and that the purified ryanodine receptor could be reconstituted in planar lipid bilayers. The observed Ca^{2+} channel activity is similar to a Ca^{2+} channel activity in the native SR (Imagawa et al., 1987; Hymel et al., 1988; Lai et al., 1988). The finding that the spanning structure itself is the Ca^{2+} release channel suggests a direct association of the Ca^{2+} release channel with the T-tubule.

The proposal that the voltage sensor molecule in the T-tubule is the dihydropyridine (DHP) receptor was based on the findings that DHP inhibits charge movement and SR Ca^{2+} release in parallel (Rios & Brum, 1987). Furthermore, injection of the expression vector containing cDNA encoding the $\alpha 1$ subunit of DHP receptor of rabbit skeletal muscle into myotubes from dysgenic mice restores EC coupling (Tanabe et al., 1988). Therefore, the two key constituents of EC coupling, i.e., the voltage sensor and the SR Ca^{2+} release channel, have been putatively identified as the DHP receptor and the junctional foot protein, respectively. If this is so, one might expect that these two constituents would have a direct communication or strong linkage between them. However, thus far no such interaction has been reported.

Within this context, studies were carried out to investigate the nature of the triadic architecture.

Previously, it has been reported that the triad junction can be disrupted by French press treatment (Caswell et al., 1979), hypertonic salt (Caswell & Brandt, 1981*a*,*b*) or proteolytic cleavage (Cadwell & Caswell, 1982). However, the more extensive investigation of the disruption of the triads reported here indicates that a fraction of the triads is unusually refractory to the known breakage procedures. We describe the pharmacological and morphological properties of those triadic vesicles which are resistant to breakage.

Materials and Methods

Membrane Preparations

TC/triads were prepared from adult rabbit back muscle by differential and gradient centrifugation according to the procedures described earlier (Caswell, Lau & Brunschwig, 1976) in the presence of the following protease inhibitors: 200 μM PMSF, 1 μM leupeptin and 1 μM pepstatin A. For the preparation of T-tubules, light terminal cisternae (LTC) and heavy TC (HTC), TC/triads were passed through a French press at 8000 psi followed by the immediate addition of 0.3 M K cacodylate and centrifuged in a Sorvall TV850 vertical rotor for 2 hr at 150,000 $\times$ *g*. The lower sharp band of rejoined triads was passed through a French press at 6000 psi and layered on a continuous sucrose gradient of 12.5–65% sucrose (wt/wt) as described before (Brandt, Caswell & Brunschwig, 1980). Protein determination was carried out by the method of Bradford (1976) using bovine serum albumin as a protein standard.

Preparation of Strong Triads

LTC were prepared either from rejoined triads or more recently from TC/triads by French press treatment followed by density gradient centrifugation. LTC is the band of intermediate density between T-tubules and HTC. LTC (4–6 mg/ml) was layered on top of a continuous linear sucrose gradient of 12–30% sucrose (wt/wt). The sample was centrifuged in a Beckman SW 41 rotor for 45 min at 50,000 $\times$ *g*. Fractions (0.5 ml) were collected from the top of the tube and analyzed for protein, [^{3}H]PN200-110 binding activity, and polypeptide composition by SDS-PAGE.

Ligand Binding Assays

All ligand binding assays were carried out by incubating samples in 0.5 ml of incubation mixture for 1 hr at room temperature or at 37°C for the ryanodine assay. Reactions were stopped by diluting the sample with 3 ml of wash buffer followed by filtration through millipore GSWP (0.22 μm) filters. Filters were washed three times with wash buffer and counted in 4 ml of toluene-based scintillation cocktail containing 45% Triton X-100. For the specific binding estimate, the nonspecific binding was substracted from the total binding.

For [^{3}H]PN200-100 binding, 20 μl aliquots from gradient unless specified in the figure legends were incubated in 20 mM Tris Cl, pH 7.0, 1 mM $CaCl_2$, 0.5 nM [^{3}H]PN200-110 in the absence or presence of 2 μM PN200-110 (control). For the Scatchard analysis, 5 μg vesicles were incubated with 0.1 nM to 20 nM [^{3}H]PN200-100. The wash medium was 0.2 M choline Cl, 20 mM Tris Cl, pH 7.4.

Standard incubation conditions for [^{3}H]ryanodine binding were 50 μl aliquots from gradient in 150 mM K gluconate, 1 mM $CaCl_2$, 10 mM Tris MOPS, pH 7.0, 1 mM Tris ATP, 2.7 nM [^{3}H]ryanodine in the absence or presence of 10 μM ryanodine (control). For Scatchard analysis, 50 μg of sample were incubated with a medium containing 0.6 M choline Cl, 50 mM HEPES, pH 7.4, 12 mM Tris ATP, 1 mM $CaCl_2$ and 0.4 nM to 24 nM [^{3}H]ryanodine. The wash medium was the incubation medium but without ATP and ryanodine.

Incubation conditions for [^{125}I]iodocyanopindolol binding were 50 μl aliquots from gradient in 50 mM Tris Cl, pH 7.4, 150 mM NaCl and 10 pM [^{125}I]iodocyanopindolol in the presence or absence of 10 μM isoproterenol. The wash medium was 25 mM Tris Cl, pH 7.4. Ouabain binding was assayed using 50-μl sample from the gradient in 120 mM NaCl, 40 mM Tris Cl, pH 7.4, 1 mM Tris EGTA, 10 mM $MgCl_2$, 50 nM [^{3}H]ouabain, and 0.2 mg SDS/mg protein in the presence or absence of 10 mM Tris ATP. Wash medium was 100 mM KCl.

For the ouabain entrapment assay, sacrospinalis muscle was infiltrated with Ringer's solution containing [^{3}H]ouabain (25–50 μCi) prior to excision from the animal (Caswell, Lau & Brunschwig, 1976). Aliquots from the gradient were diluted with 0.25 ml H_2O and then mixed with 4 ml of scintillation cocktail containing 45% Triton X-100.

Cleavage of Vesicles

Proteolytic cleavage of vesicles by Ca^{2+}-activated protease or trypsin was carried out as follows: vesicles were washed once and resuspended with medium free of protease inhibitor. Calpain digestion was carried out in 1 ml volume containing 1–1.2 mg vesicles, 250 mM sucrose, 10 mM Tris MOPS, pH 7.0, 3 mM dithiothreitol, 1 unit (from Sigma) or 10 μg (from Dr. R. Mellgren) calpain in the presence or absence of 1.5 mM $CaCl_2$ for 30 min at room temperature. Termination of digestion was made by addition of EGTA (2.5 mM final concentration). Reaction suspension were centrifuged on a 15–60% sucrose gradient for 16 hr at 120,000 $\times$ *g*. For trypsin digestion, vesicles were resuspended in a solution containing 250 mM sucrose, 2 mM histidine, pH 7.4, 1 mM $CaCl_2$ at a protein concentration of 1 mg/ml. Vesicles were incubated with trypsin at a concentration indicated in the figure legends at 37°C for 10 min. The reaction was terminated by the addition of threefold excess of soybean trypsin inhibitor and chilling on ice. Digested vesicles were immediately centrifuged on a 15–55% sucrose gradient or sedimented through 30% sucrose to assess the breakage of the junction. Control experiments were carried out identically except for the omission of trypsin.

Cleavage of vesicles by hypertonic salt treatment was carried out by incubating vesicles with 0.6 M NaSCN or 0.6 M Na gluconate on ice for 30 min before layering the vesicles on a continuous sucrose gradient. In control experiments, vesicles were incubated in the absence of salt on ice for 30 min. Gradients were centrifuged in a Beckman rotor SWS 41 for 16 hr at 120,000 $\times$ *g*.

Electron Microscopy

Vesicles for thin sectioning were centrifuged in a Beckman airfuge for 5 min at 5 psi. The pellet was let sit for 30 min before the

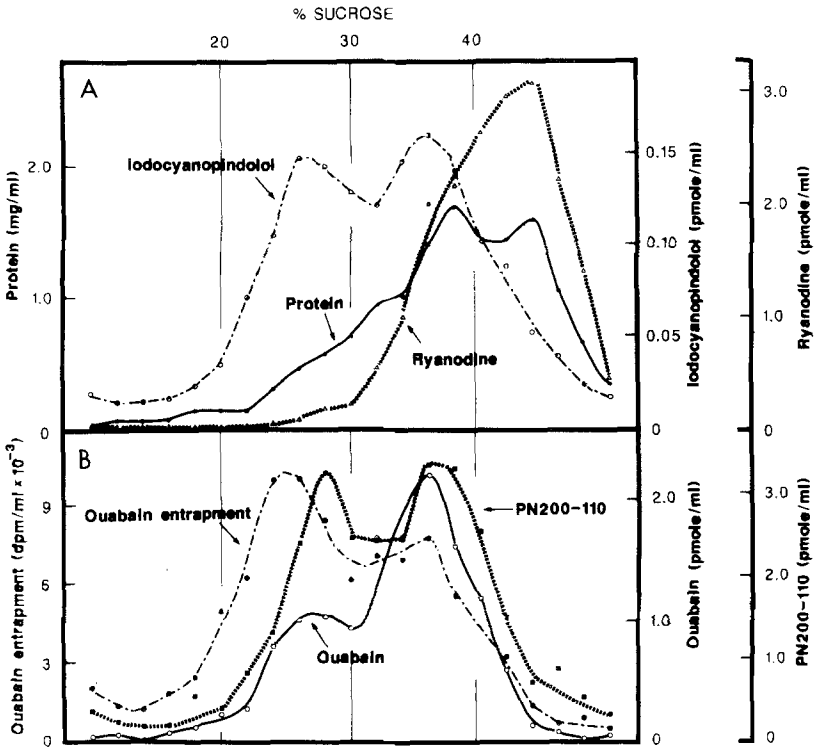

Fig. 1. Distribution of [^{3}H]PN200-110, [^{3}H]ouabain, [^{125}I]iodocyanopindolol and [^{3}H]ryanodine binding activities among the muscle subcellular fractions. Rejoined triads were passed through a French press at 6000 psi and centrifuged on a 12.5–65% sucrose gradient. Ligand binding assays were carried out using 50-μl samples from the fractions with an exception of 15-μl samples for [^{3}H]PN200-110. Ouabain entrapment was assayed by counting 50-μl aliquots in scintillation cocktail as described in Materials and Methods

fixation with tannic acid-glutaraldehyde and processed further as described before (Brunschwig et al., 1982). Thin sectioning of the intact muscle fiber from rabbit was carried out as described before (Caswell, Lau & Brunschwig, 1976).

Other Protocols

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the procedure of Laemmli (1970) using 5–15% acrylamide gradient gels. The protein bands were visualized by staining with 0.2% Coomassie Blue followed by destaining.

Materials

Trypsin (type I) and soybean trypsin inhibitor were obtained from Sigma. [^{3}H]PN200-110, [^{3}H]ouabain, and [^{3}H]ryanodine were from New England Nuclear. [^{125}I]iodocyanopindolol was from Amersham. Calpain was initially purchased from Sigma and later was a gift from Dr. R. Mellgren, Ohio Medical College. SDS-PAGE molecular weight standards were obtained as a kit from Sigma.

Results

Distribution of DHP Receptor in the Subfractions of Skeletal Muscle Microsomes

Caswell et al. (1979) have shown previously that triads can be mechanically broken by French press treatment and be subsequently reformed by treatment with salts of weak acids such as K cacodylate. The rejoining process is quite specific for the association between the T-tubule and terminal cisternae. It also permits isolation of triads enriched in the junctional components. To determine the disposition of T-tubular markers among membrane fractions, rejoined triads were subjected to French press treatment at 6000 psi and the extrudate separated on a continuous sucrose density gradient, which was assayed for PN200-110 (PN), ouabain, iodocyanopindolol (CYP) and ryanodine binding (Fig. 1). The protein pattern shows the two well-defined peaks at the isopycnic point of light terminal cisternae (LTC, at approximately 36 to 38% sucrose) and of heavy terminal cisternae (HTC, at approximately 42% sucrose). The T-tubules appear as a rising shoulder at approximately 22 to 26% sucrose. Ryanodine, a specific ligand for SR Ca^{2+} release channel, shows a single peak which matches with the HTC in position and shape. However, the distribution of PN, one of the 1,4-dihydropyridines which inhibit voltage-sensitive T-tubule Ca^{2+} channels (Borsotto et al., 1985) shows two peaks: one at the isopycnic point of free T-tubules and the other at that of LTC. The second peak in the LTC region is similarly observed with the Na^+-K^+ ATPase inhibitor, ouabain, and the β adrenergic antagonist, CYP, both of which are markers for external membrane. There is a quantitative discrepancy between

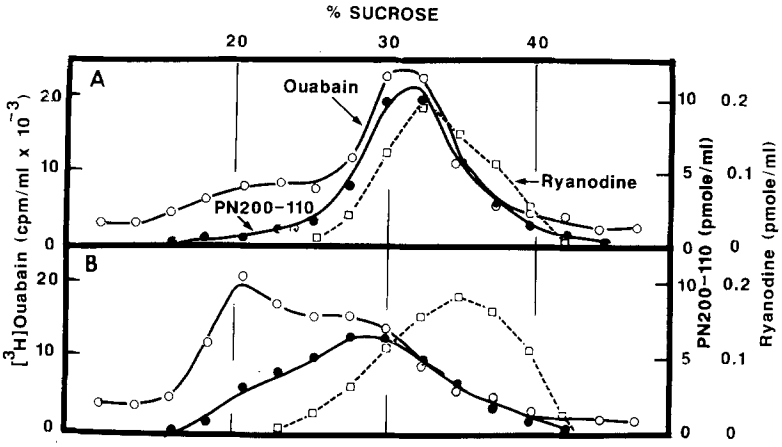

Fig. 2. Calpain digestion of TC/triads: effects on the distribution patterns of [^{3}H]PN200-110, [^{3}H]ouabain and [^{3}H]ryanodine. TC/triads (1.2 mg) were incubated with 1 unit of calpain (Sigma) at room temperature as described in Materials and Methods. (*A*) EGTA (2.5 mM) was added at the time of calpain administration. (*B*) Digestion was allowed for 30 min before adding EGTA. The digested vesicles were centrifuged on linear 15–50% sucrose gradients at 50,000 × *g* for 16 hr. [^{3}H]PN200-110 and [^{3}H]ryanodine bindings were measured in the presence of 1 nM [^{3}H]PN200-110 and 2.7 nM [^{3}H]ryanodine, respectively

ouabain binding and entrapment: ouabain entrapment shows a larger peak in the free T-tubule region than in the LTC, whereas ouabain binding shows the opposite finding. This may be accounted for by the fact that the ouabain entrapment assay may include free ouabain entrapped in the sealed T-tubule during homogenization of the muscle as well as that bound to Na^+-K^+ ATPase. This second peak of the external membrane marker activities is consistently observed in all preparations treated with the French press.

A similar distribution pattern is observed when the triad junction is broken by the Ca^{2+} activated protease, calpain (Fig. 2). Calpain is a cysteine protease that has a neutral pH optimum and requires Ca^{2+} for catalytic activity (Murachi et al., 1981). Calpain hydrolyzes the junctional foot protein selectively with little effect on other SR proteins (Seiler et al., 1984). Triad vesicles were treated with calpain in the presence (Fig. 2*B*) and absence (Fig. 2*A*) of Ca^{2+} at room temperature for 30 min and subsequently centrifuged on a continuous sucrose density gradient. The rather low isopycnic point of the TC/triads observed in this experiment might have been caused by the extraction of calsequestrin by EGTA. The comparison of the two conditions shows that if calpain was inactive, the PN, ouabain, and ryanodine binding activities almost superimposed, which is indicative of the T-tubular attachment to the TC. After activation of the calpain, the ryanodine binding moved to denser regions of the gradient implying the release of T-tubule from the TC. Under these conditions, calpain digestion does not cause any significant loss of the ryanodine binding. Concomitant with the shift of ryanodine binding, ouabain entrapment and PN binding appear in the lighter regions of the gradient at the density of free T-tubule. However, significant number of sites for PN and ouabain binding still remain in the TC region as in Fig. 1.

Identification of the Organelle Which Contains High PN Binding Activity in LTC

Previous morphological and biochemical studies on the LTC suggested that the majority of LTC vesicles are from the nonjunctional domain of the TC possibly generated by fragmentation of TC by the French press (Brandt et al., 1980). To understand the nature of the membrane which shows high PN binding activity in the LTC region, the LTC band was resubjected to three different means of junctional breakage: French press, protease digestion using trypsin or calpain, and hypertonic salt treatment using 0.6 M NaSCN or 0.6 M Na gluconate. After treatment, potential components were separated by a sucrose density gradient centrifugation. The release of the T-tubule from the TC was determined by measuring the ouabain entrapment or binding, or by assaying PN activity. Figures 3 to 6 depict the ouabain or PN activity profiles after the various breakage treatments of LTC described above.

In Fig. 3, LTC was subjected to French press treatment at 6000 psi to test whether repeating the same mechanical breakage procedure has any effect on the ouabain or PN activity profiles. Compared to the control (Fig. 3*A*), French pressed LTC (Fig. 3*B*) showed a more diffuse protein peak suggesting further fragmentation of the vesicles. However, the ouabain and PN binding activities are almost completely superimposed on the protein peak and there is no activity shifted to the lighter region of the gradient.

Figure 4 shows the ouabain entrapment profile and the electrophoretic pattern of the LTC after calpain digestion. In this experiment, calpain was added only to the vesicles shown in Fig. 4*B*. When calpain was omitted (Fig. 4*A*), the gel shows the high M_r junctional foot protein (JFP) (1st arrow) and

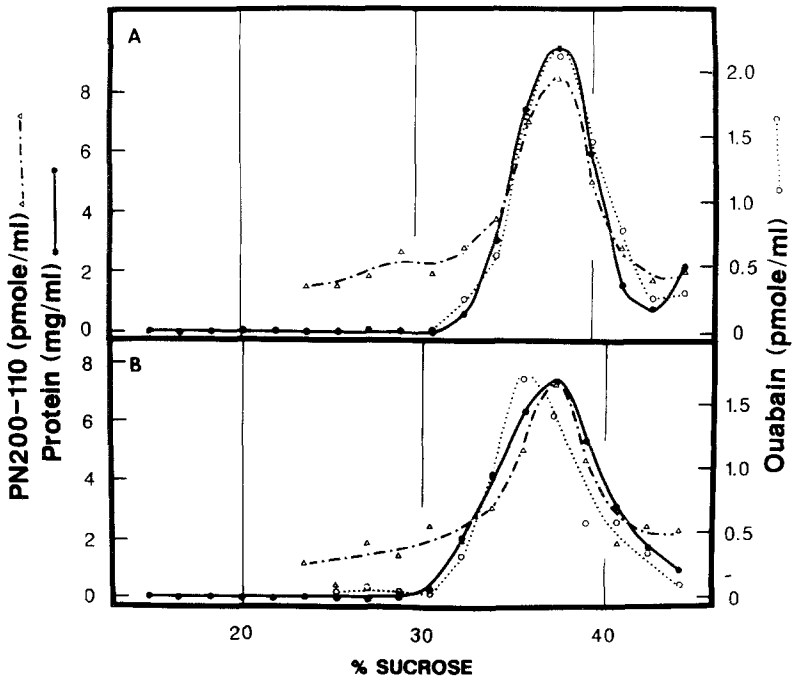

Fig. 3. Distribution of [^{3}H]PN200-110 and [^{3}H]ouabain binding activities after French press treatment of LTC. LTC were centrifuged in (*A*) the absence or (*B*) presence of French press treatment at 6000 psi on a 15–50% sucrose gradient at 50,000 × *g* for 16 hr. [^{3}H]PN200-110 and [^{3}H]ouabain binding were measured as described in Materials and Methods

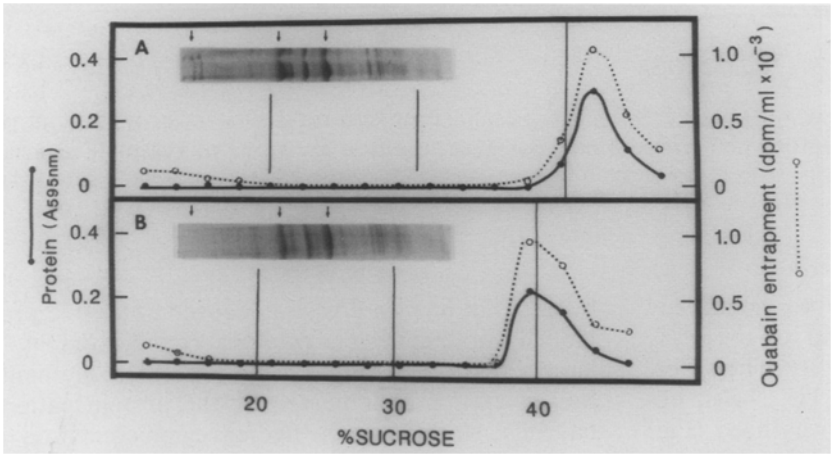

Fig. 4. Effect of calpain digestion of LTC on the distribution of [^{3}H]ouabain entrapment. Calpain digestion was performed similarly as in Fig. 2 but digestion mixtures were directly centrifuged on 15–60% sucrose gradients at the end of reactions without adding EGTA. (*A*) Calpain was omitted in the mixture. (*B*) Calpain (gift from Dr. R.L. Mellgren) was added at a protein to calpain ratio of 100 and incubated at room temperature for 30 min. Insets are Coomassie blue-stained gels of SDS-PAGE of the ouabain peak fractions (20 μg of protein per lane). From the left, arrows indicate 400-, 100- and 62-kDa protein which are foot protein, Ca^{2+}-ATPase and calsequestrin, respectively

other SR proteins (Ca^{2+} ATPase, 2nd arrow; calsequestrin, 3rd arrow). The protein and ouabain activities are superimposed as expected for intact triads. In Fig. 4*B*, where calpain was added, the gel shows the disappearance of the high M_r band with no discernible change of the pattern of other proteins. The protein peak is shifted a little into the lighter sucrose region, possibly suggesting a decrease in mass due to proteolysis. The ouabain distribution almost completely overlaps with the protein; no activity is detected in the free T-tubule region. It is possible that calpain may generate nicks in the foot protein leaving most of the protein mass and the structure of the junction intact. However, it should be noted that under almost identical conditions using TC/triads, calpain digestion caused a shift of about 60% of ouabain activity from the triads into the free T-tubule region (Fig. 2*B*).

In Fig. 5, the PN activity profiles in the LTC (right panels) after hypertonic salt treatments are compared with those of TC/triads (left panels). Figure 5*A* shows a single overlapping peak of protein and PN binding activities at approximately 36–38% sucrose in the control vesicles. Treatment with 0.6 M NaSCN treatment (Fig. 5*B*) causes release of extrinsic proteins from the vesicles as implied by the small protein peak at the top of the gradient. The protein peak in the heavy region becomes lighter (32–35% sucrose). SCN^- treatment caused a shift of part of the PN activity from TC/triads into the free T-tubule region although well-defined peaks were not observed because of overlap of the free T-

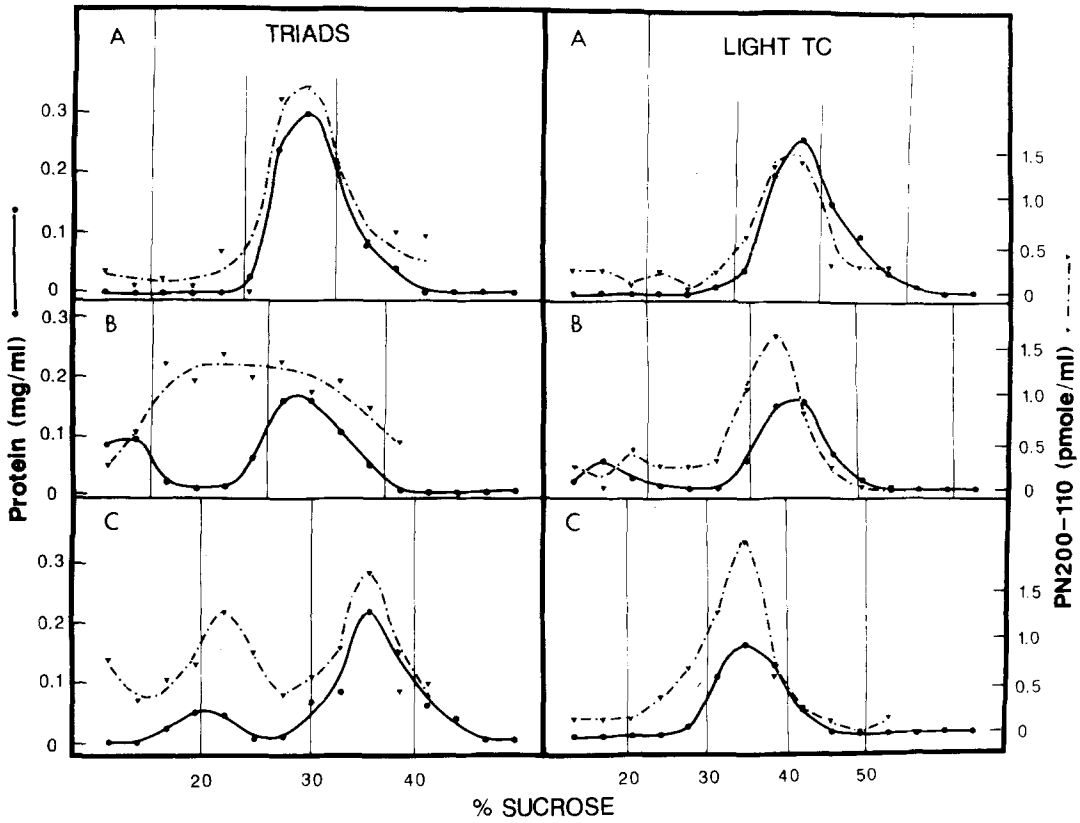

Fig. 5. Effect of hypertonic salt treatment of TC/triads and LTC on the distribution of [^{3}H]PN200-110 activity. Rejoined triads or LTC (1 mg) in 250 mM sucrose and 2 mM histidine, pH 7.4 were incubated on ice for 30 min (*A*) without adding any salts (control), (*B*) in the presence of 0.6 M Na SCN, or (*C*) 0.6 M Na gluconate, before the centrifugation on 15–55% sucrose gradients at 50,000 × *g* for 16 hr. [^{3}H]PN200-110 binding was assayed as described in Materials and Methods

tubule with intact triads. By contrast to the marked effect on TC/triads, SCN treatment of the LTC shows no significant shift of PN activity. The difference in salt effect on triads and LTC is also observed when 0.6 M Na gluconate was used (Fig. 5*C*). Gluconate$^-$ treatment of the TC/triads produces a significant PN peak in the free T-tubule region. However, Na gluconate treatment of LTC fails to shift the PN activity. The previous three figures therefore all support the view that the PN binding activity present in LTC is not caused by an incomplete breakage of a homogenous fraction of triads by the French press but rather by the presence of two distinct PN binding fractions.

Cadwell and Caswell (1982) reported that 0.01 mg/ml trypsin at 37°C for 10 min broke the junction approximately 80%. This is associated with the loss of the visible feet (Kawamoto, Brunschwig & Caswell, 1988). Trypsin (0.01 or 0.03 mg/ml) was used to examine whether extensive trypsinization can cause a shift of ouabain activity in the LTC to that of free T-tubules (Fig. 6*A*). At these high concentrations, trypsin may degrade any exposed proteins including the DHP receptor; therefore ouabain entrapment was measured as a marker for T-tubules. That trypsin digestion caused extensive proteolysis of the vesicle proteins is indicated by a large amount of protein at the top of the gradient. Some vesicles as indicated by the protein pattern have also shifted into the lighter region compared to control. The distribution of the ouabain entrapment at 0.01 mg/ml trypsin shows that most of the ouabain remains in the protein peak in the TC region. However, a significant peak appears in the free T-tubule region. This ouabain peak in the T-tubule region becomes more prominent when the trypsin concentration is increased threefold with a corresponding decrease of the ouabain in the TC region. These observations suggest that the ouabain or PN binding activity in LTC reflects the presence of T-tubules associated with TC rather than to a T-tubule fraction of high buoyant density. The electrophoretic pattern from these trypsin digestions shows extensive breakdown of a number of proteins (Fig. 6*B*, lanes *4* and *7*). In particular, the total disappearance of the intact JFP (arrow head) and its high molecular weight breakdown products and nearly complete disappearance of Ca^{2+}-ATPase are observed. Trypsin hydrolyzes protein with less protein specificity than calpain. It is noteworthy that

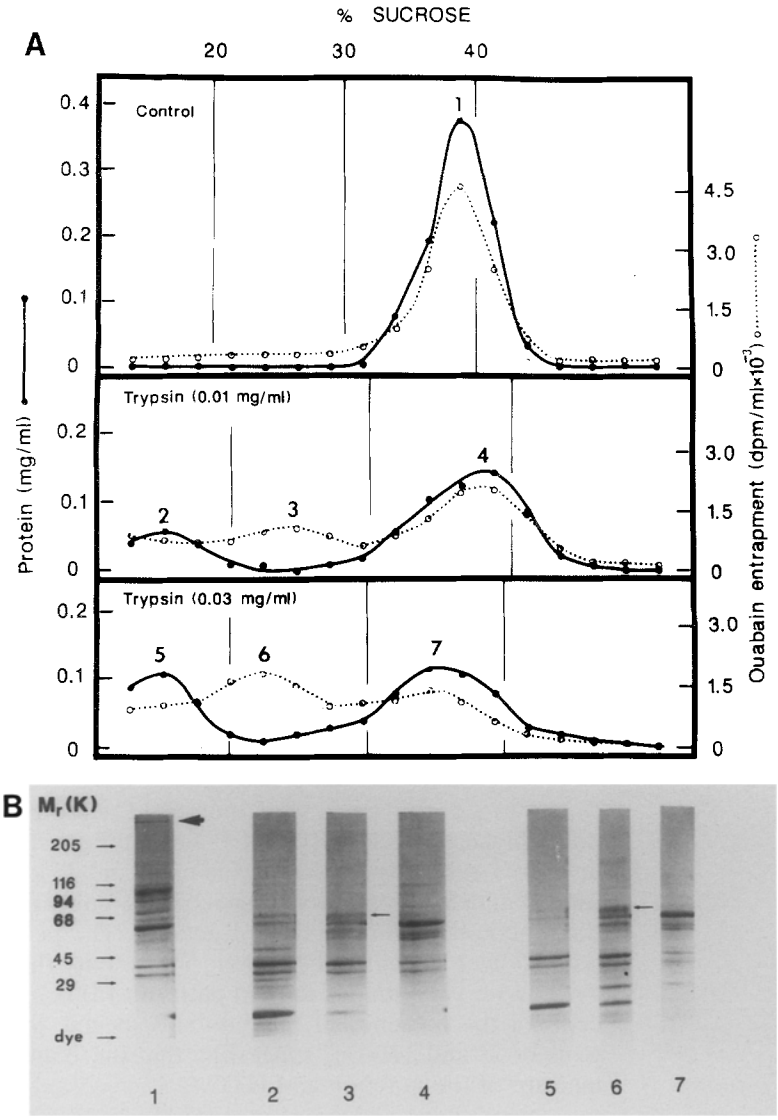

Fig. 6. Effect of trypsinization of LTC on the distribution of [^{3}H]ouabain entrapment. (*A*) Protein and [^{3}H]ouabain entrapment profile of LTC in control and 0.01 and 0.03 mg/ml trypsin digestion. Vesicles (1 mg) in 1 ml of 250 mM sucrose, 2 mM histidine, pH 7.4 and 1 mM $CaCl_2$ were incubated with trypsin at 37°C for 10 min. Reactions were terminated by adding trypsin inhibitor and vesicles were centrifuged at 50,000 × *g* or 16 hr as described in Materials and Methods. In a control, trypsin was omitted. (*B*) Coomassie blue-stained gel of SDS PAGE of gradient fractions on a 5–15% gradient gel. Each lane number corresponds to the individual peak No. indicated in (*A*). Lanes *1, 4, 5* and *7*: 20 μg of each peak fraction. Lanes *2, 3* and *6*: total pooled fractions of each peak. Arrow head indicates the junctional foot protein

trypsin allows the partial shift of the ouabain activity into the density of free T-tubule. However, despite the total disappearance of the JFP (lane *4*), ouabain is still significantly resistant to release from the TC. The gel lanes corresponding to the position of the free T-tubule band (lanes *3* and *6*) show a protein band (arrow) is detected immediately above the position of calsequestrin. This is likely to be the T-tubule specific 72-K dalton band which appears to be resistant to trypsin digestion.

Rate Zonal Purification of the Vesicles with High PN Binding Activities in LTC

The vesicles which have high PN binding activities in LTC were enriched by rate zonal centrifugation. Figure 7 depicts protein and PN activity profiles after a rate zonal centrifugation of LTC on a 12 to 30% sucrose gradient at 50,000 × *g* for 45 min. The protein profile shows a large single peak in the light fractions with a tailing into the heavy fractions and a small amount of sedimentation. In contrast to the protein pattern, the PN binding activity profile does not show a peak corresponding to the large protein peak. High activity of PN binding is observed in the middle and heavy gradient region where the protein concentration is very low. This finding is more clearly revealed when the PN binding is expressed as specific activity. It suggests that the vesicles with high PN activity are minor constituents in the LTC and that rate zonal purification allows substantial enrichment of the PN binding vesicles. The protein composition of PN rich fractions (pooled fractions from No. 10 to 17), low PN activity light fractions (pooled fractions No. 3 and 4), and the pellet of the rate zonal centrifugation were analyzed on a 5–15% SDS polyacrylamide gradient gel with LTC, T-tubule and HTC (Fig. 8). Compared to the TC/tri-

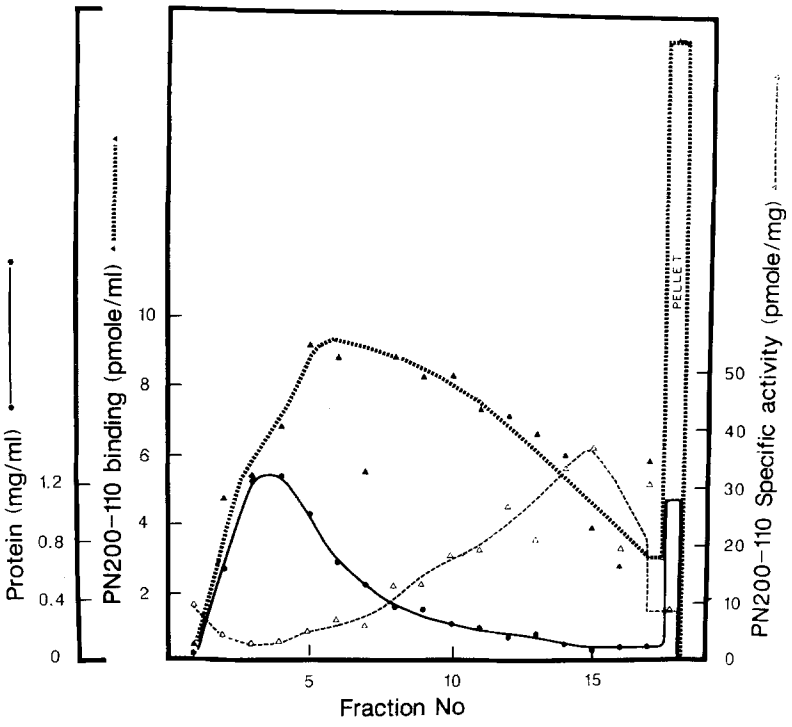

Fig. 7. Rate zonal centrifugation of LTC. LTC were centrifuged at 50,000 × *g* for 45 min on a 12–30% sucrose gradient in a Beckman rotor SW 41. Protein and [^{3}H]PN200-110 binding activity were assayed as described in Materials and Methods

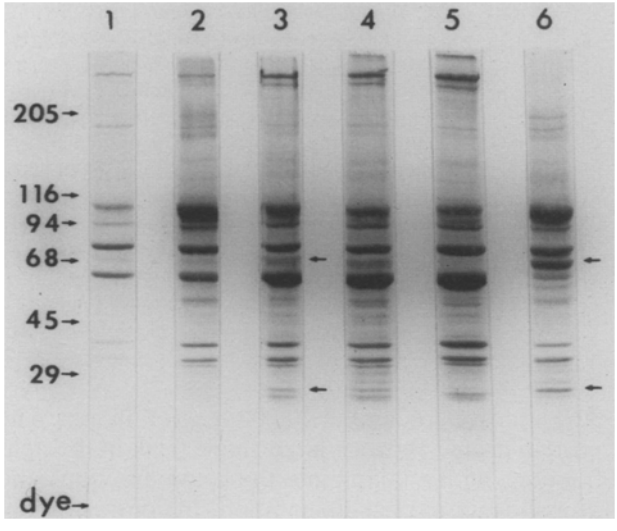

Fig. 8. Coomassie blue-stained gel of SDS-PAGE of the rate zonal centrifugation fractions. Lane *1*: TC/triads, *2*: fraction 3, *3*: combined fractions from No. 10 to 17, *4*: pellet, *5*: HTC, *6*: T-tubule. Each lane was loaded with 20 μg of protein with an exception of lane *1*

ads (lane *1*), the light fraction which has low PN activity (lane *2*) is distinguished by a relatively high content of Ca^{2+}-ATPase with M_r = 100,000 and a very weak JFP band, suggesting that these vesicles are mostly from the nonjunctional fragments of the triads. On the other hand, the PN rich fraction (lane *3*) shows a lower amount of the 100-kDa protein, but a higher content of the JFP and calsequestrin and the T-tubule specific proteins of 72 and 27 kDa (upper and lower arrows, respectively) indicating the junctional nature of these fractions. The pellet with a lower but still considerable PN activity (lane *4*) shows a very similar protein pattern to HTC except for the presence of the T-tubule specific proteins of 72 and 27 kDa, suggesting that these are a mixture of the junction and HTC.

Comparison of the Effect of Trypsinization of the TC/Triads and Strong Triads

The release by trypsin digestion of the T-tubule from the TC/triads and the rate zonal purified PN rich fraction of LTC (LTC*, strong triads) were compared in Fig. 9 by measuring ouabain activity sedimented through 30% sucrose. The ouabain profile for the TC/triads shows a biphasic pattern. Trypsin at low concentration causes marked decrease of the ouabain in the pellet. Increasing trypsin concentration causes a further decrease of the pelleted ouabain, but to a progressively smaller extent. These data are consistent with the possibility that TC/triads are a composite mixture of the junctional mixtures which differ in resistance to trypsinization. By contrast, the junction of the strong triads appears to be much more resistant to disruption by trypsin. The trypsin concentration required

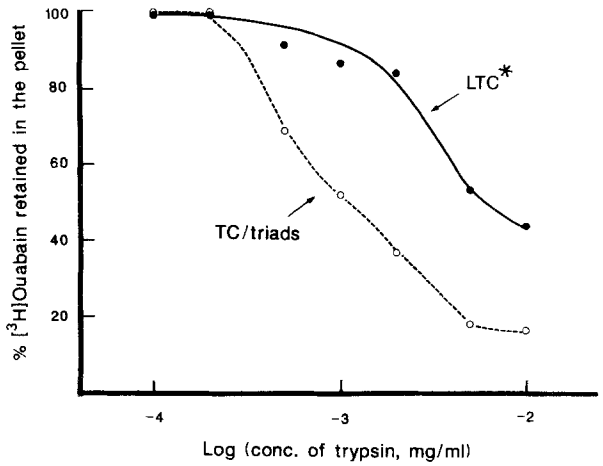

Fig. 9. [3H]ouabain retained in the pellet after trypsinization of TC/triads and LTC*. LTC* denotes rate zonal purified strong triads. Trypsinization was performed and centrifuged through 30% (wt/wt) sucrose in an airfuge as described in Materials and Methods. Percent [3H]ouabain in the pellet is expressed relative to a control experiment in which no enzyme was added

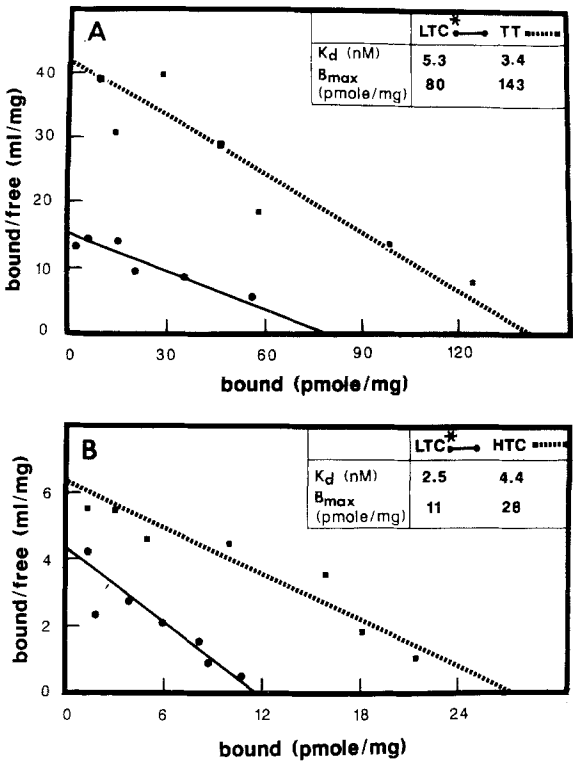

Fig. 10. Scatchard plots of (*A*) [3H]PN200-110 and (*B*) [3H]ryanodine. LTC* denotes the rate zonal purified [3H]PN200-110 rich fractions (Fig. 7*B*, lane *3*). T-tubule and HTC were prepared from triads by French press treatment as described in Materials and Methods. Binding assays were carried out exactly as described in Materials and Methods

to release 50% of the ouabain into the supernatant (ED_{50}) from TC/triads and strong triads differs by a factor of 9 with an ED_{50} of 10^{-3} mg/ml and 9×10^{-3} mg/ml, respectively.

Scatchard Analyses of PN and Ryanodine

Scatchard plots of PN binding in the rate zonal purified PN-rich fraction of LTC, now termed strong triads, and T-tubule are shown in Fig. 10*A*. For both preparations, the plots show a single class of receptors with a similar K_d value of 3 to 5 nM. The B_{max} values of the strong triads and T-tubule are 80 and 143 pmol/mg of protein, respectively. Since the T-tubule in both types of triads is a minor component on a protein basis, the similarity of the B_{max} values suggests that PN receptors are enriched in the T-tubule of the strong triads compared to that of the weak triads. To evaluate the stoichiometric ratio of DHP receptor to foot protein, Scatchard analysis of ryanodine binding was performed on the strong triads and HTC (Fig. 10*B*). The K_d values of strong triads and HTC, 2.5 and 4.4 nM, respectively, indicate that the affinity of the receptors is not significantly different in these vesicles. The density of the receptors shows B_{max} value of 11 and 28 pmol/mg in the strong triads and HTC, respectively. Using the B_{max} values of PN and ryanodine from Fig. 10, a ratio of DHP receptor to ryanodine receptor was calculated in the strong and weak triads. In the strong triads, the ratio of DHP receptor to foot is approximately 7. The calculation for the ratio in weak triads is more complicated: the ratio for the total binding sites of DHP and ryanodine receptors must be calculated with an assumption that the T-tubule and HTC obtained by French press treatment of the TC/triads are from the weak triads. The yield of the T-tubule and HTC from the rejoined triads are about 1 and 10 mg, respectively. Therefore, the stoichiometric ratio of DHP receptor to ryanodine receptor is approximately 0.5 in the weak triads. There may be some contribution of ryanodine binding from the free HTC which was not associated with the T-tubule before the French press treatment. This is unlikely to represent a major contaminant since most of the ryanodine binding is found at higher isopycnic densities after French press treatment, an observation consistent with the view that they have become separated from the lighter T-tubule by the procedure.

Electron Microscopy

Figure 11*a* is a representative electron micrographic field of the strong triads. It shows a number

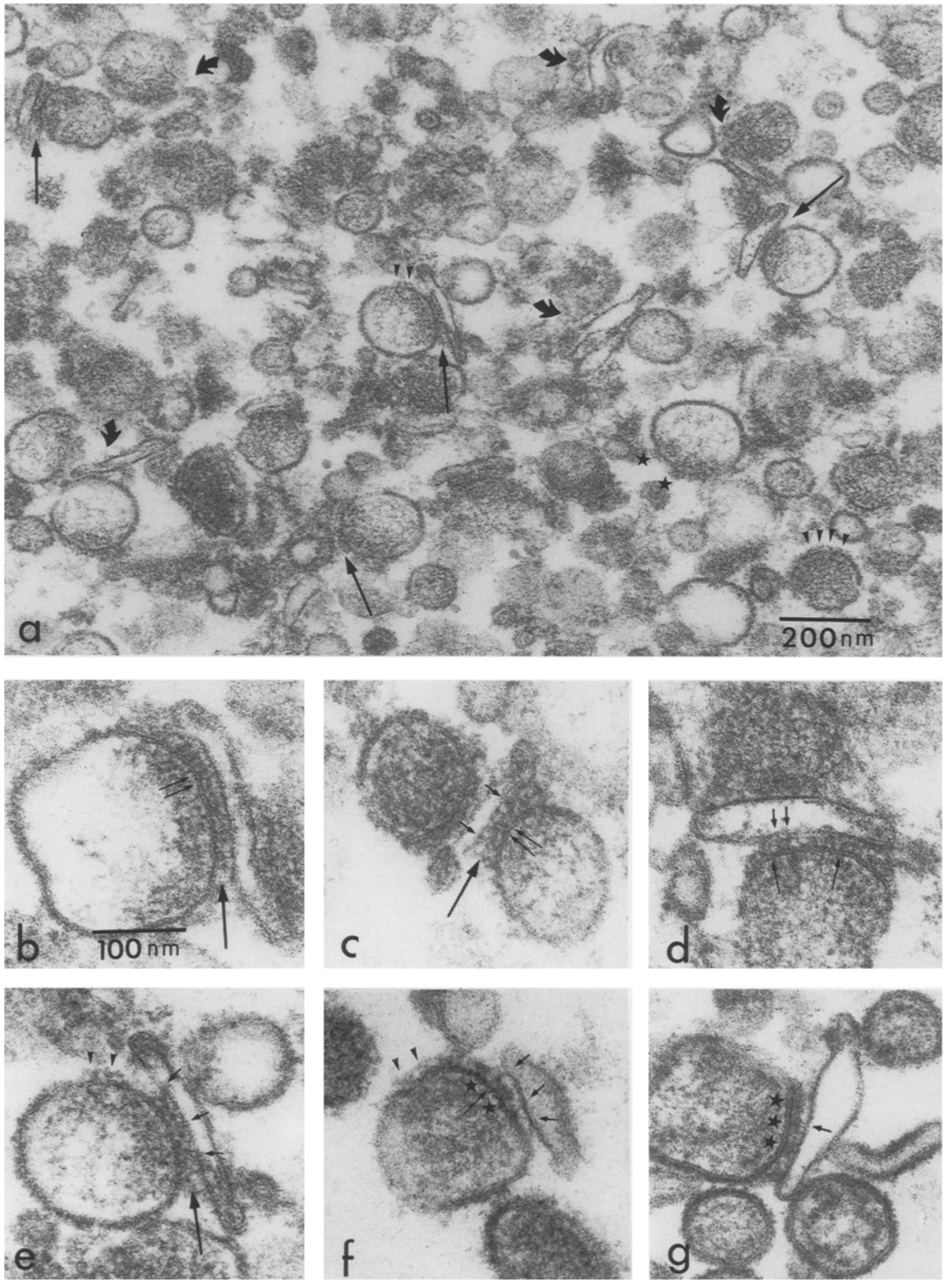

Fig. 11. Thin-section views of the strong triads. Symbols used are as follows: curved arrows for the triads/diads, stars for recognizable foot, long arrows for a central density which runs parallel to T-tubule membrane, medium arrows for thin bridges which span full gap distance, short arrows for the thin protrusions connecting T-tubule to a central density and arrow heads for the Y-shaped protrusion from TC

of the triads/diads in which junctions are preserved after the French press treatment (curved arrows). Most of the junctions appear to be well formed having a large area of contact between the T-tubule and TC. Frequently, a line of electron dense spots at a regular interval or of an apparently continuous ribbon of electron density is located in the junction equidistant between the T-tubule and TC membrane (long arrows). Thin projections are seen on the TC membrane (arrow heads). Distinct feet described as a square structure do not appear to be a major component in the junction, although these are seen occasionally in free HTC (stars).

Figure 11*b–g* are high magnification images showing the detailed fine structure of the junction. The central density (long arrows) is more apparent and the thin protrusions (short arrows in Fig. 11*C–G*) from both T-tubule and TC appear to be connected to the central density resulting in a fence-like appearance. Occasionally, thin bridging structures which completely span the full gap distance are seen (medium arrows in Fig. 11*b–d* and *f*). The pairing of the bridges appears to be variable; in some case, bridges appear to be a pair with a translucent core, but also are seen as single line. In Fig. 11*F*, a single bridge which is sandwiched by feet (stars) is seen. The average gap distance is 15–16 nm whereas the height of the foot above the TC membrane is only 12 nm. When the feet are present in the gap (Fig. 11*f* and *g*), most of the feet do not reach to the T-tubule surface. Instead, there are thin filamentous connections from the T-tubule to the top of the foot. In TC vesicles not attached to the T-tubule, Y-shaped protrusions on the TC membrane are frequently observed (arrow heads in Figs. 11*e* and *f* and 12*a* and *b*). The total height of the Y structure is 12 nm, similar to that of the foot. However, the thickness of the Y protrusion is 5–7 nm. Figure 12*c* is a thin-section image of the major light fraction of the LTC after rate zonal centrifugation. The vesicles are uniformly round and small with a luminal density much lighter than those of the HTC. These morphological differences between the two vesicle fractions from the rate zonal centrifugation are consistent with the separation of the strong triads from the major light fraction which has a low specific activity of DHP receptor.

Figure 12*d* shows a thin-section view of the TC/triads for comparison with the strong triads. A considerable range of junctional contact is seen varying from attachment apparently at one site (double arrow) to a more extensive connection through distinct or fused feet (open arrow). Feet (stars) are also observed extensively in TC without apparent attachment to T-tubule. The characteristic central ribbon of electron density seen in strong triads is only seen occasionally in TC/triads. The TC/triads are expected to represent a mixture of weak and strong triads. An example of a distinct strong triad in the preparation is shown in Fig. 12*e*. It shows the usual findings which were seen in the thin-section views of the isolated strong triads such as a central density (long arrows), bridges (medium arrows) and projections from the T-tubule (short arrow). The intact muscle fibers were also processed for thin sectioning. Figure 12*f–j* shows triadic structure which resembles those of the strong triads. In Figure 12*f*, the junction appears to be formed by apparently distinct material on one side from the other. The right side gap shows a prominent central density (long arrow) and thin protrusions connecting the central line to the T-tubule (short arrow). The left side gap appears to be formed by two less well-defined electron dense material which could be described as feet (stars). In Fig. 12*g*, clearly recognizable thin projections from the T-tubule (short arrows) are shown together with bridges seen on the other side of junction (medium arrows). In Fig. 12*h*, a central density (long arrows) is prominent on both sides. Again, projections from the T-tubule are observed (short arrows). In Fig. 12*i* and *j*, electron dense protrusions from the TC which can be described as feet are seen at a regular interval (stars). Usually the height of feet is shorter than the junctional gap which is 12–15 nm. The top of the feet appears to touch each other resulting in a central line. Projections from the T-tubule are frequently observed to be connected to a central density (short arrows) and sometimes to be connected to a thin projection from TC (medium arrow in Fig. 12*i*).

Discussion

Since EC coupling of the skeletal muscle occurs at the triad, the precise knowledge about the triadic structure and the molecular interactions involved in the formation of the junction are essential to understand the mechanism. There have been a number of morphological studies both on the isolated triads and in the intact muscle fiber. However, existence of more than one population of triads has not been reported thus far. We have here presented evidence demonstrating the heterogeneity of isolated triads, now termed strong and weak triads.

The distribution of activities of external membrane markers such as PN, ouabain or CYP, observed in the LTC after French press treatment of triads can be accounted for by the following possibilities: (*i*) incomplete breakage of a homogeneous preparation of triads, (*ii*) a fraction of T-tubule which exhibits higher density, and (*iii*) two fractions

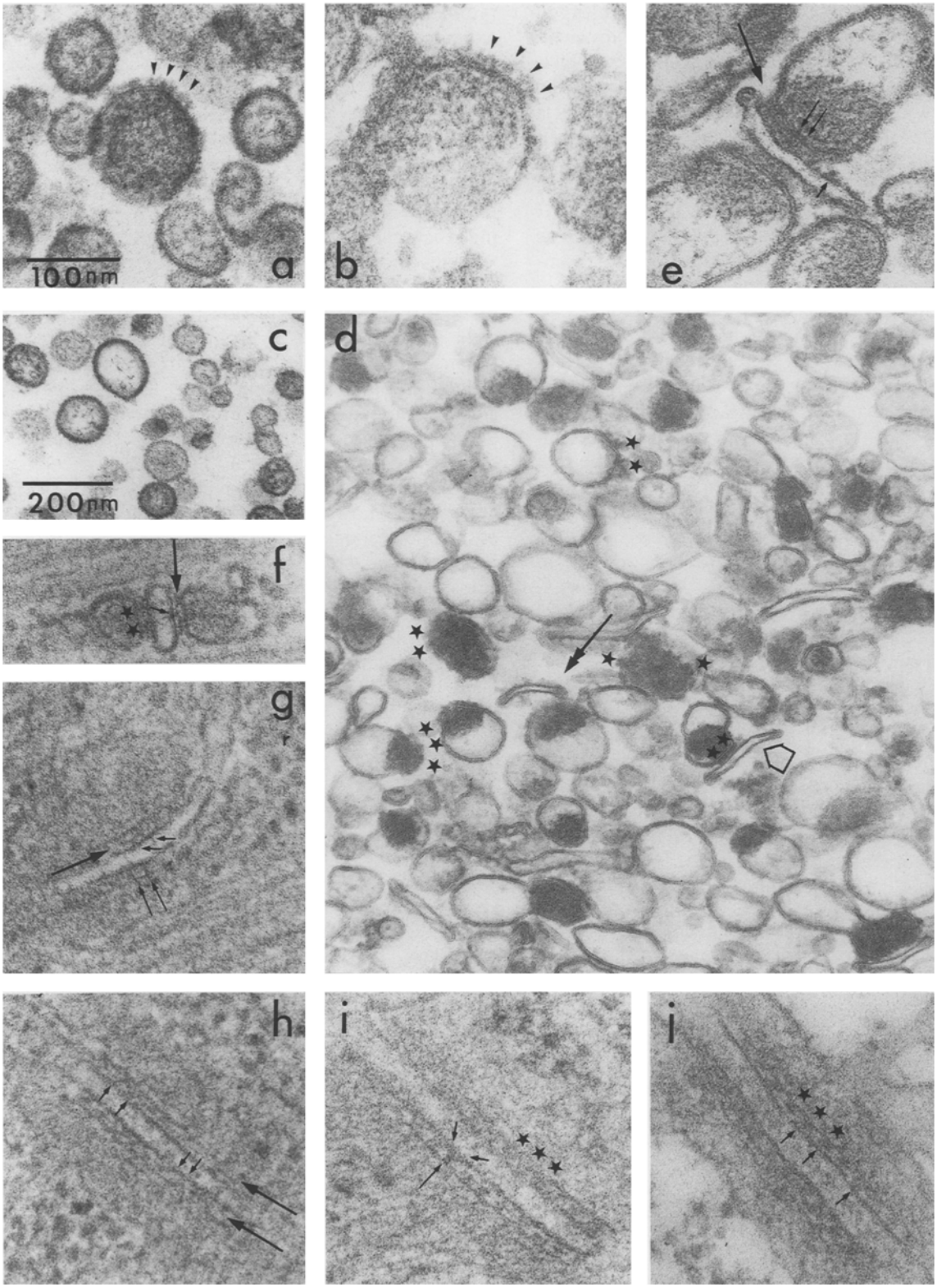

Fig. 12. (*a*) and (*b*) Thin-section views of the free HTC in the strong triad preparation. (*c*) The major light fraction of LTC with low DHP binding activity. (*d*) and (*e*) TC/triads. (*f*)–(*j*) Intact muscle fiber. All symbols used are same as in Fig. 11. (*e*) Represents a potential strong triad observed in TC/triads preparation

of triads which are different in susceptibility to mechanical or enzymatic cleavage. To distinguish these possibilities, we carried out the same breakage procedures using isolated LTC since if there is incomplete breakage of homogeneous triads, repeating the procedure would be expected to break the junction to a similar extent. Our data obtained from the French press, calpain digestion and hypertonic salt treatment (Figs. 3–5) clearly indicate that these procedures which are capable of releasing T-tubules from TC do not have any effect on the distribution pattern of the external membrane marker binding activity. Therefore, the first possibility of the incomplete breakage of homogeneous triads appears very unlikely. Moreover, the data are also inconsistent with a continuum of susceptibility to breakage since this could not account for a complete retention of marker in the LTC after the second breakage procedure. The second possibility can be ruled out by the trypsin experiment (Fig. 6). Trypsin shifted part of the ouabain activity, which has been recognized as a T-tubule marker (Caswell et al., 1976) into the region of isopycnic point of the free T-tubules. Therefore, we have concluded that the existence of receptors for external membrane ligands in the LTC is due to the presence of the T-tubule associated with TC and these remaining junctions are refractory to the breakage procedures.

Recently, Block et al. (1988) reported that clusters of orderly groups of tetrads occur in the junctional T-tubule replica of freeze fractured triads. The spacing and disposition of these T-tubule tetrads are such that they match the position of alternate feet. They proposed that the DHP receptor is one particle of the tetrad, and direct molecular coupling between four DHP receptor molecules would exist with every other foot. According to their model, the stoichiometry of DHP receptor to ryanodine receptor would be 2 if there were one ryanodine binding site per one foot (Lai et al., 1988). Our estimates of 7 and 0.5 in the strong triads and weak triads, respectively, do not match with their number. It is not clear whether this disagreement is due to the difference in the experimental approaches—morphological *vs.* pharmacological, uneven migration or proteolysis of receptors during the isolation procedures. Alternatively, molecular association of the T-tubule tetrad and ryanodine receptor involves an unidentified linkage which differs from their model.

Our data suggest that isolated triads are a composite mixture of weak and strong triads with significantly different bonding forces. In the weak triads, the junction is likely to be formed by ionic interactions of triadic proteins since the hypertonic salt treatment disrupts the junction (Fig. 5). The possible composition of the weak triad is discussed in the accompanying paper (Brandt et al., 1990).

The biochemical properties of strong triads raise a question as to whether the JFP plays a major role in junction formation: (*i*) calpain does not have any detectable effect on the strong triads, whereas trypsin which hydrolyzes many other proteins in addition to the foot protein can release the T-tubule from TC in weak triads; (*ii*) despite the total disappearance of the JFP and its high molecular weight proteolytic fragments by trypsinization in gels, still significant T-tubule activity represented by ouabain entrapment is seen in the density of triads; and (*iii*) total binding sites of the ryanodine receptors in the strong triads are 2.5 times less than those in the weak triads.

The following features distinguish the morphology of the strong triads: (*i*) a relatively low density of feet observed as protruding structure with a height of 10 nm from the TC membrane and a thickness of 20–27 nm (Ferguson, Schwartz & Franzini-Armstrong, 1984; Saito et al., 1984); (*ii*) a strongly defined electron-dense ribbon or series of spots parallel to the apposing membranes; (*iii*) pillars or bridges containing a translucent core which join the TC to the central density (sometimes pillars are seen extending the full distance to the T-tubule membrane); (*iv*) thin filamentous structures joining the T-tubule to the central density; (*v*) an average junctional gap of 15 nm; and (*vi*) the presence of protrusions of a base thickness of 5–7 nm and a height of 12 nm which flare out at their full height in the nonjunctional region of TC. Many of these findings were also observed in the intact muscle fiber indicating that these properties are a genuine reflection of the morphology of these triads rather than an effect associated with the isolation procedures. Our thin-section images of the strong triads resemble Somlyo's bridges (Somlyo, 1979) and Eisenberg and Gilai's pillars (Eisenberg & Gilai, 1979). Kelly (1969) and Franzini-Armstrong and Nunzi (1983) described a central density bisecting the junctional gap. Franzini-Armstrong and Nunzi (1983) ascribed these morphologically distinguishable structures as a single entity of foot and proposed that the difference in sectioning was responsible for the variable appearance. The section thickness for the views of strong triads and TC/triads is approximately the same. Most of the strong triad junctions are occupied by a central density and display the presence of bridges with infrequent feet. However, TC/triads frequently show solid square feet and only occasionally are bridges and a central density visible. Therefore, it is unlikely that the predominance of the central density or pillars in the strong triadic gap is due to a technical artifact of sectioning.

Our sections are approximately 50 nm thick and one may calculate that if the feet are intact tetrad structures one would observe only a single subunit in less than 10% of feet. In our views of the strong triads, we see these thin projections much more frequently. Therefore, we conclude either that the protruding structures are not JFP or that the protein is present in the dissociated monomeric forms in the intact vesicles. The central ribbon may occur through the flaring of these structures at their heads so that a continuity of protein is formed similar to the proposal of Franzini-Armstrong and Nunzi (1983). The filamentous material between the T-tubule and central ribbon may be identical to protruding structures previously described on the surface of isolated T-tubule (Brunschwig et al., 1982). The wide junctional gap described in our preparation has precedence in intact muscle. Somlyo observed a gap of 18 nm where bridges were seen in toadfish (Somlyo, 1979), while Eisenberg and Gilai (1979) found a separation of 12 nm in frog resting muscle.

The possible composition of strong triads is discussed in the accompanying paper (Brandt et al., 1990). Two simple hypotheses are: (*i*) that the junction of strong triads contains different molecular entities than weak triads, and (*ii*) that a considerable morphological alteration has occurred between the junctions in which normal feet are observed and the strong triad junction. Such a morphological change might include reorganization of the subunits of the JFP together with an enhanced separation of the two membranes. Eisenberg and Eisenberg (1982) have proposed that a transition of feet into pillars accompanies muscle activation.

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